Nanohydroxyapatite shape and its potential role in bone formation: an analytical study

Priya Kalia1, Gema Vizcay-Barrena2, Jian Ping Fan3, Alice Warley2, Lucy Di Silvio1,† and Jie Huang3,†

1Biomaterials, Biomimetics and Biophotonics Division, Dental Institute, King’s College London, Tower Wing, Guy’s Hospital, London SE1 9RT, UK
2Centre for Ultrastructural Imaging, King’s College London, New Hunt’s House, London SE1 1UL, UK
3Department of Mechanical Engineering, University College London, Torrington Place, London WC1E 7JE, UK

Bone cells (osteoblasts) produce a collagen-rich matrix called osteoid, which is mineralized extracellularly by nanosized calcium phosphate (CaP). Synthetically produced CaP nanoparticles (NPs) have great potential for clinical application. However few studies have compared the effect of CaP NPs with different properties, such as shape and aspect ratio, on the survival and behaviour of active bone-producing cells, such as primary human osteoblasts (HOBs). This study aimed to investigate the biocompatibility and ultrastructural effects of two differently shaped hydroxyapatite [Ca10(PO4)6(OH)2] nanoparticles (HA NPs), round- (aspect ratio 2.12, AR2) and rice-shaped (aspect ratio 3.79, AR4). The ultrastructural response and initial extracellular matrix (ECM) formation of HOBs to HA NPs were observed, as well as matrix vesicle release. A transmission electron microscopy (TEM)-based X-ray microanalytical technique was used to measure cytoplasmic ion levels, including calcium (Ca), phosphorus (P), sodium (Na) and potassium (K). K/Na ratios were used as a measure of cell viability. Following HA NP stimulation, all measured cytoplasmic ion levels increased. AR2 NPs had a greater osteogenic effect on osteoblasts compared with AR4 NPs, including alkaline phosphatase activity and matrix vesicle release. However, they produced only a moderate increase in intracellular Ca and P levels compared with AR4. This suggests that particular Ca and P concentrations may be required for, or indicative of, optimal osteoblast activity. Cell viability, as measured by Na and K microanalysis, was best maintained in AR2. Initial formation of osteoblast ECM was altered in the presence of either HA NPs, and immuno-TEM identified fibronectin and matrilin-3 as two ECM proteins affected. Matrilin-3 is here described for the first time as being expressed by cultured osteoblasts. In summary, this novel and in-depth study has demonstrated that HA NP shape can influence a range of different parameters related to osteoblast viability and activity.

1. Introduction

Bone is a complex and dynamic tissue which responds to biochemical and systemic cues as well as mechanical loading [1,2]. It is a composite material which contains both organic and inorganic components (collagen and biological apatite, respectively). In developing and repairing bone, nanoscale particles of biological apatite are deposited within a mainly collagen I matrix (osteoid) that is laid down by osteoblasts [3]. Matrix vesicles (MVs), which accumulate an amorphous calcium phosphate (CaP), are also released by active osteoblasts and play a role in mineralization [4–6]. The composite structure of bone lends it a particular toughness and stiffness, providing rigidity and resistance to fracture [3]. Other basement membrane and extracellular matrix (ECM) proteins, such as fibronectin (FN), matrilins [7] and collagens IV and V, play an initial role in musculoskeletal cell attachment and ECM assembly and are essential
for healthy bone formation. However, how exposure to nanoparticles (NPs) by osteoblasts affects the deposition and organization of such ECM proteins is poorly understood.

