Electronic Supplementary Information

Calcium-mediated zoledronate loading onto carbon nanohorns

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Supplementary Information I. Preparation of the OxCNH solutions.

As-grown CNHs were prepared by CO_2 laser ablation of graphite under an argon atmosphere (1 atm) at room temperature.[1] Purity was about 95%, with impurities (micrometer-sized giant graphitic particles) comprising about 5% of the sample. To introduce the carboxyl groups, as-grown CNHs were oxidized using a light-assisted oxidation method.[2] Briefly, as-grown CNHs (20 mg) were dispersed in an aqueous solution of hydrogen peroxide (30.0–35.5%; 20 mL; FUJIFILM Wako Pure Chemical Corporation, Japan), stirred on a hot plate (hot plate temperature, 100°C; solution temperature, ca. 70°C), and simultaneously irradiated for 2.5 h using a xenon arc lamp (Lightningcure LC5; Hamamatsu Photonics K.K., Japan). After cooling to room temperature, the reaction solution was filtered and washed five times with ultrapure water. The OxCNHs were dispersed in ultrapure water by sonication. The OxCNH concentration in solution was then measured in a UV-vis spectrophotometer (UV-2450; SHIMADZU CORPORATION, Japan), based on a calibration curve of optical absorbance at 700 nm versus CNH concentration.[3] The resulting OxCNH solution was sterilized by autoclaving and then diluted with injection water (Water for Injection, Fuso Pharmaceutical Industries, Ltd., Japan) to obtain a solution containing a 2.0 mg/mL OxCNH.

Supplementary Information II. Preparation of the CaP reaction solutions and nanocomposites.

All procedures were performed under aseptic conditions. Before preparing the CaP reaction solutions containing OxCNH and ZOL, three source solutions were prepared: Ca- and P-containing solutions, and an alkalinizer solution. The Ca-containing solutions (Ca^{2+}: 8.6, 5.7, and 2.9 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 ratios of 98.72:1.28, 99.30:0.70, and 99.87:0.13 (vol%); resulting in solutions X1.8, X1.2, and X0.6, respectively. The P-containing solutions ($H_2PO_4^-$/HPO_4^{2-}: 36.0, 24.0, and 12.0 mM) were prepared by mixing Klinisalz® (Fuso Pharmaceutical Industries, Ltd.) and Dibasic Potassium Phosphate Injection 20mEq Kit (Terumo Corporation, Japan) at ratios of 94.69:5.31, 97.15:2.85, and 99.59:0.41 (vol%); resulting in solutions X1.8, X1.2, and X0.6, respectively. Alkalinizer solution (HCO_3^-: 167 mM) was prepared by mixing MEYLON® Injection 7% (Otsuka Pharmaceutical Co., Ltd.) and injection water at a 20:80 vol%. The source solutions were prepared in centrifuge tubes at room temperature, and then placed in a dry bath set to 18°C.

Next, 0.250 mL OxCNH solution (2.0 mg/mL), 0.250 mL ZOL 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 to a 15 mL centrifuge tube. The final solution (10 mL) was mixed immediately by shaking several times. The resulting CaP reaction solutions containing OxCNH and ZOL had constant concentrations of OxCNH (50 μg/mL) and ZOL (73.5 μM) (Table 1). The tubes were tightly sealed and then placed in an incubator set to 25°C to allow precipitation. After 30 min, the products were collected by centrifugation (6,000 rpm, 5–10 min) and washed with injection water.

Supplementary Information III. Details of the cell viability assay, confocal microscopy, and measurement of cellular uptake, reactive oxygen species (ROS) assay, and caspase activity in RAW264.7 cells.

