ORIGINAL ARTICLE

Cytocompatible Carbonate Apatite Derived From Gypsum Granules For Bone Regeneration

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ABSTRACT

Introduction: Synthetic bone substitutes, carbonate apatite derived from gypsum (CAG) granules, are developed for bone reconstruction, offering improved surface area and regeneration. The aim of this study is to fabricate CAG granules through a dissolution-precipitation reaction by utilizing gypsum precursors. **Methods:** Set gypsum, which is known as calcium sulphate dihydrate granules with 200-355 µm was immersed in a mixed solution of disodium hydrogen phosphate (Na_2HPO_4) and sodium hydrogen carbonate ($NaHCO_3$) salt solution for 24, 48 and 72 hours of treatment time. **Results**: FTIR analysis confirmed the successful incorporation of carbonate and phosphate ions into the gypsum structure, as evidenced by increasing carbonate peaks with longer treatment durations. XRD further verified carbonate substitution within the apatite lattice, while FESEM revealed progressive structural modifications, including increased porosity over time. Degradation studies showed that CAG-24H exhibited slower degradation compared to CAG-48H and CAG-72H. Cytotoxicity testing demonstrated that CAG-24H was noncytotoxic at a concentration of 100 mg/ml, with over 80% cell viability observed. Additionally, CAG-24H promoted enhanced cell proliferation over a 9-day period. **Conclusion:** The findings indicate that CAG shows controlled degradation. CAG-24H, with its excellent cytocompatibility and ability to enhance cell proliferation, has the potential to be a promising candidate for bone graft substitutes.

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INTRODUCTION

Recent advances in biomaterials research focus on developing synthetic bone substitutes that not only replicate the mineral composition of bone but also mimic its complex hierarchical structure. These scaffolds can be engineered to deliver bioactive molecules, such as growth factors, that enhance osteogenesis, further improving the material's efficacy in promoting bone healing (1). To achieve these functional attributes, the composition of synthetic bone substitutes typically includes calcium (Ca), phosphate (P), and carbonate ions (CO_3^{2-}), which are integral to the mineral phase of human bone. These components play a vital role in mimicking the natural mineral environment and supporting the physiological processes involved in bone repair and regeneration (2).

We introduce a unique approach by systematically fabricating smaller-sized CAG (200–355 µm), which have not been explored in prior studies. This study correlates treatment duration (24, 48, and 72 hours) with both carbonate incorporation and cytotoxicity, providing deeper insights into the biocompatibility and degradability of the granules. The emphasis on nontoxicity through detailed cytotoxicity assessments using human osteoblast cells further distinguishes this work from earlier research, making it a novel contribution

to the field of bone tissue engineering.

MATERIALS AND METHODS

Sample preparation and characterization

Calcium sulphate hemihydrate (CSH; $CaSO_4 \cdot \frac{1}{2}H_2O$; Wako Pure Chemicals, Japan) was mixed with distilled water and set for 24 hours to form calcium sulphate dihydrate (gypsum; $CaSO_4 \cdot 2H_2O$). The gypsum was crushed and sieved into 200-355 µm granules, then immersed in $NaHCO_3$ and Na_2HPO_4 (Fisher Scientific, UK) for 24, 48, and 72 hours (CAG-24H, CAG-48H, CAG-72H) to induce phosphorization and carbonation, forming carbonated apatite (CO_3Ap) granules. The CAG was filtered, rinsed, dried, and characterized by Fourier-transform infrared spectroscopy (FTIR; Perkin Elmer Spectrum 2000, USA), X-ray diffraction (XRD; Bruker, D8 Advanced, Germany) and field-emission scanning electron microscopy (FESEM; Leo 1525, Germany) to determine its physiochemical composition before in vitro degradation and biological testing. Ethical approval from the Health Campus (USM) was obtained prior to the commencement of the study [Reference No: USM/IACUC/2023/ (142)(1274)].

Degradability studies

Around 1g of CAG-24H, CAG-48H and CAG-72H were immersed in 20 ml of PBS and placed in a shaking water bath (Lab Companion, Korea) at 37°C for various immersion times (1, 3, 5, and 7 d). At the pre-determined time points, the CAG samples were filtered using a qualitative filter paper (medium filtration; retention range: 7µm-11µm; GVS Filter Technology, Italy) from PBS and dried overnight at 100 °C prior to weight measurement until a mass change of <0.1 % occurred between mass determinations (3).

Cell culture

The hFOB 1.19 cells (CRL-11372™, ATCC®, USA) were cultured in DMEM/F-12 (Gibco™, Thermo Fisher Scientific) with Geneticin™ and 10% fetal bovine serum. Cells were incubated at 34°C in a 5% CO_2 humidified atmosphere, with the medium changed every 3 days aseptically.

