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Electrohydrodynamic deposition of nanotitanium doped hydroxyapatite coating for medical and dental applications

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Abstract Nano-sized titanium containing hydroxyaptite has been prepared, the particle size of nanoTiHA was shown to be 12–20 nm in width and 30–40 nm in length, smaller than that of nanoHA. X-ray diffraction analysis revealed the phase purity of nanoTiHA produced. Antimicrobical assays demonstrated that nanoTiHA has excellent growth inhibitory properties, and is able to inhibit the growth of all bacterial strains tested, both Gram-negative and Gram-positive species, including multi-antibiotic resistant EMRSA 15 and EMRSA 16 'superbugs'. Biocidal activity against all four *Staphylococcus spp* was also shown at the concentration tested. Nanostuctured TiHA coating was successfully deposited onto Ti surfaces using EHDA spraying under optimized processing conditions with the thickness of the coating being further controlled by the spraying time. All of the nanoTiHA coated Ti surfaces were able to support human osteoblast (HOB) cell attachment and growth. The coating thickness did not significantly influence the proliferation of HOB cells on nanoTiHA coatings, while the ability of nanoTiHA coating to support HOB cell differentiation was demonstrated from the alkaline phosphatase activity. Our study showed that nanoTiHA has excellent anti-bacterial properties and the thin nanoTiHA coating was also able to support the attachment, growth and differentiation of HOB cells. Therefore, nanoTiHA coating could pave the way for the development of the next generation of dental and orthopedic implants by offering anti-infection potential in addition to osteoconductivity.

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1 Introduction

Hydroxyapatite (HA) is one of the most extensively used synthetic calcium phosphates for bone replacement because of its chemical similarities to the inorganic component of bone and teeth. Biological apatites are characterized by nanometer sized crystals, poor crystallinity, non-stoichiometry and a variety of ionic (cationic and anionic) substitutions. Substitution in the apatite structure for Ca, PO_4 or OH groups result in changes in properties, such as solubility. Carbonate, fluoride and silicon substituted HA have been widely used as bone grafts and bioactive coatings on metallic implants due to their attractive bioactivities [1–5]. Recently, titanium containing HA (TiHA) has been produced via a chemical co-precipitation route [6], which demonstrated a high in vitro bioactivity by the fast formation rate of bone-like apatite following immersion in simulated body fluid. In vitro culture with primary human osteoblast (HOB) cells revealed that TiHA encouraged the attachment of HOB cells, and cells maintained their osteoblastic morphology with more visible filapodial attachment. TiHA also supported the proliferation of HOB cells and higher cellular activity was observed on TiHA with the incorporation of 0.8%Ti. Surface texture with

submicrometer scaled topography of TiHA was found to provide a favorable surface for the growth of HOB cells with a larger amount of extracellular matrices produced by HOB cells on TiHA in comparison with that on HA alone [6], which suggests that TiHA could act as alternative bioactive coating on metallic prostheses.

Titanium and its alloys have achieved great success as medical implants [7]. However, titanium and its alloys are not particularly osteoconductive, and hence research has been directed towards modifying the surface of metallic materials using methods, such as alkali and acid treatments to produce amorphous titania functional groups to induce the nucleation and formation of bone-like apatite, which in turn provides the most favorable surface for bone cell differentiation in vitro and bone bonding in vivo. Additionally, titania/HA composite coatings have been developed to achieve improved bio-activity [8, 9].

It is thus hypothesized that TiHA will serve as a favourable alternative to HA as coating materials for dental and orthopaedic application. Particularly, as a recent study has also demonstrated a bactericidal function of TiHA using a different processing route [10]. Bacterial infection is a significant cause of failure for both orthopaedic and dental implants, and there are an increasing number of patients requiring medical devices, such as artificial joints and implants to enable everyday activities with an ever ageing population. TiHA coated implants can therefore offer a solution of new prostheses more resistant to infection, to benefit millions of young and aged patients.