III-1. Cell viability assay

A medium used for culturing RAW264.7 cells (Section 2.3) was mixed with each specimen solution (**OxCNH-CaP1.8-ZOL**, **OxCNH-CaP1.2-ZOL**, **OxCNH-Ca-ZOL**, OxCNH, or ZOL) at a ratio of 9:1 (vol%). The specimen solutions were prepared for use as follows: (1) nanocomposite (**OxCNH-CaP1.8-ZOL**, **OxCNH-CaP1.2-ZOL**, or **OxCNH-Ca-ZOL**) solutions were prepared by dispersing the nanocomposite (obtained from a single batch; 10 mL of CaP reaction solution) in 1 mL of injection water, which was then diluted 3-, 5-, 10-, 20-, 30-, 50-, 100-, 200- and 300-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. (3) ZOL solutions (2.5, 5.0, 10, 25, 50, 100, 250, and 500 μg/mL) were prepared by diluting ZOMETA® for i.v. infusion 4 mg/5 mL (Novartis Pharma K.K., Japan) with injection water. Because the specimen solutions were mixed with medium at ratios of 1:9 (vol%), the final concentrations of each specimen in the medium were one-tenth of the original concentrations. Injection water and an actinomycin D solution (final concentration in medium: 1 μg/mL; FUJIFILM Wako Pure Chemical Corporation) were used as negative and positive controls, respectively.

RAW264.7 cells (7.5×10^3 cells; 0.1 mL/well; $n = 4$) were seeded into 96-well plates and incubated at 37°C for 48 h. The culture medium was exchanged for 0.1 mL of medium supplemented with the test specimens, and the cells were incubated for an additional 3 or 24 h. Then, 10 μL of Cell Counting Kit-8 (CCK-8) solution (DOJINDO LABORATORIES, Japan) was added to each well, and the plate was placed for 1 h in a CO_2 incubator at 37°C. For each specimen, the corresponding control was prepared using the same procedure but without adding CCK-8 solution to the well. Optical absorbance was measured at 450 nm in a microplate reader (iMark™; 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 absorbance was normalized against the negative control (defined as a value of 100) and expressed as % cell viability relative to the control.

III-2. Cell observation by confocal microscopy

RAW264.7 cells (1.0×10^5 cells; 2 mL/dish) were seeded into glass-bottom dishes (35 mm diameter) and incubated at 37°C for 48 h. The culture medium was then exchanged for 2 mL of medium supplemented with the test specimens and the cells were incubated for an additional 3 or 24 h. Then, the cells were washed twice with phosphate buffered saline (PBS; FUJIFILM Wako Pure Chemical Corporation).

For LysoTracker staining, fresh medium containing 50 nM LysoTracker Red DND-99 (Thermo Fisher Scientific, USA) was added and the cells were incubated for ca. 30 min. The stained cells were then observed under a confocal microscope (LSM 5 PASCAL; Zeiss, Germany).

Annexin V/propidium iodide (PI) staining was performed using an annexin V-FITC apoptosis detection kit (BioVision, Inc., USA). After washing twice with PBS, 0.5 mL of binding buffer containing fluorescein-conjugated Annexin V (annexin V-FITC) and PI was added to the cells, which were then observed under a confocal microscope (LSM 5 PASCAL).

III-3. Bradford protein assay, and measurement of uptake of OxCNH by RAW264.7 cells

RAW264.7 cells (1.0×10^5 cells; 2 mL/well; $n = 3$) were seeded into 6-well plates and incubated at 37°C for 48 h. The culture medium was then exchanged for 2 mL of medium supplemented with the test specimens and the cells were incubated for an additional 3 or 24 h. Then, the cells were washed twice with PBS, and lysed in cell lysis reagent (0.4 mL), CelLytic™ M (Merck KGaA, Germany) containing a 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. The cell number in each well was estimated using the Bradford protein assay as follows: supernatants (0.01 mL) were added to each well of a 96-well plate, followed by addition of 0.25 mL of Bradford reagent (Merck KGaA). The plates were the incubated at room temperature for 10 min. Absorbance was measured at 595 nm in a microplate reader (iMark™) and then converted to cell number. This was done using a calibration curve generated by plotting absorbance against known cell number, counted using a cell counter (Tali™ Image-Based Cytometer; Thermo Fisher Scientific).

