Biomimetic Highly Ordered Apatite Coatings for Dental Implants

Sherif Elsharkawy¹, Sara Gamea², Natalia Karpukhina³, and Maisoon Al-Jawad⁴

¹King's College Hospital Charity ²King's College London ³Queen Mary University of London Barts and The London School of Medicine and Dentistry ⁴University of Leeds School of Dentistry

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Abstract

Bioactive coatings on metallic 'titanium' or ceramic 'sapphire' implants are known for their potential to promote bone biocompatibility, osseointegration, and long-term survival. In this study, we have investigated the effect of the surface topography of titanium and sapphire at different temperatures on the chemistry, morphology, organization, and coverage of the synthesized apatite coatings. We use a wet chemical method with a fluoride rich calcium-phosphate solution, to induce bioactive coatings onto etched titanium, non-etched titanium, polished and unpolished sapphire at 37, 70, and 90@C. Fluoridated hydroxyapatite formation is detected across all temperatures using FTIR, synchrotron XRD, and MAS-NMR. Surface topography and temperature changes play a crucial role in the organization and coverage of the apatite crystals. Well-defined hexagonal nanocrystals are observed across each of the conditions, in the range between 35-81 nm. At lower temperatures, self-assembled organized nanocrystals appear to grow out from spherical structures, creating highly-ordered apatite architectures. However, at 90@C, the nanocrystals seem to lack the hierarchical organization and appear to be arranged randomly. This work demonstrates a promising avenue for modifying implant surfaces with highly-ordered apatite-based coatings at physiological conditions.

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Sherif Elsharkawy^{1,2,3,4,5*}, Sara Gamea^{1,6}, Natalia Karpukhina⁵, Maisoon Al-Jawad^{5,7*}

- Centre for Oral, Clinical, and Translational Sciences, Faculty of Dentistry, Oral & Craniofacial Sciences, King's College London, London, SE1 9RT, United Kingdom
- 2. Restorative Dentistry, Dental Directorate, Guy's and St Thomas' NHS Trust, London, SE1 9RT, United Kingdom
- 3. London Centre of Nanotechnology, London, WC1H 0AH, United Kingdom
- 4. Institute of Bioengineering, School of Engineering and Materials Science, Queen Mary University of London, London, E1 2DP, United Kingdom
- 5. Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London, E1 2DP, United Kingdom
- 6. Tanta University, Faculty of Dentistry, Department of Restorative Dentistry, Tanta, 31111, Egypt
- 7. School of Dentistry, University of Leeds, Leeds, LS2 9JT, UK Correspondence:

*Email : sherif.elsharkawy@kcl.ac.uk *Email : m.al-jawad@leeds.ac.uk

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ABSTRACT:

Bioactive coatings on metallic 'titanium' or ceramic 'sapphire' implants are known for their potential to promote bone biocompatibility, osseointegration, and long-term survival. In this study, we have investigated the effect of the surface topography of titanium and sapphire at different temperatures on the chemistry, morphology, organization, and coverage of the synthesized apatite coatings. We use a wet chemical method with a fluoride rich calcium-phosphate solution, to induce bioactive coatings onto etched titanium, non-etched titanium, polished and unpolished sapphire at 37, 70, and 90@C. Fluoridated hydroxyapatite formation is detected across all temperatures using FTIR, synchrotron XRD, and MAS-NMR. Surface topography and temperature changes play a crucial role in the organization and coverage of the apatite crystals. Well-defined hexagonal nanocrystals are observed across each of the conditions, in the range between 35-81 nm. At lower temperatures, self-assembled organized nanocrystals appear to grow out from spherical structures, creating highly-ordered apatite architectures. However, at 90@C, the nanocrystals seem to lack the hierarchical organization and appear to be arranged randomly. This work demonstrates a promising avenue for modifying implant surfaces with highly-ordered apatite-based coatings at physiological conditions.

INTRODUCTION

The use of implants to repair and replace damaged hard tissues is a growing field of research. Even though implants have a very high success rate, implant failures do still happen. Their success is governed by early osseointegration stage, which in turn is responsible for long-term clinical success.¹ Different materials have been used as dental implant material including, pure titanium, Ti6Al4V alloy, stainless steel, vitreous carbon, and single crystal aluminium oxide (alumina).²

In order to improve the biological response at the bone-implant interface and subsequently promote healing, the implant surface is typically changed on a variety of lengthscales.³Numerous research studies ^{4–7} supported the hypothesis that titanium implant roughness enhances osteoblastic development, bone apposition, mechanical interlocking, and stress distribution around implant, leading to faster osseointegration. Nevertheless, several issues with corrosion, wear, and unfavourable tissue interactions have been documented.⁸ It is crucial to prevent corrosion of human body metals since it can compromise their mechanical integrity and biocompatibility.⁹ Unwanted ions from implants could enter the body through corrosion and surface film breakdown causing adverse biological reactions and lead to mechanical failure of the device.¹⁰ On the other hand, the bioactivity of titanium, in biological environment is poor.¹¹

