Supplementary Materials for

Supervariate gel transforms into various biominerals in salt solutions

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Materials and Methods

Materials

$CaCl_2$ (purity ≥ 97%, Sigma-Aldrich), $MgCl_2$ (purity ≥ 95%, Sigma-Aldrich), Na_2CO_3 (purity ≥ 99.5%, Sigma-Aldrich), K_2HPO_4 (purity ≥ 98%, Sigma-Aldrich), H_3PO_4 (purity ≈ 85%, Sigma-Aldrich), Calcein (purity ≥ 95%, Sigma-Aldrich), HCl (purity = 35-37%, Duksan). Human teeth were provided by the Baocheng Hospital (Shenzhen) in this study. The use of human tooth tissue specimens followed a protocol that was approved by the ethical committee of the hospital and the City University of Hong Kong and agreed upon by the patients.

Preparation of the "Mg-ACCP" gel

Four stock solutions of $CaCl_2$, $MgCl_2$, Na_2CO_3, and K_2HPO_4 (each of 0.8 M) were prepared. The Na_2CO_3 and K_2HPO_4 solutions (each 50 ml) were mixed in Beaker A (capacity 500 ml); the $CaCl_2$ and $MgCl_2$ solutions (each 50 ml) were mixed in Beaker B (capacity 500 ml). Under vigorous magnetic stirring, the solutions in Beaker A and Beaker B were quickly mixed (within a few seconds), followed by further stirring at ca 1300 r/min for 15 min, during which the clear mixture gradually turned white. The supernatant thereof was removed by centrifuge and decanting; and the obtained gel mass was washed by being dispersed in DI water and then centrifuged and decanted. The washing treatment was repeated three times. The resulting gelatinous substance is denoted the "Mg-ACCP" gel.

Preparation of the "gel+Ca", "+Ca-spnt", "+Ca-frzdry-CPP", and "+Ca-dry-HAP" samples

The wet "Mg-ACCP" gel (15 g) was dispersed in 30 ml solution of $CaCl_2$ (0.4, 0.6, 0.8, 1.0, 1.5, or 6.7 M) under the Vortex agitation. After standing for 15 hrs, the white suspension was centrifuged. The supernatant collected from the sample treated with 6.7 M $CaCl_2$ solution was collected and labeled as "+Ca-spnt". The precipitates were washed three times (by being redispersed in DI water, centrifuged, and decanted) to remove the soluble ions. After draining the supernatant, the precipitate thus obtained was a gelatinous substance, denoted as "gel+Ca". The subsequently freeze-dried sample was named "+Ca-frzdry-CPP" and those air-dried at 60 ℃ or at room temperature "+Ca-dry-HAP".

Preparation of the "gel+P", "+P-spnt", and "+P-dry-HAP" samples

The wet "Mg-ACCP" gel (15 g) was dispersed into 30 ml solution of K_2HPO_4 (0.4, 0.6, 0.8, 1.0, 1.5, or 13.44 M) under the Vortex agitation. After standing for 15 hours, the white suspension was centrifuged. The supernatant collected from the sample treated with 13.44 M K_2HPO_4 solution was collected and labeled as "+P-spnt". The precipitates were washed three times (by being redispersed in DI water, centrifuged, and decanted) to remove the soluble ions. After draining the supernatant, the precipitate thus obtained was a gelatinous substance, named as "gel+P". The product dried at 60 ℃ was denoted as "+P-dry-HAP".

Preparation of "gel+C", "+C-spnt", "+C-dry-CaC" samples

The wet "Mg-ACCP" gel (15 g) was dispersed into 30 ml solution of Na_2CO_3 (0.4, 0.6, 0.8, 1.0, 1.5, 1.93 M) under Vortex agitation. After standing for 15 hours, the white suspension was centrifuged. The supernatant collected from the sample treated with 1.93 M Na_2CO_3 was collected and labeled as "+C-spnt". The precipitates were washed three times (by being redispersed in DI water, centrifuged, and decanted) to remove the soluble ions. After draining the supernatant, the precipitate thus obtained was a gelatinous substance, named as "gel+C". The product dried at 60 ℃ was denoted as "+C-dry-CaC".

Preparation of the "ACCP" gel

The Na_2CO_3 and K_2HPO_4 solutions (each 50 ml, 0.8 M) were mixed in Beaker A (capacity 500 ml); the $CaCl_2$ solutions (100 ml, 0.8 M) were placed in Beaker B (capacity 500 ml). Under vigorous magnetic stirring, the solutions in Beaker A and Beaker B were quickly mixed (within a few seconds), followed by further stirring at ca 1300 r/min for 15 min, during which the clear mixture gradually turned white. The supernatant thereof was removed by centrifuge and decanting; and the obtained gel mass was washed by being dispersed in DI water and then centrifuged and decanted. The washing treatment was repeated three times. The resulting gelatinous substance is denoted as "ACCP" gel.

