Electronic Supplementary Information

Bisphosphonate type-dependent cell viability suppressive effects of carbon nanohorn–calcium phosphate–bisphosphonate nanocomposites

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Supplementary Information I. Preparation of OxCNH

As-grown CNHs were oxidized using a light-assisted oxidation method.[1] Briefly, as-grown CNHs (20 mg) were dispersed in an aqueous hydrogen peroxide solution (30.0–35.5%, 20 mL; FUJIFILM Wako Pure Chemical Corporation, Japan), stirred on a hot plate (hot plate temperature 100°C, solution temperature ~70°C), and simultaneously irradiated for 2.5 h with a xenon arc lamp (Lightningcure^{TM} LC5; Hamamatsu Photonics K.K., Japan). After cooling at room temperature, the reaction solution was filtered and washed five times with ultrapure water. The resulting OxCNHs contained approximately 350 carboxyl groups in one carbon-nanohorn tubule.[1] The resulting OxCNHs were dispersed in ultrapure water by sonication. The OxCNH concentration in solution was determined using a UV-vis spectrophotometer (UV-2450; SHIMADZU CORPORATION, Japan) based on a calibration curve of optical absorbance at 700 nm and the CNH concentration.[2]

Supplementary Information II. Preparation of labile supersaturated CaP solutions and coprecipitation process

All procedures were performed under aseptic conditions. Before preparing supersaturated CaP solutions containing OxCNH and BP, three source solutions were prepared: Ca- and P-containing solutions and an alkalinizer solution. Ca-containing solutions (Ca^{2+}: 4.78–8.61 mM) were prepared by mixing Ringer's Solution OTSUKA (Otsuka Pharmaceutical Co., Ltd., Japan) and Calcium Chloride Corrective Injection 1 mEq/mL (Otsuka Pharmaceutical Co., Ltd.) at the volume ratios specified in Table S2. P-containing solutions ($H_2PO_4^-$/HPO_4^{2-}: 20.0–36.0 mM) were prepared by mixing Klinisalz® (KYOWA CritiCare Co., Ltd., Japan) and Dibasic Potassium Phosphate Injection 20mEq Kit (Terumo Corporation, Japan) at volume ratios specified in Table S2. Alkalinizer solution (HCO_3^-: 167 mM) was prepared by mixing MEYLON® Injection 7% (Otsuka Pharmaceutical Co., Ltd.) and injection water at a volume ratio of 20:80. The source solutions were prepared in centrifuge tubes at room temperature and placed in a dry bath set to 18°C.

In a 15 mL centrifuge tube, 0.250 mL OxCNH solution (2.0 mg/mL), 0.250 mL BP solution (2.94 mM), 7.674 mL Ca-containing solution, 0.917 mL P-containing solution, and 0.909 mL alkalinizer solution were added sequentially. The final solution (10 mL) was immediately mixed by shaking the tube several times. The resultant labile supersaturated CaP solutions containing OxCNH and BP were tightly sealed in the tubes and then placed in a 25°C incubator to allow precipitation. After coprecipitation for 30 min, the products were collected by centrifugation (6,000 rpm, 5–10 min) and washed with injection water.

Table S1. Concentrations of calcium and phosphate ions in X1.0, X1.2, X1.4, and X1.8 solutions

	Concentration (mM)			
	X1.0	X1.2	X1.4	X1.8
Ca^{2+}	3.68	4.41	5.15	6.62
$H_2PO_4^-/HPO_4^{2-}$	1.83	2.20	2.57	3.30

Table S2. Injection solutions and volume ratios for preparing Ca- and P-containing solutions

	Volume ratios			
	X1.0	X1.2	X1.4	X1.8
Ca-containing solution Ringer's Solution OTSUKA : Calcium Chloride Corrective Injection 1 mEq/mL	99.49 : 0.51	99.30 : 0.70	99.11 : 0.89	98.72 : 1.28
P-containing solution Klinisalz® : Dibasic Potassium Phosphate Injection 20mEq Kit	97.96 : 2.04	97.15 : 2.85	96.33 : 3.67	94.69 : 5.31

Supplementary Information III. Cell viability assay, celular uptake quantity determination, reactive oxygen species (ROS) assay, and fluorescence microscope observation

