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Hydroxyapatite nanoparticle-containing scaffolds for the study of breast cancer bone metastasis

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Abstract

Breast cancer frequently metastasizes to bone, where it leads to secondary tumor growth, osteolytic bone degradation, and poor clinical prognosis. Hydroxyapatite $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ (HA), a mineral closely related to the inorganic component of bone, may be implicated in these processes. However, it is currently unclear how the nanoscale materials properties of bone mineral, such as particle size and crystallinity, which change as a result of osteolytic bone remodeling, affect metastatic breast cancer. We have developed a two-step hydrothermal synthesis method to obtain HA nanoparticles with narrow size distributions and varying crystallinity. These nanoparticles were incorporated into gas-foamed/particulate leached poly(lactide-co-glycolide) scaffolds, which were seeded with metastatic breast cancer cells to create mineral-containing scaffolds for the study of breast cancer bone metastasis. Our results suggest that smaller, poorly-crystalline HA nanoparticles promote greater adsorption of adhesive serum proteins and enhance breast tumor cell adhesion and growth relative to larger, more crystalline nanoparticles. Conversely, the larger, more crystalline HA nanoparticles stimulate enhanced expression of the osteolytic factor interleukin-8 (IL-8). Our data suggest an important role for nanoscale HA properties in the vicious cycle of bone metastasis and indicate that mineral-containing tumor models may be excellent tools to study cancer biology and to define design parameters for non-tumorigenic mineral-containing or mineralized matrices for bone regeneration.

Keywords

Bone metastasis; Breast cancer; Calcium phosphate; Hydroxyapatite; Nanoparticle; Scaffold

1. Introduction

The bone microenvironment is defined by a composite matrix that consists primarily of organic collagen and inorganic mineral. Bone mineral is closely related structurally to geologic hydroxyapatite (HA) ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$); however, bone apatites are less crystalline, more soluble, and vary in their molecular composition as carbonate ions often substitute for the hydroxide and phosphate ions ($\text{Ca}_{10-2x/3}(\text{PO}_4)_{6-x}(\text{CO}_3)_x(\text{OH})_{2-x/3}$) [1]. Furthermore, the size and crystallinity of bone apatite crystals change during development,

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Appendix. Supplementary data

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growth, and as a result of bone pathologies [2,3]. While HA is largely known for its role in conferring structural and mechanical properties to bone, it also functions as a bioactive material that directly regulates the behavior of both normal and transformed cells [4,5]. For example, HA has been shown to enhance normal bone formation [6] and to alter growth and expression profiles of bone metastatic tumors [5,7]. Nevertheless, it remains unclear whether or not apatite crystal properties directly modulate the pathogenesis of bone diseases, in general, and bone metastasis, in particular. This lack of understanding is partly due to a paucity of culture systems that recreate 3-D tumor microenvironmental conditions to study changes in tumor cell behavior as a function of varying HA nanoscale properties. Therefore, we sought to utilize synthetically prepared HA nanoparticles to determine the impact of particle size and crystallinity on mammary cancer cell activity.

The vast majority of advanced stage breast cancers metastasize to the skeleton, forming secondary tumors and interfering with bone remodeling to create predominantly osteolytic lesions [8]. These lesions can lead to severe bone pain, pathological fracture, hypercalcaemia, and an overall poor clinical prognosis [9]. Osteolysis due to bone metastasis is linked to tumor-derived soluble factors, such as interleukin-8 (IL-8) and parathyroid hormone related peptide (PTHrP), that de-couple the homeostatic balance between bone formation and bone resorption [8,10]. In particular, IL-8, a molecular mediator of osteolysis [5,10] and tumor progression [11,12], is dramatically upregulated in breast cancer tissue relative to normal breast tissue in patients [13], and in breast cancer cells that preferentially metastasize to bone rather than lungs [14].

Though the exact mechanisms by which breast cancer cells preferentially target bone and induce pathological remodeling remain unclear, mounting evidence implicates the bone microenvironment and specifically HA in this pathogenesis. For example, mammary tumor cells increase expression of osteoclastogenic factors upon colonizing bone *in vivo* [14]. Additionally, IL-8 secretion by breast cancer cells is enhanced through cell-HA interactions, and stimulates osteoclast-mediated osteolysis [5,10]. These studies illustrate the important role that bone mineral might play in influencing osteolytic metastasis. Since the size and crystallinity of biogenic apatite nanocrystals change as a function of age and disease [2,3], further study is required to evaluate the role of these nanoscale properties of HA itself on metastatic bone disease.

Tissue engineered (TE) systems, originally created for regenerative medicine, can be used as tools to recreate tumor microenvironmental conditions *in vitro*. Tumor cells seeded in scaffold-based TE systems assemble into tumor-like tissues [11] and exhibit phenotypes that more closely mimic *in vivo* behavior as compared to conventional two-dimensional (2-D) cultures [7]. Furthermore, biologically active materials or molecules, such as mineral particles or proteins, can be incorporated into TE scaffolds and presented to cells in a spatially controlled manner [15]. Bone-like TE systems, including silk-based scaffold systems [16], decellularized osteoblast matrices [7], and polymer-HA composites [5,6,17] have been created to mimic certain features of bone, and several of these systems have been used to study tumor cell behavior [5,7]. Since bone metastasis can modify the crystallinity and particle size of the mineral component of bone [2], a controllable platform is needed to independently interrogate cellular responses to a wide range of HA particle sizes and crystallinities in a pathologically-relevant culture microenvironment.