Plasma spraying has been commercially used to produce HA coated metallic implants since the 1980 s [11]. More recently, various low temperature thin film techniques have been developed to extend the clinical application of HA coated implants where conventional thermal plasma spraying has been unable to deliver the required outcome, such as biomimetic, magnetron sputtering and electrohydrodynamic atomization (EHDA) deposition [12–19]. Compared with the conventional plasma spray coating technique, EHDA deposition is a simple, economical, ambient temperature process. Basically, the EHDA set up consists of a nozzle (needle) connected to a high voltage supply and a ground electrode. A suspension or solution of calcium phosphate is fed to the nozzle at a controlled flow rate. By carefully controlling the voltage, a stable cone-jet mode can be obtained, which can regularize the break-up of the jet to generate fine and uniform droplets of a few micrometers in size, and lead to the deposition of a bioactive calcium phosphate coating on metallic implant substrates. In this study, nanoTiHA coating on Ti by EHDA spraying was prepared and evaluated as regards to osteoconductive and antimicrobial properties.

2 Materials and methods

2.1 Preparation and characterization of nanoTiHA

TiHA containing 0.8wt% of Ti was synthesised using a reaction between calcium hydroxide ($Ca(OH)_2$) and orthophosphoric acid (H_3PO_4) (AnalaR grade, BDH, UK), where tetraethylorthotitanate was used as the source of Ti, with the Ca/(Ti + P) ratio kept at 1.67. Orthophosphoric acid and tetraethylorthotitanate solutions were added dropwise to Ca solution under continuous stirring at room temperature. The stirring was maintained for a further 4 h after complete addition of the reactants, the TiHA gel was then aged for a week.

TiHA crystals were collected on 3 mm copper grids and their morphology was examined using a JEOL transmission electron microscope. The crystal structure of TiHA was analysed with a Philips PW1730 X-ray diffractometer using CuK_α radiation. The data were collected from 20 to 50° with a step size of 0.05° and a scan time of 6 s was used.

2.2 Microbiological evaluation of nanoTiHA

In this study, the ability of nanoTiHA to inhibit the growth of both gram-positive and gram-negative bacterial strains was assessed, *Staphylococcus aureus* Oxford, Epidemic meticillin-resistant *Staphylococcus aureus* 15 (EMRSA 15), EMRSA 16, *Staphylococcus epidermidis, Pseudomonas aeruginosa* PAoI, *Escherichia coli* NCTC 9001 and *Proteus mirabilis* were tested.

2.2.1 (a) Growth inhibition assays

Bacteria were grown overnight in a shaking incubator (200 rpm, 37°C). Following incubation, the optical density (OD) of the cultures was adjusted to 0.1 ($\lambda = 540$ nm). 12.5 µl of each OD-adjusted bacterial culture was added to eppendorf tubes containing 750 µl of 5% nanoTiHA in phosphate buffered solution (PBS) and 250 µl of tryptone soya broth (TSB). 200 µl of each tube were transferred into a 96-well microtitre plate, and the OD at $\lambda = 540$ nm measured. The tubes were then placed on a shaking incubator (200 rpm, 37°C) for 24 h. The OD of the tubes after the incubation time was measured again by transferring 200 µl of each into a 96-well microtitre plate. OD values were compared to growth control without nanoparticles.

2.2.2 (b) Biocidal activity

10 µl of each OD-adjusted bacterial culture (as above) were added to eppendorf tubes containing 600 µl of 5%

nanoTiHA/PBS and 200 µl of TSB, and the tubes were placed on the shaking incubator (200 rpm, 37°C). After 24 h incubation, a 2 µl drop from each tube in duplicate was inoculated onto two separate nanoparticle-free tryptone soya agar (TSA) plates, and placed in the incubator. Colony growth was assessed after 24 h.

2.3 EHDA deposition of nanoTiHA on Ti

The nanoTiHA gel was dispersed in ethanol under sonification to ensure the homogeneity of nanoTiHA suspension for EHDA spraying.

