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Rapid wet chemical synthesis for ³³P-labelled hydroxyapatite – An approach for environmental research



J. Wolff^{a,b,*}, D. Hofmann^b, W. Amelung^{a,b}, H. Lewandowski^b, K. Kaiser^c, R. Bol^b

^a Institute for Crop Science and Resource Conservation (INRES) – Soil Science and Soil Ecology, University of Bonn, Nussallee 13, 53115, Bonn, Germany

^b Institute for Bio- and Geosciences – IBG-3: Agrosphere, Forschungszentrum Jülich GmbH, 52425, Jülich, Germany

^c Soil Science and Soil Protection, Martin Luther University Halle-Wittenberg, von Seckendorff-Platz 3, 06120, Halle (Saale), Germany

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ABSTRACT

Editorial handling by Prof. M. Kersten Keywords: Hydroxyapatite ³³P labelling Mineral solubility Crystallinity variations Mineral P tracer Apatite is the principal primary phosphorus (P) source in the environment; yet there is no consensus on how it can be synthesized for controlled microcosm studies, particularly not in labelled form. Here, we present a methodology that allows for the production of stoichiometric ³³Phosphorus (³³P)-labelled hydroxyapatite powders produced by a simple and fast wet chemical procedure, with different precursor compounds and at different reaction (25, 40, 60 and 80 °C) and calcination (100 and 200 °C) temperatures. The resulting morphological structures were analysed by Raman spectroscopy, X-ray diffraction (XRD), and scanning electron microscopy (SEM). The results showed that rapid synthesis of hydroxyapatite is successful using ³³P-labelled diammonium hydrogen phosphate and calcium nitrate with a Ca/P ratio of 1.67 in less than 30 h. Crystallinity increased with increasing reaction temperatures. Solubility tests confirmed a strong pH dependency for all hydroxyapatites at pH values < 3.7. To our knowledge this is the first procedure that can rapidly synthesize radioactive labelled and chemically pure hydroxyapatite of different crystallinities: It can be easily modified to allow for labelling with other isotopes, such as ⁴⁴Ca or ¹⁸O, in order to provide hydroxyapatite in reproducible manner for investigating the availability and uptake of P from apatite in future soil and environmental studies and beyond.

1. Introduction

Apatites are known to be the primary mineral source of phosphorus (P) in soils and sediments (Chen et al., 1997). Fluor-, chlor- and hydroxyapatites may rarely occur in form of individual grains but mostly as accessory phases which are omnipresent in metamorphic and igneous rocks (McClellan and Van Kauwenbergh, 1990; Nezat et al., 2007; Syers et al., 1967). In addition to mineral apatites, biological apatites are present in the environment, e.g., in mammal residues of bones and other hard tissues, consisting of about 65 wt% apatite (Wang et al., 2006). Even though both forms are members of the 'apatite' family, mineral and biological forms differ in certain characteristics (Tas, 2000a, 2000b). Biological apatite is formed by biomineralization, which, in contrast to mineral apatite, leads to very small crystals (250 Å \times 30 Å) of low crystallinity and non-stoichiometric Ca/P ratios (Kalita and Verma, 2010; Villacampa and García-Ruiz, 2000).

Hydroxyapatite $(Ca_{10}(PO_4)_6(OH)_2)$, an isomorph of the naturally abundant fluorapatite, is widely used as a biomaterial because of its

bioactive, biodegradable, and osteoconductive properties (Chen et al., 2002; Narasaraju and Phebe, 1996). In the past, researchers were targeting the preparation of hydroxyapatite for biomedical implant applications and bone regeneration purposes (Chen et al., 2002; Hench, 1991; Mengeot et al., 1973). To synthesize the different forms of hydroxyapatites, numerous methods have been developed over the last decades, including the solid-state (Sonoda et al., 2002), sol-gel (Fathi and Hanifi, 2007) and precipitation techniques (Zhang and Zhang, 2011). None of these techniques were optimised for the specific requirements as needed for production of radioactively labelled apatite forms that mirror natural (Ca/P ratio of 1.67) hydroxyapatites. While synthesis of hydroxyapatites has been achieved for medical purposes, their application and quantification in soils and other environments is still challenging.

