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# The synthesis and characterisation of nanophase hydroxyapatite using a novel dispersant-aided precipitation method

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**Abstract**

The synthesis of nanophase hydroxyapatite (nHA) is of importance in the field of biomaterials and bone tissue engineering. The bioactive and osteoconductive properties of nHA are of much benefit to a wide range of biomedical applications such as producing bone tissue engineered constructs, coating medical implants or as a carrier for plasmid DNA in gene delivery. This study aimed to develop a novel low-temperature dispersant-aided precipitation reaction to produce nHA particles (<100 nm) which are regarded as being preferable to micron-sized agglomerates of nHA. The variables investigated and optimised include the reaction pH, the rate of reactant mixing, use of sonication, order of addition and concentration of the primary reactants, in addition, the effect of employing poly(vinyl alcohol) (PVA) surfactant and Darvan 821A® dispersing agent during the reaction was also examined. It was found that by fine-tuning the synthesis parameters and incorporating the dispersing agent, monodisperse, phase-pure nano-sized particles under 100 nm were attained, suitable for clinical applications in bone regeneration.

**Keywords**

Hydroxyapatite, Nanoparticle synthesis, Particle size, Darvan dispersant, Suspension

## 1. Introduction

Tissue engineering endeavours to develop biological substitutes that maintain, improve or restore tissue and organ functionality damaged through disease, trauma or congenital abnormalities [1]. In particular, bone tissue engineering seeks to replace bone in areas such as non-union fractures, after cancer resection and general trauma by using a cell seeded construct which typically involves the use of a suitable scaffold

combined with cells and appropriate growth factors [2]. If successful, this approach would be an alternative to the use of autografts (current 'gold' standard) and allografts with which many problems exist such as shortage of donors and donor sites, immune responses and disease transmission [3,4]. Currently however, a tissue engineered bone graft replacement has not yet been optimised, despite many promising advances.

Nanotechnology, or the use of nanophase materials, may provide a solution by mimicking the properties of natural bone which is a nanocomposite. It consists primarily of a protein-based soft hydrogel template (collagen and non-collagenous proteins) and a hard inorganic ceramic phase of which hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HA) is the chemical and crystallographic template. Typically, 70 % of the bone matrix contains nanocrystalline HA which is 20-80 nm long and 2-5 nm thick [5]. Many investigations of nanophase materials to date have illustrated their potential for bone repair. For example, increased osteoblast adhesion on nano grained materials in comparison to conventional (micron grained) materials has been reported [6,7]. Osteoblast proliferation *in vitro* and long-term functions were also enhanced on ceramics with grain or fibre sizes less than 100 nm [7,8]. In addition to osteoblast responses, modified osteoclast behaviour has also been documented on nanophase ceramics [6] and *in vivo* studies have demonstrated increased new bone formation on metals coated with nHA compared to conventional apatite [9].

Bioceramics have been extensively researched for use in engineered bone grafts, and amongst them hydroxyapatite (HA) is at the forefront of investigation due to its excellent biocompatibility and bioactive properties [10]. Clinically it is used in the form of powders, granules, coatings, dense and porous blocks, as a non-viral carrier for plasmid DNA gene delivery as well as in various biocomposites [11-15]. However,

micron-sized HA exhibits poor bioresorbability and brittle characterisitics, [16,17] and so the study of nanophase HA (nHA) has become more imperative [18-20].

Various methods are reported for the production of HA, including wet chemical precipitation, sol-gel processing, solid-state reactions, chemical vapour deposition, hydrothermal and reverse micro-emulsion techniques [10,21-25]. Of these methods, wet precipitation is the most common due to its simplicity and low cost which also makes it suitable for industrial production. However, difficulties arise during the synthesis of HA [26], the resultant particles are in the micron range with a wide particle size distribution, low surface areas and widespread agglomerate formation. There is an inability to effectively control flocculation of particles as they are created in aqueous solutions and often an extensive ball-milling step is required to separate the agglomerated particles [27]. Alternatively, the use of surfactants in precipitation reactions has been investigated with moderate success, albeit they do not overcome the necessity of time consuming ageing and heat treatment stages [28-31]. In this current work, Poly(vinyl alcohol) (PVA) was chosen as a surfactant due to its biocompatibility and established use in biomedical applications [32,33], whilst, Darvan 821A® which is a dispersing agent has been reported in literature to disperse commercial HA particles in aqueous solutions [34], however, its use during the synthesis phase of HA particles has not been previously explored.