2. Materials and methods

2.1. Particle preparation

HA nanoparticles were synthesized through a two-step process in which a typical precipitation reaction of a calcium salt with a phosphate salt was followed by hydrothermal

aging of the precipitate to obtain particles with a narrow size distribution (Fig. 1). All chemicals for these reactions were obtained from Sigma Aldrich and used as received. A solution of $(\text{NH}_4)_2\text{HPO}_4$ (90 mL, 10 mM) was added drop-wise into a solution of $\text{Ca}(\text{NO}_3)_2$ (150 mL, 10 mM) under rapid stirring at 4 °C in an ice-water bath for a final calcium to phosphate ratio of 1.67 (A1-A3 particles). The pH of the starting solutions was adjusted to 9–9.5 with 0.1 M NH_4OH . In a second reaction, particles were synthesized from a different set of precursors. A solution of Na_2HPO_4 (90 mL, 10 mM) was added drop-wise into a solution of CaCl_2 (150 mL, 10 mM) under rapid stirring at 4 °C until the calcium to phosphate ratio was 1.67 (B1 particles). The pH of the starting solutions was adjusted to 9–9.5 with 0.1 M NaOH . In both cases, the reactions were allowed to proceed for 1 hr, and then stirred at 20 °C for an additional 12 hr. The resulting opaque suspensions were concentrated 3-fold by centrifugation (Thermo Scientific Sorvall Legend RT + Centrifuge, 3600 g, 5 min) of 45 mL aliquots. After centrifugation, two-thirds of the clear supernatant was decanted. The remaining suspension (15 mL) was sonicated for 30 min (Bransonic 1510R-MT), placed into a pressure vessel (Parr Instrument Company 276AC-7304), and aged at 180 °C for the indicated time periods (Table 1) in an oven (GS Blue M Electric model no. OV12A). After aging, the particles were removed from the pressure vessel, washed with NH_4OH (0.1 M) and deionized water ($\text{DI-H}_2\text{O}$) to remove soluble salts, rinsed with acetone, and then dried at 20 °C (A2, A3, and B1 particles). Poorly crystalline particles were collected following the precipitation reaction of $\text{Ca}(\text{NO}_3)_2$ and $(\text{NH}_4)_2\text{HPO}_4$. The precipitate was washed with NH_4OH and $\text{DI-H}_2\text{O}$, rinsed with acetone, and dried at 20 °C (A1 particles). Commercial HA nanopowder obtained from Sigma Aldrich (provider specified particle size <200 nm) was used for comparison.

2.2. Particle characterization

Particles were characterized by Transmission Electron Microscopy (TEM) for shape and morphology, and X-ray Diffraction (XRD) and Fourier Transform Infrared (FTIR) Spectroscopy for crystallinity and phase information. For TEM, particles were suspended in acetone and dropped onto a carbon-coated copper TEM grid (Electron Microscopy Sciences). Samples were examined by brightfield TEM (FEI Tecnai T-12 Spirit, 120 kV), and particle sizes were determined by image analysis of the TEM images through ImageJ (NIH). Dried particles were examined via powder XRD (Scintag Inc. PAD-X theta-theta X-ray Diffractometer, $\text{CuK}\alpha$ 1.54 Å, accelerating voltage 45 kV, current 40 mA, continuous scan, 2.0 deg/min). Particle sizes were determined from the peak broadening of the {002} peak of HA (25.88°) by application of the Scherrer equation using a Al_2O_3 standard (Software: JADE 9, Materials Data, Inc.). For FTIR (Mattson Instruments 2020 Galaxy Series FT-IR), KBr pellets were prepared from dried particles and spectra acquired (res 4.0 cm^{-1} , 253 scans). Particle crystallinities were determined from the splitting factor obtained via normalizing the sum of the absorbance at 562 cm^{-1} and 602 cm^{-1} to the minimum between the doublet following Weiner and Bar-Yosef [18].

2.3. Scaffold fabrication

Porous mineral-containing scaffolds were fabricated by a gas-foaming/particulate leaching technique as previously described [5,6]. Briefly, 4 mg of poly(lactide-co-glycolide) (PLG) particles (Lakeshore Biomaterials, ground and sieved, average diameter 250 μm), 4 mg of PLG microspheres (formed through a double emulsion process, average diameter 5–50 μm), 8 mg of HA nanoparticles (A1-A3, B1, SIG), and 152 mg of NaCl (J.T. Baker, sieved to a diameter of 250–400 μm) were mixed and subsequently cold pressed in a Carver Press (Fred S. Carver). The resulting matrices (8.5 mm diameter, 1 mm in thickness) were exposed to high-pressure carbon dioxide gas (800 psi) inside a non-stirred pressure vessel (Parr Instruments 4677). Following rapid release of pressure that allowed polymer foaming, scaffolds were soaked in $\text{DI-H}_2\text{O}$ for 24 hr to leach out porogenic NaCl particles. Non-

mineral-containing (NM-) scaffolds that did not contain HA were also fabricated as controls. Prior to cell culture, scaffolds were sterilized in 70% ethanol for 30 min and washed five times in sterile PBS.

