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Rapid Mix Preparation of Bioinspired Nanoscale Hydroxyapatite for Biomedical Applications

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Abstract:	Hydroxyapatite (HA) has been widely used as a medical ceramic due to its good biocompatibility and osteoconductivity. Recently there has been interest regarding the use of bioinspired nanoscale hydroxyapatite (nHA). However, biological apatite is known to be calcium-deficient and carbonate-substituted with a nanoscale platelet-like morphology. Bioinspired nHA has the potential to stimulate optimal bone tissue regeneration due to its similarity to bone and tooth enamel mineral. Many of the methods currently used to fabricate nHA both in the laboratory and commercially, involve lengthy processes and complex equipment. Therefore, the aim of this study was to develop a rapid and reliable method to prepare high quality bioinspired nHA. The rapid mixing method developed was based upon an acid-base reaction involving calcium hydroxide and phosphoric acid. Briefly, a phosphoric acid solution was poured into a calcium hydroxide solution followed by stirring, washing and drying stages. Part of the batch was sintered at 1000 °C for 2 h in order to investigate the products' high temperature stability. X-ray diffraction analysis showed the successful formation of HA, which showed thermal decomposition to β-tricalcium phosphate after high temperature processing, which is typical for calcium-deficient HA. Fourier transform infrared spectroscopy showed the presence of carbonate groups in the precipitated product. The nHA particles had a low aspect ratio with approximate dimensions of 50 x 30 nm, close to the dimensions of biological apatite. The material was also calcium deficient with a Ca:P molar ratio of 1.63, which like biological apatite is lower than the stoichiometric HA ratio of 1.67. This new method is therefore a reliable and far more convenient process for the manufacture of bioinspired nHA, overcoming the need for lengthy titrations and complex equipment. The resulting bioinspired HA product is

	suitable for use in a wide variety of medical and consumer health applications.
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TITLE:
Rapid Mix Preparation of Bioinspired Nanoscale Hydroxyapatite for Biomedical Applications

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SHORT ABSTRACT:
This paper describes a novel method for the rapid manufacture of high quality bioinspired nanoscale hydroxyapatite. This biomaterial is of great significance in the manufacture of a wide range of innovative medical devices for clinical applications in

orthopedics, craniofacial surgery and dentistry.

LONG ABSTRACT:
Hydroxyapatite (HA) has been widely used as a medical ceramic due to its good biocompatibility and osteoconductivity. Recently there has been interest regarding the use of bioinspired nanoscale hydroxyapatite (nHA). However, biological apatite is known to be calcium-deficient and carbonate-substituted with a nanoscale platelet-like morphology. Bioinspired nHA has the potential to stimulate optimal bone tissue regeneration due to its similarity to bone and tooth enamel mineral. Many of the methods currently used to fabricate nHA both in the laboratory and commercially, involve lengthy processes and complex equipment. Therefore, the aim of this study was to develop a rapid and reliable method to prepare high quality bioinspired nHA. The rapid mixing method developed was based upon an acid-base reaction involving calcium hydroxide and phosphoric acid. Briefly, a phosphoric acid solution was poured into a calcium hydroxide solution followed by stirring, washing and drying stages. Part of the batch was sintered at 1000 °C for 2 h in order to investigate the products' high temperature stability. X-ray diffraction analysis showed the successful formation of HA, which showed thermal decomposition to β-tricalcium phosphate after high temperature processing, which is typical for calcium-deficient HA. Fourier transform infrared spectroscopy showed the presence of carbonate groups in the precipitated product. The nHA particles had a low aspect ratio with approximate dimensions of 50 x 30 nm, close to the dimensions of biological apatite. The material was also calcium deficient with a Ca:P molar ratio of 1.63, which is lower than the stoichiometric HA ratio of 1.67 like biological apatite. This new method is therefore a reliable and far more convenient process for the manufacture of bioinspired nHA, overcoming the need for lengthy titrations and complex equipment. The resulting bioinspired HA product is suitable for use in a wide variety of medical and consumer health applications.

INTRODUCTION:
There is a great clinical need for advanced biomaterials with enhanced functionality in order to improve the quality of life for patients and to reduce the healthcare burden of a global aging population. Hydroxyapatite has been widely used in medical applications for many years due to its good biocompatibility. Recently, there has been an increased interest in the use of nanoscale hydroxyapatite (nHA), particularly for mineralized tissue regeneration in medicine and dentistry. The mineral found in bone and tooth enamel is calcium-deficient, multi-substituted, nanoscale hydroxyapatite. Estimates for the size of biological nHA platelets report dimensions of 50 nm x 30 nm x 2 nm[1], with even smaller structures described in immature bone[2]. Contrastingly, the mineral in tooth enamel is 10 to 100 times larger than that found in bone tissue in both length and width[3,4]. Synthetic nHA might be better termed bioinspired rather than biomimetic, as we are seeking to translate observations regarding the characteristics of natural materials into medical technologies with improved performance. It has been suggested that bioinspired nHA may be more favorable in bone and tooth tissue regeneration applications due to its similarity to naturally occurring mineral[5].

There are various methods which have been reported to prepare nHA including

hydrothermal[6], spray-dry[7] and sol-gel[8] techniques. Of these, the wet precipitation method is considered a relatively convenient method for the production of nHA. The published nHA wet precipitation methods generally include a titration step when mixing calcium and phosphorus chemical precursors[9-14]. However, these approaches are associated with a number of disadvantages including lengthy and complex processes combined in some cases with the need for expensive equipment. Commercial production may be even more complex, with patents describing sophisticated reactors for manufacture of high quality medical grade nHA[15]. Despite this, the neutralization reaction between calcium hydroxide and phosphoric acid is advantageous due to the lack of noxious chemical by-products.

The relationship between processing conditions and the morphology of the nHA product has been reported for slow titration reactions. Specifically, for titration methods involving calcium hydroxide and phosphoric acid, an elevated temperature appeared to favor the preparation of particles with a low aspect ratio[13]. This work was extended considerably by Gentile *et al.*[16], who demonstrated the relationship between temperature and other processing conditions on the quality of nHA products from a wide range of methods. He concluded that the wet chemical precipitation method of Prakash[13] made the highest quality products, but it should be noted that the results were dependent upon technically challenging and slow/ mixing processes. The original Prakash titration step takes over one hour. However, longer titration times may be required for larger batches to be prepared.

