INTRODUCTION

Bone is a composite structure that is composed of calcium phosphate mineral, type I collagen, water and extracellular matrix proteins that interact over several levels of hierarchy from the macroscale to nanoscale. Early crystallographic studies on bone biominerals identified that they are calcium deficient and carbonate substituted biosynthesized hydroxyapatite (BHAp) (Trautz, 1955; Zapanta-LeGeros, 1965). The shape of the nanocrystals is generally plate-like with particle size in the range of 30–50 nm (length), 10–30 nm (width) and 2–10 nm (thickness) (Heywood et al., 1990; Sakae et al., 2015). The extensive shape and small size of BHAp have triggered high research interest in its mechanism of crystallization in terms of nucleation and growth. Understanding the formation of BHAp in a biological environment is essential for studies in bone healing, remodelling and regeneration and provides a sound foundation for advancing future endeavours in the design of bone biomaterials (Combes et al., 2016). Additional benefits can be derived from these studies in material design related to drug and gene delivery, nutritional supplements, health products and analytical tools to probe nanoscale phenomena such as hydroxyapatite based Quartz Crystal Microbalance (QCM) adsorption systems with enhanced sensitivity (REF).
Currently, the formation mechanism of plate-like BHAp provides an intriguing enigma. The classical theory of crystal growth is unable to fully resolve the mineralization of BHAp (Olszta et al., 2007). The transient precursor phase to bone apatite is one of the oldest hypotheses regarding hydroxyapatite nanocrystal formation (Robinson & Watson, 1955). Amorphous calcium phosphate (ACP) precursor transition model for BHAp formation was proposed to occur via the recrystallization of ACP clusters agglomerate or via the transformation of ACP to octacalcium phosphate (OCP) to BHAp based on X-ray diffraction and spectroscopic data (Gower, 2008; Habraken et al., 2013; Mahamid et al., 2008, 2010; Nitiputri et al., 2016; Tertuliano & Greer, 2016; Xie et al., 2014). HRTEM on chicken bone composites showed the presence of very small crystals. The authors speculated that these small crystals could be BHAp nanocrystals but contrast limitations hindered a more definitive determination (Cuisinier et al., 1995). Wang et al. (2018) studied the nucleation and growth of calcium phosphate precipitated from solution using a very unique in situ liquid phase TEM technique which facilitates the observation of nucleation and growth phenomena and cluster aggregation processes in real time. These processes highlight particle attachment events that mimic growth mechanisms that have been reported for the growth of several nanoparticle systems including calcium carbonate, titanium oxide and iron oxide (Banfield et al., 2000; Gehrke et al., 2005; Ma et al., 2004).

Crystal growth was thought to follow the classical picture of crystallization where particles grow by the addition of atoms, ions or molecular units. Crystal growth by the classical theory results in a faceted crystal with morphology that is relatively consistent with the crystal structure (Di Pretoro & Manenti, 2020). However, studies on biomineral crystals have reported observations of nanoparticles that have different crystal morphology from the shapes expected based on classical crystal growth theory. In the last 20 years, several studies on biominerals and ceramics have reported non-classical crystallization mechanisms which are based on the aggregation and/or self-assembly of primary nanoparticles (Gehrke et al., 2005; Niederberger & Cölfen, 2006; Penn & Banfield, 1998; Revealed et al., 2010; Takasaki et al., 2016). Oriented aggregation was reported which involved the crystallographic alignment of primary crystalline units to form secondary particles. This process could result in secondary particles as an intermediate state called mesocrystal (Revealed et al., 2010).

Transmission electron microscopy (TEM) techniques have been heavily used to study crystal growth such as high-resolution transmission electron microscopy (HTREM), cryogenic TEM and electron diffraction. HRTEM has been the favourite technique for resolving images at the atomic level, and it has been successfully used for the observation of aggregate-based nanoparticle growth and the detection of defects such as dislocations, misorientation, twins and stacking faults (Penn & Banfield, 1998; Zhang et al., 2009; Zhou & Greer, 2016). The employment of fast Fourier transform (FFT) on selected regions of HRTEM image results in diffractograms that show the specific Bragg vectors that contribute to image formation. Periodicities in a region in the HRTEM image produce sharp spots in the FFT that can be used to interpret the intensity and position of the spots. These powerful techniques have been used for the detection of the oriented aggregation mechanism and assessing the mesocrystalline nature of nanoparticles by highlighting the misorientation effects and defects resulting from such aggregative crystal growth mechanisms (Banfield et al., 2000; Ma et al., 2004).

