Polarization of hydroxyapatite: Influence on osteoblast cell proliferation

D. Kumar, J.P. Gittings, I.G. Turner, C.R. Bowen, A. Bastida-Hidalgo, S.H. Cartmell

Abstract
Hydroxyapatite (HA) has been used clinically to treat bone defects. However, modifications of the surface properties of HA could improve and control bone matrix deposition and localized host tissue integration. The aim of this study was to investigate the effect of developing a surface charge on HA discs with respect to osteoblast activity in vitro. HA discs (12 mm x 2 mm) were sintered in either air or water vapour. The HA discs were then electrically polarized (positive and negative surfaces) or non-polarized (controls) and seeded with MC3T3-E1 cells. Polarized HA sintered in water vapour was shown to retain six times more charge than polarized HA sintered in air. Picogreen analysis demonstrated that at 4 h cell number was significantly higher on the negatively and positively charged HA surface (water sintered) in comparison to the non-charged water and air-sintered HA controls. At 7 days there was a significant increase in cell number on the negatively charged HA (air sintered) sample in comparison to the negatively charged water vapour sintered HA sample and the non-charged water vapour sintered control sample. Also at 7 days, the picogreen data showed a significant increase in cell number on the positively charged water-treated HA sample in comparison to both the air- and water-treated HA non-charged control HA samples. An alamarBlue assay at 7 days demonstrated significant cell metabolic activity on the charged surfaces (both positive and negative) in comparison to the non-charged HA and the tissue culture plastic controls. This study demonstrated that all of the HA discs tested supported cell viability/attachment. However, cell attachment/proliferation/metabolic activity was significantly increased as a result of developing a charge on the HA surface.

Keywords:
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Polarization
Surface charge
Osteoblasts
Electrical properties

1. Introduction

Drawbacks to current repair strategies for patients suffering from bone disease/trauma include tissue availability and donor site morbidity. Bone tissue engineering is an emerging technique that offers potential solutions to these problems. Scaffolds may be used to support and encourage cellular activity and promote faster healing. Hydroxyapatite (HA) is a calcium phosphate ceramic that has been used clinically and has been shown to have bioactive, osteoconductive and biocompatible properties [1–3]. It may be possible, however, to further enhance HA with respect to bone integration using treatments that have the potential to improve cell proliferation and thus improve implant integration and wound healing.

Recently, studies have demonstrated that polarizing sintered bioceramics, and thus creating a residual permanent charge on opposite sides of the material, can influence the response and activity of the cells it supports [4–6]. It has been shown that sintering HA samples under water vapour improves the conductivity of the samples when compared to those sintered in air [7,8]. The presence of water vapour in the atmosphere during sintering restricts the dehydration of OH⁻ ions and, as a result, due to the rotation and movement of OH⁻ ions within the sub-lattice channels, protonic conductivity is increased [9]. In air, partial dehydration of the OH⁻ ions leaves vacancies within the structure, and this predominantly determines the conductivity. HA has an ionic crystal structure with an OH⁻ ion sub-lattice that is essentially one-dimensional [10]. The chemistry of calcium phosphates is extremely complex, with a series of possible related inter-substitutions giving rise to various “impure” and/or calcium deficient apatites. The process that dominates the polarization effect in HA has been termed “nomadic polarization”, and is brought on by protonic mobile charges due to partial dehydration in the OH⁻ sub-lattice. This applies not only to protons but also to large negative ions, such as OH⁻ ions [10–13]. The monoclinic to hexagonal phase transition between 200 and 250 °C also allows the protons to rotate and orientate themselves to become aligned with the OH⁻ channels [14]. At a still higher temperature, e.g. 350–450 °C, the OH⁻ ions acquire enough thermal energy to reorientate themselves independently of their OH⁻ ion neighbours in the chain. This can also be aided by an applied electric DC field.

A number of studies have investigated the effect of electrical charges and voltages on the biological response to polarized HA. However, there is no consensus in the literature as to whether po-
sitive or negative surface charges are more beneficial to this re-
response. A number of authors report an increase in cell numbers re-
corded on negatively charged HA surfaces [15,16] with up to 10 times more cells found attached to the charged surface when com-
pared to the electrically neutral surfaces [17]. In contrast to this, 
increased cell numbers have been found on positively charged 
HA surfaces compared to negatively charged HA surfaces 2 days 
after seeding [18], with fewer cells counted on the negative sur-
faces compared to the neutral surfaces. After 4 days no difference 
was observed between the positively and negatively charged sur-
faces [18].