Single-crystal zirconia and alumina endosteal dental implants have been investigated over the last 30 years in animal^{12,13} and human studies^{14,15} Akagawa et al.¹⁶found that single-crystal alumina exhibits less tissue response compared to titanium and therefore showed more favourable biocompatibility, due to it being highly inert, non-toxic, more corrosion-resistant, and in a high chemical oxidation state. Moreover, they found uniformly organized collagen fibers at bone- alumina implant interface that resemble those found in the periodontal ligaments, hence alumina implants exhibited a good peri-mucosal seal. On the other hand, concerns have been raised by clinicians regarding the high risk of alumina implant fracture during surgery.¹⁷

There are multiple physical techniques that can be employed in order to modify the implant surface including, plasma-spraying, grit blasting, and acid etching. Furthermore, implant surfaces can be biochemically modified by a hydroxyapatite (HAp) coating. Coating of metallic implants with thin layers of bioactive materials merges mechanical advantages of the metal with the excellent biocompatibility of the surface layer. Moreover, the coatings can shield the implants against corrosion, limiting the release of metallic ions into the body.¹⁸Coating dental implants with a material such as HAp has greatly enhanced the bone healing process around the implant due to its bioactivity, osteoconductivity, and osteoinduction properties and it has a similar chemical composition to bone tissue forming a chemical bond.¹⁹ Aebli et al.²⁰ concluded that HAp coating of dental implants significantly enhances their early fixation and osseointegration, as they found that bone-implant contact with HAp coated implants was significantly higher after 2 and 4 weeks (139 % and 48 % higher respectively) compared to uncoated ones.

Hierarchical mineralized structures are found in biomineralized tissues such as teeth and bones,²¹ Wu et al.²² have grown ordered apatite crystals that show improved mechanical properties, excellent bioactivity,

and biocompatibility, however the mechanisms that enhance the biocompatibility of ordered over disordered surfaces are not yet well understood. Additionally,^{23,24} synthesized successfully ordered (two-level hierarchy) fluorapatite (FAp) crystals on metallic substrates, which were aligned parallel to each other, and their c-axes were perpendicular to the substrate. Moreover, they investigated its effects on cell adhesion, growth and mineralization. They found that ordered FAp crystals have higher cellular attachment and bond strength to the substrate compared to disordered crystals. Moreover, FAp ordered crystals were found to stimulate higher expression of bone mineralization markers compared to the uncoated metallic surfaces. Hence, it can significantly accelerate the osseointegration.²⁵

A simple wet chemical method is considered a novel biomimetic method to coat dental implants,²⁶ this method has many benefits such as coating the implant with fluoridated HAp, which is thought to reduce osteoclastic maturation, inhibit phagocytosis²³ and it is less soluble than HAp.²⁷ Furthermore, it has the ability to synthesize ordered crystals, which are self-assembled into higher hierarchical structures mimicking the biomineralized tissues such as bone.²⁸ Ordered crystals showed better mechanical properties, excellent bioactivity and biocompatibility compared to disordered.²⁴ Additionally, the wet chemical method can be processed at near physiological conditions, so it is cost-effective, and it has a great potential to be applied clinically compared to hydrothermal methods.²⁹ However, the crystal growth behaviour is dependent on various parameters that control the wet chemical method including temperature, substrate, and surface topography used to grow the apatite crystals on.³⁰Therefore, there is a need to develop inexpensive, dense, highly organized apatite-based implant coatings with improved mechanical, biological and physicochemical properties, as well as controlled morphology.

In this study, we aim to optimize the synthesis of ordered biomimetic fluoridated HAp crystals on titanium and sapphire substrates, study the effect of substrate topography and temperature on the morphology, chemistry, organization, and coverage of the synthesized apatite crystals.

MATERIALS AND METHODS

Materials:

This study includes commercially pure titanium discs; 99.6% pure, 0.7 mm thick and 15 mm in diameter supplied from Goodfellow Corporation (Coraopolis, PA, USA). Sapphire discs (length: 5 mm/ width: 5 mm / thickness: 0.5 mm) supplied from MTI Corporation with <0001> orientation (c-plane) and polished to 1 Å. The isoelectric point of titanium is approximately 6.2,³¹ while sapphire is a single crystal, where its isoelectric point is about 5.5.³²

Discs Preparation:

For etching titanium discs, a 1:1 ratio of hydrogen peroxide 50% GPR RECTAPUR (H2O2) (VWR International Ltd, Leicestershire, UK) and H2SO4 49-51% (Fluka Analytical[®], Sigma-Aldrich[®], St. Louis, MO) was used; by adding 10 ml H2SO4 very slowly to a beaker of H2O2 then left for 24 hours. Then the discs were removed, rinsed thoroughly with plenty of water and dried using air and filter papers.³³

Cleaning of Sapphire

Sapphire single crystal substrates were cleaned as follows, discs were placed in a Teflon holder inside a (40 ml) beaker filled with deionized water (DW), then left in a sonicator bath (Kerry Ultrasonics, Guyson Int. LTD, North Yorkshire, UK) for 15 minutes. Then, discs were placed in sodium dodecyl sulphate (SDS) 4% w/v solution and left in the sonicator for 15 minutes. Subsequently the discs were washed and left in DW for a further 15 minutes in the sonicator in order to remove any last traces of contamination. Finally, discs were air dried and sterilized by using ultraviolet ozone cleaner (UVOCS® Inc., Lansdale, PA, USA) for 30 minutes.