To quantify the amount of OxCNH in the cell lysates, the supernatant remaining after the Bradford protein assay and the black sediment (collected by centrifugation as described above) were redispersed in 0.5 vol% Triton™ X-100 (Merck KGaA) by sonication (Q125; WAKENBTECH Co., Ltd., Japan): amplitude, 40%; time, 20 min; 1 sec on/1 sec off mode.

Dispersion of OxCNH throughout the solution was homogeneous and stable. The OxCNH concentration in the redispersed solution was estimated by measuring optical absorbance at 700 nm in a UV-vis spectrophotometer. A calibration curve of known CNH concentration versus absorbance (700 nm) was used to estimate the concentration of OxCNH, as previously described.[3]

III-4. Detection of ROS

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. The culture medium was then exchanged for 1 mL of medium supplemented with the test specimens and the cells were incubated for an additional 24 h. Then, the cells were washed twice with PBS and incubated with 1 mL 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, 10 μM; Thermo Fisher Scientific) at 37°C for 30 min. The cells were then washed three times with PBS and lysed in cell lysis reagent (0.2 mL, CelLytic™ M containing protease inhibitor cocktail). The resulting cell lysates were centrifuged at 18,000 *g* for 10 min at 4°C to remove solid material such as OxCNH. The fluorescence of the supernatant (0.1 mL) was measured in 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-3). Absorbance at 595 nm was measured in a microplate reader (iMark™) to estimate the relative amounts of protein. Fluorescence intensity was normalized to the amount of protein and divided by that of the control.

III-5. Caspase 3/7 activity assay

RAW264.7 cells (7.5×10^3 cells; 0.1 mL/well; $n = 3$) were seeded into 96-well plates and incubated at 37°C for 48 h. The culture medium was then exchanged for 0.1 mL of medium supplemented with the test specimens and the cells were incubated for an additional 24 h. Then, the cells were washed twice with PBS and incubated for 30 min with 0.1 mL of MEM and 0.1 mL of Caspase-Glo 3/7 reagent. The resulting cell lysates were centrifuged at 18,000 *g* for 10 min at 4°C to remove solid material such as OxCNH. The luminescence of the supernatant (0.1 mL) was measured in a microplate reader (Infinite® F200 PRO). In parallel, the Bradford protein assay was performed as described above (Supplementary Information III-3). Absorbance at 595 nm was measured in a microplate reader (iMark™) to estimate the relative amount of protein. Luminescence intensity was normalized to the amount of protein and divided by that of the control.

Supplementary Information IV. SEM images and EDX spectra.