The overall goal of this study was to develop and optimise a novel aqueous, low-temperature rapid precipitation reaction to create non-aggregating particles of nHA for incorporation into polymeric scaffolds for tissue engineering applications. The aims were to determine the role of reaction pH, the rate of reactant mixing, use of sonication, order of addition and concentration of the primary reactants on the

formation of nHA. In addition, the effect of using a surfactant (PVA) and dispersing agent (Darvan 821A®) on the final product was also examined. In particular, the synthesis route presented here is developed with the specific aim of producing a calcium phosphate material of the required phase (hydroxyapatite) and suitable morphology (monodisperse, non-aggregating particles <100 nm) for incorporation into polymeric scaffolds for tissue engineering applications. Potentially, the nHA particles could also be utilised as non-viral carriers for gene delivery applications due to the mild (low temperature) and rapid aqueous-based reaction conditions in which they are synthesized [35,36].

## 2. Materials and Methods

### 2.1 Materials

The chemicals used in this study were acquired as follows: Analytical reagent grade calcium chloride dihydrate (100 %) from Fisher Scientific (Pittsburgh), sodium phosphate tribasic dodecahydrate, minimum 98 % and sodium hydroxide anhydrous both from Sigma (Dorset). Darvan 821A® was provided by R.T. Vanderbilt (Norwalk, CT). Darvan is an ammonium based dispersing agent with polyacrylic acid as the active agent. Its molecules are negatively charged along their length and they attach to particles causing them to repel each other resulting in a disperse nanophase precipitate suspension. Poly(vinyl alcohol) (Sigma, Dorset) was used in solution as a surfactant. A Ca/P ratio of 1.67 was maintained throughout the study by adding reactants in the appropriate stoichiometric ratio to ensure the formation of hydroxyapatite.

### 2.2 Preparation of nHA particles

We based our initial work on that of Kumta *et al.* [13], aqueous solutions of $CaCl_2.2H_2O$ (0.41 M) and $Na_3PO_4.12H_2O$ (0.25 M) were prepared using double distilled $H_2O$. NaOH was added to the $PO_4$ precursor to control the pH at 10.5. These were mixed using drop feeding at a rate of 18,000 ml/hr and adding the Ca precursor to the $PO_4$ precursor. The precipitation reaction occurred immediately under stirring, according to Equation 1. The solution was then centrifuged for 99 mins at 4000 rpm and washed to remove the NaCl by-product. The precipitate was resuspended using sonication to yield a non-aggregated nHA suspension, or freeze-dried to generate a fine powder. These particles will be referred to as our controls herein; all other variants are compared to these samples.

Equation (1)

$$10CaCl_2.2H_2O + 6Na_3PO_4.12H_2O + 2NaOH \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 20NaCl + 92H_2O$$

2.3 Synthesis parameters

The following parameters were varied in order to optimise the production of nHA, while maintaining a final stoichiometric Ca/P ratio of 1.67. The concentration of the initial reactants (Ca concentration: 0.41 M to 0.001 M, $PO_4$ concentration: 0.25 M to 0.0006 M), the order (Ca into $PO_4$ and *vice versa*) and the rate of their addition (5, 10, 300 and 18,000 ml/hr) were altered to establish what effect they had on nHA particle formation. In addition, the pH of the reaction was varied from 8 to 12.5 using NaOH to establish the effect of pH on the particle size distribution. The use of PVA (1, 3 and 6 % w/v) and Darvan (0.1 to 1 % v/v) was also investigated by adding the respective solutions to the $PO_4$ reactant prior to conducting the precipitation reaction. Each factor was varied while keeping all others constant to investigate their role independently. The use of sonication was also examined in conjunction with the other factors.