Wet chemical method

This method was undertaking following;³⁴ (0.1046 g) of HAp powder was added to a (100 ml) of deionized water, then (0.0084 g) of sodium fluoride was added with continuous stirring. Nitric acid (69%) was added

drop wisely into the solution very slowly until the powder was completely dissolved. Ammonium hydroxide solution (30%) was added drop wisely until the pH was readjusted to pH 6.0, then each substrate was placed in the bottom of beaker and incubated for eight days using a temperature-controlled incubator (LTE Scientific, Oldham, UK) at 37, 70 and 90@C using a water bath (Fisherbrand®, Leicestershire, UK). After incubation of solutions, precipitation on the base of the beakers were collected for further analysis.

Fourier transform infrared spectroscopy (FTIR)

FTIR analysis was conducted using the FTIR Spectrum GX (PerkinElmer®, Waltham, MA, USA). The powder sample was placed over the IR window to cover it with a metal cover and the sample was scanned. The program was set to take the average of ten scans, to analyse the samples at a wavenumber of 4000 cm-1 to 450 cm-1 in respect to % of transmittance.

Synchrotron X-Ray Diffraction (S-XRD)

The specimens were examined using the S-XRD beamline XMaS (BM28) at the European Synchrotron Radiation Facility (ESRF, Grenoble, France) using an X-ray energy of 15 keV (equivalent to a wavelength of $\lambda = 0.82$ Å) and a beam spot size of 50 µm. Two-dimensional diffraction images were collected every 30 seconds with a 2048 × 2048 pixel MAR CCD detector mounted behind the specimen, perpendicular to the X-ray beam and centred on it. A sample to detector distance of 132 mm allowed a 2 theta (2 ϑ) range of 5-30° which is equivalent to 2 ϑ range of 9-58° on a lab diffraction machine with CuK α radiation of λ =1.54 Å. A diffraction pattern from a calibration standard sample (LaB6) was also collected to allow data reduction and analysis.

After diffraction images were collected for each specimen, two-dimensional diffraction patterns from the powder were pre-processed using the FIT2D software.³⁵ Each 2D diffraction image was normalized to the incident flux and integrated over the entire 360° azimuthal range to create 1D Intensity vs. 2 ϑ diffraction patterns for Rietveld refinement with the GSAS Rietveld refinement software. Instrument parameters of X-ray wavelength, sample-to-detector distance, detector tilts, and intrinsic peak shape were determined by refinement of a LaB6 powder standard. Then for each specimen the phases present, their relative proportions and lattice parameters were refined, and values extracted. The quality of the refinement was determined by least squares methods where the goodness of fit increases as chi-square (χ 2) approached unity, where χ 2 is the ratio of the weighted R-factor and the expected R-factor squared.

Magic angle spinning-nuclear magnetic resonance (MAS-NMR)

In order to investigate the fluoride (F^-) interactions, present in the precipitates, solid-state 19F MAS-NMR analysis was conducted using a 14.1 Tesla spectrometer (600 MHz Bruker, Coventry, UK) at a Larmor frequency of 564.5 Mega Hertz (MHz) under spinning conditions of 22 kilo Hertz (kHz) in 2.5 mm rotor. The spectra were acquired with a single-pulse experiment of 60 seconds recycle duration, by using a fluorine free background probe. The 19F chemical shift scale was calibrated using the -120 ppm peak of 1M of NaF solution along with trichloro-fluoro-methane, as a second reference. Spectra were acquired for 4 hours with accumulation of 240 scans. Data was deconvoluted using DMFIT program.³⁶

Substrate Preparation

Commercially pure titanium discs (Goodfellow Corporation, Coraopolis, PA, USA) were either unetched or etched using a 1:1 ratio of hydrogen peroxide 50% and sulphuric acid 50% for 24 hours, then the discs were removed, rinsed thoroughly with plenty of water and dried using air and filter papers.²¹ On the other hand, polished Sa (P-Sa) and (U-Sa) single crystal substrates were cleaned using a sonicator bath (Kerry Ultrasonics, North Yorkshire, UK) and were sterilized using ultraviolet ozone cleaner (UVOCS[®] Inc., Lansdale, PA, USA).

Scanning Electron Microscope (SEM)

Substrates were mounted on aluminium stubs via self-adhesive tape and were coated by Agar auto sputter coating machine (Agar Scientific, Essex, UK) using gold/palladium target disk. The morphology of synthetic

apatite surface along with the organization of crystals and their dimensions were analyzed using SEM (FEI Inspect F, Hillsboro, OR, USA) at the following magnifications; 300X, 1000X, 2000X, 5000X, 10 000X, 40 000X, 80 000X and 150 000X, with working distance (WD) about 10 mm and 3.5 spot size. Images were then transferred to ImageJ (NIH, US) for quantitative analysis.