2.4. Microscopic characterization of scaffolds

Scaffolds were characterized through TEM, scanning electron microscopy (SEM) and brightfield light microscopy to assess particle distribution throughout the polymer matrix. For TEM (FEI Tecnai T-12 Spirit, 120 kV), scaffolds were embedded in an UV-active Quetal resin (EM Sciences, FortWashington, PA) and sectioned with a microtome (Sorvall Porter-Blum MT2-B Ultra-Microtome) prior to analysis. For SEM, scaffolds were fractured to expose inner pore surfaces and mounted on aluminum SEM stubs (Electron Microscopy Sciences) with carbon tape in a manner that allowed subsequent imaging of the exposed surfaces. Particle distribution on PLG surfaces were examined on exposed pore surfaces within the scaffold that were unaffected by the sample preparation (i.e., not on the fractured surface) and imaged uncoated using a Field-Emission SEM (Zeiss LEO 1550, 1 kV). Brightfield microscopy of scaffolds was performed on an Axio Observer.Z1 (Zeiss).

2.5. Analysis of protein adsorption on scaffolds

To quantify the degree of protein adsorption as a function of HA nanoparticle characteristics, scaffolds were incubated in complete DMEM (cDMEM ; i.e., DMEM [Invitrogen] supplemented with 10% fetal bovine serum [Tissue Culture Biologicals] and 1% penicillin/streptomycin [Invitrogen]). Following 30 min of incubation, scaffolds were washed twice in PBS (Invitrogen) and then sonicated (Branson Sonifier 150) in RIPA buffer (Sigma). After centrifugation, total protein in the supernatant was quantified using a Bicinchonic Acid (BCA) Kit (Thermo) and colorimetric analysis on a plate reader (Tecan M1000) according to manufacturer's instructions. Additionally, scaffolds were incubated in DMEM supplemented with 20 µg/mL of fibronectin (Sigma) and adsorbed fibronectin was analyzed by immunofluorescence. Briefly, scaffolds were fixed with 10% formalin, rinsed twice in PBS, and then incubated with a polyclonal rabbit anti-fibronectin antibody (Sigma). After a second wash with PBS containing bovine serum albumin, scaffolds were incubated with an Alexa Fluor 488 secondary antibody (Molecular Probes). Whole-mount scaffolds were imaged using an epi-fluorescence microscope (Zeiss Axio Observer.Z1).

2.6. Cell culture

Human MDA-MB231 breast cancer cells (ATCC) were maintained under standard culture conditions (37 °C, 5% CO₂) in cDMEM. For 3-D cell culture, scaffolds were statically seeded with 1.5 million MDA-MB231 cells and subsequently maintained under dynamic culture conditions on an orbital shaker for up to 10 days as previously described [5].

2.7. Analysis of ion content in media from scaffolds

To determine changes in calcium and phosphate content in media during culture, acellular NM- and A1-scaffolds were incubated in cDMEM under standard culture conditions (37 °C, 5% CO₂). The media was harvested after 2 days, digested in 5% nitric acid, and analyzed with inductively coupled plasma spectroscopy (ICP; Thermo ICAP 61E Trace Analyzer). Additionally, ICP analysis was performed with media harvested from tumor cell-seeded NM- and A1-scaffolds after 2 days of 3-D culture.

2.8. Characterization of tumor cell behavior

To measure tumor cell growth, tumor constructs were harvested 30 min after seeding (day 0) and again after 72 hr. Constructs were washed five times in PBS and mechanically disintegrated, before being sonicated (Branson Sonifier 150) in lysis buffer (25 mM Tris–

HCl, 0.4 M NaCl, 0.5% SDS). Subsequently, lysates were centrifuged, and the supernatant was assayed for DNA content using PicoGreen® assay (Invitrogen) and a fluorescence plate reader (Tecan M1000) according to manufacturer's instructions. Protein secretions in media from constructs were also analyzed 72 hr after seeding. Briefly, breast cancer cell-seeded scaffolds were transferred to fresh well plates 24 hr prior to harvesting media for analysis, and culture medium was changed to DMEM containing 1% FBS. IL-8 of harvested media was measured via ELISA (R&D) according to manufacturer's instructions and normalized to DNA content to account for differences in cell number. For analysis of IL-8 expression, RNA was collected by dissolving tumor cell-seeded scaffolds in TRIzol® (Invitrogen) and then extracting total RNA according to manufacturer protocol. Total RNA (1 µg) was then reverse transcribed to cDNA (High Capacity cDNA Reverse Transcription Kit with random hexamers [(Applied Biosystems)]), and subjected to real-time RT-PCR (25 ng template, run in triplicate) using SYBR green detection (Quanta) on an Applied Biosystems 7500 System. Primer sequences for human IL-8 (fwd: 5'-agaaaccaccggaaggaaccatct-3', rev: 5'-agagctgcagaaatcaggaaggct-3') and β-actin (fwd: 5'-aatgtggccgaggactttgattgc-3', rev: 5'-aggatggcaagggacttctgtaa-3') were synthesized by IDT Technologies. β-actin was used as an endogenous loading control gene, and relative quantification was performed using the $\Delta\Delta C_t$ method as previously described [19].