A comprehensive study on the formation mechanism of BHAp nanocrystals by employing HRTEM is essential to the enhanced understanding and design of bone biominerals. Wilson Jr. et al. (2012) showed the effectiveness of demineralized crushed crab shells in stimulating bone growth in ectopic regions. The idea of using crab shell was based on the similarities between bone and crab shell integument, on the successful induction of bone by demineralized bone matrix, and on the influence of nacre in stimulating bone growth. Here we study the mechanism of formation of plate-like biological apatite nanocrystals in a biological environment. HRTEM images of in vivo mineralization regions from a rat model revealed unique crystalline features that were used to analyse the nanocrystalline size, phase, orientation and misorientation defect structure. Our results help to support the hypothesized that the growth of BHAp follows the oriented aggregation mechanism of primary nanocrystals to form secondary particles of mesocrystalline nature.

2 | METHODS

2.1 | Experimental setup

These data that were analysed in this work were produced from an investigation on the ectopic biominalerization of BHAp in Sprague-Dawley rats (Omokanwaye et al., 2015). In summary, Carapace integument from Callinectes sapidus (Chesapeake blue crab) specimens was removed and cleaned by deionized water. The specimens were crushed by commercial grinder and diminished by a mortar and pestle. The crushed specimens were sterilized in 70% ethanol and allowed to dry. The demineralization was achieved by soaking 2 g of the crushed specimens in 20 ml HCl for 1–3 h. A subcutaneous incision was made in the abdominal region of anaesthetized 28 day old Sprague-Dawley rats, and 10–20 mg of the demineralized specimens were implanted. Tissues from the implantation site were retrieved after an ageing period of up to 8 weeks. The samples were prepared for microscopy in sequential dehydration steps starting in higher ethanol/water solutions until finally dehydrated with 100% ethanol. The samples were cut by a diamond knife to reach an adequate thickness (approximately 90 nm thin) for TEM analysis. High-resolution electron microscopy and electron nanodiffraction (END) images of the sample were obtained by using a JEM 2100 transmission electron microscopy (TEM).

2.2 | Image processing

Diffractograms obtained by applying the FFT on HRTEM images were used for the analysis of crystal nature and planar directions.
The digital image processing was performed by DigitalMicrograph™ software. FFT image processing of different crystals was performed to measure lattice spacing and observe local misorientation of the nanoparticles. The process involves taking the Fourier transform of a region of the image that shows periodic lattice to view the power spectrum of that region. The Fourier transform was based on the calculated intensity at specific position \( r \) in the real image by assuming perfect crystal orientation.

Produced with diffraction data based on the original nanoparticle’s recent microscopes. Simulated HRTEM images were then produced with diffraction data based on the original nanoparticle’s recent microscopes. Simulated HRTEM images were then produced with diffraction data based on the original nanoparticle’s recent microscopes.

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Aberration (1.2 mm) was unchanged due to limited effect on an amplitude (Fourier component of the Bragg vector \( g \) and can be defined by a phase (\( \varphi_g \)), where \( H_g(\vec{r}) \) at specific position is \( H_g(\vec{r}) = A_g(\vec{r}) e^{i\varphi_g(\vec{r})} \) (Hytch, 1997). The software allowed for direct measurement of the Bragg spots spacing and angles observed in the diffractograms. The measured data were indexed and compared with the American mineralogist crystal structure database. END image of the primary particles was automatically indexed by measuring distances and angles of the Bragg spots by DigitalMicrograph™ software. The measured ratios were compared with the American mineralogist crystal structure database and with Diffraction pattern obtained from simulation (see Section 2.3). Finally, particle dimensions were determined by IMAGEJ software, Fiji plugin (Schindelin et al., 2012).

2.3 HRTEM simulation

Since the HRTEM images were of a very small nanocrystals, the simulations were performed to complement the images of the mineralized tissue. The structural of stoichiometric hydroxyapatite was used due to the similarity with the structural parameters of BHAp. HRTEM and diffraction simulations were performed by JEMS software (Stadelmann, 1987). HAp was simulated based on the multislice method, and trial and error were considered for different crystal thickness and defocus. Field emission gun with 200 kv TEM was used for the simulation. The spherical aberration coefficient \( C_s \) was set to 0.7 mm, and the chromatic aberration (1.2 mm) was unchanged due to limited effect on recent microscopes. Simulated HRTEM images were then produced with diffraction data based on the original nanoparticle's orientation.