Enamel repair test

The native teeth were washed with DI water and ethanol each 3 times and dried in air. To mimic natural erosion, the teeth were soaked in H_3PO_4 solution (37%) for 10 sec followed by ultrasonic washing with DI water for 3 times and then dried in air (**Fig. S7**). The "Mg-ACCP" gel was smeared onto the damaged part followed by soaking in the $CaCl_2$ solution (6.7 M). A piece of Si wafer with a weight of 200 g on top was placed onto the gel-coated part to enhance close contact between gel and the etched enamel. After approximately 10 hrs, the wafer and weight were removed, and the enamel was air-dried in an oven at 60 ℃ for 1 hr. The treated tooth was finally thoroughly rinsed using DI water and air-dried at room temperature.

Confocal imaging

The Ca^{2+} in the soaking solution was labelled by calcein. First, the untreated portion was coated with nail polish as a control for comparison. After the repair test, the enamel was studied under confocal microscope. Fluorescence images were taken using an inverted confocal laser scanning microscope (Nikon-Eclipse 90i) under illumination with a 488 nm laser. All images were analysed using image analysis software (NIS-Elements).

General cell culture

Bone marrow mesenchymal stem cells (BMSCs) were cultured in low glucose Dulbecco's Modified Eagle Medium (DMEM, Invitrogen), supplied with 10% heat-inactivated fetal calf serum (Invitrogen) and 1% antibiotic-antimycotic agent (Invitrogen), in an atmosphere of 5% CO_2 at 37

^{o}C. The culture medium was substituted every three days, and the cell confluence was kept under 85%.

Cell viability assay

Cytotoxicity of "Mg-ACCP" was investigated via Cell Counting Kit-8 (CCK-8) test. The BMSCs were seeded in a 96-well plate (1×10^4 each well) for 24 hrs with DMEM. The DMEM was then removed, refilled with a DMEM solution mixed with the "Mg-ACCP" gel (wt.% of the freeze-dried gel: 1, 2, or 5 mg/mL), and incubated for another 72 hrs. A blank DMEM was used as control. CCK-solution (10μL) was then added to each well of the plate, and further incubated for another 120 min. The absorption at the wavelength of 450 nm was measured on a microplate reader (Beckman Coulter DTX 880).

Cell morphology and density evaluation

Cell morphology and density evaluation were conducted via hematoxylin and eosin staining (H&E staining). The BMSCs were incubated in a DMEM solution mixed with "Mg-ACCP" (wt.% of the freeze-dried gel: 1, 2, or 5 mg/mL) for 72 hrs. A blank DMEM was used as control. The cells were fixed in 10% formalin solution for 20 min and stained by soaking in the hematoxylin solution for 3 min, and then rinsed by water for 1 min. The sample were then differentiated in 1% acid ethyl alcohol and blued in 0.2% ammonia solution for 15 sec. Finally, the samples were stained with eosin solution for 60 sec and dehydrated through an ascending concentration of ethyl alcohol. The photographs were taken using an optical microscope (Leica DMI3000).

Osteogenic assay

Alkaline phosphatase (ALP) assay was applied to characterize the osteogenic effects of "Mg-ACCP". Three groups (negative control, positive control, and the "Mg-ACCP" gel) were tested. For the negative control group, the BMSCs were cultured with the DMEM. For the positive control group, the BMSCs were cultured with osteogenic differentiation medium (ODM) which was composed of DMEM, 50 μg/mL ascorbic acid (Sigma, USA), 10 mM β-glycerol phosphate (MP Biomedicals, France), and 100 nM dexamethasone (Santa Cruz, UK). For the "Mg-ACCP" sample, the BMSCs were cultured with ODM supplied with 5 mg/mL "Mg-ACCP".

The BMSCs were seeded in a 24-well dish at a density of 1×10^5 cells/cm^2 and cultured with DMEM first. When the confluence reached 80%, each group was refreshed with the corresponding culture medium. On Day 4, 7, and 10, the cells were stained with ALP staining solution (Beyotime, China). The photographs were taken on an optical microscope (Leica DMI3000).

Meanwhile, the cells were lysed for conducting ALP activity assay (Beyotime, China). The absorbance was measured through a microplate reader (Beckman Coulter DTX 880) under the illumination with the wavelength of 405 nm. The quantitative assay was triplicated to avoid contingency. The data was analyzed through a T-Test to examine the result's significance (P value).