Transmission electron microscope (TEM)

Samples were analyzed using JEOL JEM 2010 (JEOL Ltd., Tokyo, Japan) to assess the morphology of the crystals and their assembly. D-spacings (lattice fringes) belonging to HAp crystal structures were determined and calculated, a plot of a surface retrieved from ImageJ software shows the peaks in graph that represents the lattice periodicities in the form of alternation between white and grey value which has been counted.

RESULTS AND DISCUSSION

FTIR Characterization

After 8 days of mineralization (Figure 1), the precipitates were collected from the different temperatures 37, 70, and 90°C and measured together with commercially available HAp powder (HAp Captal[®] R). FTIR analysis was used to characterize the chemical composition of the synthesized powders as shown in Table 1. We observed a wide range of phosphates (PO_4^{3-}) bands that correspond to the typical apatite structures.



Figure 1. FTIR spectra of synthesized apatite at different temperature. (b) Table shows the peak positions of different assignments related to PO_4^{3-} and OH bonds at different temperatures.

Across all temperatures, we detected the high intensity bands in the range 561-606 cm⁻¹ that are assigned to the v4" out of plane and v4"' bending modes of the PO_4^{3-} unit. Moreover, the intense infrared bands between 1023-1099 cm⁻¹, which are attributed to the PO_4^{3-} v3' and v3"' stretching vibrations, were observed. Furthermore, infrared bands between 960-965 cm⁻¹, known as the PO_4^{3-} v1 symmetric stretching vibrational mode were observed.

Weak infrared peaks were also seen at 865-870 cm⁻¹ for the precipitate formed at 90[°]C and for the HAp Captal[®] R spectra respectively, which corresponds to the carbonate groups (CO₃²⁻). This confirms the previous findings of Okazaki³⁷ who stated that the 875 cm⁻¹ corresponds to CO₃²⁻ groups substituted with PO₄³⁻ tetrahedron (type B carbonated apatite), and when shifted to a lower frequency, it indicates higher

degree of fluoridation. This is due to the conformation of the carbonate groups in the lattice are significantly affected by the F^- content.

Table 1 : Peak positions of different assignments related to PO_4^{3-} , OH, and CO_3^{2-} bands at different temperatures.

Assignment (cm ⁻¹				
) Temperature	$37 \ ^{*}\mathrm{C}$	$70 \ ^{*}\mathrm{C}$	90 $^{*}\mathrm{C}$	HAp Captal [®] R
4" (P-O Bending)	564.93	561.78	563.93	561.13
4""	604.78	602.63	606.34	601.41
OH Liberation				631.12
1 (Symmetric P-O	963.80	965.95	965.43	962.3
Stretch)				
3' (P-O Stretch)	1028.29	1026.14	1029.44	1023.91
3"'	1097.08	1094.93	1098.56	1092.78
CO3 ²⁻			865	870
OH Stretch				3574

Similarly, a carbonate stretch vibration was also observed on HAp Captal[®] R at 1420 cm⁻¹. On the other hand, hydroxyl (OH) peaks that correspond to OH liberation and OH stretch were only detected on the HAp Captal[®] R at 631 and 3574 cm⁻¹ respectively (Supplementary S1), however were absent in all our test groups at different temperatures. This observation gives an indication of the OH group substitution in the lattice, which would be in that case substituted with F- ions, and the coupling to F-in our study was enough to mask the OH group agreed with the observation seen in previous studies.^{38,39} In contrast, Chen et al.³⁴ used a wet chemical method, where they changed the pH, while keeping the temperature constant; they found that with increasing pH, more CO_3^{2-} and OH⁻ have been incorporated into the lattice, on the contrary, our study did not find similar trend as a function of temperature. On the other hand, at the high temperature group (90?C), we observed the presence of CO_3^{2-} , this can be explained by the inclusion of CO_2 in the solutions at the high temperature. Wang et al,⁴⁰ reported that the relative intensity of CO_3^{2-} vibration compared to phosphate vibration increase with time, whilst, in our study, the time constant was kept constant, therefore it will be useful in the to vary the reaction time to test this hypothesis, as this part is beyond the scope of our experiment.

