**The interplay between crystallinity and the levels of Zn and carbonate in synthetic microcalcifications directs thyroid cell malignancy**

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**Abstract**

One of the key challenges in diagnosing thyroid cancer (TC) lies in the substantial percentage of indeterminate diagnosis of thyroid nodules undergoing ultrasound-guided fine-needle-aspiration (FNA) biopsy for cytological evaluation. This delays definitive diagnoses and treatment plans. In a recent development, our research demonstrated that hydroxyapatite microcalcifications (MCs) aspirated from thyroid nodules hold the potential to aid nodule diagnosis based on their composition. Mainly, Zn-enriched MCs have emerged as potential cancer biomarkers. However, a pertinent question remains: Is the elevation in Zn content within MCs a consequence of cancer, or do the Zn-enriched MCs encourage tumorigenesis?

To address this, we treated the TC human cell line MDA-T32 with synthetic MC analogs consisting of hydroxyapatite crystals with varied pathologically relevant Zn fractions and assessed the cellular response. The MC analogs exhibited an irregular surface morphology similar to FNA MCs observed in cancerous thyroid nodules. These MC analogs display an inverse relationship between Zn fraction and crystallinity, as shown by X-ray diffractometry. The zeta potential of the non-Zn-bearing hydroxyapatite crystals was negative, which decreased once Zn was incorporated into the crystal. The MC analogs were not cytotoxic. The cellular response to treatment with these crystals was evaluated in terms of cell migration, proliferation, the tendency of the cells to form multicellular spheroids, and the expression of cancer markers. Our findings suggest that if thyroid MCs play a role in promoting cancerous behavior *in vivo*, it is likely a result of the interplay between crystallinity and Zn and carbonate fractions in MCs.

1. **Introduction**

Mineral deposits form in the human body as part of the skeletal system, but also due to tissue abnormalities, biomaterial implantation, and disease [1–4]. In certain cancers, such as breast, ovarian, and thyroid, mineral deposits called calcifications form in the tumor microenvironment [5]. These calcifications can be as large as a few millimeters and can be detected during cancer screening methods like mammography and ultrasonography [6]. Regarding calcifications smaller than 100 m, termed microcalcifications (MCs), higher resolution techniques such as histopathology, vibrational spectroscopy-based chemical mapping, and electron microscopy are required for characterization [7–11]. Most MCs are calcium phosphate crystals in the form of non-stoichiometric hydroxyapatite (HA), with a tendency for ion substitution [12]. For instance, carbonate may substitute either phosphate or hydroxyl ions within the lattice, while mono and divalent cations can substitute calcium [8,13]. In breast MCs, there is a correlation between the crystal properties and tissue malignancy; the more malignant the tissue, the higher the crystallinity [13] and the fractions of Na, Mg, and Zn found in the MCs [10,14,15], and the lower the carbonate fraction [10].

Motivated by the connection between MC composition and breast tissue malignancy, several previous studies have focused on characterizing the composition of thyroid MCs. The underlying hypothesis was that thyroid MC composition could potentially offer valuable insights to clinicians regarding the malignancy of thyroid nodules. This is highly important due to the current diagnostic protocols for thyroid nodules, which rely on ultrasonography and fine needle aspiration for cytology (FNAC), yielding up to 30% indeterminate results [16]. However, in *ex vivo* studies involving surgically removed thyroid nodules, no discernible correlation was found between the crystal composition of MCs and tissue malignancy [17,18]. Recently, our research showed that the composition of MCs isolated from residual material following FNAC, correlates with thyroid nodule diagnosis. Specifically, more than 90% of MCs originating from cancerous nodules contained Zn, while most MCs from benign nodules lacked Zn [19].

Zn is involved in many cellular processes, including cell signaling, proliferation, invasion, apoptosis, immune responses, and inflammation [20–22]. The correlation between the presence of Zn within thyroid FNA MCs and thyroid nodule malignancy can be interpreted through a few plausible avenues. Cancerous thyroid nodules could induce the deposition of Zn-enriched MCs, possibly facilitated by an elevated Zn concentration within the nodule, stemming from disease-related dysfunctional Zn transport. Alternatively, Zn-enriched MCs forming in nodules could contribute to cancer progression. Moreover, a combination of these mechanisms is also possible, i.e., a vicious cycle in which cancerous nodules cause the deposition of Zn-enriched MCs, subsequently generating more cancerous cell behavior.

In that light, a possible mechanism through which Zn incorporation into the MCs might trigger cancerous cellular behavior is through changes in the properties of MC crystals. Calcium substitution with Zn can change crystal phase, crystallinity, particle sizes, zeta potential, specific surface area, porosity, solubility, and morphology compared to stoichiometric HA [23–27]. The extent to which Zn is incorporated into the hexagonal apatite crystal is inversely related to both crystallinity and the lattice parameters ‘a’ and ‘c’, because the ionic radius of Zn2+ is smaller than that of the Ca2+ cation [26,27]. The morphology of stoichiometric HA is of non-faceted particle aggregates, and the particle size either remains constant or changes with an increase in Zn fraction depending on the crystallization protocol [25–27].

Such changes in particle dimensions and crystal morphology can affect the attachment of proteins and cells to the mineral [28] and potentially affect disease progression. The adhesion of proteins to apatite crystals depends on the protein conformation as well as an interplay of crystal particle sizes, surface topography, roughness, porosity, pore size, charge, and functional groups [28]. Conditions that promote increased protein adsorption are linked with a favorable cellular attachment onto the crystal surface. This can be attributed to the binding of cell adhesion proteins to the crystal. Carbonated apatite crystals selectively adsorb specific pathogenic proteins, and a higher Zn fraction in apatite crystals leads to a decrease in the adsorption of the model acidic protein bovine serum albumin (BSA), even though the specific surface area increases with Zn content [23]. The surface roughness of the apatite crystal, which depends on the crystallinity and particle sizes, affects protein adhesion and cell attachment to the crystal surface [29] even more than the surface chemistry [30]. For example, bone marrow cell adhesion and proliferation on apatite crystals increase with the crystal surface roughness [31].

MCs isolated through FNA from malignant thyroid nodules contain 0.5-2.7 wt% Zn and are composed of aggregated particles [19]. In order to examine how the Zn content within thyroid nodule MCs impacts cancer progression, the current *in vitro* study evaluates the response of human thyroid cancer cells when exposed to synthetic MC analogs with varying Zn and carbonate fractions, zeta potentials, surface roughness, and crystallite sizes. The MDA-T32 cell line obtained from the malignant thyroid tissue of a 74-year-old male with papillary thyroid carcinoma (PTC), the most common histological subtype of TC, provides a robust *in vitro* framework for investigating mineral-cell interactions. By treating MDA-T32 cells with MC analogs, we aim to improve our understanding of the impact of Zn content on TC aggressiveness.