Previous studies of natural bone suggest that the mineral component of bone is composed of apatite platelets with a thickness of 2–7 nm, length of 15–200 nm and width of 10–80 nm. These particles have also been described as being ‘needle’-shaped [3]. CaP NPs, such as hydroxyapatite (Ca₁₀(PO₄)₆(OH)₂, HA), can be artificially synthesized to be as small as the nanosized-HA plates found in native bone tissue (approx. 50 nm) [8]. These NPs potentially have an important role in bone tissue engineering, forming nanosized building blocks for bone tissue repair and/or replacement. Thian et al. evaluated the bioactivity of electrohydrodynamic atomized nanophosphate [9], as well as studying nanostructured HA coatings produced using magnetron sputtering and electrohydrodynamic spraying [10–13]. Nanoapatite has also been observed to form in various shapes, and with different aspect ratios, when synthetic HA has been incubated in simulated body fluid that contains similar ion levels to blood plasma [14]. The aspect ratio of the particles may also influence the dissolution of a degradable ceramic such as HA. However, the stimulatory effect of differently shaped CaP NPs on active osteoblasts, and the effect of uptake on intracellular ion levels has not previously been studied. In addition, a thorough, ultrastructural investigation into the response of osteoblasts to HA NPs of different shapes, as well as their influence on subsequent ECM formation, is also lacking. The idea that different NP shapes may differentially influence cell behaviour has been measured using apoptosis and proliferation assays [15], as well as protein expression analysis [16] and stains for NP uptake in a macrophage cell line [16–19]. An experiment testing two differently sized rod-shaped HA NPs on an osteoblast-like (osteosarcoma) cell line found that although the smaller particles caused a significant amount of cells to apoptose, there was no difference in alkaline phosphatase activity [20]. Another study found that rod-shaped particles caused primary osteoblasts to decrease their proliferation after 3 days of culture compared with spherical-shaped. A change of morphology was also visible under scanning electron microscopy (SEM) [21]. The effect of different-shaped NPs on the intracellular ion content of primary osteoblasts, as measured using TEM-based microanalysis, as well as ultrastructural and ECM changes other than NP internalization [22] have not, to the authors’ knowledge, been previously investigated [18]. Whether NP treatment enhances MV formation and release is currently unknown. With bone regeneration being the ultimate goal of using HA NPs, our hypothesis was that the osteoblast proliferation, function and ECM production would respond to HA NPs, and that this response would be sensitive to HA NPs having two different shapes and aspect ratios. In addition to traditional biochemical assays for measuring cell proliferation, we also used a highly sensitive, TEM-based microanalysis technique that measures cytoplasmic ion concentrations, including potassium/sodium (K/Na) ratio [23,24]. To gain further information of the effects of HA NP exposure on osteoblast physiology and differentiation, microanalysis was also used to measure intracellular calcium (Ca) and phosphorus (P) levels, as a complement to other measures such as alkaline phosphatase (ALP) activity. The effects of HA NPs on the ultrastructural composition of osteoblasts (MVs and other intracellular compartments), and early matrix and MV release was studied.

2. Material and methods

2.1. Nanoparticle production

HA NPs were synthesized using the precipitation method. A total of 200 ml 0.3 M phosphoric acid (Aldrich) was slowly dripped into in 200 ml 0.5 M Ca hydroxide suspension under shear stress (250 and 4500 r.p.m.). The pH was adjusted to above 11 by adding ammonium hydroxide. The resultant precipitate was then left at room temperature and aged for 7 days [25].

2.2. Nanoparticle characterization and analysis

2.2.1. Fourier-transformed infrared spectroscopy

Fourier-transformed infrared spectroscopy was carried out on a Perkin Elmer Spectrum One FTIR spectrometer, with a Perkin Elmer Universal ATR sampling accessory. Perkin Elmer Imaging Suite version 4.1 was used to collect the data, between the range of 6000–250 cm⁻¹, and the background signal was subtracted from sample data.

2.2.2. Inductively coupled plasma mass spectroscopy

Quantification of media ion concentrations (Na, K, Ca and P), using inductively coupled plasma mass spectroscopy (ICP-MS), was carried out to obtain an approximate measure of the concentration of ions released by HA NPs of different shapes, which osteoblasts would be exposed to during in vitro tests. For this, 10 μg HA NPs (rice- and round-shaped, abbreviated as AR2 and AR4) were incubated in 10 ml supplemented osteoblast media (see below) at room temperature for 24, 48, 96 and 144 h (7 days), in polypropylene tubes. Samples were kept rotating for the entire incubation time (Denley Spiramix 10 roller system). The supernatant media was removed to fresh tubes and diluted 1/20 with distilled water and analysed using ICP-MS. The ICP-MS instrument used was a Perkin Elmer/Sciex Elan 6100DRC, with a Perkin Elmer A993+ autosampler using Perkin Elmer Elan v. 3.3 software. Argon was used as the plasma gas. The elements and isotopes monitored were: 39K, 23Na, 44Ca and 31P. For all elements except Ca, the most abundant isotope was monitored, and standard curves made up with the same isotope. For Ca, the most abundant isotope, ⁴⁰Ca matches the mass of the plasma gas, argon (⁴₀Ar). In order to distinguish between Ca and Ar, the ⁴⁰Ca isotope was monitored instead. A reaction cell was used for Ca, with ammonia flowing at 0.7 ml min⁻¹. Each isotope was monitored for 4 s, and five such replicates were averaged to produce the ‘measured intensity’ reading quoted in the results. Standards were prepared by spiking the cell culture medium with the elements to be measured at concentrations of 0.1, 1, 10, 100, 1000 and 10 000 μg l⁻¹.