III-1. RAW264.7 cell viability

A medium was mixed with each specimen (**OxCNH-CaP-BPs**, OxCNH, or BPs) at a ratio of 9:1 (vol/vol). Each specimen was prepared for use as follows: (1) **OxCNH-CaP-BP** solutions were prepared by dispersing **OxCNH-CaP-BPs** (obtained from a single batch, 10 mL supersaturated CaP solution) in 1 mL injection water, which was then diluted 3-, 10-, 30-, and 100-fold with injection water; (2) OxCNH solutions (5, 17, 50, and 167 μg/mL) were prepared by diluting a 2.0 mg/mL OxCNH solution with injection water; and (3) IBN, ZOL, or PAM solutions (2.5, 5.0, 10, 25, 50, 100, 250, and 500 μg/mL) were prepared by diluting Bonviva® (1 mg/1 mL), ZOMETA® for i.v. infusion (4 mg/5 mL), or PAMIDRONATE DISODIUM (15 mg/5 mL) with injection water. Because the specimens were mixed with medium at ratios of 1:9, the final concentrations of the specimens in the medium were $1/10^{th}$ of the original concentrations. As negative and positive controls, injection water (negative control) and actinomycin D (positive control, 1 μg/mL final concentration, FUJIFILM Wako Pure Chemical Corporation) were used instead of specimens.

RAW264.7 cells (1.5×10^4 cells/0.1 mL/well, n = 4) were seeded into 96-well plates and incubated at 37°C for 24 h, and culture medium was then exchanged for 0.1 mL medium supplemented with specimen. Cells were incubated for an additional 24 h, and subsequently, unwashed cells were observed using an optical microscope (IX71; Olympus Corporation, Japan). After observation, 10 μL Cell Counting Kit-8 (CCK-8) solution (DOJINDO LABORATORIES, Japan) was added to each well, and the plate was placed in a CO_2 incubator at 37°C for 1 h. For each specimen, the corresponding control was prepared following the same

procedure but without adding CCK-8 solution to the well. Optical absorbance was measured at 450 nm using a microplate reader (iMark^{TM}; Bio-Rad Laboratories, Inc., USA). The absorbance of the control (no CCK-8 solution) for each treatment was subtracted from the absorbance of the experimental group to compensate for the color of OxCNH. The resultant substantive absorbance was normalized against the negative control (defined as a value of 100) and expressed as cell viability relative to control.

III-2. Cellular uptake of CNH in RAW264.7 cells

RAW264.7 cells (8.0×10^4 cells/2 mL/well, n = 4) were seeded into 6-well plates and incubated at 37°C for 48 h, and culture medium was then exchanged for 2 mL of medium supplemented with specimen. After incubation for 24 h, cells were washed twice with phosphate buffered saline (PBS; FUJIFILM Wako Pure Chemical Corporation) and lysed in cell lysis reagent (0.4 mL), CelLytic^{TM} M (Sigma-Aldrich Co. LLC., USA) with protease inhibitor cocktail (Nacalai Tesque Inc., Japan). The resulting cell lysates were centrifuged at 18,000*g* for 10 min at 4°C to remove solid materials such as OxCNH. Protein concentrations were estimated using the Bradford assay as follows. The supernatants (0.01 mL) were added to each well of a 96-well plate, 0.25 mL Bradford reagent (Sigma-Aldrich Co. LLC.) was added, and the plates were incubated at room temperature for 10 min. Optical absorbance was measured at 595 nm using a microplate reader (iMark^{TM}) and converted to cell numbers. For conversion from absorbance to cell numbers, we used a calibration curve generated by a plot of absorbance versus known cell numbers, which were counted using a cell counter (Tali^{TM} Image-Based Cytometer; Thermo Fisher Scientific, USA).

To quantify OxCNH in cell lysates, the residual supernatants after the Bradford assay and the black sediments (collected by centrifugation as described above) were redispersed in 0.5%

vol/vol Triton™ X-100 (Sigma-Aldrich Co. LLC.) by sonication (Q125; WAKENBTECH Co., Ltd., Japan) at 40% amplitude for 20 min in the 1 sec on/1 sec off mode. In the resulting redispersion solution, OxCNH dispersion was homogeneous and stable. The OxCNH concentration in the redispersed solution was estimated by measuring optical absorbance at 700 nm using a UV-vis spectrophotometer. To estimate the OxCNH concentration from absorbance, a calibration curve of known CNH concentrations and absorbance (700 nm) was used as previously reported.[2]

III-3. ROS detection

RAW264.7 cells (4.0×10^4 cells/1 mL/well, n = 4) were seeded into 24-well plates and incubated at 37°C for 48 h, and culture medium was then exchanged for 1 mL medium supplemented with the specimen. After incubation for 24 h, cells were washed twice with PBS and further incubated with 1 mL 10 μM 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Thermo Fisher Scientific) at 37°C for 30 min. Cells were then washed three times with PBS and lysed in cell lysis reagent (0.2 mL, CelLytic™ M with protease inhibitor cocktail). The resulting cell lysates were centrifuged at 18,000*g* for 10 min at 4°C to remove solid materials such as OxCNH. The fluorescence of the supernatant (0.1 mL) was measured using a microplate reader (Infinite® F200 PRO; Tecan Group Ltd., Switzerland) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. In parallel, the Bradford protein assay was performed as described above (Supplementary Information III-2). Absorbance was measured at 595 nm using a microplate reader (iMark™) to measure the relative amount of protein. The fluorescence intensity was normalized to the amount of protein and divided by that of the control.