S-XRD Characterization

The Miller indices (hkl) of all samples were investigated as a function of temperature, suggesting the presence of HAp, as its main characteristic Bragg peaks were seen as 002, 211, 112 and 300 (Figure 2a). All synchrotron XRD patterns at different temperatures; 37° C, 70° C and 90° C demonstrate the presence of mixed phases within the samples. As the temperature increased, the diffraction patterns tended to show more similarities to the control group. For example, our test groups exhibit the presence of the typical high intense HAp peaks such as 002, 211, and 300. Those peaks indicate the presence of HAp (purple tick marks- Figure 2a-d), which were well-fitted to our calculated data. On the other hand, changes in the relative intensities between 102 and 210 were observed, in comparison to the control sample. This observed change in the relative intensities seemed to be higher at the 37[°]C, followed by 70[°]C, and 90[°]C respectively. This change may be attributed to the presence of calcium fluoride (CaF₂) (light blue tick marks – Figure 2b-d). Furthermore, the broadening of 222 reflection of HAp seemed to be inversely proportional with the increased temperature, as more evident at both $37^{?}$ C and $70^{?}$ C. Those previous observations indicate the presence of a different phase along the HAp, which is the calcium fluoride (CaF_2) . This is due to the diffraction reflection from 111 plane of CaF₂, which overlaps with the reflection of 102 plane of apatite, causing the relative peak variation with 210 plane. As well as the reflection from 220 plane overlaps over the 222 apatite plane leading to the broadening of the associated peak. Therefore, the presence of CaF₂ at all temperatures was confirmed as calculated in Table 2, however, with different ratios. Shapkin et al.⁴¹ studied the formation of FAp from both the intermediate amorphous phase and CaF_2 , as a function of time using synchrotron X-ray diffraction. They observed similar overlapping events of apatite and CaF_2 , as reported in the current study.



Figure 2: (a-d) S-XRD of synthesized apatite Graph showing the diffraction pattern of HAp Captal[®] R, control, $37^{?}$ C, $70^{?}$ C and $90^{?}$ C respectively, miller indices (hkl) are shown above each corresponding peak, crosses: observed data, red line: calculated diffraction, tick marks: 2ϑ peak positions and black line: difference curve.

Temperature	Lattice Parameters (Å)*	Lattice Parameters (Å)*	CaF ₂ (Wt%)
	HAP	CaF ₂	
37 ?C	$a = 9.637 (\pm 0.1\%) c =$	a=5.602 (± 0.1%)	12.8 $(\pm 0.2\%)$
	$7.061 \ (\pm \ 0.1\%)$		
70 ?C	$a = 9.6303 (\pm 0.3\%) c =$ 7.0646 (± 0.4%)	a=5.6137 (± 0.6%)	$11.8 (\pm 0.5\%)$
90 ?C	a= 9.6326 (\pm 0.1%) c= 7.0734 (\pm 0.3%)	a=5.614 (± 0.3%)	$6.4~(\pm~0.3\%)$
HAn Cantal® B	$c = 7.0651 (\pm 0.1\%)$		0

Table 2 : The effect of temperature on lattice parameters of HAp and CaF_2 phases, and the weight of CaF_2 .

They concluded that the process of FAp formation, constitutes three steps, which is also in good agreement with the modified evolutionary theory of apatite crystals that confirm the presence of CaF_2 as a precursor for FAp formation. Both a- and c-lattice parameters of HAp phase were Rietveld refined and calculated as a function of temperature along with a-lattice parameter of CaF_2 phase. Contraction of a-axes of samples, were seen to be about -0.05 Å compared to the HAp control sample. This change might be explained by the fluoride-hydroxyl substitution within the apatite structure, due to the smaller size of the F⁻compared to OH⁻, in agreement with.^{38,39} Slight expansion of the c-lattice parameter in 90[?]C was evident compared to the control HAp sample, as the CaF_2 wt% follows a decreased trend with increasing temperature (Figure 2b-d). In order to quantify the amount of HAp and compare it with S-XRD data, ¹H MAS-NMR would be fundamental in future work along with those already gathered data in order to quantify the amount of each

phase, HAp, FAp and CaF₂ relatively.

19F MAS-NMR Characterization

¹⁹F chemical shifts peak positions of apatite grown at different temperatures were investigated to identify their chemistry and F⁻ interactions. Figure 3 shows the peak positions for the mineralized powders at the different temperatures; 37[°]C, 70[°]C, and 90[°]C. Using DMFIT, NMR peaks deconvolution detected 5 peaks, two major crystalline phases could be found, the first being FAp at approximately -103.5 ppm and the second being CaF_2 having a peak at approximately -109 ppm. Furthermore, a -95 ppm peak was observed, which is identified as a CO_3 peak. It was observed that at $37^{?}C$, the reaction is driven kinetically between Ca^{2+} ions and F⁻ ions forming CaF_2 as it is more kinetically stable than FAp. On the other hand, with increasing the temperature the reaction will be driven thermodynamically as the FAp formation is favoured and is more thermodynamically stable than CaF_2 . CaF_2 crystals in the latter case may act as a reservoir of Ca²⁺ and F- ions that would react with free phosphate groups in solution to form FAp crystals. Moreover, the NMR peak -95 ppm that corresponds to the CO32-, was more evident at 90?C compared to the other temperatures. In comparing to the FTIR results, at this temperature, CO32- stretch vibrations were also seen unlike the other two temperatures, this might give an indication that this weak peak found in the NMR spectra could be related to different interaction of F- ions inside apatite lattice when CO32- is included. Exploration of this hypothesis might be useful in future studies. Scholz et al.⁴³ also identify an isotropic 19F chemical shift of -108.6 ppm of CaF2 that was synthesized mechanochemically using high-energy ball milling. Additionally, it was also found that by increasing the time of milling the 19F line width resonance increases and subsequently its amount decreases. However, Chen et al.³⁴ have used a similar wet chemical method at 70?C, where they found only one peak of FAp at -103 ppm while the CaF2 was totally absent which is considered as a contradictory finding to our MAS-NMR result. This could be explained by the time of incubation, as they incubated the samples for 5 days compared to 8 days.