The results of in vivo experiments are also contradictory. One 
study reports enhanced osteoblast activity resulting from both po-
sitively and negatively polarized HA plates implanted in calvarial 
bones of rats [6]. This was coupled with a decrease in osteoclast 
activity, which was attributed to the increase in bone formation 
on the negative surfaces due to the accumulation of Ca\(^{2+}\) ions on 
the surfaces. Two further studies implanted polarized HA in canine 
femora and rat tibiae, respectively [19,20]. In the first of these, the 
surface charge on polarized HA was shown to influence protein 
adsorption onto the HA surface in vivo layer which was thought 
to result in improved osteoconduction on both positively and neg-
avatively charged surfaces. The second study corroborated these 
results when an increase was found in the number of mature 
mature osteoblasts in bone formed on charged HA surfaces, irrespec-
tive of the polarity of the charge.

Some of the apparent contradictions between both in vivo and 
in vitro studies will arise from variations in the experimental meth-
ods used, cell types employed and analysis techniques utilized in 
addition to variations in animal models, implant sites and dura-
tions of implantation. Direct comparison of the results from these 
studies is therefore difficult. However, there is evidence that the 
surface charge, whether positive or negative, can affect the bio-
 logical response.

This paper describes the in vitro response of MC3T3-E1 bone 
cells to polarized hydroxyapatite – in particular, analysis of short 
(4 h) and long-term (7 day) responses of cell attachment, prolif-
eration and metabolic activity. A detailed electrical and composi-
tional study of the materials used is reported elsewhere [21]. It 
was found that the polarization of the samples was enhanced by 
the use of water vapour during sintering; the level of charge mea-
sured in the HA discs sintered in water was found to be six times 
greater than that for samples sintered in air. The aim of this study 
was to investigate the effect of the charge retained in polarized HA 
disks, sintered in either air or water, on osteoblast local attachment 
and proliferative capability with respect to potentially improving 
clinical treatment strategies.

2. Materials and methods

2.1. HA sample manufacture

Dense hydroxyapatite discs (12 mm diameter × 2 mm height) 
were fabricated using a commercially available precipitated calcium 
phosphate powder (TCP130, Thermphos UK Ltd.). After ball 
milling, the powder was sieved and compacts were cold pressed 
at 80 MPa for approximately 30 s, followed by sintering either in 
water vapour or air at 1300 °C for 4 h with a heating rate of 
60 °C h\(^{-1}\). Characterization of the materials has been reported else-
where [21,22]. In summary, the phase composition indicates that the 
majority of the material (75%) remains as HA, with the remain-
der composed of TCP phases. For the air-sintered material an aver-
age grain size of 3.7 μm was measured, whereas the dense and 
porous materials sintered in water vapour had smaller average 
grain sizes of 2.3 and 2.4 μm, respectively. Typical density was 
93% theoretical [22]. The microstructures appeared very similar 
in both cases, leading to the conclusion that it was reasonable to 
compare their electrical properties on an equal basis.

2.2. High-temperature polarization of HA ceramics

After sample preparation, specimens were placed between two 
platinum electrodes. This assembly was then placed in an electric 
 furnace and heated to the desired polarization temperature of 
400 °C. Once the polarization temperature had been reached a DC 
electric field of 3 kv cm\(^{-1}\) was applied for 1 h. The electric furnace 
was programmed to cool at a rate of 2 °C min\(^{-1}\) with the DC field 
still applied until room temperature was reached.

Once the samples had been polarization, the degree of polariza-
tion was determined from the thermally stimulated discharge cur-
rent (TSDC). This involved reheating a polarized sample to 
depolarize the material and measuring the depolarization current 
density as a function of temperature using a Keithley 6514 elec-
trometer. Table 1 defines the sample nomenclature used for the 
polarized and non-polarized control samples.

2.3. Cell culture

MC3T3-E1 osteoblast-like cells were cultured in culture medium 
consisting of alpha-minimal essential medium (Biosera L0476), 10% fetal calf serum (Biosera S1900), 1% 1-glutamine (Biosera G7513 2 mM), 1% antibiotics and antimitotics (Biosera L0010, 100 U ml\(^{-1}\) penicillin and 100 mg ml\(^{-1}\) streptomycin), 50 μg ml\(^{-1}\) ascorbic acid, 10 mM β-glycerophosphate and 10\(^{-8}\) M dexamethas-
none (Sigma-Aldrich).