Biomineralization assay

Alizarin red S (ARS) staining was conducted to investigate the biomineralization of "Mg-ACCP". In this experiment, 3 groups were set up, including a negative control group, a positive control group and an experimental group. For the negative control group, the BMSCs were cultured with normal DMEM. For the positive control group, the BMSCs were cultured with osteogenic differentiation medium (ODM) which is composed of normal DMEM, 50 μg/mL ascorbic acid (Sigma, USA), 10 mM β-glycerol phosphate (MP Biomedicals, France) and 100 nM dexamethasone (Santa Cruz, UK). For the experimental group, the BMSCs were cultured with ODM supplied with 5mg/mL "Mg-ACCP". The BMSCs were seeded in a 24-well dish with a 1×105 cells/cm2 density and cultured with normal DMEM for all the groups first. When the confluence reached 80%, each group was refreshed with the corresponding culture medium. At day 21, the cells were stained with 1% (wt/v) Alizarin red S solution (ARS, Solarbio, China) at pH value of 4.2. Observing and capturing images were performed on an optical microscope (Leica DMI3000). Quantification analysis was performed by dissolving the stained samples in a solution of 70% methanol and 10% acetic acid in distilled water. The absorption of 450 nm was measured via a microplate reader (Beckman Coulter DTX 880). The quantitative assay was triplicated to avoid contingency. The data was analyzed through a T-Test to examine the result's significance (P value).

Characterization

X-ray diffraction (XRD) patterns were collected on an X-ray powder diffractometer (BRUKER SRD-D2 Phaser) using Cu-Kα radiation (λ = 1.5406 Å) (scanning rate of 10°/min). The Rheometer used was Kinexus pro^{+} with a test gap of 1 mm, a frequency range of 0.1-100 Hz, and the oscillatory mode (22-25℃, 40-45%). Transmission electron microscope (TEM) images were collected on FEI Talos F200S (FEI Super-X EDS Detector). The scanning electron microscope (SEM) images and spectra were obtained using a field-emission SEM (Philips XL-30) equipped with an energy-dispersive X-ray (EDX) detector operating at 20 kV. Nanoindentation tests were collected on a G200 nanoindentation instrument (Agilent Technologies, CA, USA). A constant strain rate of 0.2 nm s^{-1} was maintained during the loading process. The applied load force and the depth of continuous penetration into the sample during indentation were monitored by a computer. Microindentation was tested on the Fischer HM2000XY Micro-Hardness Tester. Fourier-transform infrared spectroscopy (FTIR) data were collected from a PerkinElmer FTIR Spectrometer. Raman spectra were collected from the WITec RAMAN alpha 300R.