Methods for mineralogical analysis in soils are, e.g., X-ray diffractions (XRD) and near-edge synchrotron-based X-ray spectroscopy. XRD can be used to identify apatites in petrographic thin sections of rocks (Nezat et al., 2007). This method is however insufficient for apatite

E-mail address: jwolff@uni-bonn.de (J. Wolff).

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^{*} Corresponding author. Institute for Crop Science and Resource Conservation (INRES) – Soil Science and Soil, Ecology, University of Bonn, Nussallee 13, 53115, Bonn, Germany.

quantification in soils because they only occurred in trace amounts, i.e., this technique can hardly provide information on soil apatites and thus be utilized as method to derive P dynamics in soils. Further, quantification limitations also apply to bulk soil digestions due to apatites being included in weathering-resistant minerals such as silica (Nezat et al., 2007). Near-edge synchrotron-based X-ray spectroscopy may identify apatite hotspots in soil (Kruse et al., 2015); yet, this methodology is laborious and far from being routine and thus not widely applicable.

Werner et al. (2017) recently highlighted that there is still only little scientific information about the spatial micro-distribution patterns of soil P minerals due to the lack of suitable analytical methods. In this context, labelling with the two distinct radioactive isotopes of P, i.e. ³²P and ³³P, has proven to be a useful tool to trace the release of phosphate ions from soil into soil solution and further trace the uptake by plants (Frossard et al., 2011). Therefore, labelling apatite minerals with radioactive P isotopes as a tracer for plant P acquisition, especially in combination with novel methods such as radioactive imaging (Bauke et al., 2017), may greatly enhance our knowledge on P cycling in soils and its uptake into biota.

The principal goal of this research was to synthesize ³³P-labelled hydroxyapatite of different degrees of crystallinity by wet chemical precipitation for environmental research. Relevant literature (> 80 research articles) was assessed according to important criteria for a simple, safe and fast production of radioactive labelled hydroxyapatite with a Ca/P ratio of 1.67 [cf. Table S2; Supplementary material]. Based on this selection, a modified method of Wang et al. (2010) seemed most suitable for our purposes. Using this modified method, we were able to rapidly synthesize ³³P-labelled hydroxyapatite powders of different degrees of crystallinity at various temperatures (25-80 °C). The effects of controlling factors during the synthesis were recorded and the produced powders were subsequently analysed using Raman spectroscopy, X-ray diffraction and scanning electron microscopy (SEM). The different hydroxyapatite powders were tested for their solubility kinetics at different pH values by photometric determination of the amount of P released into solution. To the authors' knowledge, this is the first attempt to synthesize ³³P-labelled hydroxyapatite of different crystallinity as tracer for environmental P cycling.

2. Experimental section

2.1. Materials

CaO (Sigma Aldrich, Germany), H_3PO_4 (Sigma Aldrich, Germany), HNO_3 (Sigma Aldrich, Germany), $NH_3 \cdot H_2O$ (Merck, Germany), $NH_2CH_2CH_2OH$ (Sigma Aldrich, Germany), C_2H_5OH (Merck, Germany), $HOC(COOH)(CH_2COOH)_2$ (Sigma Aldrich, Germany) and $HOC(COONa)(CH_2COOH)_2$ (Sigma Aldrich, Germany) were used without any further purification. 700 µl of $H_3^{-33}PO_4$, equal to an activity of 295 MBq with 420 kBq/µl, were purchased from Hartmann Analytics, Germany.