To summarize, while the influence of several factors including temperature have now been studied extensively, almost no attention has been directed at reducing the complexity and associated time needed to perform titration-based methods. The aim of this study was therefore to investigate the effects of applying a rapid mix approach to the manufacture of a bioinspired nHA, and to fully characterize the resulting materials. If successful, a simplified rapid mix approach would have great benefits for laboratory researchers and industry alike where costs of manufacturing could be substantially reduced without comprising quality.

PROTOCOL:

[Figure 1 here]

1. Rapid Mix Production of Nanoscale Hydroxyapatite

1.1) Prepare 5 g of nanoscale hydroxyapatite at a calcium to phosphorus molar ratio of 1.67.

1.1.1) Add 3.705 g of calcium hydroxide to 500 mL deionized water and stir on a magnetic stirrer plate for 1 h at 400 rpm.

1.1.2) In a separate beaker, add 3.459 g of phosphoric acid (85%) to 250 mL deionized water.

1.1.3) Pour the phosphorus solution into the stirring calcium hydroxide suspension at a rate of approximately 100 mL/s. Cover beaker with parafilm.

1.1.4) Leave the suspension to stir for 1 h at 400 rpm.

1.1.5) Take the beaker off the stirrer plate and leave to settle overnight.

1.2) Wash the suspension by pouring off the supernatant, adding 500 mL deionized water, and stirring for 1 min at 400 rpm. Repeat this step three times in total, with 2 h between each wash.

1.3) Leave nHA suspension to settle overnight.

1.4) Pour off the clear supernatant and place the settled nHA suspension in a drying oven set to 80 °C.

1.5) When dry, place the dried nHA into an agate mortar and pestle and grind until fine.

1.6) Place 2.5 g of produced nHA powder in an alumina crucible and sinter powder at 1000 °C for 2 h using a ramp rate of 10 °C/min. After the heat treatment, leave the nHA to cool in the furnace.

1.7) Store powders in a vacuum desiccator.

2. Characterization of Nanoscale Hydroxyapatite

2.1) X-ray diffraction (XRD) using transmission mode diffractometers

2.1.1) Place a small amount (i.e. less than 200 µL) of poly(vinyl alcohol) (PVA) glue on acetate film and mix with a small amount (i.e. less than 100 mg) of nHA powder.

2.1.2) Treat with a hot air gun until dry.

2.1.3) Mount the sample into a sample holder and load onto a transmission mode X-ray diffractometer with Cu K_α radiation.

2.1.4) Use diffractometer settings of 40 kV and 35 mA, with a 2θ range of 10-70° according to manufacturer's protocol.

2.1.5) Analyze the resultant XRD patterns. Use the following XRD cards for phase identification: Hydroxyapatite: 9-432. β-tricalcium phosphate: 04-014-2292.

2.2) Transmission electron microscopy (TEM)

2.2.1) Place a small amount of powder (i.e. less than 10 mg) in a bijou and add approximately 3 mL ethanol.

2.2.2) Ultra-sonicate sample for 15 – 30 minutes until a homogenous suspension is

observed.

2.2.3) Pipette a small amount of solution (i.e. less than 1 mL) onto a 400 mesh copper grid with carbon film, and allow to dry.

2.2.4) Image samples at an accelerating voltage of 80 kV according to manufacturer's protocol.

2.3) X-ray fluorescence (XRF) service by the Materials and Engineering Research Institute (MERI) at Sheffield Hallam University

2.3.1) Combine 0.8 g nHA powder with 8 g of lithium tetraborate.

2.3.2) Melt mixture in a platinum-gold alloy crucible using a furnace set to 1200 °C.

2.3.3) Analyze resultant samples in an XRF spectrometer to determine the elemental composition of the samples according to manufacturer's protocol.

2.4) Fourier-transform infrared spectroscopy in attenuated total reflectance mode (FTIR-ATR)

2.4.1) Perform 64 background scans from 4000 – 500 cm^{-1} with a resolution of 4 cm^{-1} using manufacturer's protocol.

2.4.2) Place a small amount (i.e. less than 100 mg) of nHA powder on top of the diamond in the attenuated total reflectance mode adapter and compress onto the surface of the diamond using the screw top.

2.4.3) Perform 32 scans from 4000 – 500 cm^{-1} with a resolution of 4 cm^{-1} with the background scans subtracted from the sample scans according to manufacturer's protocol.

REPRESENTATIVE RESULTS:

XRD patterns (Figure 2) showed the precipitation of a pure HA phase with broad peaks, indicating a relatively small crystallite size and/or amorphous nature. After high temperature sintering, β-tricalcium phosphate (β-TCP) was detected, alongside a main phase of HA. The sharpening of the diffraction peaks, i.e. a reduction in the full width half maximum, indicated an increase in the crystallite size after sintering.

[place figure 2 here]

FTIR-ATR spectra (Figure 3) confirmed the formation of a HA phase by the characteristic phosphate and hydroxyl bands[17,18]. In detail, the bands were assigned as follows: 3750 cm^{-1} (OH^- stretch ν_{OH}); 1086 and 1022 cm^{-1} (PO_4^{3-} ν_3); 962 cm^{-1} (PO_4^{3-} ν_1); 630 cm^{-1} (OH^- libration δ_{OH}); 600 and 570 cm^{-1} (PO_4^{3-} ν_4). In the unsintered sample the additional peaks were assigned as follows: broad peak centred around 3400 cm^{-1} (absorbed water molecules); 1455 and 1410 cm^{-1} (CO_3^{2-} ν_3); 880 cm^{-1} (CO_3^{2-} ν_2). The absorbed water and carbonate groups observed in the unsintered powder were

removed during the high temperature sintering stage. The sintering process also sharpened the hydroxyl and phosphate bands which was manifested by a greater peak to trough distance.

[place figure 3 here]

TEM images (Figure 4) showed the formation of nanoscale particles with approximate dimensions of 50 nm by 30 nm. The particles had a low aspect ratio (particle length / particle width) of around 1.7. The size and shape of the nanoscale products were of similar dimensions to biological apatite[1].