**Materials and Methods**

* 1. **Clinical samples and MC isolation from FNA material**

The clinical sample collection and analysis protocol was reported elsewhere [19]. Briefly, as part of routine FNAC procedures, MCs were isolated from the material aspirated from thyroid nodules. The FNA procedure was carried out at the endocrine unit of Soroka University Medical Center (SUMC). Patients with thyroid nodules and an indication for FNAC, aged 18 years or older, who provided informed consent, were enrolled. Patient information was anonymized and assigned codes for analysis. Clinical decisions were established based on thyroid ultrasound and cytological findings. This study was pre-approved by the institutional review committee (approval number 190-17-SOR, 27 Sep 2017). The fixed FNA material was treated with 2 mL of 70% ethanol (Bio-Lab Ltd), centrifuged at 4,000 rpm for 5 minutes three times, and immersed in deionized water for two hours at 37°C. After centrifugation at 20,000 rpm for 10 minutes, the supernatant was removed, and a solution containing 1.2 wt% sodium hypochlorite (Liel POWER) in deionized water was added to the pellet for overnight incubation. The pellet underwent three washes with deionized water before being preserved in absolute ethanol (Bio-Lab Ltd).

* 1. **Cell culture**

The MDA-T32 (ATCC® CRL-3351™) human thyroid cancer cell line was used. MDA-T32 is a cell line exhibiting epithelial-like morphology isolated from the thyroid of a 74-year-old white male patient with PTC. The cells were cultured in RPMI-1640 media (1% Penicillin/Streptomycin, 5% Fetal Bovine Serum, ATCC) at 37 °C and 5% CO2 with refreshing media twice a week. The cells were regularly tested for mycoplasma contamination. To prepare the stock solutions with media and different MCs, the crystals were ground using a pipette tip in 70% ethanol and then sonicated with an ultrasonic cleaning apparatus (SONO SWISS) for 15 minutes to minimize their size. Subsequently, the particles were sterilized by immersing them in 70% ethanol and dried under UV light in a biosafety cabinet for at least 20 minutes until they were completely dry. For cytotoxicity, cell migration, proliferation, multicellular spheroids formation and cancer marker expression experiments, the MDA-T32 cells were cultured in RPMI-1640 media supplemented with MC analogs with varying fractions of Zn.

* 1. **Synthesis of Zn-containing hydroxyapatite crystals**

MC analogs were HA crystals synthesized via an aqueous precipitation reaction of calcium nitrate (Ca(NO3)2∙4H2O), ammonium phosphate ((NH4)2HPO4), and zinc nitrate (Zn(NO3)2∙4H2O) purchased from Sigma Aldrich (St. Louis, MO, USA). Deionized water (18.2 MΩ) was used for all aqueous solutions.

To synthesize the 0% Zn HA crystals, 50 mL of a 40 mM ammonium phosphate solution was added drop-wise into 150 mL of 13.3 mM calcium nitrate solution for a final concentration of 10 mM, and was stirred at 300 RPM in room temperature for 24 hours. The pH of the starting solutions was adjusted to 9.5–10 using 25 wt% NH4OH (Bio-Lab Ltd). To synthesize HA crystals with varying Zn fractions, zinc nitrate was added to a 13.3 mM calcium nitrate solution prior to mixing for a final zinc concentration of 0.1 mM, 0.2 mM, 0.5 mM, and 1 mM. The resulting opaque suspensions were centrifuged (4000 RPM, 5 min). To remove soluble salts, the remaining material was washed with 15 mL of a 100 mM NH4OH solution and deionized water, rinsed in absolute ethanol (Bio-Lab Ltd), and dried overnight at 50°C.

* 1. **XRD and FTIR crystal phase characterization**

X-ray diffractometry (XRD) was performed on a Panalytical EmpyreanII powder diffractometer (K radiation, =1.54 Å) equipped with an X’Celerator detector in 1D linear mode and operated at v=40 kV, I= 30 mA. Samples were mounted for X-ray analysis in a front-loading, zero-background quartz X-ray Powder Diffraction sample plate and hand-ground using an agate mortar and pestle as needed to improve result statistics. Data were collected in Bragg-Brentano reflectance geometry from 5-70° 2θ, with a 0.0334° step size and a 40 s count time per step. Initial phase identification analysis was performed using the Match! phase identification software version 2.1.1 in conjunction with the International Center for Diffraction Data (ICDD) Powder Diffraction File (PDF-4+) database (2022 release). Further Rietveld refinement of the XRD results was performed using the Panalytical HighScore Plus XRD data interpretation software suite, version 5.1. Refinement analysis for all samples was applied to the same HA standard phase (ICDD #: 01-080-7085; a = 9.45 Å and c = 6.90 Å) with a maximum iteration of 400 cycles and a solver tolerance of 1.0 e-4. Refinement included the hexagonal HA unit cell parameters 'a' and 'c', as well as crystallite size (X-ray coherence length, Å). No internal standard was added during the XRD measurements. The crystallite size is reported through an external LaB6 instrumental broadening standard, which enables the computation of the instrumental broadening parameters. Using the instrumental broadening parameters, the sample crystallite size, computed by Rietveld refinement of the complete diffractogram, can be computed without an internal standard and acts as a proxy for sample crystallinity [27].

Fourier transform infrared spectroscopy (FTIR) measurements were carried out using a Nicolet iS5 FTIR instrument. The isolated FNA MCs were mixed with 200 mg of KBr (Sigma-Aldrich) to form a homogenous powder that was then pressed into a pellet for analysis. The FTIR instrument was operated in the 450–4000 cm−1 range, with 32 scans performed at a resolution of 8 cm−1. The synthetic MC analogs were measured on the same FTIR instrument with an iD7 attenuated total reflection (ATR) accessory with a monolithic diamond ATR crystal (Thermo Fischer). For these samples, the FTIR instrument was operated within the 520–4000 cm−1 spectral range, employing 16 scans at a resolution of 8 cm−1. Plotting and spectral analyses were performed using Origin Pro 2020 software. The ratio of carbonate to phosphate was determined by integrating the area under the peaks ranging from 850 to 900 cm−1 (*v*2 CO32-) and 900 to 1200 cm−1 (*v*1,3 PO43-).

* 1. **Surface characterization and elemental analysis of the crystals**

To determine the mineral particle size and morphology, the crystals were mounted on a conductive carbon adhesive tape that was applied on top of a scanning electron microscopy (SEM) stub. A field-emission SEM (Verios 460L, Thermo Fisher) was used to image the particles with a 2-3 kV voltage. Energy-dispersive X-ray spectroscopy (EDS) measurements to determine the elemental composition were conducted with a voltage of 15 kV, averaging the entire particle surface.