III-4. Osteoclast viability

For differentiation into osteoclasts, RAW264.7 cells (1.0×10^3 cells/well, n = 8) were seeded in 96-well plates and cultured for 4 days in the presence of RANKL (200 ng/mL). After differentiation into osteoclasts, cells were cultured in α-MEM supplemented with each specimen for 24 or 48 h. Medium containing no specimen was used as a negative control. After aspiration of culture medium to exclude the influence of specimens, 10% alamarBlue reagent (Remel, USA) in culture medium was added to each well, and cells were allowed to metabolize the dye for 60 min. Changes in fluorescence were detected using a microplate reader (AF2200; Eppendorf, Germany) at excitation and emission wavelengths of 535 nm and 590 nm, respectively. The resultant fluorescence intensity was normalized against that of the negative control (defined as a value of 100) and expressed as cell viability relative to control.

III-5. Cell observation by fluorescence microscopy

RAW264.7 cells (3.1×10^3 cells/well) cultured on 8-well μ-Slides (ibidi GmbH, Germany) were exposed to each specimen for 24 or 48 h under conditions described above (Supplementary Information III-4). After two PBS washes, cells were incubated with nuclear stain (Bisbenzimide H33342 Fluorochrome Trihydrochloride DMSO Solution; Nacalai Tesque, Inc.) and lysosome stain (Cyto Painter Lysosomal Staining ab 138895; Abcam, UK) for 30 min prior to imaging. After two washes, cells were visualized using a fluorescence microscope (BZ-X710; Keyence Corporation, Japan).

Supplementary Information IV. Product SEM images

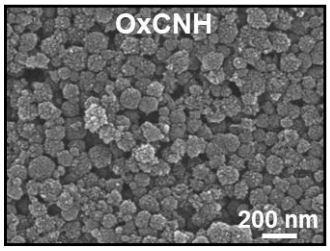

Figure S1. SEM image of OxCNH.