[place figure 4 here]

Quantitative chemical analysis of the nHA powder by XRF (Table 1) allowed the calcium: phosphorus ratio to be calculated as 1.63, which is slightly lower than the stoichiometric HA which has a calcium: phosphorus ratio of 1.67. XRF also showed the high purity of the nHA product with only trace amounts of other elements recorded.

[place Table 1 here]

Figure 1. Schematic diagram of rapid mix preparation of bioinspired nanoscale hydroxyapatite.
A. The phosphoric acid solution was poured into the calcium hydroxide suspension. After the suspension settled overnight, the nHA was washed with deionized water before being dried at 60 °C. The nHA was then ground in an agate mortar and pestle and sintered to investigate the thermal stability of the nHA product.

Figure 2. Crystal phase analysis of products.
X-ray diffraction (XRD) patterns of unsintered nanoscale hydroxyapatite (nHA) powder and nHA powder sintered at 1000 °C for 2 h. Peak labels: hydroxyapatite peaks ▼, β-tricalcium phosphate peaks ■.

Figure 3. Infrared spectra of products.
Fourier transform infrared in attenuated total reflectance mode (FTIR-ATR) spectra of unsintered nanoscale hydroxyapatite (nHA) powder and nHA powder sintered at 1000 °C for 2 h.

Figure 4. Nanoscale morphology of product.
Transmission electron micrographs (TEM) of nanoscale hydroxyapatite (nHA) prepared using the rapid mixing method at two magnifications.

Table 1. Quantitative chemical analysis of product.
X-ray fluorescence (XRF) results for unsintered nHA powder showed >99% purity by weight.

DISCUSSION:

Natural apatite is composed of nanoscale particles of non-stoichiometric carbonated hydroxyapatite with the approximate chemical formula of $Ca_{10-x-y}[(HPO_4)(PO_4)]_{6-x}(CO_3)_y(OH)_{2-x}$. The production of biomaterials with close chemical similarity to naturally occurring mineral has been reported to promote optimal biological responses. For instance, research on biomimetic calcium-deficient carbonated nHA has shown that it is able to stimulate proliferation and the alkaline phosphatase activity of murine preosteoblast cells to a greater degree than conventional nHA[19].

In this study, the precipitation of HA which showed partial thermal decomposition at 1000 °C (Figure 2) suggested the formation of a calcium-deficient HA. This was supported by the lower than stoichiometric Ca:P ratio (1.63) obtained with the XRF data (Table 1). It is understood that a reduced Ca:P ratio is associated with a lower thermal stability[20-23]. In this method, the rapid addition of the phosphoric acid solution rapidly lowered the pH of the reaction suspension to generate HPO_4 ions. The presence of HPO_4 groups facilitated the precipitation of calcium deficient HA, with the molecular formula: $Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$, where $0<x<1$.

The rapid addition of the phosphoric acid therefore had a marked effect on the precipitation kinetics of the reaction. As described previously, titration reactions involving calcium hydroxide and phosphoric acid carried out at room temperature tended to yield particles with a high aspect ratio[13]. For titration reactions involving these reactants, it was necessary to use an elevated temperature to produce particles with a lower aspect ratio which are more similar to biological apatite[13]. High aspect ratio particles are produced when the crystal nucleation rate is slower than the crystal growth rate[24]. For the new method developed in this study, the rapid addition of the phosphoric acid solution may have provided a larger number of nucleation sites, which resulted in the increased presence of small rounded particles as opposed to fewer particles with a larger aspect ratio. As the authors have not fully investigated the effects of slowly pouring the phosphoric acid into the calcium hydroxide suspension in order to achieve consistent results, we recommend that the phosphoric acid is poured at a rate commensurate with that shown here (approximately 100 mL/s).

During the development of this method, the authors investigated a number of incremental changes to the nHA preparation method based on Prakash *et al*.[13] including the comparison of products produced with the slow titration and the rapid addition of the phosphoric acid solution[25]. It was found that the slow titration of phosphoric acid into the calcium hydroxide suspension resulted in a product with a calcium hydroxide residue. We propose that the pH change caused by the rapid addition of phosphoric acid encouraged the dissolution of the calcium hydroxide and therefore allowed for the successful conversion of the reactants into hydroxyapatite. A comparison of products prepared using the rapid mixing method at room temperature and at elevated temperatures (60 °C) found that an elevated temperature resulted in a higher conductivity after the reaction was completed. This suggested that residual calcium hydroxide was present, which was likely due to the lower solubility of calcium hydroxide at increased temperatures. The presence of residual calcium hydroxide was undesirable as the basicity of this compound could compromise biocompatibility.

FTIR detected the characteristic phosphate and hydroxyl group activity associated with HA (Figure 3). It was noted that the spectrum for the sintered product showed sharper phosphate and hydroxyl peaks. These changes have been associated with a greater product crystallinity[26,27].The unsintered spectrum provided evidence for B-type carbonate substitution, where carbonate ions have been substituted for phosphate groups. This is in contrast to A-type substitution where carbonate ions may substitute for hydroxyl groups[17]. It has been reported that B-type carbonate substitution occurs in biological apatite[3]. However, Tampieri *et al.* reported that whilst B-type substitution was predominant in young bones, A-type carbonate substitution was increasingly present in bones of older individuals[28]. Carbonate substitution has been found to decrease the crystallinity and thermal stability of the nHA whilst increasing its solubility. These changes have been proposed to contribute to the increased bioactivity of carbonate-substituted HA[29]. Biological HA is also known to contain some of the other elements recorded in the XRF analysis (Table 1), such as magnesium, sodium and strontium[30]. The presence of these elements may also contribute to increased biological efficacy. Future work should be directed at the preparation of these nanoscale substituted apatites, and also products with increased biofunctionality such as silver-doped nHA[31]. In order to prepare substituted nHA, the element may be introduced with a corresponding reduction of the intended element to substitute for a reduction in the amount of the calcium compound when strontium, magnesium or zinc substitution is attempted[32]. Alternatively, another approach may be to add elements with the intention of providing ‘doped’ ions, which are present on the surface of the nHA without necessarily intending to substitute the element into the HA crystal lattice[31]. For these modifications to the method, it is possible to prepare mixed solutions of calcium hydroxide and silver nitrate, and to carry out the reaction in the same manner as described here.