2.9. Statistical analysis

One-way ANOVA and Student's *t*-test were used to determine statistical significance between conditions. Tukey's post test was used for pairwise comparisons. Significance between groups and NM-, A2-, and B1- scaffolds (see Table 1) are denoted by (*), (#), and (o) respectively. In all cases, $P < 0.01$ is indicated by a single symbol and $P < 0.005$ is denoted by double symbols. For all experiments, sample conditions were run in triplicate or greater. Data are presented as averages, and error bars represent standard deviations.

3. Results

3.1. Particle synthesis and characterization

Synthetic HA (sHA) with varying nanoscale characteristics can be formed via solution-based precipitation reactions, hydrothermal synthesis, electrodeposition, or sintering [1,20]. The sHA nano-particles used for the fabrication of mineral-containing scaffolds were obtained from either a two-step synthesis involving a precipitation reaction followed by hydrothermal aging or a commercial source for comparison (Fig. 1, Table 1). To synthesize poorly crystalline particles (A1), solutions of calcium and phosphate salts were mixed together rapidly at low temperature. Remaining particles (A2, A3, and B1) were prepared by hydrothermally aging a suspension of the particles from the precipitation reaction. The size and crystallinity of the particles increased with aging time (A2, A3), and could also be varied by changing the precursor salts (B1).

Particle shape and size distributions of both the sHA and commercial HA were determined by TEM (Fig. 2). The sHA particles that were formed from the initial precipitation reaction (A1) were small and poorly crystalline; however, hydrothermally aging these same particles resulted in the formation of uniform, larger, faceted, and elongated particles with a narrow size distribution (A2 and A3). Similar to previous observations [1], the length of the rod-like particles increased with aging time from 20 to 40 nm after 0.5 hr of aging (A2) to 60–170 nm after 72 hr of aging (A3). Using a different set of precursor salts and shorter aging time (1.5 hr), rod-like particles (B1), with lengths similar to A3 (50–150 nm), were also obtained. In contrast to the sHA particles, the SIG particles were spherical and exhibited a wide range of sizes (20–600 nm in diameter).

All of the synthetic particles were pure HA according to powder X-ray diffraction (pXRD) (Fig. 3). The diffraction pattern of the SIG particles, however, contained unidentified peaks in addition to those indexed to HA (ICDD PDF no. 09-0432). The pXRD also provides information regarding the particle size and crystallinity. Scherrer analysis of the {002} peak width (25.88°) revealed that the average length of the particles to be 24 nm (A1), 32 nm (A2), 103 nm (A3), 99 nm (B1), and 125 (SIG) (Table 1). The increase in the number of clearly resolvable peaks at higher angles for A3 and B1 particles suggest an increase in crystallinity compared to A1 and A2 particles.

FTIR results confirmed the increase in crystallinity for the larger particles obtained from longer hydrothermal aging times and varying precursor salts (A3, B1). The absorbance at 630 cm^{-1} is attributed to structural hydroxides in HA and is known to increase with enhanced crystallinity (Fig. 4) [21]. This absorbance was undetectable for the poorly crystalline HA (A1), and increased in intensity for nanoparticles A2, A3, and B1. The increase in crystallinity was further confirmed from calculation of the splitting factor (Table 1). The splitting factor, which quantifies the degree of splitting of the PO_4 bond bending (ν_4) peaks (562 cm^{-1} and 602 cm^{-1}), is known to increase with increasing crystallinity [18]. Interestingly, although A3 and B1 are similar in length, B1 had nearly twice as large a splitting factor, indicating differences in crystallinity between the two similarly sized particles made from different precursor salts (Table 1).

3.2. Scaffold Characterization

The micro- and nano-architecture of the scaffolds was analyzed via brightfield light microscopy, SEM and TEM. Mineral-free (NM-scaffolds), SIG-scaffolds, and A3-scaffolds were compared to assess the integration of different particles into the bulk-PLG. Incorporation of the SIG and A3 nanoparticles did not change the micro-architectural characteristics (i.e., pore size and wall thickness) of the scaffolds compared to the NM-scaffold as shown by brightfield light microscopy (Fig. S1). The distribution of both A3 and SIG particles on pore surfaces within the scaffolds was revealed by SEM micrographs. Despite the differences in particle morphology, similar surface coverage was observed for both the rod-like A3 particles and spherical SIG particles. Both scaffold types contained regions of high and low particle density, as well as areas without surface-associated HA (Fig. 5). High magnification SEM images of the A3 and SIG scaffolds (Fig. 5C, D) further illustrated that the surface topography is dictated by the morphology of the incorporated particles and reflects the rod-like and spherical morphology of A3 and SIG particles. TEM micrographs of sectioned A3-scaffolds revealed that HA particles were localized at or near the pore surfaces, with a small number of particles completely embedded within the bulk-PLG (Fig. 6). This distribution of particles suggests that a majority of the particles are accessible for interaction with seeded cancer cells.