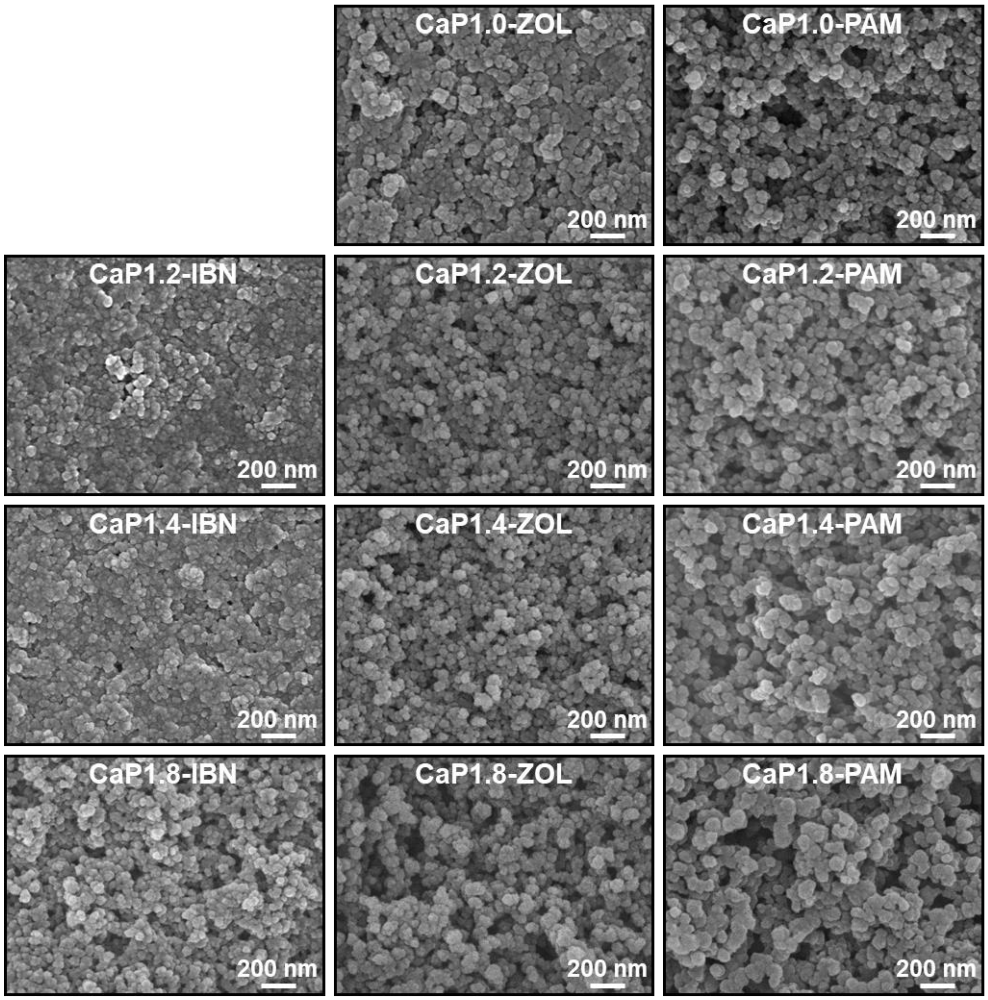

Figure S2. SEM images of **CaP-BPs**. **CaP1.0-IBN** was not collected by centrifugation due to insufficient yield.

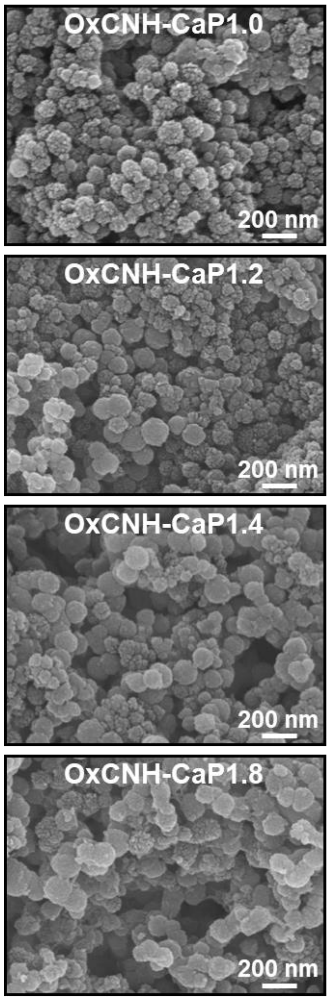

Figure S3. SEM images of **OxCNH-CaPs**.

Supplementary Information V. Compositional analysis of the products.

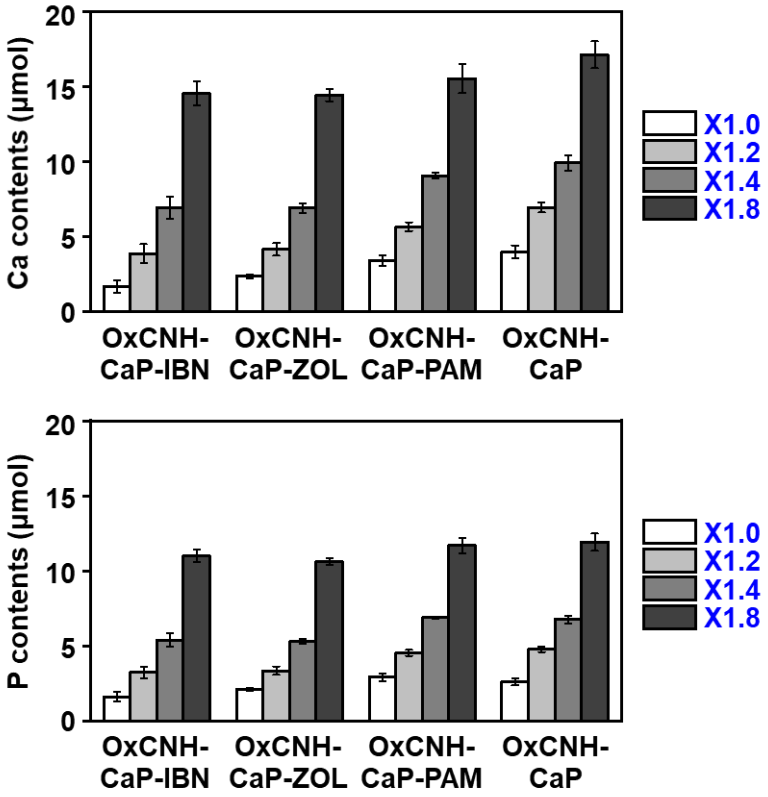

Figure S4. Contents of Ca and P in **OxCNH-CaP-BPs** and **OxCNH-CaPs** obtained from a single batch of supersaturated CaP solution (10 mL).