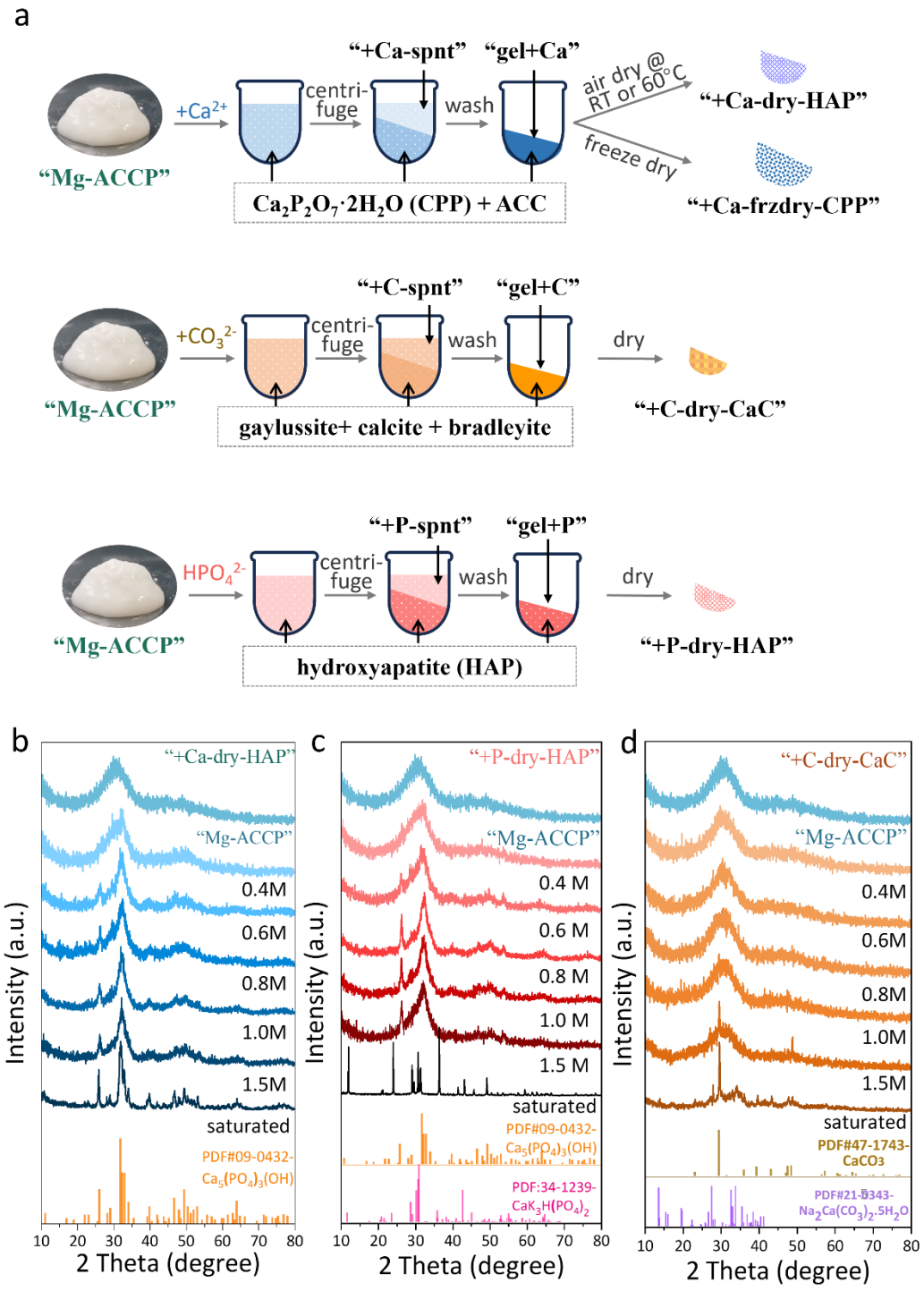

Figure S1. a), The mineralization routes of the "Mg-ACCP" gel studied in this work. b-d), XRD patterns of "+Ca-dry-HAP", "+P-dry-HAP", and "+C-dry-CaC", fabricated by soaking "Mg-ACCP" gel in different concentrations of $CaCl_2$ (0.40 to 6.7 M), K_2HPO_4 (0.40 to 13.44 M), and Na_2CO_3 (0.40 to 1.93 M). The soaking time for all the samples was 8 hrs.