2.4 Particle size determination

Particle size distributions were measured using dynamic light scattering (DLS) (ZetaSizer 3000 HS, Malvern instruments, UK). Measurements were carried out under monochromatic, coherent He-Ne laser light of fixed wavelength (633 nm) at room temperature with each size determination yielding an average particle size expressed as the mean diameter ($Z_{ave}$) together with a graph of the size range.

2.5 Electron microscopy and atomic force microscopy

Analysis of particle morphology was performed using transmission electron microscopy, (JEOL 100CXII TEM, Japan) by placing a drop of nHA suspension onto pioloform-coated copper grids and allowing it to dry.

For scanning electron microscopy (SEM) analysis, aggregated nHA samples were adhered to a thin layer of rapid setting epoxy resin on an aluminium specimen stub and then sputter coated with gold, using a Polaron Sputter Coater, to a thickness on 10 nm. Imaging was carried out using a JEOL 840 SEM, operated at 15 kV. In addition, nHA suspension was placed onto a silicon disc and dried in a vacuum oven at 60˚C for 60 mins in preparation for Atomic Force Microscopy (AFM) imaging. This was carried out using a Nanoscope 3A Multimode AFM, operated in tapping mode using silicon nitride tips over an area of 25 $mm^2$.

2.6 Physico-chemical characterisation

The nHA precipitate was freeze dried to yield a fine powder (Advantage EL, Vir-Tis Co., Gardiner, NY). The crystal phase of the product was determined using a Bruker Advance D8 X-Ray diffractometer (XRD). Cu-Kα radiation ($\lambda = 0.1542$ nm) was used and all samples were run for 2 hours in the 2θ range from 20–60˚ at a scan speed of 4

sec/step using an increment step size of 0.02. Fourier Transform Infra-Red spectroscopy (FTIR) analysis was carried out using a Spectrum One FTIR (Perkin Elmer, UK). Freeze-dried powders were mixed with potassium bromide using a mortar and pestle, before being pressed into a transparent sample. Spectra were collected between wavenumbers 4000 and 400 $cm^{-1}$.

2.7 Statistical analysis

All data was analysed for significance ($p \leq 0.05$) using one-way analysis of variance (ANOVA) tests to compare particle size means. Post hoc tests to determine significant differences between group means were performed using the Tukey test.

## 3. Results

3.1 Particle size analysis

Hydroxyapatite particles produced in section 2.2, *i.e.* the non-optimised "control" precipitation method using a Ca/P ratio of 1.67, were found to be an average of 24 μm in diameter with large deviations. Scanning Electron Microscopy (SEM) revealed that aggregates had formed during synthesis as shown in Fig. 1.

The average particle size decreased significantly after a reduction in the concentration of both the calcium and phosphate precursors. The initial calcium solution concentration of 0.41 M yielded particles that were immediately visible on precipitation. This indicated that they were many microns in size. The calcium concentration was sequentially diluted to 0.001 M at which stage the particles were no longer visible during the synthesis phase. However, the yield of HA reduced with decreasing concentration, and so a lower limit was set at this 0.001 M value (denoted concentration X, with concentration 5X having a calcium concentration of 0.005 M,

10X having a concentration of 0.01 M). Therefore, reducing the concentration of the reactants corresponded to a reduction in aggregate size; however, altering the concentration of the precursors alone was not sufficient to prevent aggregation.

Changing the order of addition of the reactants so that the phosphate solution was added to the calcium solution resulted in a significant decrease in particle size (Fig. 2). However, the rate of reactant addition was not found to have a bearing on the size of the resulting precipitate (Fig. 3). The use of sonication was also investigated during this study and was found to aid particle size reduction, although it was not sufficient to prevent aggregation independently.

The pH of the reaction was controlled by the addition of sodium hydroxide (NaOH), it was established that the particle size was lowest and most consistent at a pH between 8 and 9.25. As seen in Fig. 4, the particle size rises sharply above pH 9.5.