2.3. Osteoblast cell culture

Primary human osteoblasts (HOBs) were isolated from bone discarded during elective orthopaedic surgery, and cells grown using an explant outgrowth technique, as described previously [26]. Cells were plated onto tissue culture plastic and passaged when confluent. For proliferation, alkaline phosphatase (ALP) activity and lactate dehydrogenase (LDH) assays, cells were seeded onto tissue culture plastic according to standard culture protocols in osteogenic media (10⁻⁷ M water-soluble dexamethasone, 10 mM β-glycerophosphate and 0.02 mM ascorbic acid (all Sigma)). Briefly, 40 000 HOBs were seeded per plate and flushed with 2 ml media per well, either control media (no NPs) or with AR2 or AR4 NPs on June 17, 2017 http://rsif.royalsocietypublishing.org/ Downloaded from http://rsif.royalsocietypublishing.org/
Cell proliferation: DNA (Hoescht) assay
Total cellular DNA was measured using the Hoescht 33258 dye (B2883, Sigma-Aldrich, Dorset, UK). Briefly, this was diluted from 1 to 1.0 mg ml⁻¹ in saline sodium citrate (SSC) buffer added to each standard or sample well in the Fluoronuncate plate at a 1 : 1 ratio with sample volume. Samples were read in an Opsys MR plate reader (Dynex Technologies, UK) at 460 nm, using Revelation Quicklink software (v. 4.24). Adenine-thymidine Hoescht 33258-specific fluorescence was read at 460 nm.

2.5. Alkaline phosphatase activity
The ALP activity was used as a phenotypic marker of osteogenesis [26]. For the assay, 10 ml of substrate reagent was mixed (40 mg p-nitrophenyl phosphate, 34 mg MgCl₂·H₂O, 80 µl Triton X-100 12.5% v/v (Sigma, UK) in 0.1 M glycine (pH 10.3) and a p-nitrophenyl standard (Sigma, UK, 100 µg ml⁻¹). After 3 and 7 days of culture, samples were washed in phosphate-buffered saline (PBS), and cells lysed by adding DNase/RNase-free water followed by three freeze–thaw cycles, then transferred to Eppendorf tubes and spun in an ultracentrifuge at 10000 r.p.m. for 10 min. Samples were reacted with substrate reagent in 1 : 1 ratio in black 96-well plates (Clinplate, Thermo Fisher Scientific), sealed (pre-cut transparent microplate sealers, Greiner Bio-One, Stonehouse, Glous, UK) and shaken for 2 min (Titertek, Flow Laboratories, UK) and measured at 410 nm (reference wavelength Stonhouse, Glous, UK) and shaken for 2 min (Titertek, Flow sealed (pre-cut transparent microplate sealers, Greiner Bio-One, in black 96-well plates (Clinplate, Thermo Fisher Scientific), 10 min. Samples were reacted with substrate reagent in 1 : 1 ratio with sample volume. Samples were read in an Opsys MR plate reader (Dynex Technologies, UK) at 460 nm, using Revelation Quicklink software (v. 4.24). Adenine-thymidine Hoescht 33258-specific fluorescence was read at 460 nm.