The surface roughness of the minerals was measured using a 3D optical microscope (New View 200 Zygo Scanning White Light Microscope). The minerals were uniformly distributed on a glass slide, and surface maps (72 µm x 54 µm) were acquired. To eliminate the form, a reference surface (plane) was subtracted. The roughness parameters Ra and Rq were computed using the Zygo MetroPro software with a high filter wavelength of 1.78838 µm and a low filter wavelength of 5.45030 µm. For each mineral, at least four roughness map measurements were conducted, and the resulting Ra and Rq values are presented as the mean ± the standard deviation.

Elemental composition was analyzed using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). Three mg of the synthetic MCs were first digested in 0.2 mL of ultrapure 70% (Sigma-Aldrich) and subsequently diluted to a final volume of 10 mL. These samples were then subjected to multi-elemental trace analyses using a Spectro Arcos ICP-OES instrument equipped with a CETAC autosampler. The instrument was calibrated using a multi-elemental standard solution dissolved in 5% HNO3, spanning concentrations from 0.1 ppm to 20 ppm. The ICP-OES instrument was operated under 1400 W plasma RF power, 13 L/min coolant gas flow, 1 L/min auxiliary flow, 0.75 nebulizer flow, 30 rpm pump speed, and a 15 s flush time between samples. All elements were detected in radial mode, with integration employing three points per peak and one point for background measurements. To determine the weight fraction in the crystal (wt%), the ICP-OES results (ppm) were multiplied by the initial volume to derive the element's weight (mg). This weight was divided by the initial mass of digested crystals.

* 1. **Zeta potential measurements**

Zeta potential measurements were conducted using the Zetasizer Nano ZS (Malvern Instruments Ltd.). Synthetic MC analogs were dispersed in PBS to achieve a final crystal concentration of 0.1 mg/mL. The zeta potential was measured at 37°C following 30 minutes of ultrasonication. The measurement was independently conducted three times.

* 1. **Assessment of cell malignancy potential**

**Cytotoxicity of MCs**

96-well plates were seeded with 25,000 MDA-T32 cells per well for cytotoxicity assessment. After 6 hours of culture, sterile minerals were added to each well at a 0.2 mg/mL concentration. Control cells were cultured without added minerals. For MTT-based cytotoxicity measurement, after 20 hours of culture, 20 µL of MTT (5 g/L) in PBS was added for 4 hours. Then, the media was aspirated, and the formed formazan crystals were dissolved in 100 µL DMSO. The plates were shaken for one hour for the complete dissolution of the formazan and the absorbance at 570 nm was measured using a Synergy H1 plate reader (BioTek). The experiment was carried out three times with distinct MC analog batches, with twelve wells for each condition in each experiment. The absorbance in the presence of MC analogs was normalized to the absorbance of the control (without MC analogs).

For live/dead assay, the cell medium was removed 24 hours after seeding, and the wells were washed with PBS. The PBS was aspirated and 70 μL of a staining solution containing calcein AM (1 μM) and ethidium homodimer-1(10 μM) was added. The cells were imaged after 20 minutes using a Nikon ECLIPSE Ti2-U inverted microscope equipped with a DS-QI2 mono-cooled digital camera with a 10× objective. The experiment was repeated three times.

**Wound healing**

A wound healing assay was conducted to assess the impact of the MC analogs on the migration of MDA-T32 cells. Cells were seeded in 6 individual μ-Dish 35 mm Ibidi plates (Munich, Germany) with a wound healing divider at a density of cells/mL with a volume of 70 µL per well. After 6 hours of culture, sterile minerals were introduced to each well at a 0.02 mg/mL concentration. Control cells were cultured without added minerals. The cells were then cultured until they reached a confluency of 95% (20 hours). Then, the dividers were removed to simulate a tissue wound, marking the starting point (time 0) of the experiment. The closure of the gap was imaged at specific time intervals using a 4× objective, and the speed of wound closure was quantified with the ImageJ software [32]. The experiment was performed six independent times.

**Cell proliferation**

To determine the effect of MC analogs on cell proliferation, 200,000 MDA-T32 cells per well were seeded in 6-well plates. After 6 hours of culture, sterile minerals were added to each well at a 0.02 mg/mL concentration. Control cells were cultured without added minerals. After 72 hours of incubation, the media was aspirated, and the cells were rinsed with PBS. Then, each well was treated with 1 mL of a 0.1% crystal violet (CV) solution in deionized water. The cells were washed repeatedly using deionized water until the water turned clear. Following overnight drying, the plates were imaged using a Nikon Eclipse Ci-L Ergo R2S Fi3 microscope equipped with a DS-Fi3 camera and a 4× objective. The CV was then dissolved in 1 mL of acetic acid. Synergy H1 plate reader (BioTek) was used to measure the absorbance at 570 nm. By comparing the cell count to the count of untreated cells (control), the relative proliferation was determined. The experiment was carried out three times with two biological replicas each time.

### **Western blotting**

The expressions of ERK, p-ERK, AKT, and EGFR were studied in cell monolayers cultured in T-25 flasks for 72 h in the presence of MC analogs with varying Zn fraction. The cells were trypsinized and X cells from each condition were collected into a 1.5 mL centrifuge tube, washed with PBS, and centrifuged for 5 min at 5 rpm and at 4⁰C. The cells were lysed by adding 100 µL of ice-cold RIPA buffer (50 mM Tris (Sigma Aldrich), 150 mM NaCl (Sigma Aldrich), 0.5% Triton (Sigma Aldrich), 0.1% SDS (Sigma Aldrich) containing 10% protease inhibitor cocktail (Sigma Aldrich) and incubated on ice for 40 min. The cell debris was pelleted by centrifugation for 10 min at 15,000 rpm and at 4⁰C, and the supernatant was collected. The protein concentration of the lysates was determined using the BCA protein assay (Pierce). The samples for Western blotting were prepared by mixing 15 µg protein from each cell lysate with Laemmli Sample Buffer, boiling it for 5 min, and then chilling on ice. The proteins were separated by electrophoresis on 10% agarose gel and transferred to a nitrocellulose membrane. The membrane was incubated for 1 h in a blocking buffer of PBST (PBS (Sigma Aldrich) supplemented with 0.1 v/v % Tween 20 (Sigma Aldrich) and 5% (v/w) dry skim milk (Sigma-Aldrich). The membrane was washed three times with PBST for 5 min and then incubated with primary antibody diluted in PBST supplemented with 2% skim milk. The anti-X antibody (sc-33684, Santa Cruz Biotechnology, Dallas, TX, USA) was diluted 1:50 (v/v) in PBST supplemented with 2% skim milk prior to use, and incubated with the membrane for 3 h. The anti β-Actin antibody (sc-47778) was diluted 1:400 (v/v) and incubated with the membrane for 1 h. Next, the membrane was washed three times with PBST and incubated for 1 h at 25°C with a secondary, anti-mouse HRP-conjugated antibody (115-035-003, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1:10,000 (v/v) in PBST supplemented with 0.5% (v/w) skim milk. To finalize the procedure, the membrane was washed three times with PBST and incubated with an ECL Western blotting reagent (1,705,060, Bio-Rad, Hercules, CA, USA) for 5 min in the dark. Images were collected by chemiluminescence, using the Fusion FX imaging system (Vilber Lourmat, Collégien, France). The experiment was performed with three independent biological replicates.