HA discs were autoclaved at 120 °C for 25 min for sterilization 
before cell seeding, at a temperature where no thermally stimu-
lated discharge current was observed and well below the polariza-
tion temperature (400 °C). They were then individually placed 
into the wells of a 24-well plate with either the negative or positive 
charged surface facing upwards. Cells were added to the top of each 
HA disc (to either a negatively charged surface, a positively charged 
surface or an uncharged control surface) at a density of 750,000 cells 
in a 150 μl culture medium suspension per HA disc. Samples were 
incubated for 1 h at 37 °C under 5% CO\(_2\). One and a half millilitres of 
culture medium was added to each sample prior to further incu-
bation. The culture medium was changed every 2–3 days.

2.4. Picogreen assay

At 4 h and 7 days after initial cell seeding, a picogreen assay was 
performed on the samples. The assay (P7589 Invitrogen) was per-
formed according to the manufacturer’s instructions. Briefly, 
100 μl of each sample was added to each well of a 96-well plate 
in duplicate. A further 100 μl of picogreen dye was then added to 
each well. Samples were analysed using a fluorescent plate reader 
(Victor 1420 multi-label counter) at 485/535 nm wavelength.

DNA from three different locations on each HA disc sample was 
measured (Fig. 1). These three locations were: Location 1, cells sus-
pended in culture medium; Location 2, cells adhered to the HA disc 
(cells were lysed in situ on the disc to obtain the DNA value of cells 
adhered to HA surface); and Location 3, cells adhered to the tissue 
culture plastic control (TCP CTL) well surface (cells were also lysed

<table>
<thead>
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<th>Table 1</th>
<th>Nomenclature for HA samples.</th>
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<tr>
<td>HA sample</td>
<td>Non-polarized (control)</td>
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<tr>
<td>Sintered in air</td>
<td>AIR CTL</td>
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<tr>
<td>Sintered in H(_2)O</td>
<td>H(_2)O CTL</td>
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in situ in this sample group). These three regions were analysed by removing the medium from each sample well and transferring to 1.5 ml vials. These supernatants were then microcentrifuged to pellet any suspended cells. The excess medium was removed and discarded. One millilitre of lysis TE buffer (provided in picogreen kit) was added to each cell pellet. This process analysed Location 1 as identified in Fig. 1.

After removal of the medium, the HA discs (plus any adhered cells) were transferred to new, individual wells of a 24-well plate (to analyse Location 2 as seen in Fig. 1). One millilitre of TE buffer was also added to these samples. The last cell adherence location (Location 3, as seen in Fig. 1) was analysed by adding 1 ml of TE lysis buffer to each well. This allowed any cells adhered to the inside of tissue culture plastic wells to be detected using the picogreen assay. All samples for analysis underwent a freeze/defrost cycle in order to ensure full cell lysis. Prior to analysis with picogreen, the samples were homogenized further by vortexing the TE buffer plus sample and scraping any surfaces to which cells had adhered using individual sterile pipette tips. For each sample group, six samples were taken at each time point.

2.5. AlamarBlue

At 7 days, the HA discs were removed from the incubator and rinsed with phosphate-buffered saline. One millilitre of alamarBlue working solution was added to each sample prior to incubation for 90 min at 37 °C (protected from light). After incubation, 100 μl of the alamarBlue solution was transferred to a 96-well plate (Falcon) in triplicate for each of the samples and read on the fluorescence plate reader (Cytoflour, Perceptive Biosystems) at 530 nm excitation, 590 nm emission. Six measurements were performed for each sample group. The following seven sample groups were analysed:

- Air+ = % viability measured from cells seeded onto positive surface of air-treated hydroxyapatite disc.
- Air− = % viability measured from cells seeded onto negative surface of air-treated hydroxyapatite disc.
- AIR.CTL = % viability measured from cells seeded onto non-charged surface of air-treated hydroxyapatite disc.
- H2O+ = % viability measured from cells seeded onto positive surface of water vapour treated hydroxyapatite disc.
- H2O− = % viability measured from cells seeded onto negative surface of water vapour treated hydroxyapatite disc.
- H2O.CTL = % viability measured from cells seeded onto non-charged surface of water vapour treated hydroxyapatite disc.
- TCP.CTL = % viability measured from cells seeded onto tissue culture plastic surface (seeded into well of 96 well plate).