In conclusion, this paper reports a novel, rapid, and substantially improved method for the preparation of bioinspired nHA. For this method, the rapid mixing of the chemicals takes less than 5 seconds which is a marked reduction in time compared to titration reactions typically requiring hours of careful monitoring. It has great potential for use in biomaterial development due to its relative simplicity and low cost compared to currently used industrial nHA manufacturing methods. In particular, this new method is superior to continuous flow processes or hydrothermal techniques due to significantly lower start-up equipment costs. Also, the inherent complexity of current commercial systems results in lengthy research and development times and substantially increased manufacturing costs.

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DISCLOSURES:
The authors have nothing to disclose

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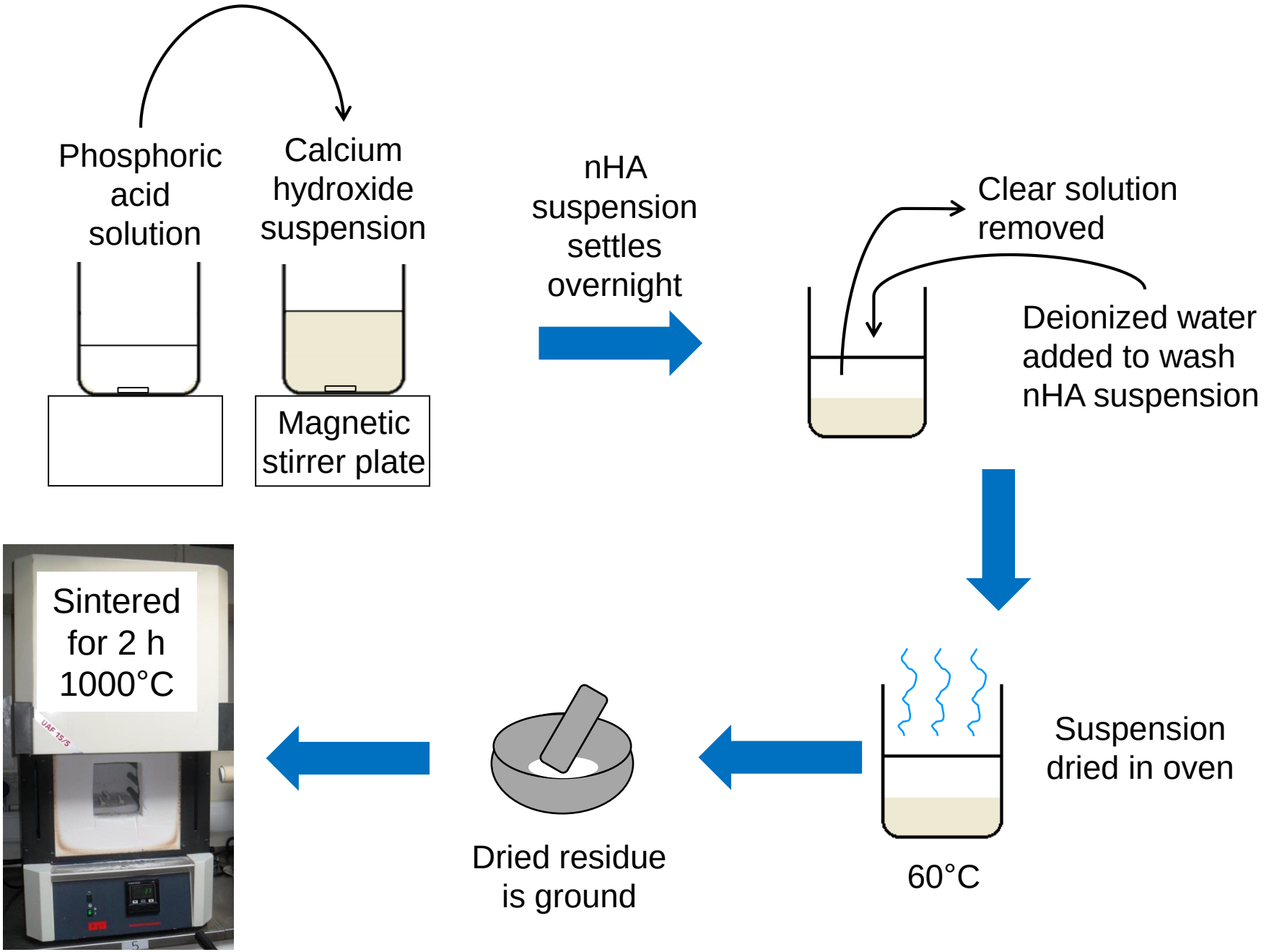