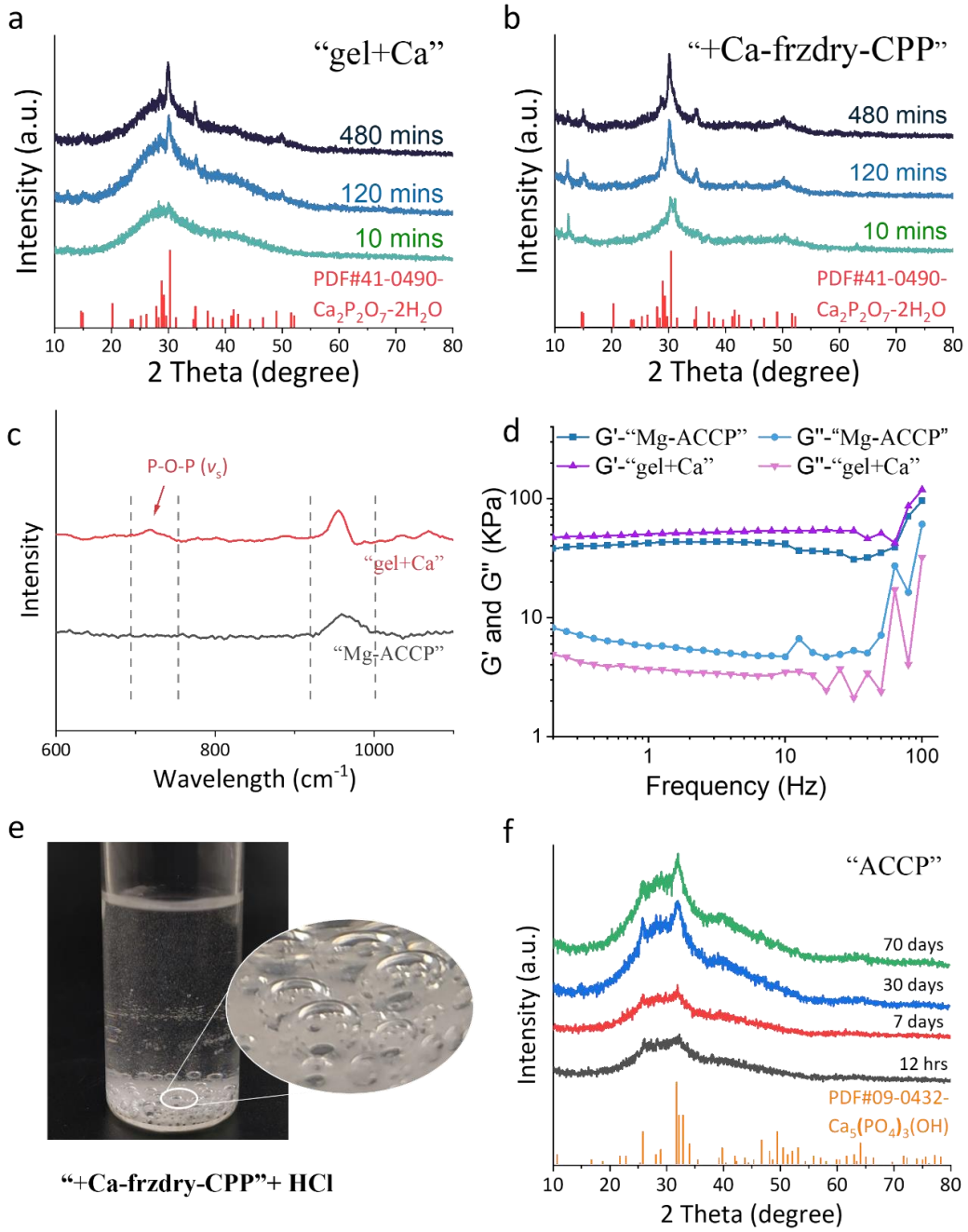

Figure S2. a-b) XRD patterns of "gel+Ca" and "+Ca-frzdry-CPP" prepared with different soaking time (10, 120, and 480 min) in the $CaCl_2$ solution (6.7 M). c) Raman spectra of "gel+Ca" and "Mg-ACCP". d) Plots of G' (storage modulus) and G'' (loss modulus) versus shear frequency for the "Mg-ACCP" gel and "gel+Ca" (prepared via soaking in 6.7 M $CaCl_2$ solution for 480 min). e) Photograph showing ample CO_2 bubbles generated with "+Ca-frzdry-CPP" (prepared via soaking in 6.7 M $CaCl_2$ solution for 480 min) dissolved in a solution of HCl (15 wt%). f) Time-evolving XRD patterns of the wet "ACCP" gel collected after it was stored under ambient conditions for different time durations.

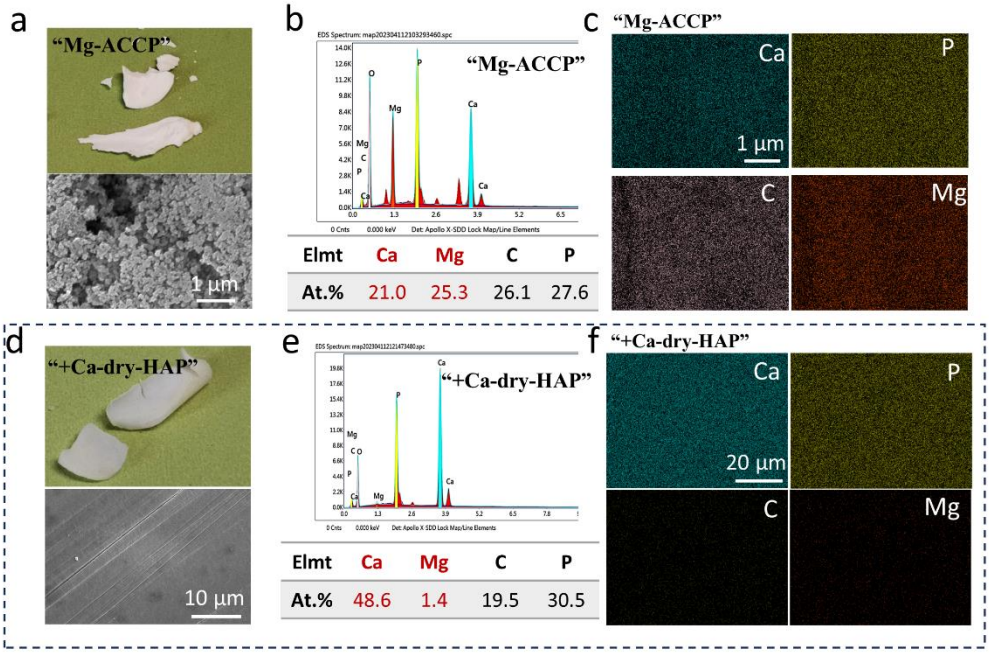

Figure S3. Photographs, SEM images, EDS spectra, and elemental mapping for Ca, Mg, C, P of "Mg-ACCP" (a-c), and "+Ca-dry-HAP" (prepared via soaking in 6.7 M $CaCl_2$ solution for 480 min) (d-f).