NanoTiHA coating on Ti was prepared using the EHDA spraying set up reported previously [19], with a stainless steel needle held in epoxy resin connected to the power supply, and a syringe pump used to control the flow rate. A stainless steel needle with an inner diameter of ~500 µm was used. The nanoTiHA suspension was fed to a nozzle with a flow rate of up to 20 µl/min, and subjected to an electric field to obtain a stable fine jetting. Ti discs were ground/polished with silicon carbide paper (upto p2500) prior to spraying. The Ti substrate also served as the ground electrode during the spraying process.

Freshly prepared nanoTiHA suspension was syringed to the needle at a flow rate of 20 µl/min, with the distance between substrate and needle at 20 mm, the high voltage was applied between the needle and the ground electrode to obtain a stable cone-jet spraying mode for nanoTiHA deposition. The spraying time was 30 and 120 s for thin and thick coatings, respectively.

NanoTiHA coated Ti discs were also subjected to heat-treatment under an Argon atmosphere at a temperature of 500°C for 2 h at a rate of 5°C/min.

The microstructure of nanoTiHA coated samples was subsequently examined using a JEOL field emission scanning electron microscope.

2.4 In vitro biological responses

A primary human osteoblast (HOB) cell model was used for evaluation of cellular responses to nanoTiHA deposition. HOB cells (2×10^4cells) were seeded directly onto nanoTiHA coated substrates and incubated at 37°C in a humidified air atmosphere with 5% CO_2.

2.4.1 (a) Cell proliferation and differentiation

The proliferation of HOB cells on the nanoTiHA coating during 21 days of culturing was determined using the alamarBlue™ proliferation assay (Serotec, Oxford, UK) with an Opsys plate reader (570/630 nm, Dynex Technologies, US) and compared with that of tissue culture plastic (Tcp) and nanoHA controls. The differentiation of HOB cells on nanoTiHA coating was assessed using the alkaline phosphatase (ALP) assay. 4-nitrophenol phosphate (pNPP) was added into cell lysates buffered to pH 10.3 and the absorbance measured at 5, 10, 15 and 30 min using an Opsys plate reader (405/670 nm) and compared to a standard curve.

2.4.2 (b) Live/dead fluorescent staining

Live and dead fluorescent staining was used to assess the cytotoxicity of nanoTiHA coatings. After seven days of culture, HOB cells were then stained with calcein AM and counterstained with ethidium bromide homodimer (Molecular Probes) at concentrations of 10 nmol/well in PBS and incubated for 1 h at 37°C. Microscopical visualisation was carried out using an inverted Olympus IX51 microscope.

2.4.3 (c) Cell—coating interaction

After each cell culture time period, HOB cells were fixed with 1.5% gluteraldehyde in 0.1 M sodium cacododylate (NaCA) buffer and stained with 1% osmium tetroxide, dehydrated with graded ethanol (from 20 to 100%) and hexamethyldisilazane, and finally air dried. After sputter coating with gold, the sample was examined by a JEOL field emission SEM.

3 Results

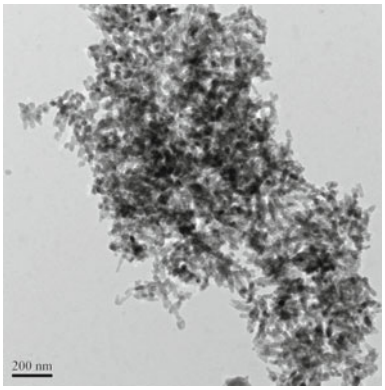
3.1 NanoTiHA

TEM examination revealed that nanometer scaled TiHA particles were produced (Fig. 1a), the size of nanoTiHA particles was about 12–20 nm in width and 30–40 nm in length. XRD revealed the presence of all the major HA peaks expected (Fig. 1b). No secondary phase was detected.