3.3. Serum protein adsorption

To assess whether the HA nanoscale characteristics modulate cellular interactions with scaffolds, we next determined the capacity for protein adsorption of the different mineral-containing (HA-) scaffolds. When incubated in serum-containing media (DMEM + 10% FBS), HA-scaffolds adsorbed significantly more protein than control NM-scaffolds, and furthermore, the scaffolds with the smallest and least crystalline HA nanoparticles (A1) adsorbed the most serum proteins, as determined by a colorimetric assay (Fig. 7A). Specifically, scaffolds with poorly crystalline particles (A1) adsorbed two-fold more serum protein as scaffolds with 103 nm long particles (A3). Furthermore, A3-scaffolds adsorbed significantly more protein than the more crystalline B1 scaffolds, yet these two particles did not vary in size. Incubating scaffolds in pure solutions of fibronectin, an adhesive serum protein that readily adsorbs onto most biomaterial surfaces [22], yielded similar trends as

indicated by fibronectin immunofluorescence (Fig. 7B). To control for changes in surface area as a function of particle size and/or shape, fibronectin adsorption experiments were repeated with scaffolds containing HA nanoparticles in amounts normalized to surface area. Under these conditions, scaffolds containing smaller, poorly crystalline nanoparticles (i.e., 20 nm long) adsorbed significantly more fibronectin relative to those with longer, more crystalline particles (i.e., 80 nm and 100 nm long) (Fig. S2A). These results suggest that HA particle characteristics, in addition to purely surface area, regulate the adsorption of serum proteins that may in turn modulate cell behavior.

3.4. Scaffold and particle integrity under culture conditions

To determine if HA nanoparticle solubility contributes to calcium and phosphate content in media, which during cell culture could lead to differences in cell behavior, the elemental composition of media harvested from scaffolds was analyzed via ICP (Fig. 8). A1-scaffolds were used for this experiment since poorly crystalline HA has increased solubility compared to more crystalline HA [1] and would be most susceptible to potential dissolution. Analysis was performed on both cell-seeded and cell-free scaffolds, revealing that the calcium concentrations remained unchanged for all conditions and matched concentrations found in cDMEM. Similarly, non-seeded scaffolds exhibited unfettered phosphorous levels equal to concentrations found in cDMEM. In contrast, phosphorus concentrations increased for both the NM and A1-scaffolds that were seeded with cells. This increase was attributed to the presence of the cancer cells rather than the dissolution of HA particles. Overall, our findings show that any observed effects of mineral-containing scaffolds on cell behavior are related to cell-material interactions with HA nanoparticles and not the presence of increased levels of soluble calcium and phosphorous.

3.5. Effect of HA crystallinity and particle size on MDA-MB231 adhesion and growth

To determine the relevance of mineral characteristics to breast cancer cell colonization and growth within bone, we seeded MDA-MB231 breast cancer cells into mineral-containing PLG scaffolds and evaluated their adhesion and proliferation as a function of HA nanoparticle characteristics. In comparison to NM scaffolds, all of the HA-scaffolds enabled significantly more cell adhesion as detected by DNA quantification following initial cell seeding (Fig. 9A). Similar to the increased protein adsorption (Fig. 7), cell adhesion was greatest within scaffolds containing small, poorly-crystalline nanoparticles (A1) as compared to larger and more crystalline sHA (A2, A3, and B1) suggesting a possible link between HA-regulated protein adsorption and cell adhesion. Likewise, significantly more cells adhered to A3 scaffolds than B1 scaffolds, suggesting that increased HA crystallinity inhibits breast cancer cell adhesion by suppressing protein adsorption. Importantly, tumor cell proliferation was also affected by the presence of different HA nanoparticles and followed similar trends as observed for cell adhesion (Fig. 9B).

3.6. IL-8 secretion by MDA-MB231 cells as a function of HA crystallinity and particle size

To evaluate the effect of HA nanoparticle characteristics on the osteolytic capability of breast cancer cells, we analyzed IL-8 secretion by MDA-MB231 cells in the different scaffolds. Interestingly, in contrast to cell adhesion and proliferation, IL-8 secretion was inversely regulated by sHA crystallinity and size. Specifically, cellular secretion of IL-8 was greatest in scaffolds that incorporated highly crystalline HA (B1) (Fig. 10A), while scaffolds that contained similarly sized, but less crystalline HA (A3) secreted significantly less IL-8. Furthermore, tumor cells cultured in scaffolds with the smaller and less crystalline particles (A1, A2) secreted less IL-8 relative to the highly crystalline particles (B1). These changes in secretion were mimicked at the expression level, as real-time RT-PCR demonstrated upregulation of IL-8 in cells seeded in scaffolds containing larger sHA crystals as compared to less crystalline sHA or NM controls (Fig. 10B). To confirm that these outcomes resulted

from nanoparticle characteristics and not a change in surface area due to particle size and/or shape, experiments were repeated with scaffolds containing HA nanoparticles in amounts normalized to surface area. In these experiments, the scaffolds containing larger, more crystalline sHA still increased IL-8 secretion (Fig. S2B). Taken together, these data suggest a role of sHA nanoparticle characteristics, beyond size, in regulating the osteolytic potential of MDA-MB231 breast cancer cells.