3 RESULTS

TEM images of the extracted samples revealed the biomineralization of the demineralized crab shell implanted. The mineralization induced a mineral phase, mineralized collagen bundles and fibres, and fibroblast as reported in Omokanwaye et al. (Omokanwaye et al., 2015). HRTEM micrographs of the mineral regions showed an aggregate of nanoparticles with spots and shape of nanocrystalline characteristics as shown in Figure 1. Some nanocrystals have a hexagonal geometry. The measured spacing from consecutive spots (d-spacing) from several nanoparticles was around 0.26 nm. FFT was performed on two nanoparticles [1 and 2] in Figure 1. The diffractograms from two nanoparticles showed Bragg spots of distances 0.26, 0.24 and 0.30 nm which corresponded to the (202), (310) and (112) planes of hydroxyapatite, respectively (Davies et al., 2012). The angles measured corresponded to the (202) spot positions in the diffractograms of nanocrystal 1 ≈ 114° while in nanocrystal 2 ≈ 107°. The average size of the nanocrystalline measured from several micrographs (not shown here) that showed similar crystalline nature and phase of BHAp is approximately 3 ± 0.5 nm.

Figure 2 shows simulated HRTEM image of the nanocrystal. The defocus was set at 61 mm with a very thin sample considered. The simulation of the stoichiometric HAp showed resemblance to the nanocrystalline in Figure 1. The atomic position of HAp can be seen projected in the simulated HRTEM. The simulation increases the reliability of HRTEM image that the primary particles in Figure 1 have the structure of hydroxyapatite viewed in the [111] orientation. END data taken from the same sample of the nanocrystal aggregates were analysed to further examine the phase of the nanoparticles.

Figure 3 depicts an indexed END image of the nanoparticle. The spot pattern and the measured spacing of END showed that the nanoparticle is indeed single crystal biological apatite. The image was taken along the [112] zone axis with Bragg spots indexed in the (110) and (111). The measured d-spacing of the (110)/(110) nanodiffraction spots is approximately 0.81 nm. In the inset, a diffraction simulation of hydroxyapatite was performed from the same zone axis as the nanodiffraction.

A region close to the aggregates was examined by HRTEM. Figure 4 shows plate-like nanocrystals with their corresponding FFT.
image. The lattice fringes of the plate-like crystals and their FFT image displayed single crystal characteristics. Distances measured between fringes of the two (A and B) crystals showed 0.81 nm spacing which corresponded to the (110)/(110) direction of the hexagonal crystal. Two regions in crystal A were further examined to visualize distortion in the lattice in more detail. The interesting results obtained from the FFT diffractions of the two regions (1 and 2) in crystal A are shown in Figure 4a. The measured angles of the Bragg spot in a (1) and a (2) showed a misalignment of about 4° based on approximate angle measurements of 55° and 59°. The particle in B is aligned along the [225] zone axis with the appearance of streaking within the (330) and (330) spots. The measured dimensions of the crystal were found to be approximately 46 nm in length and 22 in width. Another important region of misalignment in crystal B (highlighted by the arrow) shows dis-registry in the observed fringes with feature length around 4 nm. These results in A and B are comparable with defects of mesocrystal observed in the literature (Ma et al., 2004).

**FIGURE 2** (a) HRTEM simulation of the HAp nanocrystals (1 and 2) from Figure 1. (b) HRTEM simulation of the nanocrystal of HAp where the spot pattern shows similarity to (a). (c) Depiction of the atomic positions of HAp based on the simulated image where atoms shown represent Ca (red), H (green), P (blue) and O (grey)

**FIGURE 3** Indexed nanodiffraction of an area of the BHAp primary particles oriented along [112] zone axis. In the inset, an electron diffraction simulation of HAp in the same zone axis

4 | DISCUSSION

The HRTEM results combined with the nanodiffraction reveal the nanocrystalline nature of the primary particles of biological apatite. The hexagonal crystal structure of hydroxyapatite obtained from simulation matches the experimental results in the spot pattern, diffraction simulation and the measured interplanar spacing. The spacing measured of consecutive spots in the crystal corresponding to the (202) planes is 0.26 nm. The measured d-spacing for the nanodiffraction of the (110)/(110) planes is 0.81 nm. The measured d-spacing is comparable to the simulated hydroxyapatite where the calculated d-spacing of the (110)/(110) is 0.816 nm and for the (202) plane is 0.263 nm. An interesting challenge in the determination of the crystal phase of the primary particles is the similarity between the octacalcium phosphate and HAp in diffraction analysis. The crystal structure of hydroxyapatite is hexagonal, while octacalcium phosphate is triclinic (Wilson et al., 2004). The calculated interplanar spacing for the triclinic structure of octacalcium phosphate would show 0.31 nm for the 202 planes and 0.95 nm for the (110)/(110) planes. Although more statistical work is needed to determine the average measured interplanar spacing and analysis of the crystal structure of BHAp, the results showed resemblances to HAp. We have not observed any crystalline structure comparable to octacalcium phosphate in the samples.