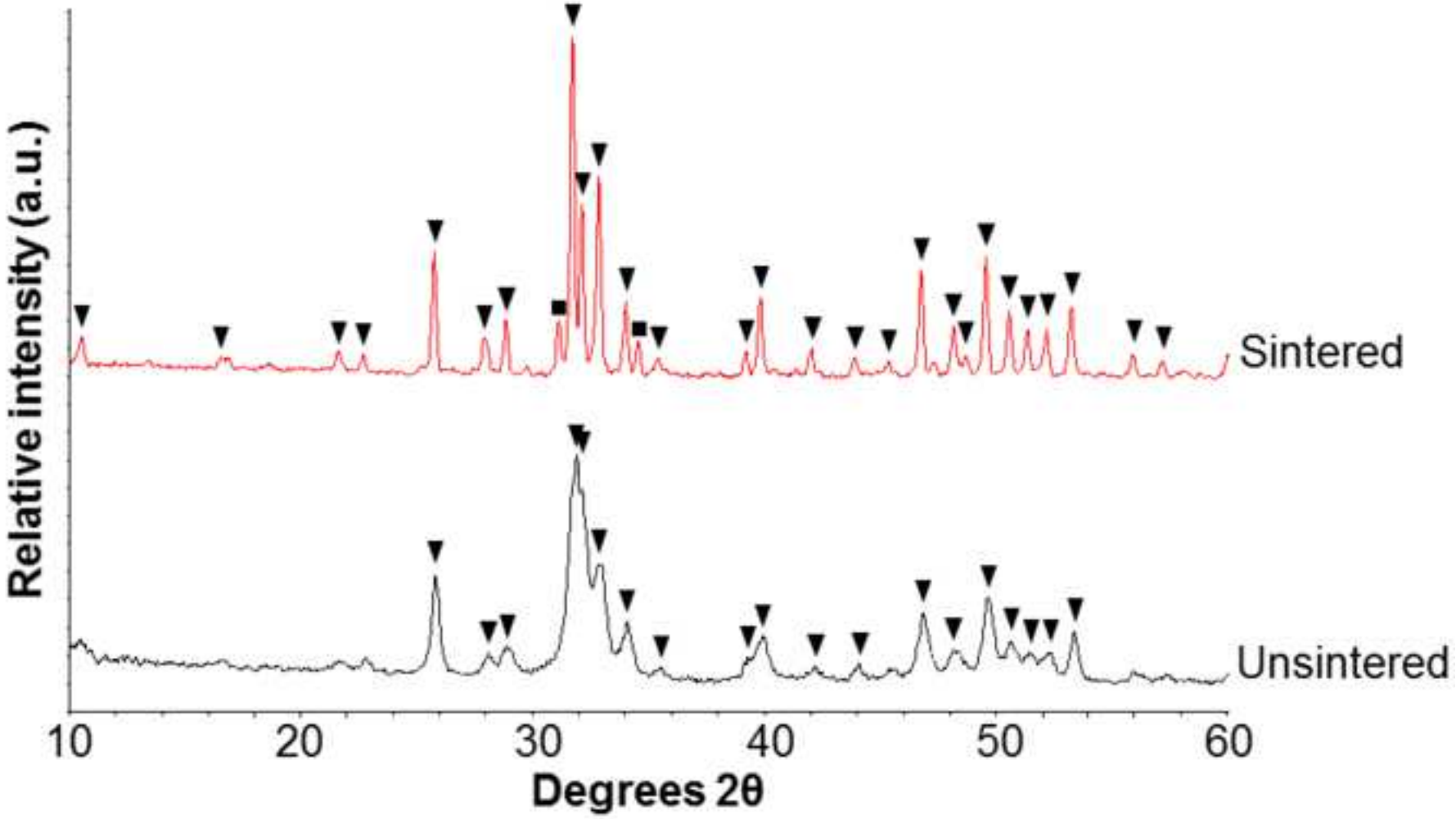

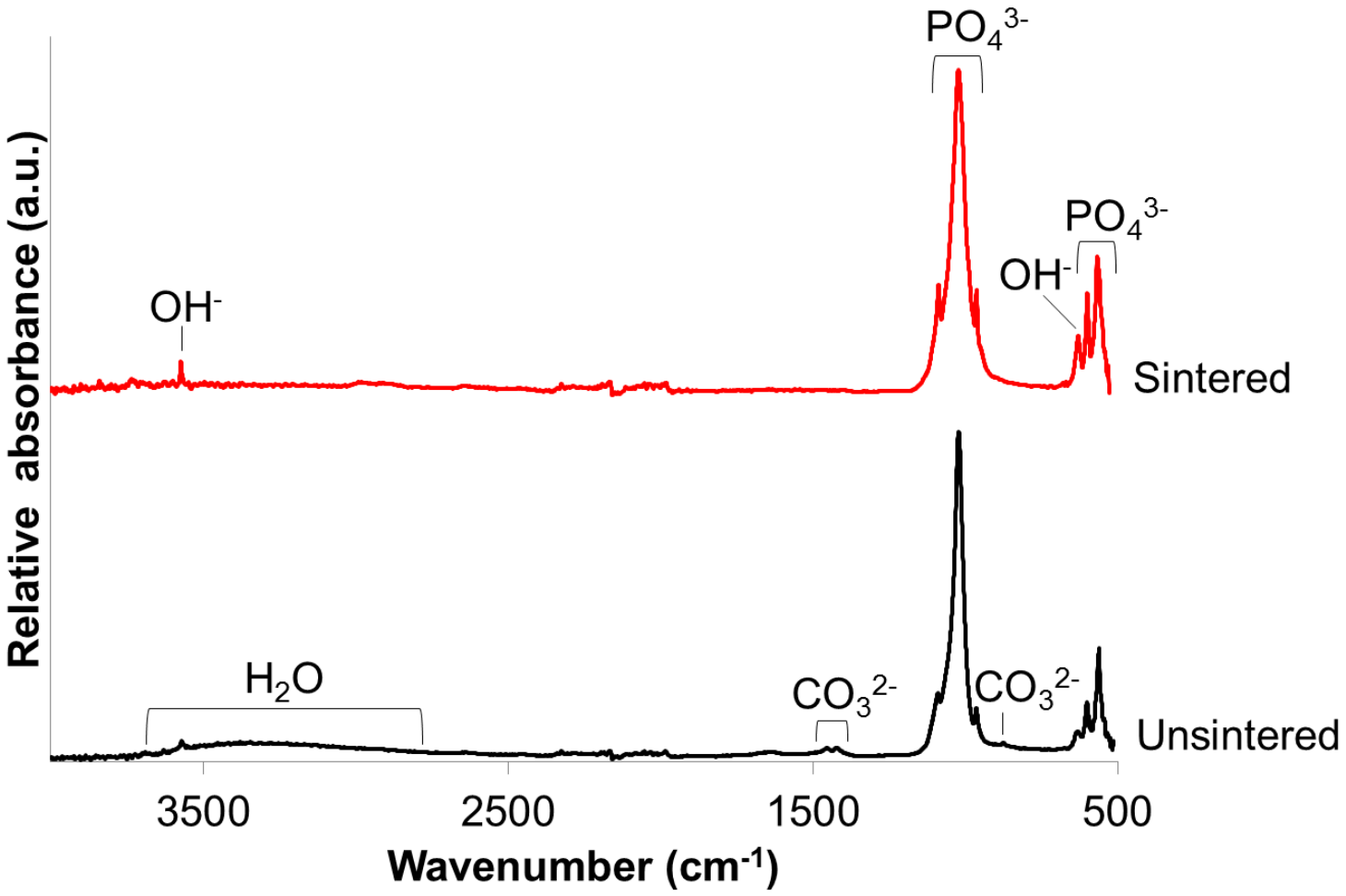

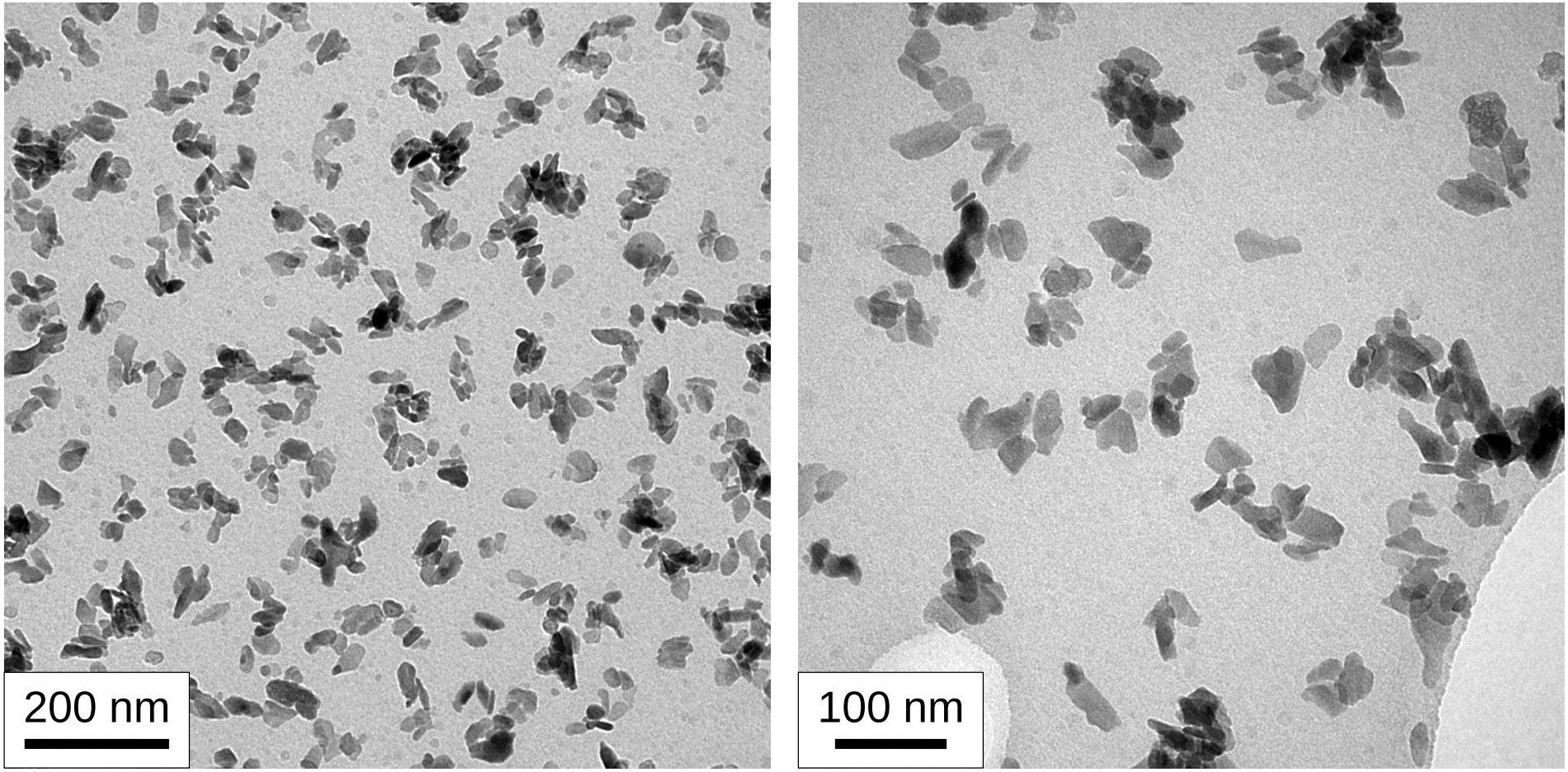

Compound	Weight %
CaO	51.52
P_2O_5	39.89
MgO	0.46
Na_2O	0.13
Y_2O_3	0.07
Al_2O_3	0.03
SiO_2	0.03
Mn_3O_4	0.03
SrO	0.02
TiO_2	0.01

Click here to download Excel Spreadsheet- Table of Materials/Equipment Table of specific materials.xlsx

Name of the Material / Equipment	Company	Catalog Number
Calcium hydroxide (purity of ≥ 96%)	Sigma Aldrich UK	31219
Phosphoric acid (85 %)	Sigma Aldrich UK	345245
STOE IP x-ray diffractometer	Phillips	
International centre for diffraction data (ICDD) PDF4+ database	International Centre for Diffraction Data	
Holey carbon films on 300 mesh grids	Agar Scientific	S147-3H
Tecnai G2 Spirit transmission electron microscope	FEI	
Lithium tetraborate	ICPH, Malzéville, France	
PW2440 XRF spectrometer	Philips	
ThermoScientific Nikolett Spectrometer	Unicam Ltd	

Comment / Description (optional)
Good laboratory practise should be used at all times including the use of appropriate personal protective equipment (i.e. lab coat and gloves)

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Title of Article: RAPID MIX PREPARATION OF BIOINSPIRED NANOSCALE HYDROXYAPATITE FOR BIOMEDICAL APPLICATIONS

Author(s): CAROLINE WILCOCK

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Institution: UNIVERSITY OF SHEFFIELD

Article Title: RAPID MIX PREPARATION OF BIOINSPIRED NANOSCALE HYDROXYAPATITE FOR BIOMEDICAL APPLICATIONS

Signature: C Wilcock Date: 5/8/16

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September 2016

Dear Dr Nguyen,

Re: Manuscript JoVE55343 / response to reviewer's comments

On behalf of the authors, I have attached our response to the reviewer's comments regarding improvements of the manuscript for publication. We were pleased that the reviewers recognised that the paper was of value to the scientific community to report our new method of preparing bioinspired nanoscale hydroxyapatite. The editorial and reviewer comments have been addressed and the manuscript has been amended accordingly.