3.2 Microbiological testing

3.2.1 (a) Growth inhibition assays

The growth inhibition assays results showed that nanoTiHA has the capacity to inhibit the growth of *Staphylococcus aureus* Oxford, EMRSA 15, EMRSA 16, *Staphylococcus epidermidis, Pseudomonas aeruginosa* PAoI, *Escherichia coli* NCTC 9001 and *Proteus mirabilis* during a 24 h incubation period ($P < 0.001$). In every case, bacterial growth was below 10% of that achieved with the positive control.



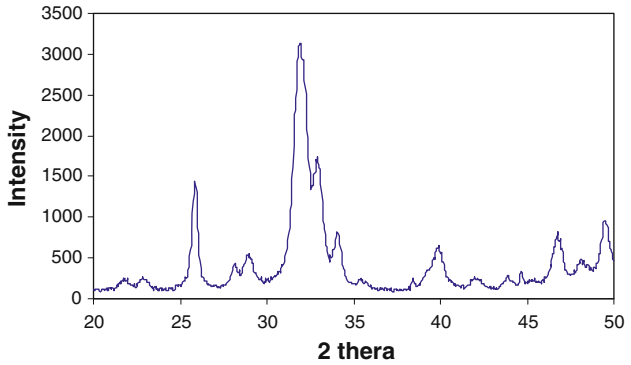


Fig. 1 **a** TEM micrograph of nanoTiHA particles, and **b** XRD patterns of nanoTiHA

Table 1 Biocidal activity of nanoTiHA. '+' microbial growth and '−' no growth/biocidal potential

Bacterial species/strains	nanoTiHA	+ve test control
S.aureus Oxford	−	+
EMRSA 16	−	+
S.epidermidis	−	+
P. aeruginosa PAoI	+	+
EMRSA 15	−	+
E.coli NCTC 9001	+	+
P.mirabilis	+	+

3.2.2 (b) Biocidal activity

The results of the biocidal activity of nanoTiHA are shown in Table 1. Compared with the positive test control, nanoTiHA showed biocidal potential against *Staphylococcus epidermidis* and *Staphylococcus aureus*, the microbial species often observed in septic implant failure. Particularly, the biocidal activity against epidemic meticillin-resistant *Staphylococcus aureus* EMRSA 15, EMRSA 16 'superbug' was observed.

3.3 NanoTiHA coating

A uniform nanoTiHA coating on Ti was obtained under an optimized processing set up, namely at a flow rate of 20 μl/min, with an applied voltage kept at 4 to 5 kV, and a distance between nozzle and Ti substrates of 20 mm. There were no cracks and no particle aggregation on the coating surface, with a coating thickness of about 2 μm for 30 s of spraying. As expected, the thickness increased to 5 μm with a spraying time of 120 s. The nanoscaled topographical feature of TiHA was observed on a deposited nanoTiHA coating, which was maintained after heat-treatment at 500°C for 2 h.

3.4 Cytotoxicity

The live/dead staining was used to assess the cytotoxicity of the nanoTiHA coating when in direct contact with HOB cells. From the fluorescent microscopy images (Fig. 2) of HOB cells cultured on nanoTiHA coating, no indication of apoptotic/necrotic cell death was found, as the HOB cells appeared to retain their membrane integrity, thus indicating that nanoTiHA coating was not cytotoxic to HOB cells.

3.5 Proliferation and differentiation of HOB cells

The proliferation of HOB cells on nanoTiHA coated Ti surfaces was measured using the alamarBlue™ assay during 21 days of culture (Fig. 3), which showed that nanoTiHA coated Ti surfaces were able to support the growth of HOB cells in vitro. There was an increase in cell numbers with time on all the nanoTiHA coated specimens, but there was no significant difference in growth as regards coating thickness (2 or 5 μm).

ALP activity of HOB cells on nanoTiHA coating after seven days was used as an early indicator of differentiation of HOB cells. There was no significant difference in ALP activity of HOB cells on nanoHA and nanoTiHA coating, in comparison with tissue culture plastic control.

SEM examination showed that HOB cells were able to attach to the nanoTiHA surface and maintained their osteoblastic morphology with numerous visible filapodia observed (Fig. 4a). HOB cells were able to proliferate on the nanoTiHA coating and multi-layers of HOB cells were seen after seven days of culture (Fig. 4b).