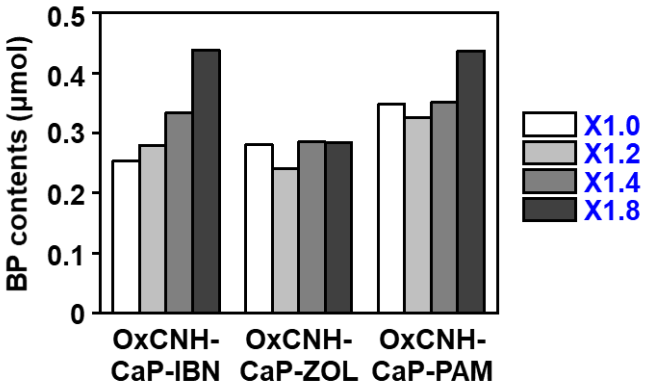

Figure S5. Contents of BPs in **OxCNH-CaP-BPs** obtained from a single batch of supersaturated CaP solution (10 mL). The BP contents in **OxCNH-CaP-BPs** were estimated based on the assumption that the P/Ca ratio of CaP in **OxCNH-CaP-BPs** would be roughly equivalent to that in the corresponding **OxCNH-CaPs**, as described in our previous report.[3] The average values of Ca and P contents (Figure S4) were used for the estimation.

Supplementary Information VI. Size distributions and zeta potentials of the products

The size (hydrodynamic diameter) distributions and zeta potentials of **OxCNH-CaP-BPs** and OxCNH in injection water and medium were measured at 20°C by quantifying dynamic light scattering (DLS) and electrophoretic light scattering (ELS) with a particle size analyzer (Zetasizer Nano-ZS, Malvern Instruments Ltd., UK). Before DLS and ELS measurements, **OxCNH-CaP-BP** solutions were prepared by dispersing **OxCNH-CaP-BPs** (obtained from 10 mL supersaturated CaP solution) in 1 mL injection water, which was then diluted 3-fold with injection water. OxCNH solution (167 μg/mL) was prepared by diluting 2.0 mg/mL OxCNH with injection water. **OxCNH-CaP-BP** and OxCNH solutions were ultrasonicated for 1 min, diluted 10-fold in injection water or medium with pipetting, and placed in the analyzer. ELS measurements were repeated three times per solution to obtain an average and standard deviation of the measured values.

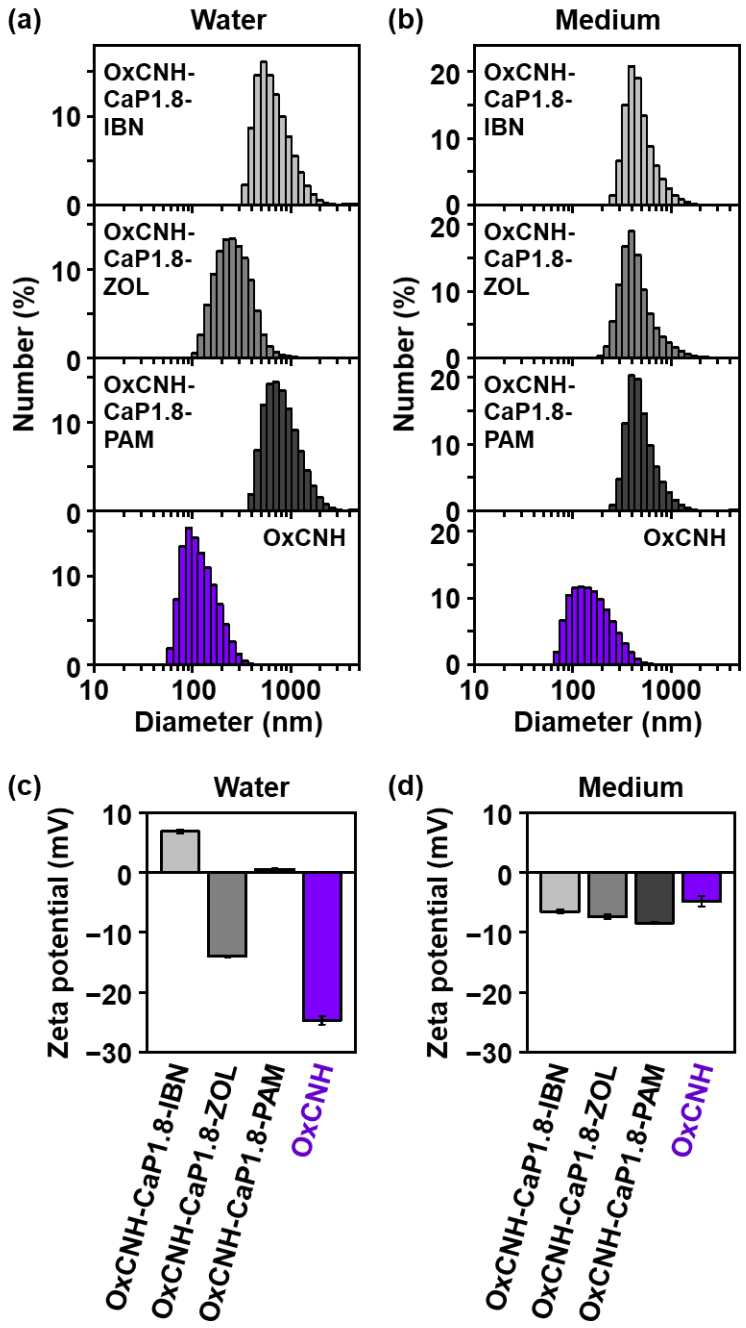

Figure S6. DLS histograms of number distributions (a and b) and zeta potentials (c and d) of **OxCNH-CaP1.8-IBN**, **OxCNH-CaP1.8-ZOL**, **OxCNH-CaP1.8-PAM**, and OxCNH in injection water (a and c) and medium (b and d).