**Multicellular spheroid formation**

The bottom of each well of the 96-well plate was coated with 70 µL of 1.5% low melting agarose dissolved in RPMI-1640 cell culture medium. Subsequently, 7,000 cells were mixed with crystals at a concentration of 0.02 mg/mL in 100 µL of RPMI medium and then seeded into each well (Day 0). The plates were incubated at 37 °C and 5% CO2 under continuous shaking to obtain low adherence environment to induce multicellular spheroid formation. After 24 hours, an additional 100 µL of cell culture medium was added to each well. The cell culture medium was renewed with fresh medium after 48 hours. The spheroids were imaged on Day 1 and Day 5 of the incubation using a Nikon ECLIPSE Ti2-U inverted microscope equipped with a DS-QI2 mono-cooled digital camera with a 10× objective. The diameters of the spheroids were measured using ImageJ software [32].

* 1. **Statistical analysis**

The data presented are expressed as the mean ± standard deviation (SD) for Zeta potential, wound healing, and cytotoxicity assays and the mean ± standard error of the mean for the proliferation assay. Variations in the wound healing and cytotoxicity experiments were assessed through one-way ANOVA analysis. Statistical significance was assigned for P values equal to or below 0.05 (\*P≤0.05). The statistical analysis was conducted using OriginPro 2020.

1. **Results**

## **The physicochemical properties of FNA MCs and of synthetic MC analogs**

To explore the effect of synthetic MC analogs on thyroid cells, we first characterized MCs that were isolated from the excess material remaining after performing FNA procedures on patients with clinically significant thyroid nodules (Fig. 1). As part of the isolation protocol, organic components were removed from the MCs. Similar to our recent findings [19], these FNA-derived MCs displayed textured surfaces consisting of conglomerates of spherical particles (Fig. 1 a). These MCs exhibit sub-micrometer particle dimensions, ranging between 30-100 nm, forming densely packed aggregates spanning tens of micrometers. EDS analysis shows Zn fractions that align with the previously reported Zn content in FNA-derived MCs from cancerous nodules, which varies from 0.7 wt% to 2.5 wt% (Fig. S1). [19]. The Ca/P molar ratios range 1.2 to 1.8, while the Zn/Ca ratio is 0.1 to 0.9. The FTIR analysis of the thyroid MCs shows their composition as carbonated apatite (Fig. 1 b). Peaks identified by FTIR correspond to phosphate and carbonate groups (v3 PO4: 1100 cm-1 and 1030 cm-1, v4 PO4: 605 cm-1 and 575 cm-1, v2 CO3: 875 cm-1), alongside a 1386 cm-1 peak linked to nitrate, and residual organic material (1600-1800 cm-1).

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**Figure 1**. Crystal characteristics of FNA-derived MCs isolated from a cancerous thyroid nodule. **a.** SEM image of the MC, along with Ca/P and Zn/Ca ratios determined by EDS analysis. **b**. FTIR spectrum of an FNA MC showing the presence of carbonated apatite.

In order to explore the potential influence of Zn on the aggressiveness of the MDA-T32 thyroid cancer cell line, we synthesized MC analogs comprising HA crystals with diverse Zn fractions. These MC analogs were designed to mimic the Zn content observed in MCs from cancerous nodules. Following the characterization of these MCs, human thyroid cells were exposed to these crystals to investigate their response *in vitro*. The crystallization of the MC analogs was carried out in aqueous solutions to synthetically mimic the pathological thyroid MCs in terms of crystal phase, Zn fraction, morphology, and particle sizes. These solutions maintained consistent concentrations of Ca(NO3)2 and (NH4)2HPO4 while integrating varying concentrations of Zn(NO3)2∙4H2O.

To accurately quantify the Zn content within the synthetic MC analogs, we employed ICP-OES (Table 1). As the concentration of Zn2+ in the synthesis solution increased, a notable decrease in the Ca2+ fraction within the final precipitate was observed, accompanied by an increase in the Zn fraction. Furthermore, as the concentration of Zn2+ in the synthesis solution increased, a corresponding slight decrease in the Ca/P ratio occurred. Concurrently, the Zn/(Zn+Ca) ratio increased, all are indicative of Zn2+ cations substituting for Ca2+ within the HA crystal structure. The P fraction also decreased as the concentration of Zn2+ in the synthesis solution increased, possibly due to nitrate or other competing anion incorporation.

**Table 1.** Elemental composition of synthetic MC analogs measured by ICP-OES, according to the Zn2+ concentration in the synthesis solution.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Zn+2 solution concentration**  **(mM)** | **Ca fraction (wt%)** | **P fraction (wt%)** | **Zn fraction (wt%)** | **Ca/P molar ratio** | **Zn/(Zn+Ca) molar ratio** |
| **0** | 35.86 | 18.73 | 0.06 | 1.48 | 0.00 |
| **0.1** | 33.74 | 17.54 | 0.74 | 1.49 | 0.01 |
| **0.2** | 30.68 | 16.11 | 1.22 | 1.47 | 0.02 |
| **0.5** | 28.02 | 15.10 | 2.62 | 1.43 | 0.05 |
| **1** | 26.83 | 14.57 | 5.21 | 1.42 | 0.11 |

The Zn fractions selected for the MC analog syntheses hold physiological relevance. Among the MC analogs, one lacks Zn, and three other MC analogs correspond to the Zn fractions from MCs found in cancerous nodules: 0.74 wt%, 1.22 wt%, and 2.62 wt%. This range closely resembles the Zn fractions detected in FNA MCs from cancerous nodules, which fall within the comparable range of 0.5% to 2.7%. Additionally, one synthetic MC has a higher Zn fraction than typically observed in thyroid nodule MCs (5.21 wt%). This experimental design examines whether physiological Zn fractions hold significance or if the connection to malignancy is linearly reliant on the Zn fraction. The crystals with 0% Zn are similar in composition to FNA MCs found in benign nodules, but their morphology differs from these MCs. Hence, they cannot be considered representative analogs of benign nodule MCs; instead, they enable examination of the influence of Zn fraction on cellular behavior together with other parameters.