A reduction in particle size is evident upon the addition of increasing amounts of PVA (Fig. 5a). However, large deviations can be seen due to irregular particle size ranges within samples. Preparing the 6 % w/v sample of PVA proved troublesome as it had a 'bubbly' consistency and although it resulted in a 26 fold reduction in aggregate size, it also led to a significant decline in the yield. Sonication was not effective when working with PVA and actually increased the particle size when compared to non-sonicated samples. This may be due to the viscous nature of the PVA solution dissipating the sonic energy.

Subsequently, nano-particles of HA were synthesized successfully by using the optimal reaction conditions (phosphate solution (0.006 M) added to the calcium solution (0.01 M) at a pH of 9.2 with 10 minutes of sonication) with the dispersing agent, 0.1% v/v Darvan 821A®. The large random sized agglomerates observed previously were replaced by uniformly sized nano-particles in suspension. In

comparison between the use of 6 % w/v PVA surfactant *vs.* 0.1 % v/v Darvan dispersant, a smaller average particle size (360 ± 194.7 nm and 140 ± 10.7 nm respectively) with a much tighter standard deviation was seen when Darvan 821A® was used (Fig. 5b). The required amount of dispersant to prevent aggregation was found to be in the range of 0.1 % to 0.5 %. A combination of reduction in particle size above 0.1 % and a significant decrease in particle yield above 0.5 % led to this determination (Fig. 6).

Sonication was required to fully disperse the nano particles at higher concentrations. Reactions without any dispersant followed by sonication yielded average particle sizes in the micron range (23.7 ± 19.3 μm) whereas, the presence of a dispersing agent prevented particle agglomeration and reduced the average particle size to 91.9 ± 0.7 nm following sonication. This is evident in Fig. 7 where the higher concentrations yield micron sized aggregates with no sonication, but the sizes could be reduced to less than 100 nm after 4 minutes of sonication. In addition, increasing the time of sonication up to 12 minutes was shown to reduce particle size further, at which stage no further significant reduction was obtained (Fig. 8).

3.2 Microscopical investigation

Transmission Electron Microscopy (TEM) was used to verify that nHA particles generated using the optimised conditions and in the presence of Darvan 821A® dispersing agent were non-aggregating rod shaped crystals approximately 30 nm by 10 nm (Fig. 9a). This is less than the corresponding DLS findings which gave an average size of 82.4 ± 11.1 nm. SEM was applied to image aggregated particles (section 3.1) while individual nHA particles were viewed using the high resolution of Atomic Force Microscopy (AFM). The samples were oven dried before imaging

causing some aggregation to occur, which is likely due to static forces arising during drying. The heights of individual particles appear to be under 50 nm and particle aggregations have approximately similar height dimensions (Fig. 9b).

3.3 Material characterisation

X-Ray Diffraction (XRD) confirmed the material to be hydroxyapatite without the presence of any other calcium phosphate phases (JCPDS 72-1243C). The crystallinity of the non-heat treated sample was determined to be 72.9 % and as shown in Fig. 10, the crystallinity can be significantly increased on calcination of the sample at 650ºC. When nHA made without Darvan is compared to nHA made with Darvan there is a close correspondence in their spectra, but with broader peaks observed in the Darvan sample (Fig. 11); this may be due to the lack of long range order of the nano crystals. There is also a small peak at 22.5˚ 2θ which is attributed to ammonium calcium phosphate (JCPDS 21-0697). This is known to decompose to HA over time at room temperature [37]. Peak broadening occurs and can be used to estimate the crystallite size using Scherrer's equation [38]:

$$X_s = K\lambda / (\beta_m \times \mathrm{Cos}\theta) \qquad (2)$$

Where $X_s$ is the crystallite size in nm, $K$ is a constant, taken for HA as 0.89, λ the wavelength of monochromatic X-Ray beam (0.154 nm); $\beta_m$ the full width at half maximum for the diffraction peak under consideration (rad) and θ is the diffraction angle (˚). Taking the (002) peak at 25.9˚ 2θ, the crystallite size for nHA made using the dispersing agent was determined to be 26.8 nm *vs.* 50.4 nm for powder made using the non-optimised control synthesis.