Please find provided our amended manuscript which demonstrates the rapid mix preparation of bioinspired nanoscale hydroxyapatite. The authors believe that this paper will benefit from JoVE's unique multimedia format in conveying the relative simplicity and ease of this method which should be of great interest to many research groups developing calcium phosphate biomaterials.

Yours sincerely,

Paul Hatton

Response to reviewer's comments

The authors would like to thank the editor and reviewers for their valuable comments for improving the paper. Each comment has been addressed in the list below detailing the changes that have been made to the manuscript.

Comments and authors responses

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Author's response to comment 1: The manuscript has been proof read.

2. Please abbreviate all journal titles.

Author's response to comment 2: All journal titles have been abbreviated as requested.

3. Formatting: Please define all abbreviations at first occurrence (ie PVA, etc.).

Author's response to comment 3: PVA has been added in full:

'poly(vinyl alcohol) (PVA)'

4. Additional detail is required:

-1.10 , 2.3.2– What vessel is used?

Author's response to comment: The appropriate vessels have been added to the manuscript:

'Place 2.5 g of produced nHA powder in an alumina crucible and sinter powder at 1000 °C for 2 h using a ramp rate of 10 °C/min.'

'Melt mixture in a platinum-gold alloy crucible using a furnace set to 1200 °C.'

-2.1.1, 2.2.1, 2.2.3 , 2.4.2– What is considered a "small amount"?

Author's response to comment: The text has been adjusted as follows:

'Place a small amount (i.e. less than 200 µL) of poly(vinyl alcohol) (PVA) glue on acetate film and mix with a small amount (i.e. less than 100 mg) of nHA powder.'

'Place a small amount of powder (i.e. less than 10 mg) in a bijou and add approximately 3 mL ethanol.'

'Pipette a small amount of solution (i.e. less than 1 mL) onto a 400 mesh copper grid with carbon film, and allow to dry.'

'Place a small amount (i.e. less than 100 mg) of nHA powder on top of the diamond in the attenuated total reflectance mode adapter and compress onto the surface of the diamond using the screw top.'

-2.1.5, 2.1.6 – What actions are performed here? It is unclear what we would film.

Author's response to comment: The actions involve using software on the computer to view the collected data with database results.

-2.3.3 – How is this done? Please provide detail if this will be filmed in detail. Provide a citation otherwise.

Author's response to comment:

This work was carried out at as a service by the Materials and Engineering Research Institute at Sheffield Hallam University, so this has now been stated in the method.

-2.4 –How are scans performed?

Author's response to comment: The parameters are inputted on a computer which then carries out the scans on the material.

5. Please remove commercial branding: 2.1.5 – ICDD PDF 4+

Author's response to comment 5: This has been removed.

6. Results: Figure 2 – the β-tricalcium phosphate peaks are not labeled in the figure as the legend describes.

Author's response to comment 6: Thank you for highlighting this. The appropriate peak labels have now been added.

7. Discussion: Please discuss the limitations of the method. Please also discuss any modifications/troubleshooting that can be performed.

Author's response to comment 7:

The authors have added the following text to discuss the limitations of the method:

'As the authors have not fully investigated the effects of slowly pouring the phosphoric acid into the calcium hydroxide suspension, in order to achieve consistent results we recommend that the phosphoric acid is poured at a rate commensurate with that shown in the video (approximately 100 mL/s).'

In order to discuss modifications that can be performed the authors have expanded the section regarding how to carry out substitutions with this method. The text below has been added:

'In order to prepare substituted nHA the element may be introduced with a corresponding reduction of the intended element to substitute for, e.g. a reduction in the amount of the calcium compound when strontium, magnesium or zinc substitution is attempted[32]. Alternatively, another approach may be to add elements with the intention of providing 'doped' ions which are present on the surface of the nHA without necessarily intending to substitute the element into the HA crystal lattice[31]. For these modifications to the method it is possible to prepare mixed solutions e.g. of calcium hydroxide and silver nitrate, and to carry out the reaction in the same manner as described here.'

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The present work describes a new method for producing calcium deficient, carbonated nano hydroxyapatite with a fast and simple method.

Major Concerns:

None

Minor Concerns:

Reviewer 1 comment 1: *HA crystals from dental and orthopaedic tissues show different morphologies and it would be good to briefly describe these differences in the introduction as applications in dentistry as well as orthopaedics are variously mentioned.*

Author's response to comment 1: The following text has been added to the introduction:

'Contrastingly, the mineral in tooth enamel is 10 to 100 times larger than that found in bone tissue in both length and width[3,4].'

Reviewer 1 comment 2: *Where previously described wet-precipitation methods for producing nHa are described, a general idea of the times for the production cycle should be mentioned: this is especially true of the Prakesh method which is given as a kind of "gold standard" technique but which the method described in the current work seeks to provide an advantageous alternative. The full cycle time for the new method described here should also be given to verify the claims regarding the current method where it is posited to constitute "a simplified rapid mix approach would have great benefits for laboratory researchers and industry alike where costs of manufacture could be substantially reduced without comprising quality".*

Author's response to comment 2:

The following text has been added to the introduction:

'The original Prakash titration step takes over one hour. However, longer titration times may be required for larger batches to be prepared.'

The following text has been added to the discussion:

'For this method, the rapid mixing of the chemicals takes less than 5 seconds which is a marked reduction in time compared to titrations reactions typically requiring hours of careful monitoring.'

Reviewer 1 comment 3: *Manufacturer details should be provided for products described by their trade name: e.g. Parafilm.*

Author's response to comment 3: This has been added to the method:

'Cover beaker with Parafilm (Bemis, USA).'

