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# Chemical and physical properties of carbonated HA affect breast cancer cell behavior

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ABSTRACT

Breast microcalcifications are routinely explored for mammographic detection of breast cancer and are primarily composed of non-stoichiometric hydroxyapatite ($Ca_{10-x}(PO_4)_{6-x}(CO_3)_x(OH)_{2-x}$) (HA). Interestingly, HA morphology and carbonate substitution vary in malignant vs. benign lesions. However, whether or not these changes (i) are functionally linked and (ii) impact malignancy remains unclear due in part to lack of model systems that permit evaluating these possibilities. Here, we have adapted a 96 well-based mineralized culture platform to investigate breast cancer cell behavior in response to systematic changes in the chemical and physical properties of HA. By adjusting the carbonate content of the simulated body fluid (SBF) solutions used during growth, we can control the morphology and carbonate substitution of the deposited HA. Our results suggest that both the combined and individual effects of these differences alter breast cancer cell growth and secretion of tumorigenic interleukin-8 (IL-8). Consequently, changes in both HA carbonate incorporation and morphology impact the behavior of breast cancer cells. Collectively, our data underline the importance of biomineralized culture platforms to evaluate the functional contribution of HA material properties to the pathogenesis of breast cancer.

**Statement of Significance**

Breast microcalcifications are small mineral deposits primarily composed of hydroxyapatite (HA). HA physicochemical properties have been of considerable interest, as these are often altered during breast cancer progression and linked to malignancy. However, the functional relationship between these changes and malignancy remains unclear due in part to lack of model systems. Here, we have adapted a previously developed a 96 well-based culture platform to evaluate breast cancer cell behavior in response to systematic changes in HA properties. Our results demonstrate that changes in HA morphology and carbonate content influence breast cancer cell growth and interleukin-8 secretion, and suggest that characterizing the effect of HA properties on breast cancer cells may improve our understanding of breast cancer development and progression.



## 1. Introduction

Breast tissue microcalcifications serve as an indicator for non-palpable breast cancer during routine mammographical screening and are a common index for *in situ* and many invasive ductal carcinomas (e.g., primarily estrogen receptor [ER] and HER-2 positive; infrequently triple negative; rarely invasive lobular) [1,2]. Moreover, not only the pure presence of breast microcalcifications, but also their specific material properties, matter with regard to clinical outcome. For example, the morphology of microcalcifications serves as an indicator for the malignant nature of mammographically detected lesions where crushed stone or casting-type microcalcifications are associated with a higher histopathological grade relative to diffusive punctate or powderish microcalcifications [3]. Furthermore, microcalcifications associated with malignant breast tissue are primarily composed of carbonated

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hydroxyapatite (HA), and the carbonate content of HA decreases with progression from benign to malignant disease [4,5]. Whether the morphologic differences and the carbonate content of HA are functionally coupled, and if these differences modulate tumor cell behavior individually and/or in a combined manner, remains largely unclear. To investigate this possible functional relationship a cell culture platform is needed that readily allows studying tumor cell behavior in response to HA with systematically varying carbonate content and morphological variations.

To understand and mimic the structure and function of biological HA, various synthetic methods have been developed. In particular, simulated body fluid (SBF) has been used to mineralize biomaterial surfaces for studies of mineral growth and cell-mineral interactions [6–9]. SBF contains inorganic ions found in human blood plasma, enables mineral deposition under near physiological conditions, and the chemical composition of the resultant HA resembles that of human bone [10,11]. Furthermore, SBF formulation, pH, and temperature can be used to readily adjust the resulting HA properties [12–14]. For example, modifying the ion composition and concentration of SBF modulates the structure of HA surfaces and ultimately cell behavior [6,15,16]. In addition, varying the carbonate concentration of SBF regulates HA particle size and crystallinity [17], and these changes can modify cell functions as demonstrated in the context of non-viral gene delivery [18]. Yet no SBF-based mineralization methods currently exist that permit studying the relationship of HA surface properties, carbonate content, and tumor cell properties.

We have previously shown that HA broadly affects breast cancer cell growth and secretion of the chemokine interleukin-8 (IL-8) [19] and that this cellular response varies with defined HA nanoparticle characteristics [20]. IL-8 is of particular interest as elevated levels of this chemotactic and inflammatory chemokine have been linked to various aspects of malignancy including increased tumor angiogenesis, invasion, and bone-metastatic capability of breast cancer [21,22]. For example, the more invasive character of estrogen (ER) negative vs. ER positive breast cancer cells is related to increased levels of IL-8 [23]. Furthermore, bone-metastatic breast cancer cells express more IL-8 relative to lung-metastatic ones [24], and varied integrin engagement may underlie these changes [25]. Interestingly, altered IL-8 secretion in response to changes in HA material properties may also be due to varied integrin engagement as HA material properties influence the adsorption of proteins in general [26,27] and adhesion molecules in particular [20]. Nevertheless, the effect of varied HA carbonate content and consequential changes in HA morphology on protein adsorption, tumor cell growth, and IL-8 secretion continues to be relatively poorly understood.