Biocompatibility test using hFOB cell line

Cytotoxicity test

For the cell viability test, CAG-24H extracts (200 mg/ml) were prepared by immersing the material into a culture medium for 24 h at 37°C. The untreated control was complete media without material. Pure extract and dilutions (100, 50, and 25 mg/ml) were added to osteoblast cells seeded at 1 x 10^5 cells/ml in 24-multiwell plates for 24 h at 34°C. Cell viability was tested at 24, 48, and 72 h using alamarBlue™ (Thermo Fisher Scientific) and absorbance was measured at 540 nm with a universal microplate reader (Bio-Tek Instruments, Winooski, USA). Four replicates were performed for each treatment (4).

Cell proliferation test

Cell proliferation was assessed using CAG-24H extracts (100 mg/ml). The unexposed control was complete media. Extracts were added to cells seeded at 1x10^4 cells/ml in 6-multiwell plates for 24 h. After 1 day, cell viability was stained with alamarBlue™ and absorbance was measured at 540 nm using a microplate reader. Three replicates were performed for each treatment. The culture was refreshed with extracts and incubated again, with staining repeated on days 3, 5, 7, and 9.

RESULTS

Physicochemical analysis

Fig. 1a shows the FTIR spectra of gypsum before and after treatment at 80°C for 24, 48, and 72 hours. The C-O peaks at 1450–1410 cm^{-1} and 872 cm^{-1} increase with longer treatment hours, indicating greater incorporation of CO_3^{2-} from $NaHCO_3$ into the gypsum matrix. The P-O bond intensities between 1000–1100 cm^{-1} and at 960 cm^{-1} increase significantly over the treatment period, showing that phosphate ion (PO_4^{3-}) from Na_2HPO_4 progressively replace sulphate ions (SO_4^{2-}). Concurrently, the S-O bond intensities at 1619 cm^{-1}, 667 cm^{-1}, and 1100 cm^{-1} decrease, indicating gypsum dissolution. To validate CO_3^{2-} incorporation, the FTIR spectrum of calcium carbonate ($CaCO_3$) was included for comparison, revealing characteristic C–O absorption

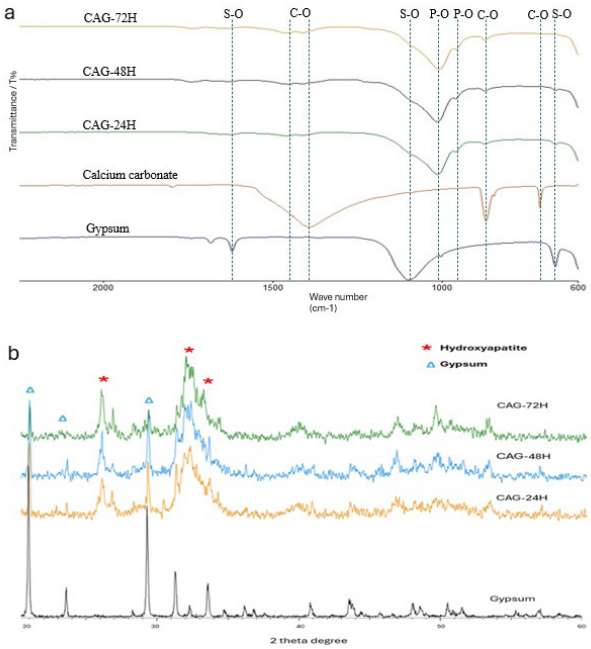

Figure 1: a) FTIR spectra of gypsum, CaCO3, CAG-24H, CAG-48H and CAG-72H. b) XRD pattern of gypsum, CAG-24H, CAG-48H and CAG-72H.

bands closely matching those observed in the treated samples, confirming the successful formation of CO_3Ap.

Further observations on the CAG samples were conducted using XRD, with the spectra matched against hydroxyapatite (HA) (JCPDS PDF No. 09-0432) and CO_3Ap (JCPDS PDF No. 35-0180). As shown in Fig. 1b, the disappearance of the gypsum peak at 29.1° is observed as the treatment duration increases, confirming the complete conversion of gypsum. Additionally, a broadening of peaks around 29–34° of 2θ suggests a strong indication of CO_3 incorporation into the apatite lattice. The transformation process facilitates the exchange of SO_4^{2-} in gypsum with PO_4^{3-}, leading to the formation of HA. These results, in combination with FTIR data, confirm the successful conversion of gypsum into CO_3Ap through ion substitution and phase transformation mechanisms.