2.6. Preparation of cells for transmission electron microscopy and immuno-transmission electron microscopy
For imaging, microanalysis and immune-TEM of HOB cultures, cells on coverslips were fixed in 0.05% glutaraldehyde in phosphate buffer, rinsed several times and then treated with 1% (w/v) osmium tetroxide in phosphate buffer. The coverslips were then infiltrated with epoxy resin (TAAB, UK) and then sectioned. Sections in the plane of the monolayers, as well as cross sections through the sample (profile view of the cell culture layers) were made. Ultrathin sections (70–90 nm) were cut using a Reichert-Jung Ultracut E ultramicrotome, mounted on mesh copper grids and contrasted using uranyl acetate and lead citrate.

For TEM immunogold labelling, cells grown on coverslips were fixed in 0.05% glutaraldehyde in phosphate buffer, washed in 50 mM glycine in phosphate buffer, rinsed briefly in phosphate buffer and water, and dehydrated through a graded series of ethanol. The coverslips were then infiltrated with epoxy resin (TAAB, UK) and then sectioned. Sections in the plane of the monolayers, as well as cross sections through the sample (profile view of the cell culture layers) were made. Ultrathin sections (70–90 nm) were cut using a Reichert-Jung Ultracut E ultramicrotome, mounted on mesh copper grids and contrasted using uranyl acetate and lead citrate.

2.7. Transmission electron microscopy, immuno-transmission electron microscopy and electron dispersion of X-rays spectroscopy
HA NPs were imaged using an FEI Tecnai T20 electron microscope, and images captured using a Gatan ultrascan camera. For TEM and immuno-TEM imaging, sections were examined on a FEI Tecnai 12 transmission microscope operated at 120 kV and images acquired using an AMT 16 000 M digital camera. For elemental analysis (electron dispersion of X-rays spectroscopy (EDS)), a Tecnai T12 microscope equipped with an EDAX X-ray detector and Genesis software was used. HA NP aspect ratios were calculated based upon measurements of length versus width of a minimum of 25 samples per group. For both side (transverse) and top views of HOB TEM sections, MVs were quantified for each group (n = 5–10). MVs released by cells at the top of cell cultures or between cell layers were quantified and averaged for each group (control, AR2 and AR4). MVs which had clustered at the plasma membrane of cells were also quantified (figure 8c).

Intracellular ion concentrations can be measured using TEM-EDS microanalysis [23]. In this study, the use of the microanalysis technique was applied as a novel measure of osteoblast response to HA NPs, over a time course. Intracellular concentration of sodium and potassium were estimated using TEM X-ray microanalysis. Cells were grown on gold TEM grids as described in Warley et al. [27]. At each time point (24 h, 48 h and 7 days), the grids were removed from the culture and washed briefly by dipping into ice cold distilled water, then cryofixed by plunging into liquid nitrogen. The grids were then freeze-dried and coated with a thin layer of carbon before analysis. Point analysis of Na, K, Ca and P in the cell cytoplasm was carried out, avoiding internalized HA NPs. Quantification was carried out using the Hall method. Elements were quantified as mmol/kg by reference to previously prepared internal calibration standards [23]. A minimum of 10 cells per group were analysed at each time point.

2.8. Statistics
A minimum of four samples were tested per group for all assays unless otherwise stated, apart from microanalysis where a minimum of 10 cells were analysed per group for each time point. For statistical analyses, SPSS v. 17.0 was used. The Mann–Whitney U non-parametric test was used to compare between two samples. For non-parametric tests, the Kruskall–Wallis non-parametric test was used to compare groups of more than two samples.

3. Results
3.1. Nanoparticle characterization and analysis
3.1.1. Fourier-transformed infrared spectroscopy
FTIR showed that both AR2 and AR4 NPs had similar spectra (figure 1). The peaks at 1023 cm⁻¹ (AR4) or 1024 cm⁻¹ (AR2) are most likely PO₄³⁻ ν₃, confirming the presence of HA. The
bands at 874 cm$^{-1}$ (both groups), and at 1416 cm$^{-1}$ (AR4) or 1421 cm$^{-1}$ (AR2) suggest the presence of CO$_3^{2-}$/CO$_2^{2-}$ [28].

3.1.2. Transmission electron microscopy and electron dispersive X-ray spectroscopy

TEM of the HA NPs showed a size of approximately 50 × 20 nm for AR4, and a rice-grain morphology (figure 2a,b), and an approximately 20 nm diameter and spherical shape for AR2 (figure 2c,d). Aspect ratios are listed in table 1. High-angle annular dark-field-scanning TEM (HAADF STEM) showed the presence of elements of high atomic number (for example, higher than H, O or C) within the HA NPs. EDS was then used to confirm the presence of Ca and P (figure 3).