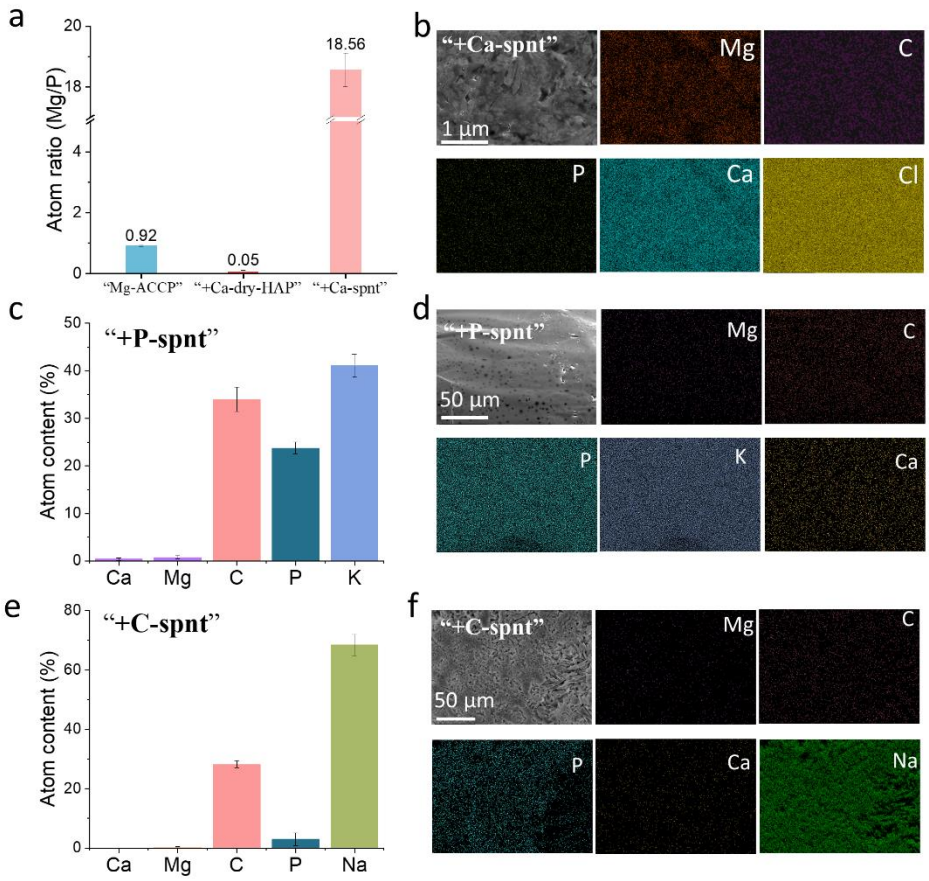

Figure S4. a) The atomic ratios of Mg:P of "Mg-ACCP", "+Ca-dry-HAP", and "+Ca-spnt". b) Elemental mapping images of "+Ca-spnt". c) Atomic contents of the different elements in "+P-spnt". d) Elemental mapping images of "+P-spnt". e) Atomic contents of the different elements in "+C-spnt". f) Elemental mapping images of "+C-spnt". Samples in (a), (c), and (e) were prepared via soaking in 6.7 M $CaCl_2$, 13.44 M K_2HPO_4 and 1.93 M Na_2CO_3 solutions, respectively, for 480 min.

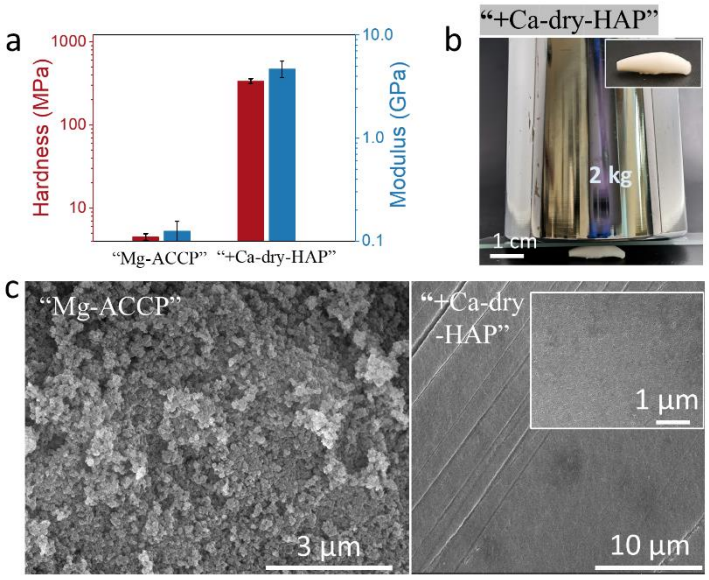

Figure S5. Microhardness and modulus values (a), photographs (withstanding a weight of 2 Kg to illustrate its strength and integrity), (b) and SEM images (c) of "Mg-ACCP" and "+Ca-dry-HAP" (prepared via soaking in 6.7 M $CaCl_2$ solution for 480 min).