Supplementary Information VII. ROS levels in RAW264.7 cells exposed to OxCNH and BPs

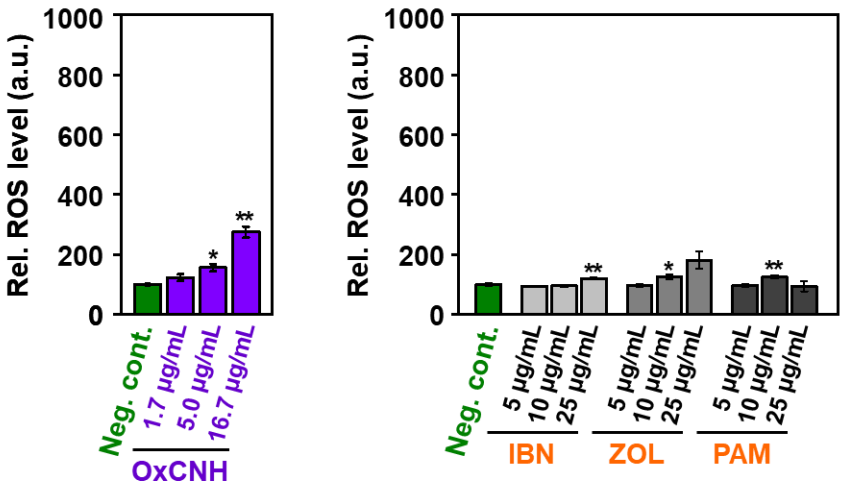

Figure S7. ROS generation in RAW264.7 cells exposed to OxCNH and BPs (IBN, ZOL, or PAM) for 24 h (average ± standard error, n = 4). Injection water was used as a negative control. *P < 0.05 and **P < 0.01 relative to negative control.

Supplementary Information VIII. Viability of osteoclasts exposed to **OxCNH-CaP1.2-BPs**, OxCNH and BPs

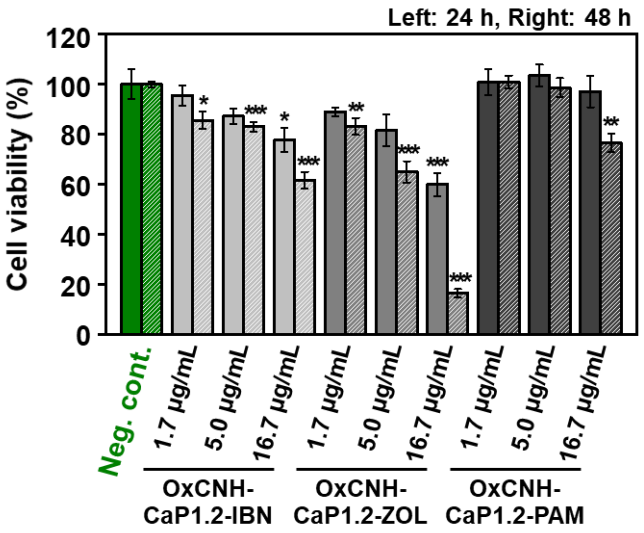

Figure S8. Cell viability of osteoclasts treated with **OxCNH-CaP1.2-IBN**, **OxCNH-CaP1.2-ZOL**, or **OxCNH-CaP1.2-PAM** for 24 h and 48 h, as estimated by fluorescence intensity of the alamarBlue assay (average ± standard error, n = 6). The estimated final OxCNH concentrations in **OxCNH-CaP1.2-BPs** media were 1.7, 5.0, and 16.7 µg/mL, as indicated in the figure (30-, 10-, and 3-fold dilutions, respectively). Medium was used as a negative control. *P < 0.05, **P < 0.01, and ***P < 0.001 relative to negative control.