The FESEM micrographs in Fig. 2 illustrate the morphological differences between untreated gypsum and treated CAG samples. Gypsum exhibits well-defined, dense, and compact plate-like crystal structures. CAG-24H retains a relatively intact morphology with fewer visible pores and a smoother surface, indicating higher structural integrity and lower porosity. In contrast, CAG-48H and CAG-72H reveal a progressively more porous and fragmented surface, with irregularly shaped granules and an increase in voids. The increase in porosity and microstructural disruption in CAG-48H and CAG-72H suggests a higher degree of transformation, correlating with their faster degradation in an aqueous environment.

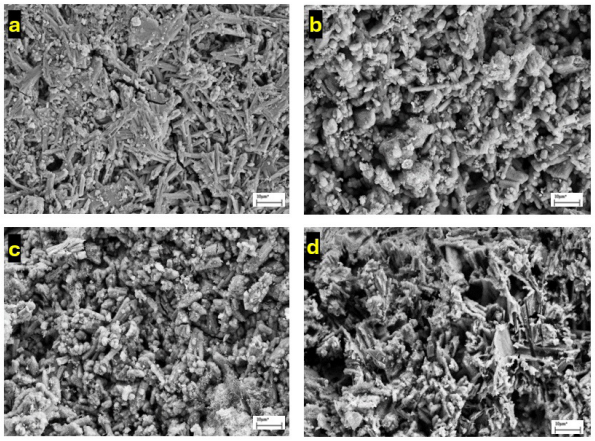

Figure 2: FESEM micrograph a) gypsum, b) CAG-24H, c) CAG-48H and d) CAG-72H (Mag: 3Kx).

Degradability study of CAG

Fig. 3 shows the degradation of CAG-24H, CAG-48H, and CAG-72H, where CAG-24H exhibits the slowest degradation. Despite having the lowest P-O and C-O bond intensities in the FTIR results, CAG-24H retains more of its original gypsum structure, resulting in higher structural integrity and lower porosity, which slows down dissolution. This is further supported by Fig. 2b, which shows a denser and less porous surface morphology in CAG-24H compared to the other treated

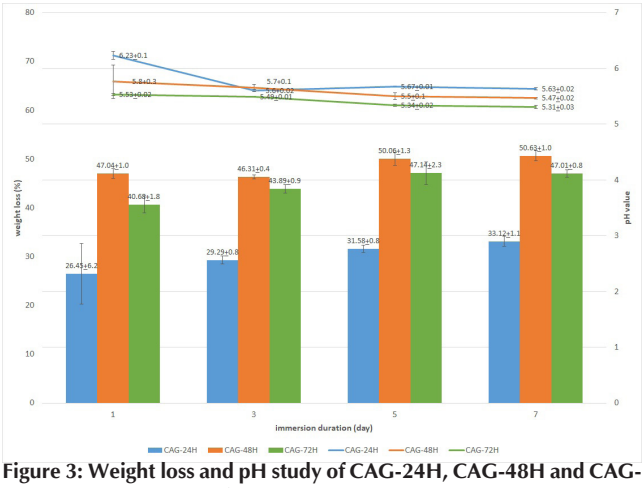

Figure 3: Weight loss and pH study of CAG-24H, CAG-48H and CAG-72H. Data are expressed as mean + SD

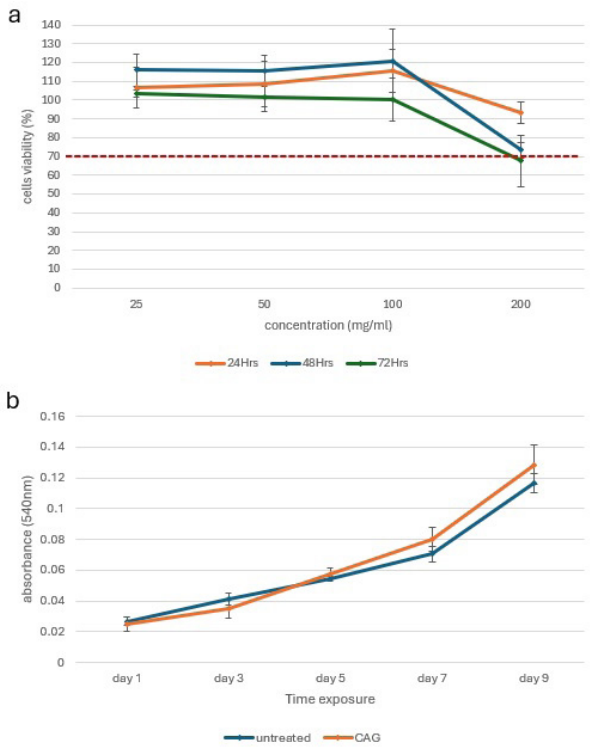

Fig. 4a: Cytotoxicity of CAG-24H exposed for different durations (24 h, 48 h, and 72 h) on hFOB cells. The red line represents ISO 10993-5 cut off level (70%). Fig 4b: Cell proliferation study over a 9-day period, comparing untreated and CAG-24H treated samples. Data are expressed as mean + SD