Here, we have adapted a previously developed mineralized culture platform [6] to systematically vary HA carbonate content and probed the resulting effects on HA morphology and the malignant potential of breast cancer cells. Our results revealed that varying the carbonate content of SBF may be used to generate HA coatings with defined surface properties and that the chemical and physical properties of these coatings correspond to the level of carbonate incorporation in HA. Importantly, the malignant potential of breast cancer cells correlates with changes in mineral properties implying that our approach may be useful to assess the relevance of HA mineral properties to breast cancer progression.

## 2. Materials and methods

### 2.1. Mineral coating formation

Poly(D,L-lactide-co-glycolide) (PLG, lactide: glycolide = 85: 15, inherent viscosity: 0.6–0.8 dL/g in chloroform, Lakeshore

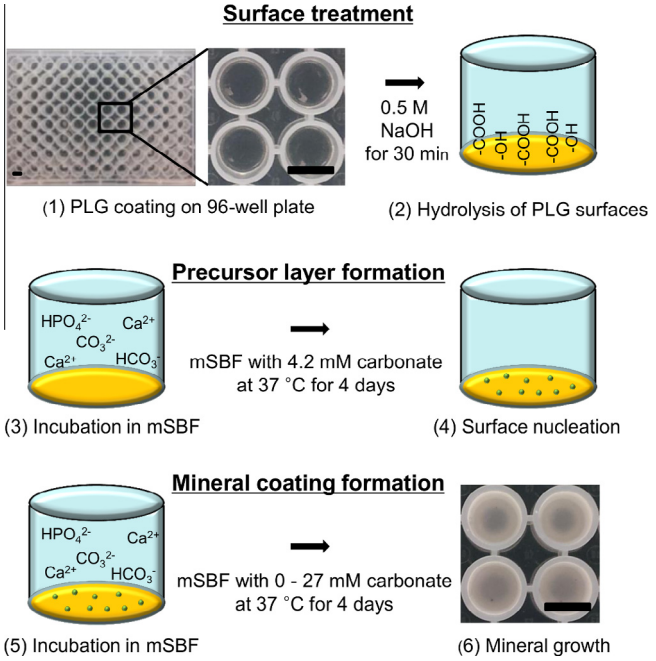


**Fig. 1.** Schematic of HA mineral formation procedure on 96-well polypropylene (PP) plates. According to previous work (Ref. [6]) HA mineral growth was induced by coating 96-well PP plates with PLG. Following partial hydrolysis of PLG, a precursor layer was formed by incubation in 4.2 mM [$CO_3^{2-}$] mSBF for 4 days. Finally, mineral coatings of varied carbonate content were formed by incubation in 0–27 mM [$CO_3^{2-}$] mSBF for 4 days. Scale bar = 5 mm.

Biomaterials) was coated on polypropylene 96-well plates by solvent casting of 100 mg/mL PLG in acetone. To accelerate mineral formation PLG-coated plates were hydrolyzed for 30 min in 0.5 M NaOH leading to the formation of carboxylate and hydroxyl groups at the surface (Fig. 1). For mineral formation, a series of modified simulated body fluid (mSBF) solutions was prepared (Table 1). All mSBF solutions contained twice as much $Ca^{2+}$ and $PO_4^{3-}$ than conventional SBF, which was first introduced by Kobubo and contains $Ca^{2+}$ and $PO_4^{3-}$ concentrations similar to those found in human blood plasma [28] while all other components (except carbonate) were dissolved at the same concentration as in conventional SBF; carbonate concentrations ranged from 0 mM to 27 mM. It should be noted that the term "carbonate" refers to both bicarbonate ($HCO_3^-$) and carbonate ($CO_3^{2-}$) ions from added sodium bicarbonate because both species co-exist at pH 6.8 in mSBF conditions. The pH of mSBF solutions was adjusted to pH 6.8 ensuring mineral formation. To initiate mineralization of the hydrolyzed PLG surfaces a precursor mineral layer was formed by incubation in 4.2 mM carbonate-containing mSBF for 4 days at 37 °C (Fig. 1). Subsequently, this solution was exchanged for mSBF with 0–27 mM carbonate for 4 days at 37 °C. All solutions were refreshed every 12 h during the course of mineral formation. Finally, mineral-coated surfaces were rinsed in deionized water and either dried for characterization of mineral properties or immediately used for cell culture.

### 2.2. Mineral coating characterization

The morphology of the mineral coatings was analyzed by scanning electron microscopy (SEM) (Tescan, Mira3 LM) following sputter coating with gold/palladium alloy (Denton Vacuum, Desk II). SEM images were taken at 5 keV. To characterize their structure and composition, mineral coatings were scraped off the PLG surface. Then, mineral coatings were ground and compressed with potassium bromide (KBr) and the resulting pellets were analyzed

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