Reviewer 1 comment 4: *This reviewer is confused by the claim that in the FTIRS data, "The sintering process also sharpened the hydroxyl and phosphate bands." I cannot see this myself and would wonder about the physico-chemical basis for such a phenomenon. FWHM in an FTIR is usually associated with resolution-based instrumental artefacts while peak height is associated with concentration. It could be that sintering increases the crystallinity which leads to a better rationalisation of the e.g. phosphate vibrational states but it would be good if the authors could please provide their interpretation for the phenomenon and further show clearly that the phenomenon is present.*

Author's response to comment 4: The authors believe that the FTIR spectra for the sintered product shows increased definition (i.e. sharpened peaks) in terms of greater peak to trough distance for the phosphate peaks and the hydroxyl peaks. It has been reported previously that these changes correspond to an increase in crystallinity[26,27].

The following text has been added to the manuscript:

In the results section:

'The sintering process also sharpened the hydroxyl and phosphate peaks which was manifested by a greater peak to trough distance.'

In the discussion:

'It was noted that the spectrum for the sintered product showed sharper phosphate and hydroxyl peaks. These changes have been associated with a greater product crystallinity[26,27].'

Reviewer 1 comment 5: *It would be of benefit to know what the sample population size was for determination of key values such as Ca:P ratio and how statistically significant the values are. Has this been determined for more than one batch? If so, what is the percentage statistical deviation between batches etc.*

Author's response to comment 5: The complete chemical analysis has been performed on one batch, therefore statistical analysis has not been carried out. Additional batches (n=3) have been prepared and quality assured using XRD and TEM.

Additional Comments to Authors:

Reviewer 1 comment 6: *If available, XRD and FTIR data from samples produced using the Prakesh method should be plotted along with your samples.*

Author's response to comment 6: Unfortunately the authors do not have the data requested, but this data is in the reference provided for the Prakash method.

Reviewer #2:

Manuscript Summary:

The paper is a useful description of a simple way to produce and characterise non-stoichiometric HA.

Major Concerns:

Reviewer 2 comment 1: *I would like the term novel to be removed from the paper. There are numerous examples of this route to produce calcium phosphates and the author should cite some of them.*

Author's response to comment 1: Whilst the authors appreciate that the reaction between calcium hydroxide and phosphoric acid has been reported numerous times for the preparation of hydroxyapatite, we are proposing a novel method based on rapid mixing to prepare consistent nanoscale hydroxyapatite; it is the method that is novel because it has not been previously reported. Other reports involve the slow titration of the chemicals which have been referenced in the paper. We therefore politely submit that the text is correct and it is important to highlight this point.

Reviewer 2 comment 2: *I would like the fact that the author used a Ca:P ratio at the start of*

1.67, I have calculated it is but it would be nice as this is key to people following this method in the future to produce a variety of calcium phosphates with varying Ca:P ratios.

Author's response to comment 2: The following text has been added to the methods:
'Preparation of calcium and phosphorus solutions to prepare 5 g of nanoscale hydroxyapatite using a calcium to phosphorus molar ratio of 1.67.'

Reviewer 2 comment 3: *To repeat this method everywhere I do think that they should possibly use deionised water. Distilling doesn't take ions out of the water and so depending on the location of the person carrying out the experiment will change the ions present in the water and these are likely to substitute into the HA. At the same time as this is being considered it would be useful to explain the purity of the calcium hydroxide used as the impurities in this will lead to the ions also in the end HA.*

Author's response to comment 3: Thank you for noticing this. We have corrected the manuscript to use the term 'deionised water' that was indeed used throughout these experiments. The purity of the calcium hydroxide used has also been added to the table of specific materials.

Reviewer 2 comment 4: *In the abstract the author really needs to rewrite this to clarify it. Stoichiometric HA would not have phases of TCP present at 1000 Degrees C but would remain a single phase. It is the fact that the route leads to a non-stoichiometric, in this case calcium deficient, HA that leads to this happening.*

Author's response to comment 4:

The authors agree that this point could be made more clearly in the abstract and so the wording in the abstract has been adjusted to clarify this point.

'X-ray diffraction analysis showed the successful formation of HA, which showed thermal decomposition to β-tricalcium phosphate after high temperature processing, which is typical for calcium-deficient HA.'

Minor Concerns:

Reviewer 2 comment 5: *I don't like the fact that the authors say to pour the phosphorous solution into the calcium solution but quote a rate. I know it's approximate but pouring it is more than suitable for describing it.*

Author's response to comment 5:

The following text was added to the discussion in light of the editor's comment regarding the discussion of method limitations.

'As the authors have not fully investigated the effects of slowly pouring the phosphoric acid into the calcium hydroxide suspension, in order to achieve consistent results we recommend that the phosphoric acid is poured at a rate commensurate with that shown in the video (approximately 100 mL/s).'
Therefore it seemed appropriate to leave the approximate rate in the method text.

Reviewer 2 comment 6: *I would also somehow make a distinction between how the way to characterise (using XRD) the material is discussed and how others might. It's an interesting way of doing it, and uses minimal powder but isn't the only way of doing so. Many labs have holders to carry out XRD with.*

Author's response to comment 6:

The authors agree with your point so we have clarified that this preparation is for transmission X-ray diffraction in the methods section:
'X-ray diffraction (XRD) using transmission mode diffractometers'

Reviewer 2 comment 7: *I think the end of the paragraph beginning on line 292 needs some backing up. Equally to what you say it could be said that at higher temperatures the reaction has become faster and so there is less time for calcium hydroxide to react.*

Author's response to comment 7:

The authors propose that since the reaction is limited by the amount of reactants, the rate of the reaction should not affect the amount of residual reactants at the end of the reaction. We have not modified the manuscript but could add this as a comment if the editor thought it was helpful.

Reviewer 2 comment 8: *I would also like you to say how much HA you intended to produce for your reaction.*

Author's response to comment 8:

This has been added to the method section:
'Preparation of calcium and phosphorus solutions to prepare 5 g of nanoscale hydroxyapatite using a calcium to phosphorus molar ratio of 1.67.'

Additional Comments to Authors:

-Overall I think this work would be useful for the overall community to see how things are done but there needs to be explanations as to alternative routes and what this would mean to the nHA

or the person trying to replicate this will just replicate what you did and not be able to vary things. The changes above would clarify things more.

The authors are grateful to the reviewers for their insightful comments, and we hope our corrections and clarifications are helpful.