Figure 3. ¹⁹F MAS-NMR spectra of grown apatite at different temperatures 37?C, 70?C and 90?C. Asterisks marks are the spinning side bands, and the red dot corresponds to weak peak at -95 ppm

Surface Topography Characterization

When a dental implant osseointegrates to living bone, it is essential that a bioactive layer is formed on its surface.⁴⁴ In our case, apatite is used as the bonding interface, while investigating the effect of topographical changes and chemistry of the substrates at different temperatures to allow the comparison between different surface properties on the morphology, organization, and dimensions of the synthesized apatite coatings. The orientation of the crystals can be controlled by the stereochemical recognition at the interface, determining the crystallographic orientation of the synthesized crystals. On the other hand, epitaxial growth produces an anisotropic strain at the interface that results in determining the shape of crystals rather than the orientation.⁴⁵ Further understanding of these concepts, crystal growth can be fine-tuned to produce advanced hierarchical biomaterials.

The surface features of all substrates employed in this study, were inspected using SEM. We showed that nonetched titanium (NE-Ti) has grooves running in different directions, this is secondary to the cutting of the samples (Figure S2a), However, etching the titanium (E-Ti) surface resulted in revealing the grain boundaries and increasing the submicron surface groove size and roughness (Figure S1b). On the other hand, irregular asymmetrical grooves were observed on the unpolished sapphire (U-Sa) surface (Figure S1c), whereas polished sapphire (P-Sa) substrate exhibited smooth surface topography (Figure S1d). After incubation of the samples in the fluoride-rich supersaturated calcium phosphate solution at different temperatures, we have observed multiple shapes and morphologies made from apatite nanocrystals (Chart 1), grown across the surfaces of our substrates, including the titanium and sapphire.

Effect of Substrate and Temperature on the Apatite Crystal Morphology and Organization .

The wet chemical method used during this study is advantageous compared to the other wet chemical methods that form apatite crystals, as this method relies on the calcium and phosphate components from one source (HAp powder). This results in controlling the molar ratio of Ca:P to be 1.67, therefore it provides the accurate stoichiometry for apatite formation. However, as other mineralization solutions supply the calcium and phosphate from different sources, which can

Chart 1. Chart showing the apatite coatings morphologies observed on the different substrates at different temperatures.

	Shape	Name	Shape	Name
а		Smooth ball-like structure with spikes	d Marine	Bowknot-like structure
b		Smooth ball-like structure without spikes	e e	Disordered crystals
c		Dandelion- like structure	f	Needle-like structure (Hexagonal)

potentially lead to the formation of non-apatitic calcium phosphate phases or salts other than calcium phosphates.⁴⁶ The Ca:F ratio of that method was calculated to be about 5:1, which is the appropriate stoichiometry for FAp.³⁴ The incubation of different substrates inside the fluoride-rich supersaturated calcium phosphate solutions, are thought to drive the occurrence of heterogeneous nucleation on the surfaces, which is known to take place faster than the homogenous nucleation, as the substrates decrease the energy required to form a nucleus.^{47,48} The morphology, coverage, and crystal sizes of apatite coatings precipitated on the substrates, is known to be dependent on the substrate's chemistry, topography, charge, grain size, lattice

geometry, and architecture.^{47,49} Concave surfaces usually have high spatial charge, enhance the assembling of ions in three dimensions, thus they are considered as good nucleation sites. While convex surfaces dissipate the charge, which then decrease the nucleation.⁴⁷ Moreover, planar surfaces were also known to act as two dimensions nucleation sites with equal distribution of charge depending on the substrates' isoelectric point itself rather than its topographical properties.⁴⁷

After mineralization, we have observed multiple organized structures made of apatite nanocrystals. For example, dandelion shaped nanocrystals (Chart 1c) were seen on all the substrates at 37*C and 70*C (Figs. 4a, b, d, e, g, h, j, and k). As well, bowknot-like structures made of assembled nanocrystals (Chart 1d), were observed on NE-Ti at 37*C on the E-Ti disc at 70*C (Figure 4a, e). Furthermore, smooth ball-like structures either with spikes (Chart 1a, Figs 4a, b, d, e, g, j, h, and k) or without spikes (Chart 1b, Figs. 4a, b, d, e, g, j, h, and k) were observed on all substrates, except on the NE-Ti, E-Ti, P-Sa at 90*C. On the other hand, disordered nanocrystals (Chart 1e) were detected on all substrates at 90*C except the U-Sa (Figure 4c, f, i, and l).

The difference in the morphology between the ball-like structures with and without the spiky features, is potentially due to the outward growth of nanocrystals from inside the ball-like structures. Kinetically, it is proposed that the ball-like structures with the spiky features, can transform at later stages into bowknots. This then leads to the morphological transformation into the dandelion structures. Therefore, those morphologies which are found mainly at 37?C could be tested as potential biocompatible material at the bone-implant interface.