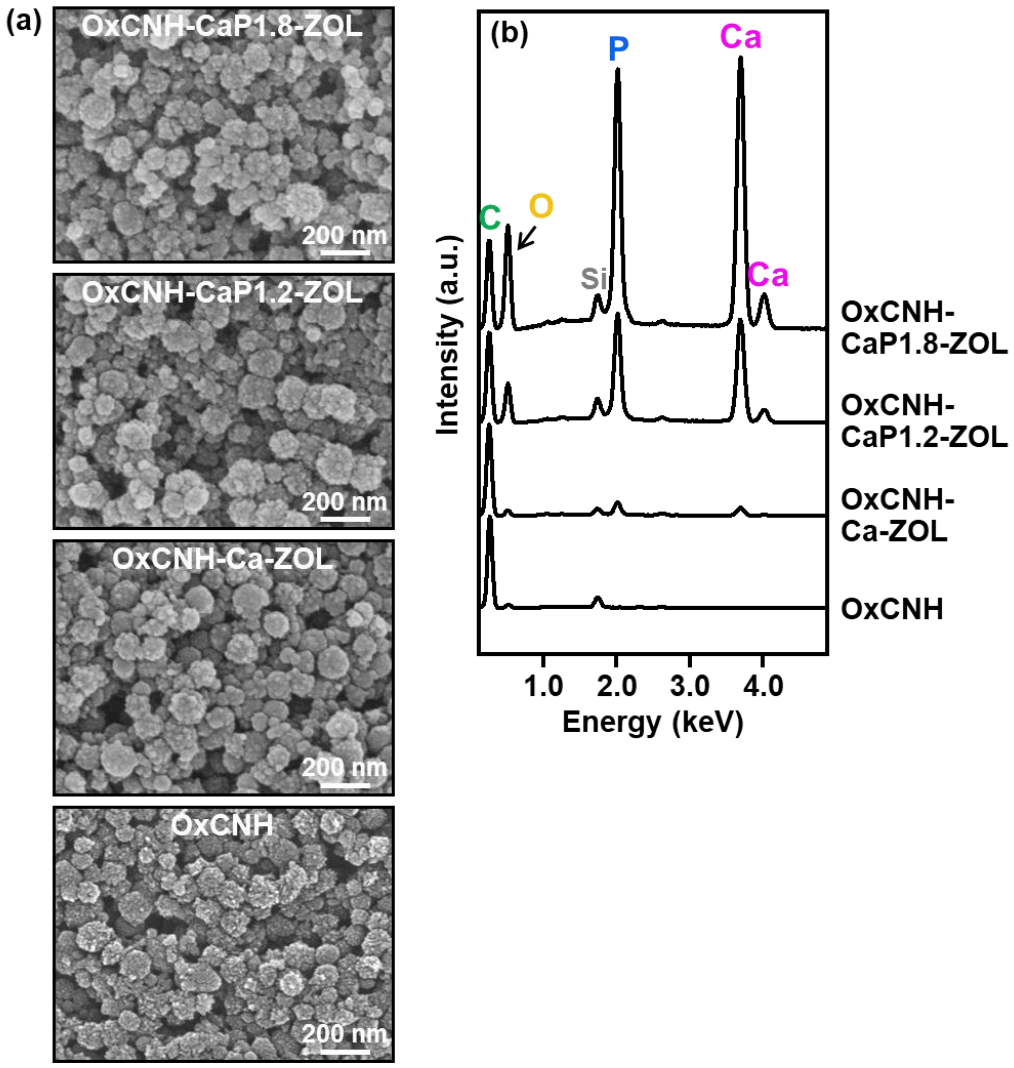

Figure S1. SEM images (a) and EDX spectra (b) of **OxCNH-CaP1.8-ZOL**, **OxCNH-CaP1.2-ZOL**, **OxCNH-Ca-ZOL**, and OxCNH. The Si peaks in (b) are derived from the silicon substrate used to mount the products. Intensity of elemental C in (b) was normalized.

Supplementary Information V. Viability of RAW264.7 cells exposed to three types of nanocomposites and OxCNH.