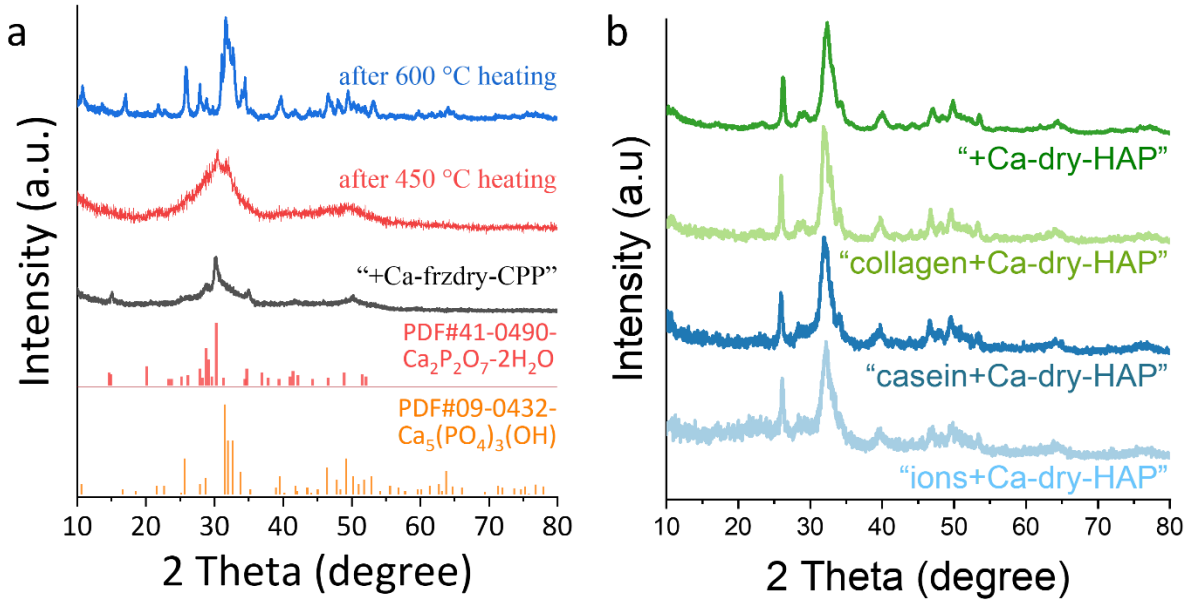

Figure S6. a) XRD patterns of the "+Ca-frzdry-CPP" (prepared via soaking in 6.7 M $CaCl_2$ solution for 480 min) sample as freeze-dried and after thermally annealed at different temperatures (450 and 600 ℃) for 2 hrs. **b)** XRD patterns of "+Ca-dry-HAP" prepared with no or different additives added into the $CaCl_2$ soaking solution. The samples were prepared by first soaking "Mg-ACCP" in 1 M CaCl2 solution without any additive or with 1 wt.% collagen, 1 wt.% casein, or a mixture of Na^+, K^+, Mg^{2+}, and Sr^{2+} (each of 0.02 M) added to the soaking solution. After soaked for 20 hrs, the samples were washed with DI water, and air-dried at 60 °C.

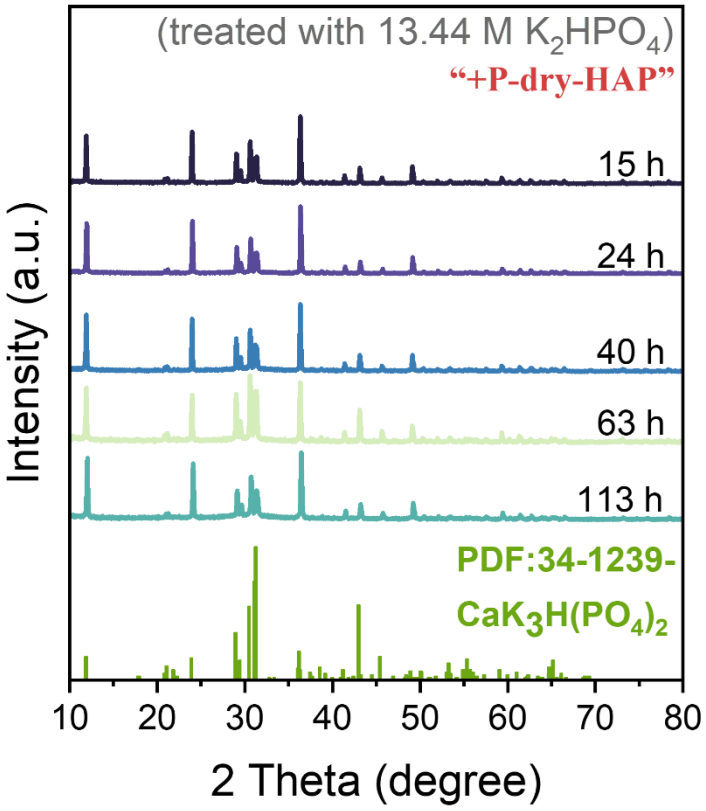

Figure S7. XRD patterns of the "+P-dry-HAP" samples produced with different soaking times in the concentrated K_2HPO4 solution (13.44 M).