Activation energy for nucleation is known to be dependent on the nucleus-substrate interactions. Crystal growth is favoured at low energies, as it decreases the overall free energy of the critical nucleus formation, thus promoting the sequential phase transformations.⁵⁰ In our scenario, spherical structures (Chart1a, b, f) were mostly seen at lower temperature as these structures are energetically favoured with less energy needed for their formation. With increasing the temperature to 70?C, self-assembled organized nanocrystals start to grow out of the ball-like structures (Chart 1c,d), with further formation of ordered apatite features. However, at high temperatures (90?C), the nanocrystals seem to lack the hierarchical organization and appeared to be arranged randomly.

Effect of Substrate and Temperature on the Coverage of the Apatite Coatings

Fujishiro et al.⁵¹ reported deposited HAp on various substrates; they found that the coverage, morphology, and adhesive strength of the coatings. For example, they found that the coverage changes considerably between different substrates, as titanium was entirely coated compared to sparsely coated alumina substrate. Furthermore, it is well known that at pH of 6, titanium substrate acts as a negatively charged surface.⁵² while sapphire substrate is either neutral or exhibits a slightly positive surface at values of the pH below the isoelectric point, sapphire has a positive charge.⁵³ Zhu et al.⁵⁴ observed that the nucleation of HAp is favoured on negatively charged substrates compared to the positively charged ones. Using image analyses, we quantified the extent of the coating coverage on the different substrates at 37[°]C, 70[°]C and 90[°]C. This quantification was conducted, to assess the effect of the different surface properties including the surface chemistry and topography on the coverage. The highest apatite coverage was found on P-Sa 37[?]C and U-Sa at 70[°]C with a percentage of $45.5 \pm 0.03\%$ and $35\pm0.02\%$ respectively. All other substrates at different temperatures showed moderate coverage between 12-29%. The enhanced apatite coverage, which is seen on the E-Ti surfaces, gives an indication that the apatite nucleation and growth is favoured due to increased grooves and pits on the surface, which in turn increase the nucleation sites.⁵⁵ and overall coverage. Holbrough et al.⁵⁶, who confirmed that the surface topography can control efficiently the nucleation sites and in turn the coverage, stated that some cavities on the substrate surface could have the same dimensions of a critical nucleus. Moreover, our results agree with Dennig and Stevenson, and Campbell et al.^{57,58} who stated that the nucleation events prefer rougher surfaces such as scratches.



Figure 4. (a-c) SEM images of the apatite coating 'wet method' on E-Ti at different temperatures 37[?]C, 70[?]C, and 90[?]C, respectively. (d-f) SEM images of the apatite coating 'wet method' on NE-Ti at different temperatures, 37[?]C, 70[?]C, and 90[?]C, respectively. (g-i) SEM images of the apatite coating 'wet method' on P-Sa at different temperatures, 37[?]C, 70[?]C, and 90[?]C, respectively. (j-l) SEM images of the apatite coating temperatures 'wet method' U-Sa at different, 37[?]C, 70[?]C, and 90[?]C, respectively. (m) Percentage of apatite coverage on Ti. (n) Percentage of crystal coverage on Sa.

A study by Arres et al.⁵⁹ reported the presence of a biomimetic HAp coating on titanium surface, which reduced the structural stiffness, is essential to improve implants biocompatibility and osteointegration. In this study, new citrate-HAP coatings were produced by a simple hydrothermal method on pure titanium surface, without requiring any additional pre-treatment on this metal surface. The formed cHAp coatings consisting of nanorod-like HAp particles, conferred nano roughness and wettability able to endow improved biological responses.

Another study by Abhijith et al.⁶⁰ investigated the effects of multiscale topography on osteogenic behaviours of titanium-based bioimplant surfaces using laser texturing. The initial cell adhesion and proliferation appear to have been improved by the combined effects of surface topography, surface physical, and chemical performance. On two distinct grades of specimens—commercially pure titanium and Ti-6Al-4V titanium alloy—micro grooves with embedded nano ripples as periodic surface features were micro-fabricated. The findings demonstrated that multiscale topography improves cell adhesion and gives osteoblast cells a clear orientation to grow in the direction of the micro grooves. The preferential integration of bone tissues on the titanium surface, together with the presence of crystalline phase and enhanced hydrophilicity, all appeared to play a significant impact.