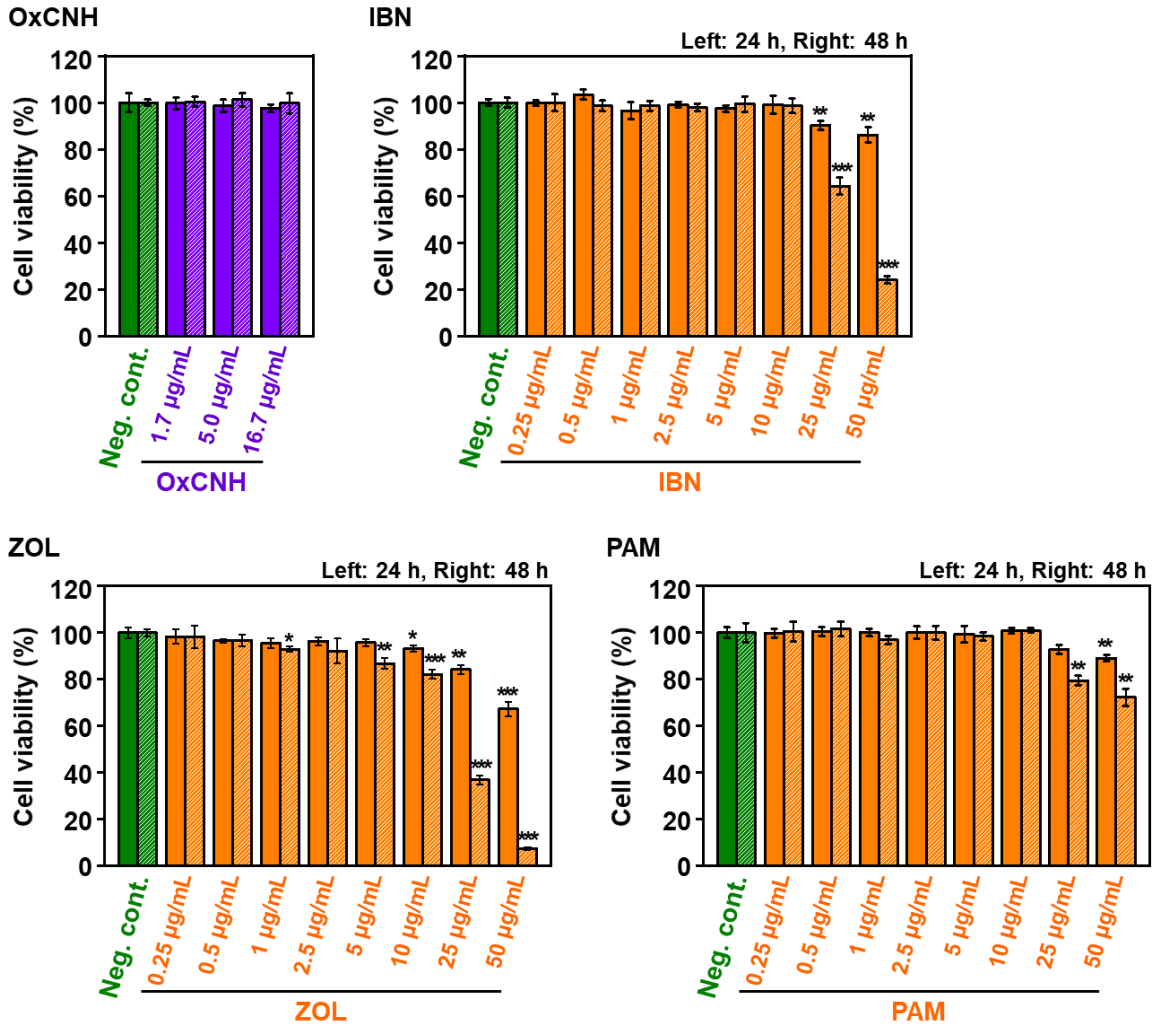

Figure S9. Effect of OxCNH and BPs (IBN, ZOL, and PAM) on osteoclast viability after incubation for 24 h and 48 h, as estimated by fluorescence intensity of the alamarBlue assay (average ± standard error, n = 6 or 8). Medium was used as a negative control. *P < 0.05, **P < 0.01, and ***P < 0.001 relative to negative control. Cell viability for OxCNH: modified with permission from ref. 3 (Copyright 2021 American Chemical Society).