4 Discussion

Hydroxyapatite is known for its osteoconductivity, as it has the ability to promote bone apposition. Chemical modification of hydroxyapatite has been demonstrated as a suitable method to enhance osteoconductive potential. The bioactivity and biocompatibility of TiHA was demonstrated previously. In this study, nanoTiHA particles have been prepared with the particle size being smaller

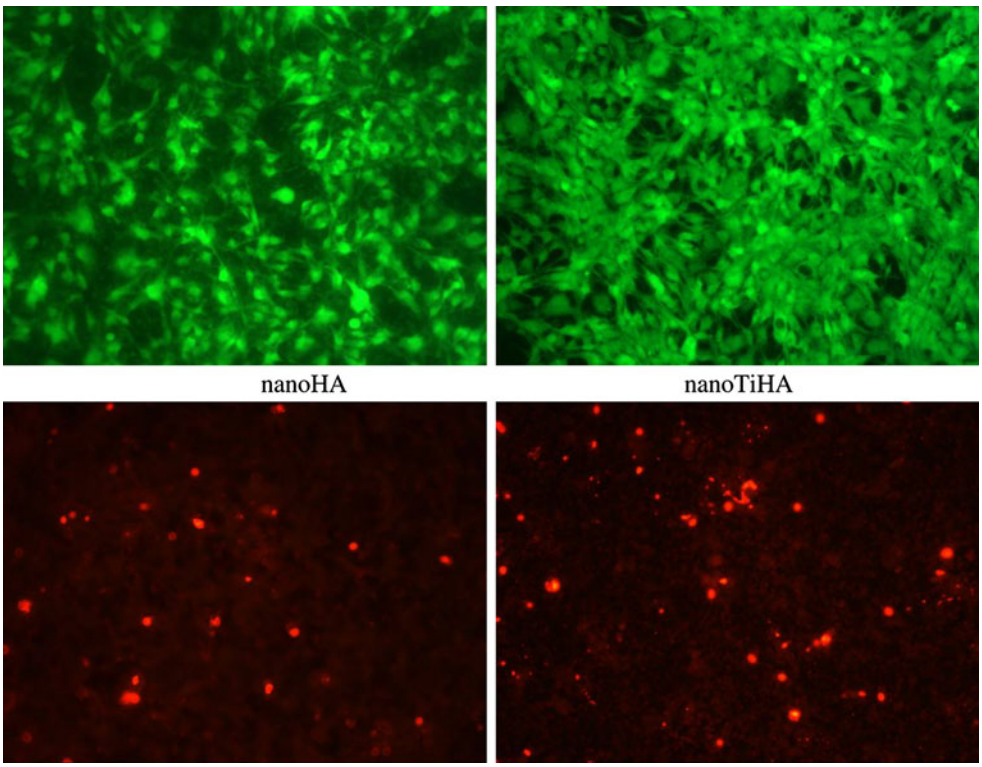


Fig. 2 Live/dead staining of HOB cells on nanoHA and nanoTiHA coatings, indicating good viability and no statistical difference in dead cell number on either surface (*top images: live signal, below: dead cells*), ×20 objective (NA0.6)

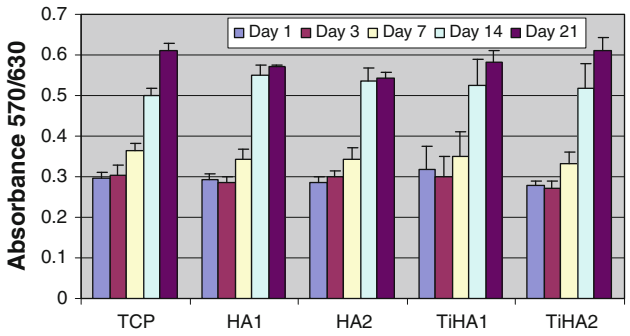


Fig. 3 Alamarblue ™ assay shows the proliferation of HOB cells on nanoHA and nanoTiHA coatings during 21 days of culture

(12–20 nm in width and 30–40 nm in length) than that of nanoHA (20–30 nm in width and 50–100 nm in length) [16], and more suitable for further applications, such as the preparation of thin TiHA coatings with nano-scaled surface topography.