2.6. Statistical analysis

Data obtained from the picogreen assay was analysed using a three-factor analysis of variance (ANOVA; between three groups of charged surface (positive (+) vs. negative (−) vs. no charge (CTL), air/water-treated (air vs. H2O) and time point (4 h vs. 7 days)). Data obtained from the alamarBlue assay was analysed using a two-way ANOVA (i) within (tissue culture plastic sample group as this sample was run against every factor) and (ii) between (charged surface (positive (+), negative (−), no charge (CTL) vs. air/ water-treated (AIR vs. H2O)) using ezANOVA software. Statistical significance was taken to be p < 0.05.

3. Results

Fig. 2 shows the typical TSDC traces from polarized HA sintered in both air and water vapour. During depolarization a peak current density of 26 nA/cm² was measured when heating to 600 °C for the water-treated scaffolds in comparison to approximately 4 nA/cm² current density from the HA discs sintered in air alone. Such peak current densities are typical of polarized HA materials and are dependent on polarization temperature, polarization electric field and sintering conditions [4]. The difference in current density for the air- and water-sintered samples was not sufficiently high to anticipate that there would be any influence on cell response.

The results from the picogreen assay can be seen in Figs. 3 and 4. The bar charts in Fig. 3 show the amount of DNA (as measured in picogreen assay) in the different sample locations. The symbol - represents DNA measurements from hydroxyapatite samples that were significantly higher in relation to the respective 4 h and 7 day samples. It also represents DNA measurements from HA samples that were significantly higher in relation to the corresponding medium and well groups (p < 0.05). The results show that the majority of cells seeded onto the HA discs were retained on the surface of the disc – between 95% and 99% of the total cell number per sample were located on the HA rather than in the medium or on the well interior that housed the disc. Fig. 3 also demonstrates the significant increase in cell number from 4 h to 7 days.

Fig. 4 shows the picogreen results from the cells adhered to the HA discs only. The bar chart shows the amount of DNA (as measured in picogreen assay) from cells seeded onto the positively charged surfaces, negatively charged surfaces and non-charged hydroxyapatite discs. The symbol - represents DNA measurements from HA samples that were significantly higher in relation to the...
4 h and 7 day samples respectively. The symbol % represents DNA measurements from the 4 h H$_2$O$^{-}$/C$^0$ samples that were significantly higher in comparison to the 4 h AIR+, AIR CTL and H$_2$O CTL groups. The symbol $ represents DNA measurements from the 4 h H$_2$O$^+$ samples that were significantly higher in comparison to the 4 h AIR+, AIR CTL and H$_2$O CTL groups. The symbol + represents DNA measurements from the 7 day AIR$^{-}$/C$^0$ samples that were significantly higher in comparison to the 7 day H$_2$O$^{-}$/C$^0$ and H$_2$O CTL groups. The symbol ^ represents DNA measurements from the 7 day H$_2$O$^+$ samples that were significantly higher in comparison to the AIR CTL and H$_2$O CTL groups. The symbol & represents DNA measurements from the 7 day AIR$^+$ samples that were significantly higher in comparison to the H$_2$O CTL group. All significances are $p < 0.05$.

Fig. 4. A comparison of DNA levels measured at 4 h and 7 days from the surfaces HA discs polarized after sintering in air (AIR$-$, AIR$^+$) or water (H$_2$O$-$, H$_2$O$^+$) and unpolarized controls (AIR CTL, H$_2$O CTL). The symbol % represents DNA measurements from hydroxyapatite samples that were significantly higher in relation to the 4 h and 7 day samples. The symbol $ represents DNA measurements from the 4 h H$_2$O$^{-}$ samples that were significantly higher in comparison to the 4 h AIR$^+$, AIR CTL and H$_2$O CTL groups. The symbol $ represents DNA measurements from the 4 h H$_2$O$^+$ samples that were significantly higher in comparison to the 4 h AIR$^+$, AIR CTL and H$_2$O CTL groups. The symbol + represents DNA measurements from the 7 day AIR$^{-}$/C$^0$ samples that were significantly higher in comparison to the 7 day H$_2$O$^{-}$/C$^0$ and H$_2$O CTL groups. The symbol ^ represents DNA measurements from the 7 day H$_2$O$^+$ samples that were significantly higher in comparison to the AIR CTL and H$_2$O CTL groups. The symbol & represents DNA measurements from the 7 day AIR$^+$ samples that were significantly higher in comparison to the H$_2$O CTL group. All significances are $p < 0.05$.