3.1.3. Inductively coupled plasma mass spectroscopy

Measurement of Ca, P, Na and K concentrations in cell culture media, using ICP-MS, showed that following incubation with
3.2. Proliferation assay
Cell proliferation, as measured by total DNA, was lower than controls in the presence of both groups of HA NPs, at 3 and 7 days compared with controls (figure 5), although this was only statistically significant for AR2, when compared to the control group ($p = 0.043$ and $p = 0.034$ at 3 and 7 days, respectively).

3.3. Alkaline phosphatase activity
ALP activity increased in the presence of NPs at 3 days, and in the AR2 group at 7 days (figure 6). This was found to be statistically significant ($p = 0.034$) for both AR2 and AR4 at 3 days, and for AR2 at 7 days ($p = 0.034$).

3.4. Transmission electron microscopy-based ultrastructural observations and matrix vesicle release, microanalysis of cytoplasmic ion concentrations and immuno-transmission electron microscopy
Transverse sections, looking at a ‘side view’ of monolayer cultures revealed a 5–10 cell thick cell layer. Osteoblasts appeared to self-organize in culture. In all groups, cells at the top of the 5–10 cell-layer structure (media-facing side, as opposed to tissue culture plastic-facing) contained higher numbers of vesicles and higher MV release was observed (figure 7), suggesting that these cells may have a greater potential for promoting bone/matrix mineralization. After 7 and 28 days, cells displayed normal morphology. NP-stimulated cell cultures appeared more active, containing a greater number of Golgi bodies, mitochondria and MVs (figure 7b), compared with controls (figure 7a). In some cells, NP-like clusters were observed at the plasma membrane, especially at the ‘top’ layer of cultures (figure 8a). In addition, intracellular osmium-stained vesicles were observed to a greater extent in NP-stimulated samples following 7 days’ culture (figure 8b,c), especially at the top layer of cells. Counting of the released MVs, at the top of the cell layers or between cell layers, found an overall decrease in vesicles released post-HA NP stimulation, although this was not statistically significant (figure 8c, $p = 0.10$). There were more MVs observed in AR2-stimulated HOB cultures than in the AR4 group, although there was no statistically significant difference observed between the two groups ($p = 0.12$).

Random, disorganized fine ‘threads’, which did not show typical collagen banding patterns (figure 7c), were observed frequently within the extracellular space of control cultures, but not in either type of HA NP cultures (figure 7d). There were also fewer ‘gaps’ visible between cells following NP stimulation. Immuno-TEM identified the ‘thread’-like fibrils as FN (see arrows, figure 9a). FN-positive staining fibrils were not as apparent in the HA NP-treated groups and did not show the banding pattern associated with collagen (figure 9b). Following rice-shaped HA NP exposure, matrilin-3 was found to form larger polymeric units [7] as identified by clusters of the immunogold secondary antibody (figure 9d). These large clusters were not visualized in the control HOB samples (figure 9c).

Measuring cytoplasmic Ca and P concentrations showed highly elevated levels in both AR2 and AR4 groups at 24 h. This was statistically significant for AR4 ($p = 0.05$ for Ca and P, table 2). The Ca and P concentration in AR4 seemed to decrease between 24 and 48 h, whereas it increased in AR2. The reverse was observed at 7 days, with the AR4 group having the highest Ca and P levels (although Ca levels were still elevated in the AR2 group).

Table 1. Length and width measurements of rice- and round-shaped HA NPs, as well as corresponding aspect ratios ($n = 25$).

<table>
<thead>
<tr>
<th>HA NP shape</th>
<th>length (nm)</th>
<th>width (nm)</th>
<th>aspect ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>rice (AR4)</td>
<td>107.8 ± 27.0</td>
<td>28.4 ± 9.1</td>
<td>3.79</td>
</tr>
<tr>
<td>round (AR2)</td>
<td>39.7 ± 6.6</td>
<td>18.7 ± 5.0</td>
<td>2.12</td>
</tr>
</tbody>
</table>
K/Na ratios measured with TEM microanalysis have been used as a sensitive indicator of cell viability and proliferation [29]. Following treatment with HA NPs for 24 and 48 h, both types of HA NPs had lowered K/Na ratios compared with controls, suggesting that they had lower viability. The change in K/Na ratio was significantly lower for AR4 group \((p = 0.05)\), indicating lower cell viability. These data did not correlate with the DNA assay results, which showed that the AR2 group had the lowest cell proliferation.