2.2. Methods

2.2.1. Method selection

In order to find the most suitable method for our purposes, 87 papers looking at different synthesis methods of hydroxyapatite were reviewed [cf. Table S2; Supplementary material]. Most frequently, precipitation methods (27% of papers), followed by hydrothermal methods (11%) and sol–gel methods (9%) were used in the past. The most common precipitation methods, also known as chemical precipitation, aqueous precipitation or wet chemical precipitation, were based on a chemical reaction of aqueous calcium solution with aqueous orthophosphoric acid solution. These reactions could be performed at different conditions (e.g., pH, temperature, etc.) in order to produce large quantities of hydroxyapatite at reasonable costs (e.g. Nayak, 2010). Due to the fact that we aimed at producing ³³P-labelled hydroxyapatite, we

focused on methods with short production periods and particularly on the absence of methodical steps that preclude work with radioactive substances for safety reasons, such as milling the samples into fine powders (risk of inhalation) or heating of liquids (risk of radioactive steam formation). We also excluded methods that did not clearly result in products stoichiometrically similar to the required Ca/P ratio of 1.67. We preferred methods synthesizing powders to those synthesizing gels to facilitate a uniform distribution of the apatites in soil matrices.

All these preconditions led to the wet chemical method described by Wang et al. (2010). They described a preparation of hydroxyapatite powders with "various morphologies" under controlled synthesis conditions, relying on different "drying methods, solvent and dispersant species, initial pH, and reaction temperatures". They reported that at an initial pH of 10, hydroxyapatite powders of different degrees of crystallinity can be obtained. We modified the described synthesis pathways to comply with radioactive work requirements by excluding elevated pressures, and, above all, by changing the preparation of solutions involved, the calcination temperatures, and the duration of different synthesis steps.

2.2.2. Synthesis of hydroxyapatite

As calcium and phosphorus sources for apatite synthesis, calcium nitrate tetra-hydrate ((Ca(NO₃)₂·4H₂O) and diammonium hydrogen phosphate ((NH₄)₂HPO₄) solutions were prepared. For a 1 M calcium nitrate tetra-hydrate solution, 100 mL of 2 M HNO₃ were slowly added to 100 mL 1 M CaO suspension under continuous stirring. For the preparation of a 1 M diammonium hydrogen phosphate solution, 50 mL 1 M H₃PO₄ were slowly added into 50 mL 2 M NH₃ and stirred for 1 h. To label the phosphate with ³³PO₄ with an activity of 295 MBq.

For the synthesis, 100 mL of the Ca(NO₃)₂ solution was added into a 500 mL double-walled three-necked synthesis reactor placed on a magnetic stirrer. A water heat pump, a thermometer, and a pH meter were connected to the reactor in order to control pH and reaction temperature. To prevent aggregation, 3 wt% of ethanolamine were added as a dispersant to the Ca(NO3)2 solution. Additional aqueous ammonia (NH3 · H2O) was used to adjust the pH to 10 if necessary. Before further proceeding with the synthesis, the Ca(NO₃)₂ mixture was heated to the intended temperature (cf. Fig. 1) under continuous stirring at 300 rpm. A dropping funnel was filled with 100 mL of the $^{\rm 33}P\text{-}$ labelled diammonium hydrogen phosphate solution. As soon as a constant temperature of the Ca(NO₃)₂ mixture was achieved, the ³³P-labelled solution was added dropwise at a rate of 2 mL/min. During the synthesis process, additional aqueous ammonia was added to maintain a pH \ge 9. The resulting suspension was stirred for 1.5 h without additional heating and then aged for another 12 h at room temperature. Thereafter, the suspension was filtered and washed twice with 30 mL deionized H₂O to remove any residues and impurities. To remove the remaining water, the filtrate was washed with 15 mL ethanol before drying for 5 h at 60 °C. The final calcination step was performed at 100 and 200 °C for 1 h in a muffle furnace.

Raman measurements were performed using a Bruker RFS 100/S FT-Raman spectrometer and the OPUS software. The excitation source was a Nd:YAG laser (1064 nm, 500 mW). All spectra were recorded at a resolution of 2° cm⁻¹. The nature of the precipitates was analysed via X-ray diffraction (X'Pert Pro, PANalytical B.V., Almelo, The Netherlands) with Cu K α -radiation ($\lambda = 1.541$ nm) in step scan mode with a step size of 0.02° 2 θ and fixed slits, using random powder mounts. Identification of mineral phases was carried out with the HighScore 4.2 software (PANalytical). Images were taken with a scanning electron microscope (Merlin, Carl Zeiss Microscopy GmbH, Germany) with acceleration voltages between 2 kV and 5 kV.