To examine distinctions in surface characteristics among the minerals, SEM was employed to visualize particle sizes and surface morphologies (Fig. 2 a-e). The particle sizes and the surface features of the MC analogs were similar to the pathological FNA-collected MCs (Fig. 1). Both FNA-collected MCs and the synthetic analogs form micron-scale aggregates from smaller particles; however, the biological MCs were more spherical and smoother than the synthetic MCs. The synthetic crystals exhibited surfaces featuring particle-like and needle-like structures, with slight differences in crystal morphology between the various Zn fractions. Notably, for 1.2 wt% Zn, the surface particles were smaller than in the other crystals. The crystal phase of the synthetic MC analogs was determined through FTIR spectroscopy (Fig. 2 f). The spectral analysis indicated the presence of phosphate and carbonate peaks, aligning with the characteristic features of carbonated calcium phosphate apatite. Carbonate to phosphate peak ratios were the highest for the HA with no added Zn (0 wt%) and the lowest for HA containing 0.74 wt% Zn (Table S1). Surface roughness measurements of the synthetic MC analogs showed differences between the crystals, with 5.2 wt% Zn being the roughest and 2.6 wt% Zn the smoothest (Table 2).

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**Figure 2**. Characterization of synthetic MC analogs with different Zn fractions. **a-e.** SEM images of MC analogs. **f.** FTIR spectra of MC analogs. **b, c, d.** MC analogs with 0.74 wt%, 1.2 wt%, and 2.6 wt% Zn that correspond to malignant MC composition **e.** MC analog with 5.2 wt% Zn that corresponds to a non-physiological, high Zn fraction.

**Table 2.** Surface roughness of the synthetic MC analogs according to Zn fraction. Rq is the root mean square roughness and Ra is the average roughness. The values are presented as the mean ± the standard deviation.

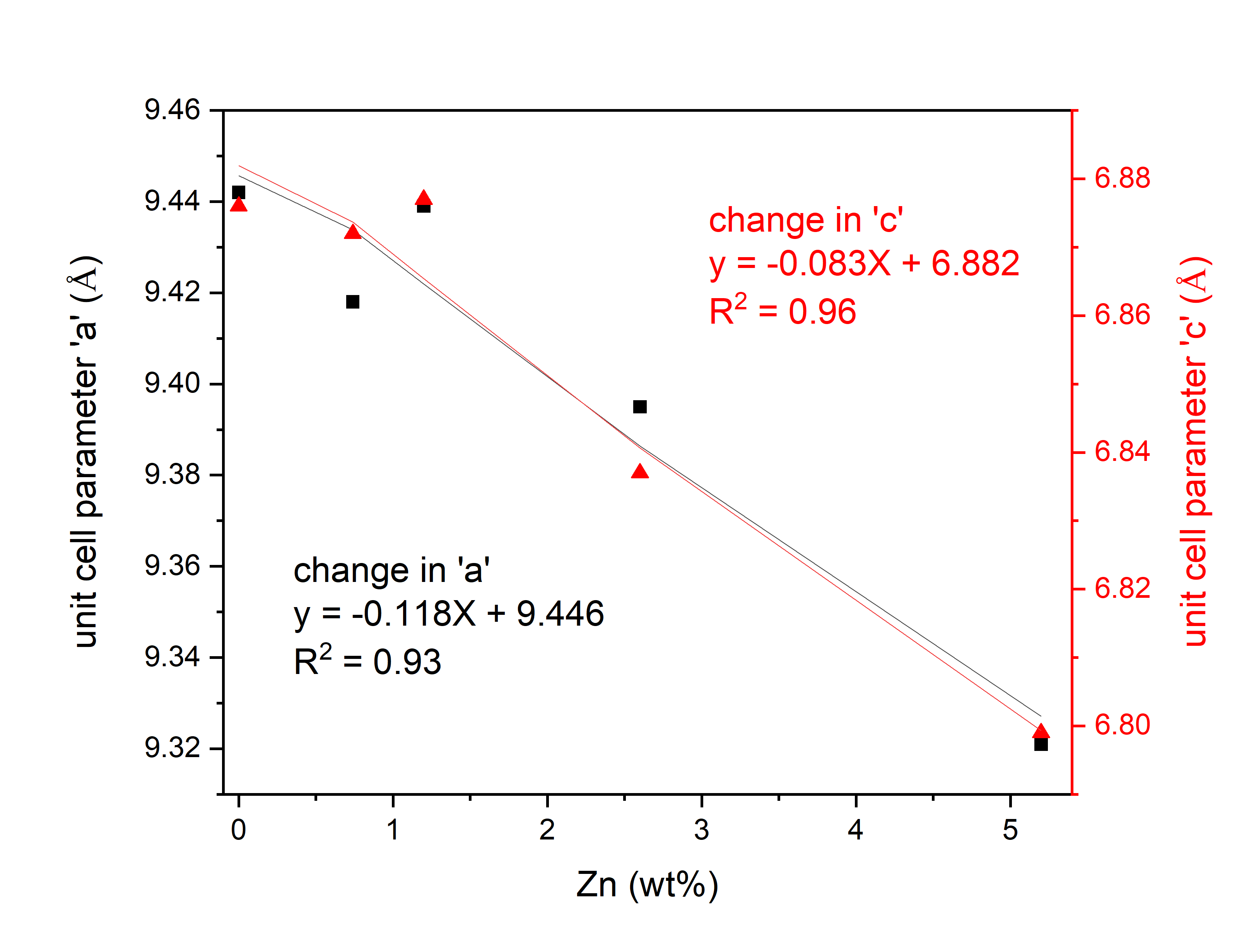
|  |  |  |
| --- | --- | --- |
| **Zn fraction**  **(wt%)** | **Rq ()** | **Ra ()** |
| **0** | 2.6 ± 0.7 | 1.7 ± 0.5 |
| **0.74** | 2.4 ± 0.3 | 1.6 ± 0.2 |
| **1.2** | 2.5 ± 0.6 | 1.8 ± 0.3 |
| **2.6** | 1.5 ± 0.5 | 1.2 ± 0.3 |
| **5.2** | 3.0 ± 0.6 | 2.1 ± 0.4 |

Overall, the synthetic MC analogs effectively mimic the crystal phase, particle sizes, bulk morphology, and composition of pathological thyroid FNA MCs. As a result, they can be used to investigate the direct effect of thyroid MCs on thyroid cancer cells in a controlled experimental setup.

The crystal structure of the MC analogs was further characterized using XRD (Table 3, Fig. 3, Fig. S2). Both computed unit cell parameters ('a' and 'c') decrease with increasing Zn fraction (Fig. 3), which has previously been reported as an indication of Zn2+ substitution for Ca2+ in the HA unit cell [26]. Additionally, the crystallite size decreases with increasing Zn fraction for most MC analogs (Table 3). The crystallite size is a proxy for sample crystallinity: the smaller the crystallite size, the less well-crystalline the sample. MC analogs exhibiting 0.74 wt% and 1.2 wt% share comparable crystallinity but diverge in their Zn and carbonate contents. This contrast establishes a platform to assess the individual influences of these factors on MDA-T32 cell line malignant properties.