Supplementary Information IX. Fluorescence micrographs of osteoclasts

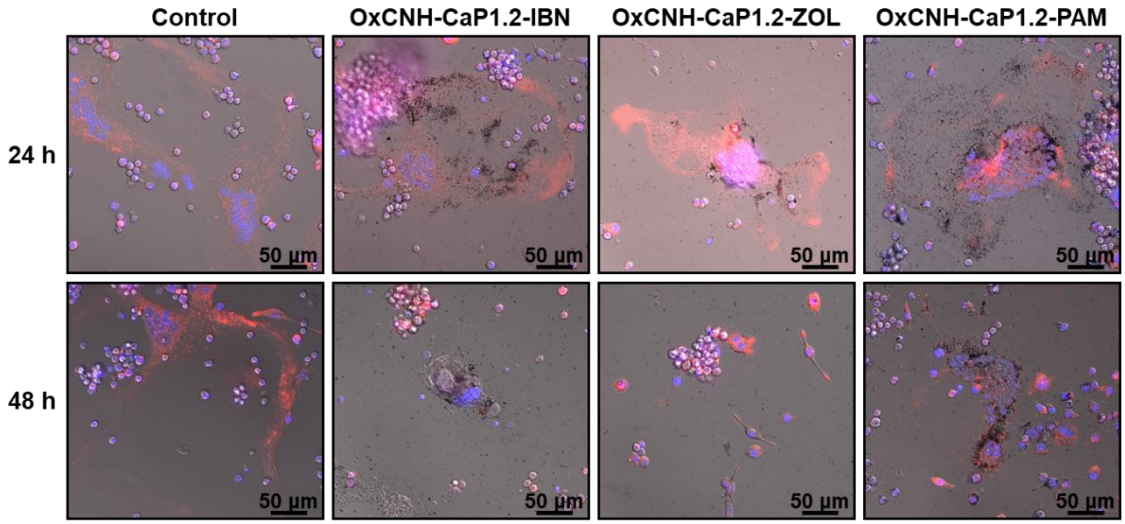

Figure S10. Fluorescence micrographs of osteoclasts after incubation for 24 h (upper) and 48 h (lower) with medium (negative control, left), **OxCNH-CaP1.2-IBN** (second from the left), **OxCNH-CaP1.2-ZOL** (second from the right), or **OxCNH-CaP1.2-PAM** (right). The estimated final OxCNH concentration in **OxCNH-CaP1.2-BP** media was 16.7 µg/mL (3-fold dilution). Nuclei and lysosomes were stained with bisbenzimide H33342 fluorochrome trihydrochloride (blue) and CytoPainter ab 138895 (orange), respectively.

Supplementary Information X. Putative mechanism of OxCNH–CaP–BP nanocomposite formation

The mechanism of OxCNH–CaP–BP nanocomposite formation in labile supersaturated CaP solutions could be similar to the mechanism proposed in our previous report of OxCNH–CaP–IBN nanocomposites.[3] First, homogeneous CaP nucleation occurred throughout the supersaturated CaP solution. BPs could be involved in the CaP nucleation stage due to the strong interaction between the anionic phosphate groups of BPs and the cationic calcium ions of CaPs. The CaP nuclei with BPs could then grow spontaneously over time to form CaP–BP nanocomposites < 50 nm (IBN or ZOL) or approximately 100 nm (PAM) (lower insets of Figures 2d, 2h, and 2l). In parallel, heterogeneous CaP nucleation occurred on the carboxyl groups of OxCNH due to interaction of calcium ions with carboxyl groups. The CaP nuclei on OxCNH grew into CaP–BP deposits that could robustly cover OxCNH surfaces.[3]

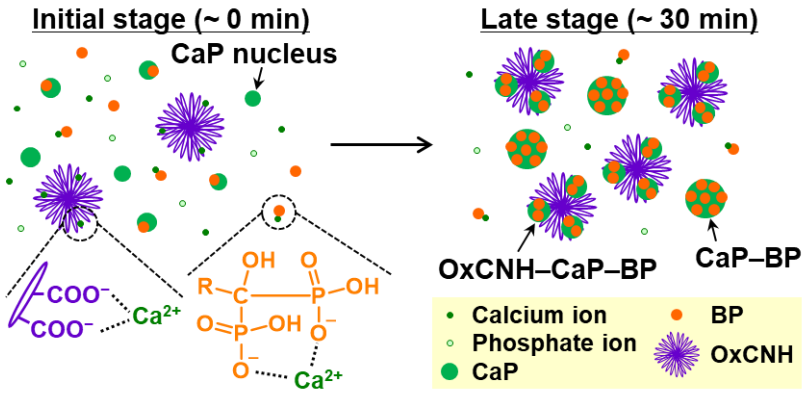

Figure S11. Schematic illustration of OxCNH–CaP–BP nanocomposite formation in labile supersaturated CaP solutions containing OxCNH and BPs. Modified with permission from ref. 3 (Copyright 2021 American Chemical Society).

References

1. M. Zhang, M. Yudasaka, K. Ajima, J. Miyawaki and S. Iijima, Light-assisted oxidation of single-wall carbon nanohorns for abundant creation of oxygenated groups that enable chemical modifications with proteins to enhance biocompatibility, *ACS Nano*, 2007, **1**, 265–272.
2. M. Zhang, X. Zhou, S. Iijima and M. Yudasaka, Small-sized carbon nanohorns enabling cellular uptake control, *Small*, 2012, **8**, 2524–2531.
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