2.2.3. Solubility tests

Solubility tests were performed using deionized water (pH > 5), acetate/lactate buffer (pH 4.3) and citric acid/citrate buffers, ranging



Fig. 1. Schematic flow chart showing the wet chemical procedure for synthesizing ³³P labelled hydroxyapatite powders.

from pH 2.6–4.1 (intermediate steps of 0.3) representing components of root exudates (Jones, 1998). Tests were performed with one measurement per minute, using the spectrophotometer Spectramax Plus 384





Fig. 3. X-ray diffraction patterns of hydroxyapatites synthesized at different reaction temperatures (25, 40, 60, 80 $^{\circ}$ C). Calcination temperature: (A) 100 $^{\circ}$ C and (B) 200 $^{\circ}$ C.

microplate reader (Molecular Devices, California) with an integrated shaker (shake duration 50 s before each analysis). The synthesized hydroxyapatite powders were evenly distributed over a 96-well plate, with a spatula of hydroxyapatite in each well. $250\,\mu$ L of the prepared solutions were given into the previously assigned wells. The data acquisition was carried out with the Softmax Pro v4.8 software



Fig. 2. Raman analysis of hydroxyapatite, synthesized at different temperatures: $a = 25 \degree C$; $b = 40 \degree C$; $c = 60 \degree C$; $d = 80 \degree C$. Highlighted are the v_1 , v_2 , v_3 , and v_4 phosphate mode regions of the hydroxyapatite compounds.



Fig. 4. Scanning electron microscopy (LSM) images of synthesized apatite powders. Reaction temperatures: (a) = $25 \degree$ C; (b) = $40 \degree$ C; (c) = $60 \degree$ C; (d) = $80 \degree$ C. Calcination temperatures: (a₁) - (d₁) = $100 \degree$ C; (a₂) - (d₂) = $200 \degree$ C. Images shown here were taken with acceleration voltages between 2 kV and 3 kV, respectively. The scales shown for images b₂ and d₁ is 2 µm, for the others 1 µm, respectively.



Fig. 5. Solubility tests of different synthesized hydroxyapatite powders in 0.1 M citric acid/citrate buffers of pH 2.9 to 3.8. No deviating changes have been detected at lower (pH 2.6) and higher pH values (4.1), respectively (data not shown).

(Molecular Devices, California).

3. Results and discussion

Previous studies applying Raman spectroscopy to 'pure' apatites revealed scattering signals of different vibrational modes in the spectral range of 0–1200 cm⁻¹ (Li et al., 2004; O'Shea et al., 1974). Carbonate, phosphate, and hydroxyl ions within hydroxyapatite appear at ranges $> ~400 \text{ cm}^{-1}$ (Klinkaewnarong et al., 2010). The Raman spectra confirm major characteristics of the four different hydroxyapatite powders synthesised via our wet chemical method (cf. Fig. 2).

The bands at vibration mode ~150 cm⁻¹ can be referred to the lattice mode region of hydroxyapatites, where three typical lines, appearing at ~68 cm⁻¹, ~144 cm⁻¹ and ~160 m⁻¹, often overlap. The bands appearing at ~430 cm⁻¹ are associated to the (ν_2) O-P-O stretching whereas the bands at ~580 cm⁻¹ and ~590 cm⁻¹ are due to the symmetric stretching of the (ν_4) O-P-O. The most characteristic and most intense band of hydroxyapatite at ~963 cm⁻¹ is due to the total stretching of (ν_1) P-O-P along with free ions of tetrahedral phosphate. The bands at vibration mode at ~1046 cm⁻¹ can be referred to the (ν_3) P-O-P stretching symmetry (Klinkaewnarong et al., 2010; Li et al., 2004; O'Shea et al., 1974). The increasing band at mode ~770 cm⁻¹ could be due to necessary additions of liquid ammonia with increasing synthesis temperatures.