4. Discussion

Previous work in the literature has described initial NP uptake by cells and their effects on cell proliferation and protein expression [16]. However, a thorough ultrastructural investigation into the effects of HA NPs on osteoblasts, especially of different NP shapes, and MV release has not previously been carried out, to the authors’ knowledge. Similarly, the use of microanalysis to understand how exposure to NPs alters the intracellular ion balance has not previously been tested in osteoblasts, which we have shown here. Furthermore, we have been able to demonstrate that exposure to NPs may be influenced by NP shape, in terms of changes to osteoblast proliferation, ALP activity and intracellular ion levels. A previous study compared ALP activity following NP exposure to two rod-shaped NPs of different sizes and did not find any significant difference between them [19,21]. Another study looking at an osteosarcoma-derived cell line found that cell proliferation was decreased in the presence of spherical-shaped HA NPs [21]. The group also reported a change in cell morphology, as observed via SEM, although this was not based on ultrastructural analysis of ECM proteins or intracellular ion concentration. This study provides additional in-depth information to these initial reports that tested HA NPs of different shapes. Two ECM proteins, which were affected by the exposure of cells to NPs, FN and matrilin-3, were identified using immuno-TEM. This work has shown that osteoblast cell layers appear to self-organize, such that the top layer of cells is highly active and contains numerous MVs. MV release by HOBs into the extracellular space was altered in the NP-stimulated groups.

Figure 4. Graphs comparing release of (a) Ca, (b) P, (c) K and (d) Na by HA NPs following 24, 48, 96 and 144 h (7 days) incubation in cell culture media, as measured using ICP-MS. Note that the media ion concentrations decreased after incubation with HA NPs at all time points.

Figure 5. Graph representing results of DNA (Hoescht) assay at 3 and 7 days post-incubation of HOBs with HA NPs (AR4 and AR2). Cells exposed to AR2 showed the lowest proliferation over the 7-day period. Statistical significance is shown with an asterisk (*) where \(p \leq 0.05\).

Figure 6. Results of ALP assay, showing a change to ALP levels following 3 and 7 days' treatment with AR4 and AR2. Statistical significance is shown with an asterisk (*) where \(p \leq 0.05\).
**Figure 7.** Images from TEM, showing in (a) and (b) a transverse view of HOB cell layers following 7 days' culture, (a) controls (no NPs) and (b) with AR2, which showed higher levels of MVs towards the top of the culture, compared with controls (see arrow); (c) thin 'threads' in the extracellular space of control osteoblast cultures (see arrow); (d) a lack of such ECM structures in the round HA NP-stimulated group. The scale bars in (a) and (b) are 1 μm; in (c) and (d) they are 500 nm.

**Figure 8.** TEM images of MVs release by round-shaped HA NP-stimulated osteoblasts, including (a) a growing cluster of MVs at the cell surface and (b) 'chains' of MVs being released into the extracellular space (scale bar, 100 nm for all images). (c) Number of MVs released by primary HOBs under control conditions and after stimulation with rice- or round-shaped HA NPs. There was no significant difference between groups, although AR2 had higher MV release than AR4.
Despite extensive research into ceramic NPs and their in vitro biocompatibility, as well as the effect of HA NPs on other cell types [18,30], this is the first work to specifically document the changes in osteoblast ultrastructure and ECM following HA NP exposure. Furthermore, it attempts to quantify cytoplasmic Ca and P, based on a microanalysis technique which has not been used with cultured osteoblasts. In terms of cultured cells, microanalysis has been limited to use in other cell types, such as red blood cells and monocytes [15,24]. In the first description of MVs, Anderson [4] used TEM, EDS and XRD techniques to detect Ca and phosphate within sections of cartilage and bone. Motskin et al. [15,30] used microanalysis to measure the Ca and P content of HA NPs engulfed within monocyte-macrophages in vitro and to study NP dissolution, as opposed to the specific measurement of cytoplasmic Ca and P concentrations. Relating the increase in ALP activity to the different intracellular Ca and P levels, we suggest that a moderate increase in Ca and P levels for 24 h, as experienced following AR2 HA NP exposure, may be stimulatory for increasing osteoblast function, for example, via increased MV CaP accumulation and release. Xu et al. [16] showed an increase in expression of Ca-regulating proteins after stimulation with needle-shaped or round-shaped HA NPs. By contrast, higher Ca and P ion levels may not be as effective, or indeed detrimental, as observed with AR4 HA NPs. The decrease in cell proliferation in the AR2 group, as detected using DNA quantification, may be a result of increased cell differentiation, as suggested in an osteoblast study by Robinson et al. [31]. A similar result was observed by Xu et al. [16] except between sharper, needle-shaped HA NPs and round-shaped HA NPs.