4. Discussion

Since bone metastasis often leads to the formation of osteolytic lesions [8] that are characterized by less crystalline and smaller apatite particles relative to healthy bone [2], studies of breast cancer bone metastasis would benefit from HA-containing scaffold systems in which particle size and crystallinity can be independently varied. While 3-D culture approaches have enhanced the physiological relevance of *in vitro* studies [11,23], models to investigate cell–mineral interactions in response to specific HA nanoparticle characteristics currently do not exist. Commercially available HA particles, which are routinely employed for the fabrication of mineral-containing scaffolds [5,6], are heterogeneous in size, shape, and chemical composition as shown by us (Fig. 2A, B) and others [24]. Gas-foamed/particulate-leached polymer composite scaffolds have previously been used to effectively present mineral to cultured cells [5,6], and we have applied this platform to present hydrothermally grown HA particles with tunable nanoscale characteristics to cells in a 3-D culture context. Specifically, we have used these scaffolds to study the effects of HA particle size and crystallinity on breast cancer cell behavior and have demonstrated that altering the crystal properties of HA may control the osteolytic and metastatic phenotype of breast cancer cells.

Several methods have previously been used to synthesize HA nanoparticles with narrow size distributions [20,25]. Many of these methods depend on the presence of surfactants or fractionation to control the size distribution, and the resulting particles have organic impurities and are produced in low yields [26,27]. Here, we describe an optimized technique for the synthesis of HA nanoparticles, which does not require additives or elaborate separation techniques to obtain narrow size distributions. The two-step process involves a wet chemical precipitation followed by hydrothermal aging and yields hydroxyapatite nanoparticles of tunable sizes, crystallinity, and, possibly, composition. Compared to conventional hydrothermal HA particle synthesis methods whereby particles are made directly in hydrothermal reactions [1,20], the division of the process into two steps ensures size uniformity by separating the nucleation and growth processes of the HA particles. The precipitation step nucleates HA seed crystals, while the hydrothermal aging step controls the growth of the seed crystals into larger particles [28]. To generate HA nanoparticles of systematically varying crystallinity and size, we varied the hydrothermal aging times (A1, A2, A3) and utilized different precursor salts (B1), while maintaining temperature and pH. Extensive characterization via TEM, SEM, FTIR, and pXRD defined the specific nanoscale properties of the resulting particles (Table 1). In particular, the importance of the choice of precursor salts is evident in comparing A3 particles and B1 particles, which exhibited similar length, but varied in crystallinity. The resulting particles ranged from having biologically relevant lengths and crystallinities (A1) [2,3] to longer dimensions and higher crystallinities (A2, A3, B1), enabling us to systematically evaluate the effects of nanoparticle size on tumor cell behavior. Incorporation of these well-defined particles into PLG scaffolds resulted in the presentation of the particles at the porous surface of the scaffolds, enabling interactions with seeded cells (Fig. 5). Such scaffold systems will be critical for the identification of parameters for the design of non-tumorigenic mineral-containing and mineralized matrices for bone regeneration.

It has been shown that HA and other calcium phosphate surfaces efficiently adsorb adhesive and serum proteins (e.g., fibronectin) that are crucial in mediating cell attachment and tissue formation [5,29-31]. Other studies with different material systems have demonstrated that nanoparticle size modulates protein adsorption [32], and our results further indicate that altering the nanoscale properties of HA affects overall protein adsorption behavior (Fig. 6). Specifically, our studies reveal that the overall magnitude of protein adsorption decreases monotonically with increasing HA crystal size. Additionally, decreasing the crystallinity of HA nanoparticles (assessed by the IR splitting factor) independent of overall size also increases the protein adsorption capacity of the scaffolds, as indicated by differences in serum protein adsorption between scaffolds fabricated from particle sets A3 and B1 (Fig. 6A). Overall available scaffold surface area may vary based on particle size and shape, and these changes may contribute to the observed differences in protein adsorption. Nevertheless, our results measured with scaffolds in which the amount of HA was normalized to surface area indicate that nanoparticle characteristics also modulate total adsorption capacity (Fig. S2A). These changes in protein adsorption may have important implications for general bone function as well as metastatic bone disease, as altered protein adsorption broadly modulates cell adhesion, proliferation, and survival of osteoclasts [33], osteoblasts [4,29,30], and breast cancer cells [5]. It is likely that adsorbed RGD-containing proteins, such as fibronectin, may be involved in these effects, as these biomolecules regulate cell attachment [34], focal adhesion formation [35], and proliferative signaling pathways [36].