**Table 3.** XRD results of the synthetic MC analogs according to Zn fraction. The solid phase Zn fraction (wt%) was measured by ICP-OES. ESD is the estimated standard deviation of the crystallite size.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Zn fraction**  **(wt%)** | **‘a’ ()** | **‘c’ ()** | **Crystallite size ()** | **ESD ()** |
| **0** | 9.442 | 6.876 | 220.9 | 0.134 |
| **0.74** | 9.418 | 6.872 | 100.6 | 1.767 |
| **1.2** | 9.439 | 6.877 | 101.8 | 0.013 |
| **2.6** | 9.395 | 6.837 | 75.3 | 0.005 |
| **5.2** | 9.321 | 6.799 | 62.1 | 0.003 |



**Figure 3.** Trends in changing unit cell parameters for MC analogs with increasing Zn fraction.

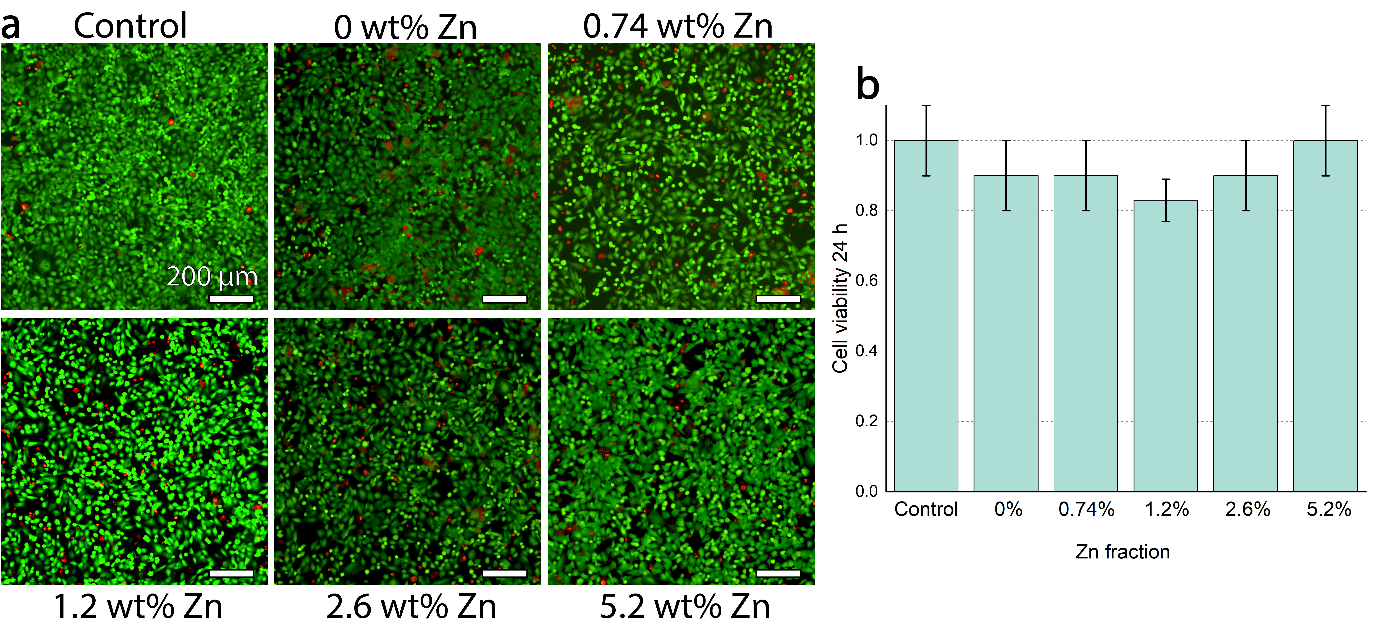
The zeta potential of the synthetic MCs exhibited a negative value across all crystal types, with the least negative value in the 0 wt% Zn samples, and the most negative in the 5.2 wt% Zn samples (Fig. 4). Intermediate zeta potential values were observed for MC analogs containing 0.74 – 2.6 wt% Zn.

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**Figure 4**. Zeta potential of HA MC analogs in PBS as a function of Zn fraction. The error bars represent the standard deviation.

## ***In vitro* cell behavior in the presence of synthetic MC analogs**

Before conducting functional assays to examine the effect of the MC analogs on cancer cell behavior, a preliminary evaluation of MC analog cytotoxicity was conducted. Cells were exposed for 24 hours to MC analogs featuring varying Zn fractions. Subsequently, the cell viability was compared with that of untreated cells, using live/dead and MTT assays (Fig. 5 a, b). None of the MC analogs displayed cytotoxic effects.



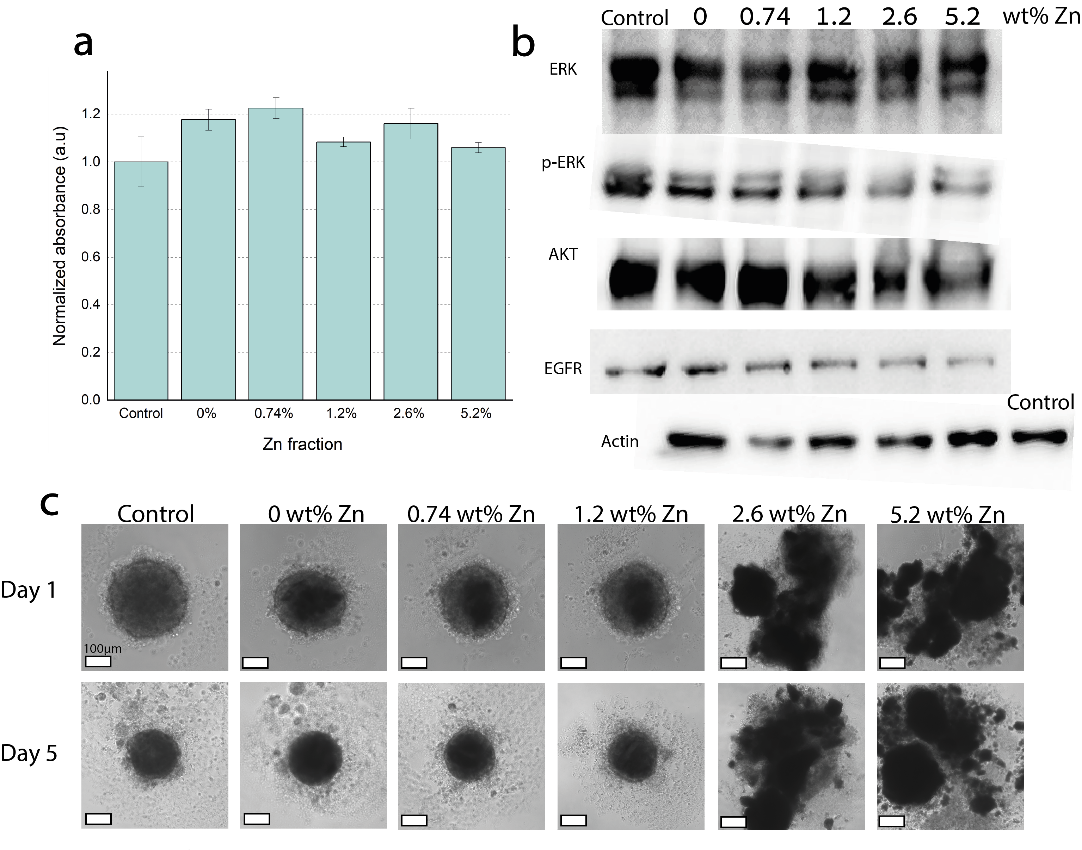
**Figure 5**. Cytotoxicity of the MC analogs according to their Zn content. **a.** Live/dead assay following 24 h treatment of MDA-T32 thyroid cancer cells with MCs analogs. Green - live, red - dead. The control is cells cultured without the presence of MC analogs. **b.** Cytotoxicity of the MC analogs measured using MTT assay following 24-hour treatment of MDA-T32 thyroid cancer cells. The absorbance of each condition was normalized to the absorbance of the control (no treatment). The error bars represent the standard deviation.