The X-ray diffraction (XRD) patterns of the different hydroxyapatite

powders are shown in Fig. 3. The diffractograms of all synthesized powders produced at 25, 40, 60, and 80 °C showed no other reflexes than those of hydroxyapatite.

The samples thus represent rather pure hydroxyapatite phases. The resolution of the XRD diffractograms increased with increasing crystallinity of the produced hydroxyapatite phases and increasing reaction temperature of the synthesis, due to more well-defined reflexes of decreasing half-height width and an overall increase in signal-to-noise ratios. The increasing crystallinity of the produced hydroxyapatite phases was best reflected by the increasing resolution of reflexes at the positions between 31 and 34 $^{\circ}2\Theta$.

The scanning electron microscopy (SEM) images of non-ground hydroxyapatite powders, produced at 25, 40, 60, and 80 °C and calcinated at 100 and 200 °C, respectively are shown in Fig. 4. In comparison to the hydroxyapatite powders described by Wang et al. (2010), we did not produce nano-sized powders but rather aggregate-type clusters with sizes of several micrometers. The different morphology might be due to a previous grinding of the samples by Wang et al. (2010), though this is not mentioned in their publication. SEM revealed that the hydroxyapatite powders synthesized at low temperature (cf. Fig. 4a₁ and 4a₂) comprised of smaller aggregates (1–6 μ m) with a porous surface structure and SEM analyses also revealed that all aggregates within a given fraction were of similar shape. At high reaction temperatures larger aggregates form and more needle-type crystals can be observed at the surfaces as opposed to at lower temperatures. As to be seen in Fig. 4b₁ to $4d_2$, more distinct needles develop with increasing reaction temperatures, which matches the increasing crystallinity of these samples. Aggregates synthesized at 60 and 80 °C (cf. e.g. Fig. 4c₁ and 4d₁) also show plane, smooth surfaces, which likely reflect higher crystallinity at higher reaction temperatures. The temperature of calcination caused only minor morphological differences between hydroxyapatites produced at the same temperature but did not result in major visible changes.

Fig. 5 displays the dissolution kinetics of the hydroxyapatite powders in different citric acid/citrate buffer solutions within the pH range 2.9–3.8. In contrast there was no dissolution of hydroxyapatite in water and lactate-acetate buffer [cf. Figure S1; Supplementary material].

All hydroxyapatite powders are stable at pH values > 3.8, which is in agreement with previous findings (Koutsopoulos, 2002). When hydroxyapatites are exposed to pH values < 3.7, the minerals dissolve within the first hour except for hydroxyapatites synthesized at 80 °C. Moreover, Fig. 5 shows that less crystalline hydroxyapatites dissolve faster than hydroxyapatites of higher crystallinity. These results support the assumption that poorly crystalline hydroxyapatites might be easier available to plants than more crystalline forms in soil, due to a more rapid dissolution.

4. Conclusion

We successfully synthesized ³³P-labelled hydroxyapatite powders using a modified approach of an established wet-chemical method (Wang et al., 2010). The new method allows a rapid synthesis of hydroxyapatites of varying crystallinity and thus is suitable to produce radioactively labelled apatite specimen for environmental research. Raman spectroscopy, XRD, and SEM confirmed that increasing reaction temperatures resulted in increasing crystallinity of the produced hydroxyapatites while calcination temperature hardly affected the surface features of the hydroxyapatites. Solubility tests confirmed that the synthesized hydroxyapatite powders dissolve at pH values < 3.7 and that dissolution rates decrease with increasing crystallinity. The (i) short preparation time, (ii) resulting close-to-natural pure hydroxyapatite phases of different crystallinity, and (iii) the absence of special equipment make this modified method suitable for producing radio-labelled hydroxyapatites of different reactivity to test biological P uptake processes under near-natural conditions. Due to the simple protocol of the method, any kind of additional labelling such as with ⁴⁴Ca or ¹⁸O is possible.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.apgeochem.2018.08.010.

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