Fourier Transform Infra-Red (FTIR) spectra of nHA synthesised both with and without the presence of Darvan reveal characteristic peaks for hydroxyapatite; 469,

562 and 603 $cm^{-1}$, with a broad phosphate band evident from 1090 to 980 $cm^{-1}$ (Fig. 12). Carbonate peaks are also present in both spectra at 870, 1415 and 1450 $cm^{-1}$.

## 4. Discussion

The rationale behind developing a quick, low temperature method for synthesizing nHA is to enable convenient production of the particles for use in bone tissue engineering applications, specifically as a filler material in a composite scaffold or a delivery vehicle for plasmid DNA [39]. The aqueous suspension is an appropriate form for these functions, although the suspension can also be freeze-dried to yield a fine nanophase powder if desired. Fine-tuning synthesis methods to construct nano-sized, non-aggregating nHA particles has proved difficult, often employing the use of surfactants in conjunction with time consuming heating and aging steps in precipitation reactions [40,41]. Results of this study indicate that controlling reaction parameters such as the concentration of initial reactants, pH and the use of sonication has important implications for the final product in terms of aggregation and particle size. Reducing the calcium and phosphate precursor concentrations correlates to a decrease in particle size although a limit is set due to the parallel reduction in product yield. This trend agreed with other studies [42] and a range of calcium concentrations, from 0.001 M (X) to 0.01 M (10X), was chosen for the rest of this study. The original synthesis method (section 2.2) specified the addition of the calcium into the phosphate precursor, however, altering this, and combining the use of sonication led to a large reduction in aggregation and thus a decrease in resultant particle size. Sonication has long been used to disperse solids in a suspension, but to work it must overcome the attractive forces between molecules. In this research, it was found that the forces between newly forming nHA particles were too strong and sonication alone was not sufficient to overpower these.

Surprisingly, in this simple chemical precipitation reaction the rate of addition of the precursor solutions did not play any significant role on the ensuing particles at optimal concentration and order of addition conditions, contrary to previous findings [14]. This may be due to the optimisation of the protocol for working with these particular starting materials and we speculate that the rate of introducing phosphate ions into a calcium ion solution is not important if the concentration is sufficient to keep the nucleation sites separated, thus preventing widespread aggregation from occurring.

Without the addition of a surfactant or dispersing agent, the synthesis required strict control over reaction pH, maintaining it between 8 and 9.5 in order to minimise particle size. It was observed that conducting the reaction above pH 9.5 formed large and polydisperse agglomerates, while reactions below pH 7.5 are known to produce other forms of calcium phosphate such as brushite ($CaHPO_4 \cdot 2H_2O$) and tri-calcium phosphate ($Ca_3(PO_4)_2$) [13]. We recommend that unaided (*i.e.* no surfactant or dispersant) precipitation reactions, synthesizing nHA, should be executed within the range pH 8-9.5.

The factor with the largest effect on aggregation and particle size was established to be the use of a dispersing agent. The surfactant, PVA, reduced the particle size range from 23.7 ± 19.3 μm to 360 ± 194.7 nm but required a large volume (6 % w/v) to do this and a large variability was obtained. In contrast to this, using the dispersing agent, Darvan, led to uniformly sized particles of under 100 nm using 0.1 % v/v. This was obtained from concentration X (Ca: 0.001 M and $PO_4$: 0.0006 M) up to 10X (Ca: 0.01 M and $PO_4$: 0.006 M) by utilising sonication. Volumes of the dispersant below 0.1 % resulted in no reduction in particle size, while volumes greater than 0.5 % significantly reduced the yield.

Examining the particles under TEM revealed rod shaped crystals approximately 30 nm in length, although this is less than 90 nm, which the DLS results indicated. AFM showed small crystals beginning to clump together during the oven drying, and these individual crystals were approximately under 50 nm in dimension.