The full range of microbial species that cause septic implant failure is not currently known, as many species cannot be successfully cultured. Particularly, there has been a worryingly high incidence of antibiotic resistance and related nosocomial infections in recent years. Therefore, both gram-positive and gram-negative bacteria species were tested in the current study, including the common microbial species observed in septic implant failure, such as *Staphylococcus epidermidis* and *Staphylococcus aureus*, as well as Epidemic meticillin-resistant *Staphylococcus aureus* 'superbug'.

Antimicrobial testing indicated that nanoTiHA has excellent growth inhibitory effects, and is able to inhibit the growth of both gram-negative and gram-positive strains, including EMRSA 15 and EMRSA 16. Gram-negative species were shown to be more resistant to the biocidal activity of nanoTiHA. Differences in cell wall structure, between Gram-negative and Gram-positive species, may account for the results observed. The mechanism of the antibacterial action of nanoTiHA is currently under further investigation.

Nanostuctured TiHA coating was successfully deposited on Ti surfaces using EHDA spraying under optimized processing conditions. The thickness of the coating was further controlled as a function of spraying time. The short spraying time (up to 2 min) used in the study has demonstrated the high efficiency of such coating technique and therefore offers a great potential for its biomedical applications.

All of the nanoTiHA coated titanium surfaces were able to support HOB cell attachment proliferation and differentiation, thus demonstrated its osteoconductive potential. The coating thickness tested (2 and 5 μm) did not significantly influence the proliferation of HOB cells on nanoTiHA coatings, both showed significant increase with time. The effect of coating thickness on the mechanical properties of nanoTiHA coating is currently under systematic investigation.

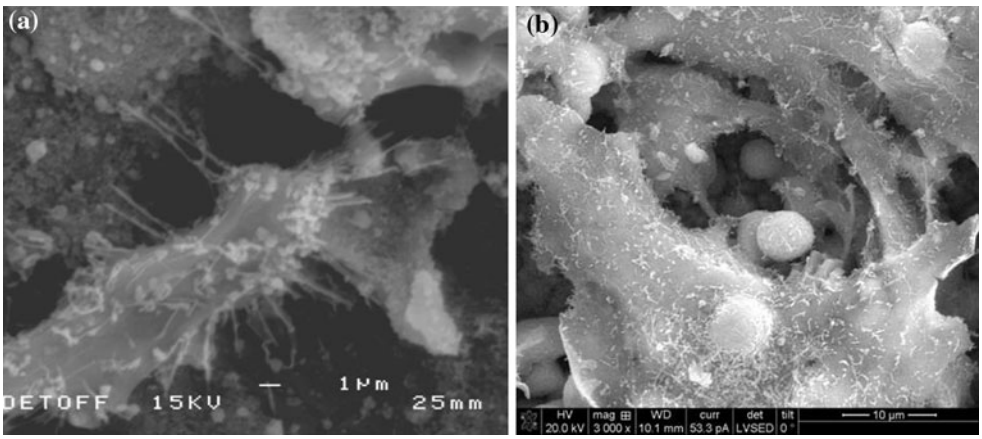


Fig. 4 Morphology of HOB cells on nanoTiHA coating after **a** 1 day and **b** 7 days of culture. HOB cells maintained osteoblast morphology, the cell attachment to nanoTiHA (**a**) and multi-layering of cells were observed after 7 days (**b**)

5 Conclusion

Our study shows that nanoTiHA has excellent anti-bacterial properties and a thin nanoTiHA coating also supported the attachment, growth and early differentiation of HOB cells. Potentially, nanoTiHA coating could pave the way for the development of the next generation of dental and orthopaedic implants by offering anti-infection properties in addition to osteoconductivity.

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