Cell behavior is affected by mineral characteristics. For example, osteoblasts respond differently to HA as compared to tricalcium phosphate (TCP) [37], and amorphous calcium phosphate and highly crystalline HA exhibit varying levels of osteoconductivity [38]. Similarly, HA and TCP elicit varying levels of differentiation in pre-osteoclasts [39] and resorptive activity in mature osteoclasts [40]. Additionally, the results of our study indicate that nanoscale mineral properties are crucial regulators of tumor cell behavior. In particular, scaffolds that contained smaller and less crystalline particles and adsorbed more adhesive proteins out of serum also supported more initial breast cancer cell adhesion and proliferation (Fig. 7A, B). While enhanced secondary tumor formation in the bone is generally attributed to resorptive release of growth factors and other soluble cues [8], our data support the idea that direct interactions with the mineral matrix could give rise to increased tumor formation and cancer cell growth in the bone microenvironment. Pathologically, our data suggest that exogenously introduced HA may regulate cellular adhesion and growth through adhesive protein adsorption, and thereby serve as a high affinity-substrate for metastasizing breast cancer cells. *In vivo*, TE bone has been observed to serve as a metastasis target [16], and recent evidence indicates that cancer cell-bone matrix interactions activate invasive and proliferative potential [7], further supporting the contention that HA may be involved in the breast cancer cell avidity for bone.

IL-8 secretion is linked closely to osteoclast-mediated osteolysis in metastatic breast cancer [10] and is known to be enhanced through cell-HA interactions [5]. Our current study suggests that the nanoscale properties of HA strongly affect overall IL-8 expression, with larger and more crystalline HA stimulating significant IL-8 upregulation in breast cancer cells (Fig. 8). This finding is somewhat surprising since IL-8 expression may be expected to correlate with protein adsorption, as integrin engagement affects IL-8 transcription in certain cancers [41]. However, upon adsorption onto surfaces, in particular crystals, protein conformation, and possibly the presentation of cellular binding sites, may change [31,42]. Therefore, it is possible that though more protein is adsorbed to the smaller, less crystalline particles, these proteins no longer have their native conformation and do not trigger IL-8 expression. It is also possible that the cell-mineral interactions affect IL-8 production by breast cancer cells through other mechanisms. For example, nanoparticles could become

loosened from the scaffold during cell growth and induce an inflammatory reaction, as environmental particles are known to activate proinflammatory signaling [43]. Although not clear from the current study, our mineral-containing system could be further employed to investigate these possibilities.

While the insights provided by our system have pathological significance, future studies can take into account the broader complexity of breast cancer and the bone microenvironment. For example, in this study we have solely tested the cellular response of a highly metastatic breast cancer cell line, but it is possible that non-metastatic breast cancer cells may also be responsive to HA. In fact, HA may promote growth and invasiveness in non-metastatic breast cancer cell lines, and this response may be clinically significant, as microcalcifications in the breast are associated with malignancies and could potentially play a role in driving tumor progression [44]. Furthermore, this platform could be used to investigate interactions between breast cancer cells and osteoblasts and osteoclasts to fully recapture the cellular aspect of bone. Both of these cell types induce the osteolytic capability of breast cancer cells [5,10,45], and both may be affected by nanoscale HA characteristics [46,47]. Mineral-containing tumor models incorporating hydrothermally-aged HA nanoparticles provide the capability to address these aspects of the bone microenvironment and thereby have the potential to improve our understanding of the molecular mechanisms underlying bone metastasis that may ultimately enable the discovery of novel therapeutic targets for patient care. Moreover, the use of sHA in culture models could broadly provide insight into basic bone biology and the potential risks of new classes of mineralized and mineral-containing matrices for bone regenerative technologies.

5. Conclusions

We have created a mineral-containing platform that recreates certain aspects of the 3-D bone microenvironment by incorporating well-defined sHA nanoparticles into a TE biomaterial scaffold. We have used this platform to investigate the effect of nanoscale HA characteristics on breast cancer cell behavior, and our study indicates that the nanoscale properties of HA play a key role in regulating breast cancer cell behavior. In conjunction with previous evidence that osteolytic lesions are sites of low-crystallinity and smaller mineral particles [2], this work suggests that altering the nanoscale properties of microenvironmental bone mineral could play a role in the formation of a metastatic niche in bone supporting enhanced tumor cell colonization and growth. Specifically, we have demonstrated that smaller, less crystalline HA particles increase cellular adhesion and growth, while larger and more crystalline HA enhances breast cancer cell expression of the osteolytic factor IL-8. Overall, our findings provide new evidence that the vicious cycle of bone metastasis is not only mediated by growth factors in the bone microenvironment, but also by the actual materials characteristics of the mineral matrix.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

HA nanoparticle synthesis schematic showing the two-step precipitation reaction to obtain poorly crystalline HA followed by hydrothermal aging of the precipitate to obtain crystalline HA nanoparticles.

Fig. 2. TEM images of the different HA particles used to prepare mineral-containing PLG-scaffolds: Commercial Sigma Aldrich particles, (shown at low [SIGa], and high [SIGb] magnification), HA particles synthesized from $\text{Ca}(\text{NO}_3)_2$ and $(\text{NH}_4)_2\text{HPO}_4$ precursors with hydrothermal aging times of 0 hrs (A1), 0.5 hrs (A2), and 72 hrs (A3), and particles synthesized from CaCl_2 and Na_2HPO_4 precursors aged for 1.5 hrs (B1).