Both FTIR and XRD analysis demonstrated that the dried precipitate is phase pure nHA. The broadness of the XRD peaks in the untreated sample is due to the small particle size leading to no long range order of crystals (similar to biological HA) within the sample, which is contrary to the well defined peaks in the heat-treated sample. The spectrum, *via* the Scherrer's equation, also reveals the crystallite size of the dispersant-aided synthesis to be 26.8 nm, much smaller than the crystallite size (50.4 nm) of the particles formed using the initial control method. This value agrees closely with the TEM and AFM imaging analysis which shows individual crystals of approximately these dimensions. The discrepancy between these results and the DLS analysis may be due to the DLS method measuring the hydrodynamic diameter of particles, which is larger than the particle size observed using microscopy.

Therefore, the use of a dispersant in this optimised protocol enables the aqueous-based production of nHA for use in many applications. Particles of nHA synthesized using this method have been incorporated into collagen scaffolds, resulting in a composite scaffold with significantly improved mechanical properties and biological response compared to a collagen only control [39]. Darvan has not been studied previously in this regard and we believe it offers much potential in overcoming aggregation of HA particles, meriting further examination with alternative synthesis methods.

## 5. Conclusions

Non-aggregating nanoparticles of HA were synthesized using a novel low temperature precipitation reaction. Time-consuming heating and ageing treatments to produce the nHA crystals were avoided by employing this procedure. The introduction of Darvan 821A® dispersing agent was the principal factor behind the prevention of agglomerate formation. Further significant factors which reduced particle size included controlling the reaction pH between 8 and 9.5, decreasing the initial concentrations of reactants, using sonication, and changing the original order of reactant addition by adding the phosphate precursor to a calcium precursor. The resultant nHA particles may potentially be used in a myriad of biomedical applications either in a suspension or powder form.

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**Fig 1: Scanning Electron Microscopy (SEM) image showing the aggregation of nanophase hydroxyapatite (nHA) particles precipitated without the use of a dispersant (Darvan).**

**Fig 2: Graph showing the relationship between average particle size and the order of addition of the calcium and phosphate precursors. Additionally, the effect of sonication is shown. (S) = sonication used (NS) = no sonication.**

**Fig 3: Graph showing the relationship between average particle size and the rate of addition of the calcium and phosphate precursors.**

**Fig 4: Graph demonstrating the effect of pH on average particle size; a more variable behaviour and larger particles are formed above a reaction pH of 9.5.**

**Fig 5: Figure showing the effect of (a) the addition of poly(vinyl alcohol) (PVA) and (b) a comparison of using 6 % w/v PVA surfactant *vs.* 0.1 % v/v Darvan dispersant on the resultant average size of nanophase hydroxyapatite (nHA) particles.**

**Fig 6: Dynamic Light Scattering (DLS) results displaying the average particle sizes of nanophase hydroxyapatite (nHA) prepared with different concentrations (% v/v) of dispersant (Darvan).**

**Fig 7: Graph showing the effect of the concentration of the initial reactants (calcium and phosphate precursors) on average particle size. Additionally, the effect of sonication (10 mins) is also displayed. X: [Ca] = 0.001 M, 5X: [Ca] = 0.005 M, 10X: [Ca] = 0.01 M, Ca/P Ratio = 1.67, S = sonication used, NS = no sonication.**

**Fig 8: Graph showing average particle size as a function of sonication time. The particles were precipitated in the presence of 0.1 % v/v dispersing agent (Darvan) at an initial calcium concentration of 0.001 M.**

**Fig 9: (a) Transmission Electron Microscopy (TEM) image showing the morphology of the nanophase hydroxyapatite (nHA) particles synthesized using 0.1% v/v Darvan as the dispersing agent and, (b) Atomic Force Microscopy (AFM) image showing how the particles tend to re-aggregate slightly due to static forces following oven drying.**

**Fig 10: X-Ray Diffraction (XRD) spectra of the as-prepared nHA synthesized with Darvan (bottom spectrum), and the same sample after calcination at 650°C (top spectrum).**

**Fig 11: The broader XRD spectrum (b) shows the presence of a small peak at 22.5° 2θ indicating the presence of ammonium calcium phosphate compared to the HA spectrum synthesized using the original "control" synthesis (a).**

**Fig 12: Fourier Transform Infra-Red (FTIR) spectra of nHA particles synthesized in the absence (a) and presence (b) of the dispersant, Darvan.**