To investigate the impact of MC analogs on thyroid cell behavior, a wound healing assay was performed to assess MDA-T32 cell migration. Images of the initial wound area were captured upon divider removal (t = 0) and after 20 hours of culture (Fig. S3 a, b). Notably, cells treated with HA containing 0.74 wt% Zn exhibited a significant increase in migration and cell coverage compared to the control untreated cells. Conversely, cells exposed to other MC analogs displayed migration and cell coverage similar to the control.

To further investigate the influence of synthetic MC analogs on cell behavior, particularly concerning cell proliferation, we conducted a crystal violet (CV) assay following a 72-hour treatment of MDA-T32 thyroid cancer cells (Fig. 6 a). Even though the statistical analysis did not show differences in proliferation with P<0.05, the results suggest that treatment with HA containing 0.74 wt% Zn increased cell proliferation.

To further evaluate the effect of MC analogs with varying Zn fractions on thyroid cancer cells, the promotion of signaling pathways controlling cellular processes such as proliferation, differentiation, motility and apoptosis was examined[33]. We analyzed the expression of extracellular-signal-regulated kinase (ERK) and its phosphorylated form (p-ERK) that take part at the MAPK pathway, and the protein kinase B (Akt) that is a part of the PI3K/Akt pathway. Additionally, the expression of epidermal growth factor receptor (EGFR), known for its association with aggressive thyroid cancers, was examined. The expressions of ERK, p-ERK, AKT, and EGFR were determined after a 72-hour exposure to the crystals (Fig. 6 b, Fig. S4). There were differences between protein expressions depending on the Zn fraction, with high variance between repetitions of the analysis, due to the cell heterogeneity. Untreated cells and cells treated with MC analogs containing 0-1.2 wt% Zn had similar expressions of p-ERK and EGFR, and a lower expression when treated with 2.6 and 5.2 wt% Zn. Untreated cells and cells treated with all MC analogs had similar expressions of AKT and ERK.

To investigate how the MC crystal properties influence the cellular ability to form multicellular spheroids, cell suspensions were mixed with MC analogs containing various Zn fractions. Then, they were seeded together in low adherence conditions that can induce the formation of 3D structures that mimic tumors (Fig. 6 c). Cells seeded without the presence of MC analogs (control) or those seeded in the presence of MC analogs containing 0-1.2 wt% Zn formed multicellular spheroids on Day 1 of the culture. On Day 5 of the culture, these spheroids had smaller diameters (Table 4). The decrease in spheroid size may be due to increased cell-cell adhesion to form compact spheroids, or migration of cells away from the spheroids. Cells that were mixed with MC analogs containing the highest Zn fractions of 2.6 and 5.2 wt%, which had the lowest crystallinity, did not form multicellular spheroids even after 5 days of incubation. Instead, they formed loose aggregates with no clearly defined 3D spherical geometry, indicating a lack of cell–cell and cell–matrix interactions.



**Figure 6.** The cellular response to the Zn content in the MC analogs in terms of the cell tendency to form multicellular spheroids, cell proliferation, and expression of cancer markers. **a.** Cellular proliferation following 72 hours of treatment with MC analogs measured with a CV assay. The cell numbers are normalized against the control, which consisted of untreated cells. The error bars represent the standard error. **b.** Western blot analysis for ERK, p-ERK, AKT, and EGFR expression following 72 h of the culture according to the Zn wt% in the MC analogs. **c.** Light microscopy images of MDA-T32 thyroid cancer cells in the presence of MC analogs with varying Zn fractions under low-adherence conditions on days 1 and 5 of incubation.

**Table 4.** The diameters of the multicellular spheroids formed in the presence of MC analogs with various Zn fractions on days 1 and 5 of the incubation. The control is cells that were seeded without the presence of MC analogs. The errors represent the standard deviation.

|  |  |  |
| --- | --- | --- |
| **Zn fraction in MC analogs** | **Average diameter, day 1 ()** | **Average diameter, day 5 ()** |
| **Control** | 320 ± 20 | 210 ± 9 |
| **0 wt%** | 280 ± 10 | 204 ± 8 |
| **0.74 wt%** | 290 ± 20 | 220 ± 10 |
| **1.2 wt%** | 280 ± 20 | 210 ± 20 |
| **2.6 wt%** | - | - |
| **5.2 wt%** | - | - |

1. **Discussion**

MC analogs made from HA containing Zn fractions ranging from 0 wt% to 2.6 wt% and even higher than physiological Zn values were synthesized. These crystals exhibited similar morphology to MCs found in cancerous thyroid nodules and had sub-micron crystallite sizes. Zn2+ ions likely substituted Ca2+ ions, resulting in crystals characterized by reduced crystallinity and unit cell parameters. This does not rule out the possibility of Zn adsorption onto the crystal surface. Additional parameters that distinguished the MC analogs included carbonate content, surface roughness, and zeta potential.