4. Discussion

Fig. 2 demonstrates the increase in charge retention of water vapour-treated HA scaffolds in comparison to air-treated HA scaffolds. The HA is polarized by a mechanism known as protonic migration which relates to the transportation of protons in the OH$^-$ columnar structure [14]. During this process the hydroxide
ions are forced to rotate and align themselves in the direction of the applied DC electric field. A current discharge during subsequent reheating of samples demonstrates that they have an associated surface charge. The water vapour-sintered discs retain a higher charge than the air-sintered samples due to the greater conductivity of the water vapour-treated discs. The presence of water vapour in the atmosphere during sintering prevents the dehydration of hydroxide ions and thus increases protonic conductivity. In contrast, in air, partial dehydration of the hydroxide ions leaves vacancies within the structure, which predominantly determine the conductivity. No information is currently available in the literature regarding the effects of how a charge of either 4 nA cm\(^{-2}\), in the case of the air-sintered samples, as opposed to 26 nA cm\(^{-2}\) for the water-sintered samples, relates to cell activity. The magnitude of the difference appears to be relatively large but, on the overall scale, the significance of the difference can only be speculated upon; further work would be necessary to derive any conclusions in this respect.

The picogreen data (Figs. 3 and 4) show that osteoblast cells adhere and proliferate on all the different types of HA discs tested. The seeding technique demonstrated a good continuity, with approximately 99% of the cells seeded having attached to the HA discs in all conditions (Fig. 3). The results show that there was a significant difference between DNA measurements for the positive and negatively charged HA samples in comparison to the HA controls, sintered in both air and water. In particular, at the 4 h time point, a significant increase was seen in the amount of DNA (thus the % viability of cells measured for the two controls differs significantly in that the air control sample has an initial cell viability measurement that is four times that of the water control sample. If this is taken as the starting point, comparison of the relative increase in % viability for the air and water samples is interesting. It can be seen that the cell viability of the polarized water vapour-sintered samples, relative to the control, has increased more rapidly than the equivalent air-sintered samples. This may warrant further investigation.

The alamarBlue assay detects cell viability via metabolic activity. As has been seen, in this assay, no overall difference was seen between the air- or water vapour-treated negatively charged HA discs after 7 days. This is in contrast to the picogreen assay, which measures the quantity of DNA present. It appears that, even though an increase in cell number was seen (Fig. 4), no difference in the metabolic activity from the cell populations cultured on the negatively charged air-treated HA disc in comparison to the cells cultured on the negatively charged water vapour-treated disc was observed (Fig. 5). These aspects should be taken into account when considering the final application intended for the HA material. For example, it may be more important to increase the cell number on a tissue-engineered construct prior to patient implantation. Alternatively, it may be more important to be aware of the cell's metabolic activity if, for example, implanting a treated HA-based construct directly into the patient.

Overall, in terms of actual cell numbers recorded, there was a measurable increase in the number of cells attaching to charged HA surfaces as opposed to non-charged HA, as well as differences in the rate of proliferation on charged HA samples. Other significant observations were apparent in relation to the % viability results measured with the alamarBlue assay. An increase in metabolic activity of the osteoblast cells in contact with charged HA was seen in all cases relative to the controls. No differences in the final measures of metabolic activity were observed for either positively or negatively charged samples, although the initial values for the two control samples differed by a factor of four.

5. Conclusions

The results demonstrate that polarizing HA discs and creating both positive and negative charges on the surfaces can influence the in vitro response and activity of osteoblastic cells. It has been shown that all the HA samples involved in this study (both charged and uncharged) supported osteoblast growth and attachment. A charge (either positive or negative) on the HA surface produced an increase in cell attachment at 4 h and cell proliferation over a 7 day period. It can also be seen that metabolic activity was significantly increased as a result of adding a charge to the HA surfaces. Both negative and positive charges present on HA surfaces demonstrated a significant increase in cell metabolic activity in comparison to the uncharged HA and tissue culture plastic controls. Thus, modifying the HA surface to increase cell proliferation and metabolic activity using the described manufacturing techniques could have great potential in improving HA interaction in terms of encouraging cellular activity and promoting faster healing.

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References