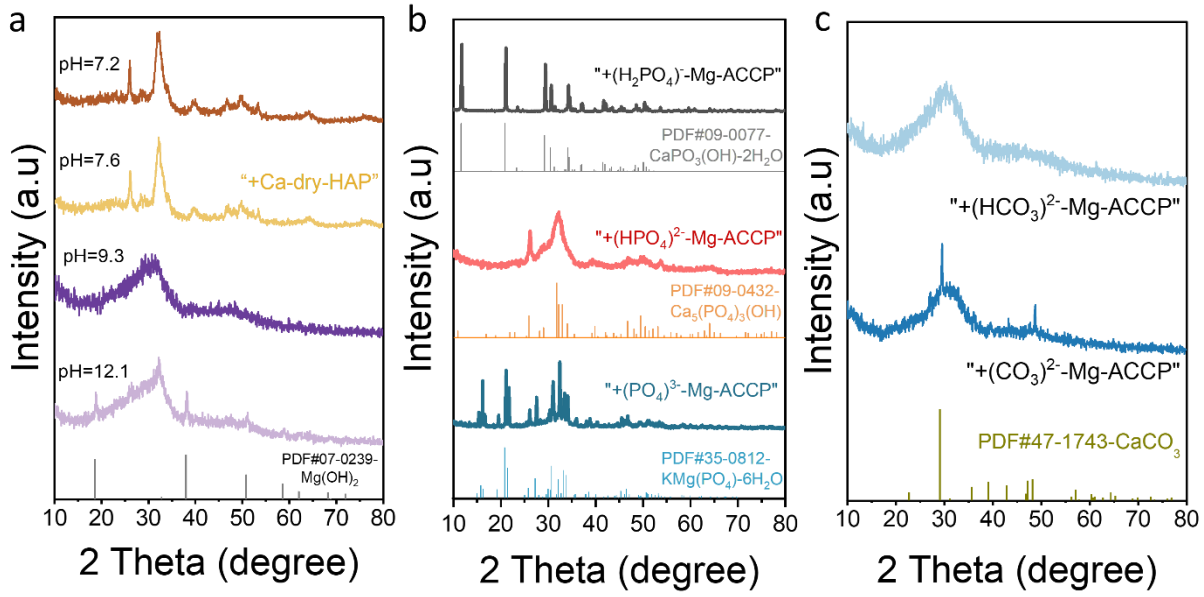

Figure S8. XRD patterns of the mineralization products obtained with different soaking solutions of varying pH values (all soaked for 20 hrs, washed with DI water, and air-dried at 60 °C): a) 1 M $CaCl_2$, pH value adjusted with HCl (10 wt.% aq.) and NaOH (0.5 M aq.) solutions; b) 1 M KH_2PO_4, K_2HPO_4, or K_3PO_4; c) 1 M $NaHCO_3$ or Na_2CO_3.

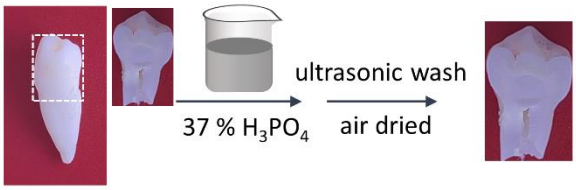

Figure S9. Photographs of the native tooth before and after the acid etching (etched for 10 sec).

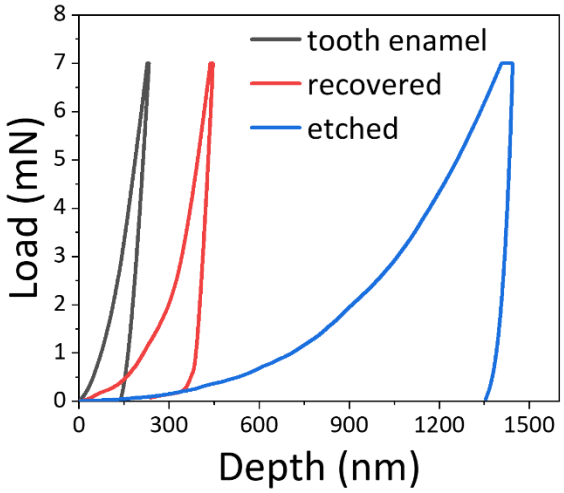

Figure S10. The nanoindentation curves for the native, acid-etched, and recovered enamels.