Although it seems that the nanocrystals are randomly oriented, particles at a close distance are almost aligned at similar crystallographic orientation. The slight misalignment of ~5–7° measured in the diffractogram means that imperfect oriented aggregation could occur in some regions like reported in the literature (Penn & Banfield, 1998). Moreover, the BHAp aggregates show attachment behaviour in the lattice fringes that results in a chain-like shape of several nanocrystals attached similar to the recently reported oriented aggregation mechanism in several biominerals (Gehrke et al., 2005; Niederberger & Cölfen, 2006). The aggregation growth process of hydroxyapatite has been reported previously in both in vitro and in vivo studies. The proposed mechanisms follow a phase transition from the aggregation and attachment of particles
of amorphous calcium phosphate, octacalcium phosphate, or even tricalcium phosphate (Cuisinier et al., 1995; Habraken et al., 2013; Mahamid et al., 2008, 2010; Tertuliano & Greer, 2016; Wang et al., 2018; Xie et al., 2014). However, the transient precursor phases have been subjects of debate due to the difficulty in observing the ACP phase under biological condition. In the other hand, the existence of a carbonated environment in the detected apatite makes it incompatible with the occurrence of OCP (Grynpas & Omelon, 2007).

As explained by several reports, the secondary crystals resulted from the non-classical aggregation-based growth could have distinctive shapes that are different from the observed in classical crystallization pathway. For example, the growth of stoichiometric hydroxyapatite results in a hexagonal rod-like crystal elongated in the c-axis direction (Aoba et al., 1984; Suchanek et al., 2018). This comes in contrast to the observed plate-like BHAp nanocrystals observed in both in biomimetic and biological settings (Cuisinier et al., 1995). Non-classical crystal growth has been extensively observed in the growth of several biominerals and other materials (Niederberger & Cölfen, 2006). The particle aggregation-based growth has been reported previously where primary building blocks self-assemble by attachment to form secondary particles. The secondary particles formed from this growth process could result in an intermediate state called mesocrystal (Sturm & Cölfen, 2017).

In this study, we propose that the primary building blocks are in fact BHAp nanocrystals that follow the oriented aggregation mechanism. This mechanism results in a plate-like crystal composed of smaller crystals aligned in the same crystallographic direction (Figure 5). The predicted misalignment resulted from the imperfect oriented aggregation of the primary nanocrystal can be identified in the secondary mesocrystals. The angles measured in the primary nanocrystals show more misalignment (~7°) in general than detected from the mesocrystals (<4°). The observed resemblance between the primary nanocrystal and the mesocrystal in crystal texture and alignment has led to identifying this mechanism. The findings of this study can help to advance our understanding of bone as a material at the nanoscale level. The classical growth mechanism proposed that larger crystals grow by ion by ion growth is not observed in this study. However, this ex situ study cannot observe the crystal growth at all time and does not exclude other mechanisms. Moreover, this
CONFLICT OF INTEREST
The authors declare no conflict of interest.

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FIGURE 5 A structure depiction of the BHAp nanocrystal aggregation. Blue: Calcium; Brown: Phosphorous; Red: Oxygen; Silver: Hydrogen

study did not detect the amorphous phase since HRTEM images cannot identify it in the presence of a crystalline structure. Further studies on biological apatite nanocrystals can help in elucidating the mechanism of crystals initiation and nanocrystalline interface.

5 | CONCLUSIONS
This study investigated the mechanism of plate-like BHAp formation from HRTEM and nanodiffraction images extracted from a biological environment. Interesting results were found regarding the imperfect oriented aggregation of nanocrystals in the size range of 3 nm to form a plate-like mesocrystal BHAp. Although the secondary crystals show single crystal fringes, particle misalignment can be observed in some regions in both the real image and the FFT diffractogram. This study contributes to the efforts to gain a better understanding of the crystallization of bone biominerals which will lead to improving the designing of bone-based biominerals and fascinating developments in technologies that are influenced by hydroxyapatite.
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