Crystal Thickness and Morphology

The nanocrystals at different temperatures showed well-defined hexagonal cross sections (Chart 1f and Figure 5a-c). Crystal size on the different substrates were quantified using Image J (Figure 5d-e). The smallest nanocrystals were observed on P-Sa at 37?C with mean 5 ± 4 nm. Moderate thickness was observed on E-Ti at 37?C, E-Ti at 90?C, and P-Sa at 90?C with mean $39.7 \text{ nm} \pm 6.55$, $35.43 \text{ nm} \pm 5.5 \text{ nm}$, and $34.45 \pm 5.76 \text{ nm}$, respectively. The largest crystals were seen on U-Sa at 90?C, NE-Ti at 37?C, and E-Ti at 90?C with mean 81.25 ± 27.23 nm, 80.46 ± 11.26 nm, and 81.05 ± 12 nm. A study by Abidi et al.⁶¹ studied HAp powder as

an implant coating material at different temperatures from 100 to 800 °C to achieve the stoichiometric using wet chemical method Ca/P ratio 1.667. The results showed that the crystal size increased with increasing the temperature however high purity of nano-HAp powders were obtained at low temperatures, it has also been reported from XRD spectra that HAp at higher temperatures exhibited good crystallinity as the peaks become narrow and sharp.

TEM Crystal Morphology Characterization

To assess the morphology, orientation, and the crystalline phase of the mineralization process, we have conducted TEM imaging of the crystals alongside with their d-spacings calculations. We have observed that the crystals exhibit a flat geometry at their ends (Figure 6a) similar to those observed with protein-based mineralization.⁶²Some lattice fringes of the crystals under the transmission of electrons have been revealed. For example, we have identified d-spacings that correspond to the typical FAp diffraction including the (102) and (002) (Figure 6a-b).



Figure 5. (a-c) SEM images showing the crystal size at different temperatures;37[?]C, 70[?]C, and 90[?]C, respectively. (d-e) Crystal size quantification on titanium and sapphire substrates, respectively.



Figure 6. (a-b) High Resolution TEM images showing typical apatite crystal structure running in a longitudinal pattern

CONCLUSIONS

This work demonstrates a potential cost-effective route to coat implant materials with highly- ordered fluoridated apatite structures using a low temperature wet chemical method. These coatings exhibit a fine control over the hierarchical organization, coverage, and size of the nanocrystals, exploiting the surface topography of the substrates. Furthermore, the results suggests that both titanium and sapphire can form bioactive coatings regardless being pre-treated or not. We believe that these hierarchical apatite coatings may play a major role in optimising the osseointegration and biocompatibility of dental and orthopaedic implants.

Supporting Information

FTIR spectra of synthesized apatite, SEM showing surface topography.

Corresponding Authors

* Sherif Elsharkawy - Centre of Oral, Clinical, and Translational Sciences, Faculty of Dentistry, Oral, and Craniofacial Sciences, King's College London, London SE1 9RT, United Kingdom; Prosthodontics Department, Guy's Hospital, Guy's and St. Thomas' NHS Trust, London SE1 9RT, United Kingdom; ORCID ID: https://orcid.org/0000-0001-9195-4982;

Email: sherif.elsharkawy@kcl.ac.uk

* Maisoon Al-Jawad - School of Dentistry, University of Leeds, Leeds, LS2 9JT, UK;

 $Email:\ m.al-jawad@leeds.ac.uk$

Authors

Sara Gamea - Centre of Oral, Clinical, and Translational Sciences, Faculty of Dentistry, Oral, and Craniofacial Sciences, King's College London, London SE1 9RT, United Kingdom; Tanta University, Faculty of Dentistry, Department of Restorative Dentistry, Tanta, 31111, Egypt; ORCID ID: https://orcid.org/0000-0002-5719-7217.

Natalia Karpukhina - Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London, E1 2DP, United Kingdom

Author Contributions

S.E. and M.A.J. conceived the project. S.E. conducted the experiments, S.E. and S.G. carried out the analysis, N.K. contributed to the MAS-NMR, and M.A.J. supervised the work.

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Conflict of interest

The authors have no conflicts of interest to declare.

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S.E. and S.G. contributed equally to this work.

Abbreviations

Å Angstrom, [@]C degree celsius, Θ theta, µg micrograms, µm micrometer, $\chi 2$ chi-square, Ca²⁺ calcium, CaF₂ calcium fluoride, CO₃²⁻ carbonate, E-Ti etched titanium, FAp fluorapatite, F⁻ fluoride, FTIR Fourier transform infrared spectroscopy, HAp hydroxyapatite, NE-Ti unetched titanium, MAS-NMR Magic angle spinning-nuclear magnetic resonance, OH hydroxyl, PO₄³⁻ phosphate, P-Sa polished sapphire, SEM scanning electron microscope, S-XRD Synchrotron X-Ray Diffraction, TEM transmission electron microscope, U-Sa unpolished sapphire.

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Table of Contents

Our study offers a potentially cost-effective way for coating implant materials with highly ordered fluoride apatite structures using a low temperature wet chemical process. These coatings direct the hierarchical organization, coverage, and size of the nanocrystals. Furthermore, our results convey that titanium and sapphire, whether pre-treated or not, can produce bioactive coatings. These hierarchical apatite coatings could play a significant role in improving the osseointegration and biocompatibility of dental and orthopedic implants.



Supporting Information



Figure S1. FTIR spectra of synthesized apatite at different temperature showing the peak position related to OH stretch bond observed on the HAp powder spectrum only.



Figure S2. (a-d) SEM showing surface topography of NE-Ti, E-Ti, U-Sa, and P-Sa, respectively.