Intracellular Ca levels have an important role in osteoblast homeostasis, regulation and apoptosis [32–34]. Interestingly, a time-dependent change in Ca$^{2+}$ levels was observed over 7 days, when cells were exposed to HA NPs. Whether this change was due to HA NPs entering into the cell cytoplasm followed by NP dissolution is yet to be determined. Motskin et al. [15] showed that microparticles and NPs made via different materials synthesis techniques had different effects on human monocyte-derived macrophages, with some showing significantly greater uptake and/or toxicity. TEM images demonstrated that HA NPs were dissolved by cells over time, whereas microparticles broke into NPs before they were dissolved by cells [15]. Using two complimentary methods of assessing cell viability and/or proliferation (DNA quantification and Na/K microanalysis), we have shown that cell viability was affected by treatment with HA NP over 24 h, 48 h and 7 days, and that HA NPs with two different shapes resulted in different microanalysis results. The effects of four differently shaped NPs (needle, spherical, long rod-like and short rod-like) on the proliferation, ALP activity, intracellular reactive oxygen species and apoptosis, has also been carried out by Xu et al. [18].

Although extracellular phosphate is essential to the regulation of mineralization [35], in this experiment we have actually observed a decrease in phosphate-media levels following incubation with HA NPs, rather than increase. Differences in, or changes to surface chemistry, with or without ion adsorption/de novo apatite formation, may have contributed to changes in NP toxicity [15], uptake, intracellular dissolution and NP surfaces, which have been linked to NP morphology [36,37], may have also contributed to the microanalysis results.

Another observation in this study was the apparent self-organization of osteoblasts following cell culture for 7 days, as demonstrated by transverse TEM sections showing that HOBs formed a four- to five-cell thick layer on tissue culture plastic. MVs, which were visualized using TEM, were much more prevalent in the top osteoblast layer than at the bottom of the cell-layer structure. Budding and release of MVs into the extracellular space were also located at the top layer of the culture. These TEM images show some evidence that osteoblasts are able to self-organize in vitro, such

**Figure 9.** Immuno-TEM of HOBs for (a) FN (control group), (b) FN (AR4), (c) matrilin-3 (control group) and (d) matrilin-3 (AR4). Gold labelling showed that FN was shown to co-localize with the fine fibrils that were decreased in the presence of HA NPs (fibrils with no apparent collagen double-banding pattern). Matrilin-3, which is important for normal endochondral ossification during skeletal development, was found to have different staining patterns following HOB treatment with HA NPs, showing increased oligomerization and/or polymerization. Arrows indicate positive immunogold labelling.
that the most ‘active’ cells are located at the top layer of the culture. In this five-cell thick in vitro culture, we have shown that by self-organizing, the HOBs have exposed their MV-releasing region of the outer membrane to the extracellular environment, especially at the top of the culture where MV release is most apparent. By observing cryo-electron microscopy of developing mouse calvaria and long bones, and using elemental analysis (EDS), Mahamid et al. [5] hypothesized that phosphate first concentrates within MVs, followed by the sequestration of Ca, forming an amorphous CaP. We have also shown the deposition of osteoid/MVs by these cells from the top of the cultures and within the extracellular space, similar to that imaged by Ecarot-Charrier et al. [38], and that their release into the extracellular space appeared to be altered by HA NPs of either morphology. The statistically significant increase in ALP activity in the round-shaped HA NP (AR2) group suggests that osteoblasts in this group may be more active compared to those stimulated with AR4 [39].