samples. In contrast, CAG-72H and CAG-48H, which exhibit higher CO_3 incorporation (C-O bonds) in FTIR, show faster degradation due to increased porosity and structural disruption, as observed in their FESEM images (Figs. 2c and 2d). The FESEM images confirm the progressive fragmentation and porosity increase in these samples, correlating with their increased degradation rates. Additionally, the pH values across all samples remained stable over the 7-day immersion period, with final pH values of 5.63 ± 0.02, 5.47 ± 0.02 and 5.31 ± 0.03 for CAG-24H, CAG-48H and CAG-72H respectively. The minimal variation in pH suggests that while structural changes influence degradation rates, the dissolution process does not significantly disrupt the surrounding medium's pH equilibrium.

Biocompatibility of CAG

Based on the results of the degradation test, CAG-48H and CAG-72H exhibited more than 50% weight loss after 5 days of immersion. CAG-24H, with a more controlled degradation rate, was chosen for further cytocompatibility testing. Fig. 4a shows the cytotoxicity of CAG-24H exposed for different durations (24 h, 48 h, and 72 h) on hFOB cells. The CAG samples are relatively biocompatible, with minimal cytotoxic effects at lower concentrations. However, at 200 mg/ml, a slight reduction in cell viability is observed, approaching the 70% threshold below which a material is classified as cytotoxic according to ISO 10993-5.

Fig. 4b presents a cell proliferation study over a 9-day period, comparing untreated and CAG-24H treated samples. Both groups show a consistent increase in cell proliferation over time, with the CAG-24H treated samples exhibiting slightly higher cell growth, particularly by day 9.

DISCUSSION

Although CO_3 incorporation rises with treatment duration, it remains less significant compared to P incorporation. As gypsum dissolves, Ca^{2+} and SO_4^{2-} ions are released and react with PO_4^{3-} and CO_3^{2-} ions, forming CO_3Ap, as reported in previous studies (5-7). The stronger attraction of Ca^{2+} for P due to its -3 charge, along with the shorter and stronger P-O bonds compared to S-O and C-O bonds, makes P the dominant factor in the structural transformation of gypsum into a P- and CO_3-rich structure (8, 9).

The treatment time influences the phase purity of CO_3Ap, with longer durations promoting complete transformation and reducing residual gypsum. Extended treatment enhances structural refinement through effective ion exchange and CO_3 incorporation, ensuring a well-ordered apatite phase without secondary phases. However, despite improved phase purity at longer durations, material selection should also consider degradation behaviour and structural stability.

The faster degradation of CAG-72H and CAG-48H samples is attributed to the higher CO_3 incorporation, which increases porosity and accelerates structural breakdown. This finding aligns with previous research indicating that higher CO_3 content in materials like carbonated HA leads to faster degradation due to enhanced solubility, greater surface area, and increased ion exchange (10). Moreover, Ayukawa et al. demonstrated that CO_3Ap exhibits enhanced bioresorption due to its low crystallinity, supporting the observation that higher CO_3 content results in more rapid degradation (11). Despite the faster degradation, the pH stability during the 7-day immersion period suggests that CO_3 incorporation does not significantly alter the pH of the surrounding environment (12). The slower degradation rate of CAG-24H is attributed to its lower CO_3 content compared to CAG-48H and CAG-72H, resulting in a more compact and stable structure that resists dissolution more effectively.

Balancing degradation with bone regeneration is crucial, as excessively fast and slow degradation can disrupt the healing process (13). CAG-24H was selected for further testing due to its controlled degradation rate, making it more suitable for potential clinical applications. The cytotoxicity results suggest that while CAG-24H is generally biocompatible, higher concentrations and longer exposure durations increase cytotoxic effects, highlighting the importance of optimizing both concentration and exposure time for safe use.

The overall trend indicates that CAG-24H treatment may enhance cell proliferation and cytocompatibility. The increased cell growth in the CAG-24H treated samples suggests that CAG could provide a favourable environment for cellular activity, potentially supporting tissue regeneration. This enhancement in proliferation could be beneficial for applications where rapid tissue integration is desired.

CONCLUSION

This study highlights the ability to control CO_3 content using gypsum and varying the treatment period. Specifically, a 24-hour treatment period (CAG-24H) resulted in a less degradable sample. Furthermore, CAG-24H at a concentration of 100 mg/mL demonstrated cytocompatibility, as it did not induce cell death after 72 hours and promoted cell proliferation over a 9-day period. Therefore, CAG-24H shows potential as a promising candidate for use as a bone graft substitute.

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