Fig. 3. pXRD patterns of particles SIG, A1-A3, and B1. All samples are confirmed to be HA. Major peaks have been labeled with HA crystal indices. Peaks not characteristic of HA pXRD pattern in SIG are indicated by (*), (ICDD PDF no. 09-0432).

Fig. 4. FTIR spectra of particles SIG, A1-A3, and B1. Positions of characteristic HA peaks are indicated.

Fig. 5. SEM micrographs of A3-scaffold (A, C) and SIG-scaffold (B,D) show the distribution of A3 and SIG particles on a fractured surface of the scaffold in which pore surfaces are visible. Regions of high particle density (*), low particle density (O), and pores in the scaffold (#) were observed for both scaffolds (A, B). Residual salt crystals are indicated (x). Arrows point to individual A3 particles (C) and SIG particles (D).

Fig. 6. TEM micrograph of an A3-scaffold shows the distribution of the particles (indicated by arrows) in the bulk (indicated by 'O') and the pores (indicated by '#') of the polymeric matrix. Inset: Higher magnification image of the area indicated by a box.

Fig. 7. (A) Serum protein adsorption on non-mineral-containing (NM) and mineral-containing (A1-A3, B1, SIG) scaffolds as quantified via colorimetric BCA analysis of scaffold lysates prepared after incubation with serum-containing cell culture media. (B) Immunofluorescence analysis of fibronectin (FN) adsorption onto non-mineral-containing (NM) and mineral-containing scaffolds containing small, poorly crystalline (A1) and highly crystalline HA particles (B1). Significance between groups and NM, A2, and B1 scaffolds are denoted by (*), (#), and (o) respectively. In all cases, $P < 0.01$ is indicated by a single symbol and $P < 0.005$ is denoted by double symbols.

Fig. 8. Calcium (white) and phosphorus (black) content of media harvested from nonmineral-containing (NM) and mineral-containing (A1) scaffolds after 2 days of culture in the presence and absence of MDA-MB231 breast cancer cells as determined by ICP. Culture media incubated for the same period of time, but not exposed to scaffolds or cells was used as a control (Media).

Fig. 9.

(A) MDA-MB231 cell adhesion onto non-mineral-containing (NM) and mineral-containing scaffolds (A1-A3, B1, SIG) as quantified via PicoGreen DNA assay of cell lysates 3 hours after seeding. (B) MDA-MB231 growth 3 days after seeding of non-mineral-containing (NM) and mineral-containing scaffolds (A1-A3, B1, SIG) as analyzed by PicoGreen DNA assay. Values are depicted relative to day 0 to account for variations in initial cell adhesion between the individual groups. Significance between groups and NM, A2, and B1 scaffolds are denoted by (*), (#), and (o) respectively. In all cases, $P < 0.01$ is indicated by a single symbol and $P < 0.005$ is denoted by double symbols.

Fig. 10.

(A) IL-8 secretion by MDA-MB231 breast cancer cells as analyzed through ELISA of conditioned media collected from non-mineral-containing (NM) and mineral-containing scaffold cultures (A1-A3, B1, SIG). Values were normalized to cell numbers as determined via PicoGreen DNA assay to account for changes in cell proliferation. (B) IL-8 mRNA expression by MDA-MB231 breast cancer cells as analyzed through quantitative real-time RT-PCR of lysates collected from non-mineral-containing (NM) and representative mineral-containing scaffold cultures (A1, B1, SIG). Values are shown as normalized to expression levels in NM scaffold cultures. Significance between groups and NM, A2, and B1 scaffolds are denoted by (*), (#), and (o) respectively. In all cases, $P < 0.01$ is indicated by a single symbol and $P < 0.005$ is denoted by double symbols.

Table 1

Summary of particle characterization, detailing precursor material, hydrothermal aging time, size of particles determined via TEM and XRD, and splitting factor determined via FTIR.

Sample ID	Precipitation Precursors	Aging time [h]	Length [nm] ^a (TEM)	Average Length [nm] ^b (pXRD)	Splitting Factor (FTIR) ^c
SIG	Commercial Sigma	N/A	20–600	125	2.07
A1	Ca(NO ₃) ₂ , (NH ₄) ₂ HPO ₄	0	*	24	2.56
A2	Ca(NO ₃) ₂ , (NH ₄) ₂ HPO ₄	0.5	20–40	32	3.61
A3	Ca(NO ₃) ₂ , (NH ₄) ₂ HPO ₄	72	60–170	103	3.99
B1	CaCl ₂ , Na ₂ HPO ₄	1.5	50–150	99	6.60

^aThe range of particle lengths observed from TEM micrographs along the *c*-axis.

* Lengths of A1 particles could not be determined from TEM.

^b Average lengths of the particles were obtained via Scherrer analysis of the {002} peak from pXRD.

^c Splitting factors were obtained from FTIR spectra by normalizing the sum of the absorbance at 562 cm⁻¹ and 602 cm⁻¹ from PO₄ bond bending (ν₄) to the minima between the two peaks.