In terms of changes observed in the ECM, which plays a crucial role in bone formation, we have shown that thin fibrils observed via TEM stain positively with anti-FN antibody, and appear to be altered in the presence of HA NPs. FN is required for collagen assembly and maintenance, as well as bone development and repair [40–42]. HA has been shown to avidly bind proteins and other ions and molecules, including FN [43–45]. It may be that the FN-binding ability of HA may have interfered with the in vitro formation of normal FN networks. It is also not known whether the changes to the ECM are linked to matrilin-3 oligomerization. Matrilins-1 and 3 have been shown to be expressed temporally within the growth plate and also have been observed to form extended filamentous networks [7]. Matrilin-3 expression has not previously been observed in cultured osteoblasts [7] and is reported here for the first time. The role of biomaterials in regulating such ECM proteins is an area of current investigation.

5. Conclusion
In conclusion, HA NPs of two different shapes, rice-shaped (AR4) and round-shaped (AR2), were shown to influence HOB proliferation, cytoplasmic ion levels, ultrastructure and ECM assembly. AR2 and AR4 were shown to adsorb Ca, P, Na and K at different levels, from cell culture media. A sensitive TEM-based microanalysis technique was used to detect subtle changes in cell viability and cytoplasmic ion levels, by measuring the K/Na ratio and Ca and P concentrations. ECM proteins FN and matrilin-3 were shown to change in organization following culture with HA NPs. Differences in ion levels were found following HA NP treatment, which corresponded to assays for cell proliferation (DNA) and osteoblast function (ALP).

### Table 2. Cytoplasmic HOB Ca, P, Na and K concentrations following 3 and 7 days’ culture, with and without HA NPs (AR4 and AR2). Differences in ion levels were found following HA NP treatment, which corresponded to assays for cell proliferation (DNA) and osteoblast function (ALP).

<table>
<thead>
<tr>
<th></th>
<th>Ca</th>
<th>P</th>
<th>Na</th>
<th>K</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>16.47±4.50</td>
<td>5.94±0.07</td>
<td>16.67±1.14</td>
<td>5.94±0.07</td>
</tr>
<tr>
<td>AR4</td>
<td>22.5±5.50</td>
<td>6.0±0.07</td>
<td>22.5±5.50</td>
<td>6.0±0.07</td>
</tr>
<tr>
<td>AR2</td>
<td>15.5±4.50</td>
<td>5.5±0.07</td>
<td>15.5±4.50</td>
<td>5.5±0.07</td>
</tr>
<tr>
<td>48 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>2.81±1.41</td>
<td>1.20±0.07</td>
<td>2.81±1.41</td>
<td>1.20±0.07</td>
</tr>
<tr>
<td>AR4</td>
<td>3.81±2.24</td>
<td>1.6±0.07</td>
<td>3.81±2.24</td>
<td>1.6±0.07</td>
</tr>
<tr>
<td>AR2</td>
<td>3.81±2.24</td>
<td>1.6±0.07</td>
<td>3.81±2.24</td>
<td>1.6±0.07</td>
</tr>
<tr>
<td>7 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>5.33±3.02</td>
<td>2.0±0.07</td>
<td>5.33±3.02</td>
<td>2.0±0.07</td>
</tr>
<tr>
<td>AR4</td>
<td>6.5±3.0</td>
<td>2.3±0.07</td>
<td>6.5±3.0</td>
<td>2.3±0.07</td>
</tr>
<tr>
<td>AR2</td>
<td>6.5±3.0</td>
<td>2.3±0.07</td>
<td>6.5±3.0</td>
<td>2.3±0.07</td>
</tr>
</tbody>
</table>

rsif.royalsocietypublishing.org J. R. Soc. Interface 11: 20140004
further analysis of NP effects on osteoblast ultrastructure and ECM production is an important and critical biological consideration for future work.

Acknowledgements. The authors would like to thank Orthopaedic Research UK (ORUK) for their generous support and Dr Giuseppe Cama for assistance with FTIR analysis of HA NPs.

References