Overall, our synthetic MC analog XRD results are consistent with previous studies where increasing the Zn fraction leads to less well crystalline minerals with smaller observed crystallite sizes, both by XRD and other methods [24–27]. The synthetic MC analogs produced for this study show XRD results dominated by a poorly crystalline HA with evidence for Zn substitution of Ca in the unit cell via a steady decrease in both the ‘a’ and ‘c’ unit cell parameters with increasing Zn substitution. This is consistent with results reported previously [26], where both HA unit cell parameters decreased with increasing Zn substitution. The results are also generally consistent with the results reported by Miyaji et al. [26] and Ren et al. [27], although both these studies observed an increase in the 'a' unit cell parameter at high Zn fractions (above 5 mol%). Inconsistencies reported in previous observations of the effects of Zn substitution on the synthetic HA structure and unit cell parameters are probably related to the flexibility of the HA unit cell and the mineral's capacity to take up and release OH-, water, and other anions in solution. This makes it difficult to definitively relate structural changes to Zn substitution [27,28]. For this reason, a pure HA control was included as the starting point in the Rietveld refinement of all the XRD results presented here. The decrease in the HA hexagonal unit cell parameters with increasing Zn fractions is thought to be related to the difference in the ionic radii between Zn2+ and Ca2+. Zn2+, at 0.74 Å has a significantly smaller ionic radius than Ca2+ at 0.99 Å and, as a result, decreases in the HA unit cell parameters are often presented as evidence for Zn substitution of Ca in synthetic HA [28]. These minor differences may have a bearing on the observed *in-vitro* tumorigenesis effects of the synthetic MC analogs, especially concerning the analog with no Zn.

The observed negative zeta potential across all MC analogs is consistent with the findings for Zn-substituted HA particles within a similar Zn fraction range, where negative zeta potentials have been reported [34]. Some variance could arise due to different synthesis protocols yielding varying morphologies, carbonate content, and crystallite sizes. Notably, the measured zeta potential values fell below 25 mV in both our case and the literature, indicating unstable particle dispersion.

When thyroid cancer cells were exposed to the non-cytotoxic MC analogs, their malignancy potential was investigated by cell migration and proliferation assays, as well as by their ability to form multicellular spheroids in low-adherence conditions and the expression of cancer markers. Cell migration and proliferation were increased the most upon exposure to MC analogs containing 0.74 wt% Zn. These MC analog crystals featured the lowest carbonate content, together with intermediate crystallinity, surface roughness, and zeta potential values. We did not observe changes in AKT and ERK signaling, as all cells had similar and high expressions independent of whether they were treated with MC analogs, or the type of mineral. However, the cells showed lower expression of the cancer markers p-ERK and EGFR when treated with MC analogs of higher Zn fractions.

The association between the ability to form multicellular spheroids and malignancy potential depends on the cell type and methodology[35,36]. While not consistently observed, it is common for cells exhibiting increased invasive behavior to form compact multicellular spheroids[37,38]. The formation of multicellular spheroids was observed for untreated cells, and in the presence of MC analogs containing 0.74 and 1.2 wt%, but not higher Zn fractions that also showed lower expression of cancer markers. This indicates that cells respond differently to varying crystal properties of MC analogs, and that most likely the formation of multicellular spheroids is associated with higher malignancy potential. The MC analogs that in their presence the cells did not form multicellular spheroids have the highest Zn fractions and lowest crystallinities. They also present both the lowest and highest surface roughness from all MC analogs, intermediate and low zeta potentials, and intermediate carbonate contents. Putting it all together, this suggests that Zn fraction and crystallinity, rather than surface roughness, zeta potential, and carbonate content are the main parameters affecting the cellular tendency to form multicellular spheroids.

There is no one clear parameter that controls the effect of HA on thyroid cancer cells *in vitro*. A complex combination of morphology, crystallite size (crystallinity), zeta potential, surface roughness, Zn and carbonate content, and most likely solubility that is a derivative of all the abovementioned parameters interacts to increase or decrease HA tumorigenesis influence *in vitro*. Our results suggest that high crystallinity and low carbonate and Zn content, which are at the range detected in cancerous FNA MCs, are associated with inducing more cancerous cellular behavior.

Earlier research has highlighted that the interaction of biological molecules is affected by the physiochemical properties of HA, encompassing factors like crystallinity, surface area, surface charge, and ion substitution [39–42]. For instance, changes in HA composition due to ion substitution can influence protein adsorption, with the specific type of ions substituted playing a role in modulating this interaction. Incorporating carbonate ions into HA crystals can alter crystal surface properties such as roughness, area, and crystallinity, consequently influencing protein adsorption and the behavior of cancer cells [43,44]. These effects are likely connected to the crystal surface charge, given that interactions between proteins, cells, and HA surfaces are primarily governed by electrostatic forces [28]. With the substitution of Zn2+ ions, the zeta potential of the synthetic MCs decreased, leading to a probable reduction in the adsorption of negatively charged proteins and plasma membranes. Hence, the less negatively charged surface of the low Zn MC analog can partially explain the more pronounced aggressive cell behavior observed. Given also that both biologic MCs and synthetic MC analogs form aggregates from smaller crystals, there is a strong possibility that aggregate crystal size and morphology, especially surface roughness, play a stronger role in generating cellular aggressiveness than crystallite size.

Nonetheless, it is important to acknowledge the limitations of these findings. For instance, in this *in vitro* study, the morphology of the 0% Zn MCs differed from the observations of morphology in benign thyroid nodules, which typically feature more faceted minerals. Additionally, the cell line employed in this study represents cancerous cells, albeit non-metastatic, and not benign thyroid nodule cells. Regarding the proliferation data, the influence of the MC analogs was not statistically significant.

Our findings rule out the notion that the previously observed correlation between elevated Zn content in thyroid FNA MCs and thyroid nodule malignancy [19] can be solely attributed to Zn-enriched MCs promoting cancerous cell behavior due to their composition. Instead, they imply an intricate interplay among crystal parameters, including Zn and carbonate levels, crystallite size (crystallinity), and zeta potential. These ideas can coexist with the possibility that the increase of Zn within cancerous thyroid nodule MCs might arise from shifting conditions within the tumor microenvironment during disease progression.

1. **Conclusions**

Synthetic MC analogs of HA with varied Zn fractions have similar morphology to malignant thyroid FNA MCs. Incorporating Zn2+ ions within the HA lattice reduces the unit cell parameters 'a' and 'c' as well as the crystallite size, indicative of lower crystallinity. When exposing thyroid cancer cell lines to these MC analogs, the response in terms of malignancy potential mainly depends on the Zn and carbonate fractions and crystallinity. High crystallinity and decreased carbonate and Zn content, which is in the range observed for cancerous FNA MCs, emerge as potential drivers of malignancy potential. Returning to the initial question regarding the relationship between the Zn content of FNA MCs and malignancy in thyroid nodules, these *in vitro* findings do not definitively determine the causality of the observed correlation. However, they highlight the significance of considering not only the Zn presence and MC morphology but also the crystallinity, surface charge, and carbonate content of the MCs.

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