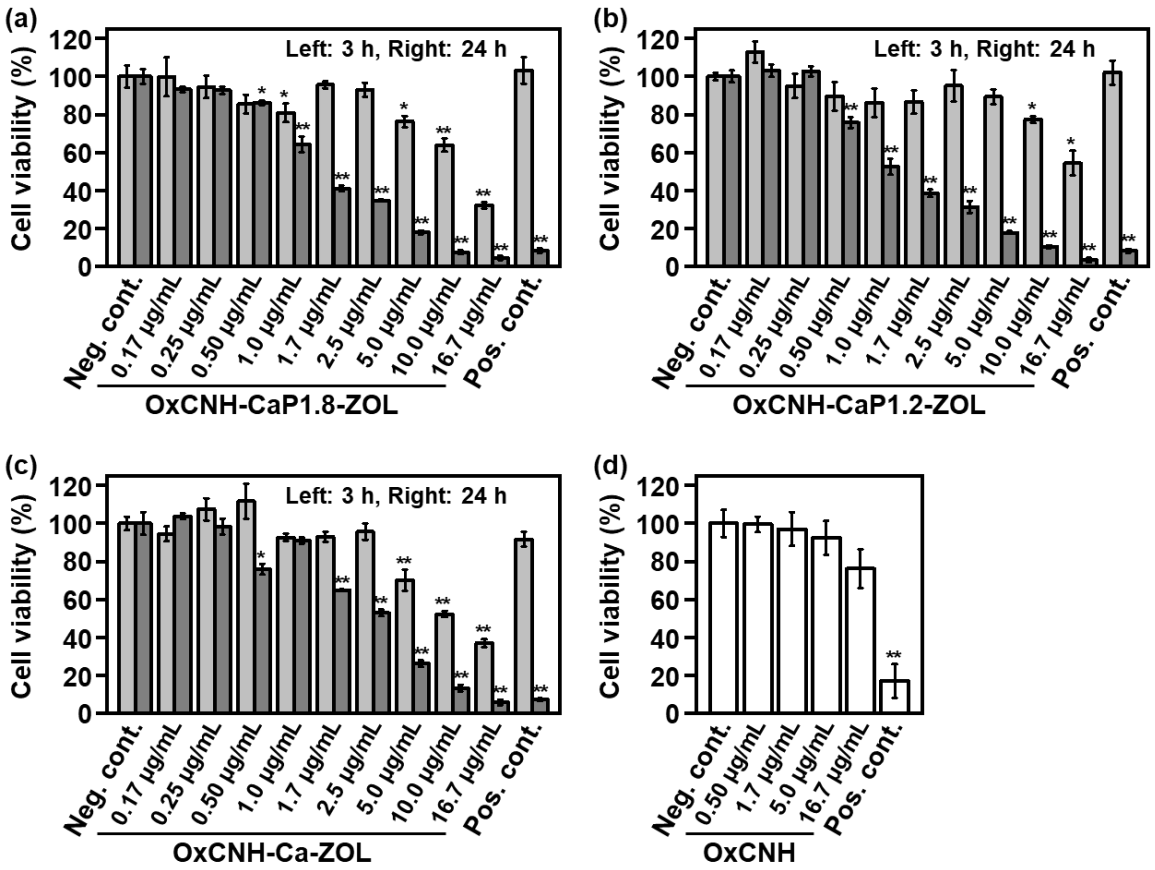

Figure S2. Viability of RAW 264.7 cells incubated with **OxCNH-CaP1.8-ZOL** (a), **OxCNH-CaP1.2-ZOL** (b), or **OxCNH-Ca-ZOL** (c) for 3 or 24 h, or with OxCNH (d) for 24 h; viability was estimated by measuring the absorbance in CCK-8 assays (average ± standard error, $n = 4$). The estimated final OxCNH concentrations in medium containing nanocomposites were 0.17, 0.25, 0.50, 1.0, 1.7, 2.5, 5.0, 10.0, 16.7 µg/mL (300-, 200-, 100-, 50-, 30-, 20-, 10-, 5-, 3-fold dilutions, respectively). The negative and positive controls were injection water and actinomycin D (1 µg/mL), respectively. * $P < 0.05$ and ** $P < 0.01$, relative to the negative control. (d): Taken from ref. 4, and presented with some modifications.

Supplementary Information VI. Concentrations of OxCNH and ZOL in each medium.

Table S1. Estimated final concentrations of OxCNH and ZOL in medium containing **OxCNH-CaP1.8-ZOL**, **OxCNH-CaP1.2-ZOL**, and **OxCNH-Ca-ZOL**

Dilution	OxCNH (μg/mL)	ZOL (μg/mL)		
		OxCNH-CaP1.8-ZOL	**OxCNH-CaP1.2-ZOL**	**OxCNH-Ca-ZOL**
300	0.17	0.057	0.054	0.030
200	0.25	0.085	0.081	0.045
100	0.50	0.17	0.16	0.090
50	1.0	0.34	0.32	0.18
30	1.7	0.57	0.54	0.30
20	2.5	0.85	0.81	0.45
10	5.0	1.7	1.6	0.90
5	10.0	3.4	3.2	1.8
3	16.7	5.7	5.4	3.0

Supplementary Information VII. ROS levels and caspase activity in RAW 264.7 cells exposed to three types of nanocomposites, OxCNH, and ZOL.

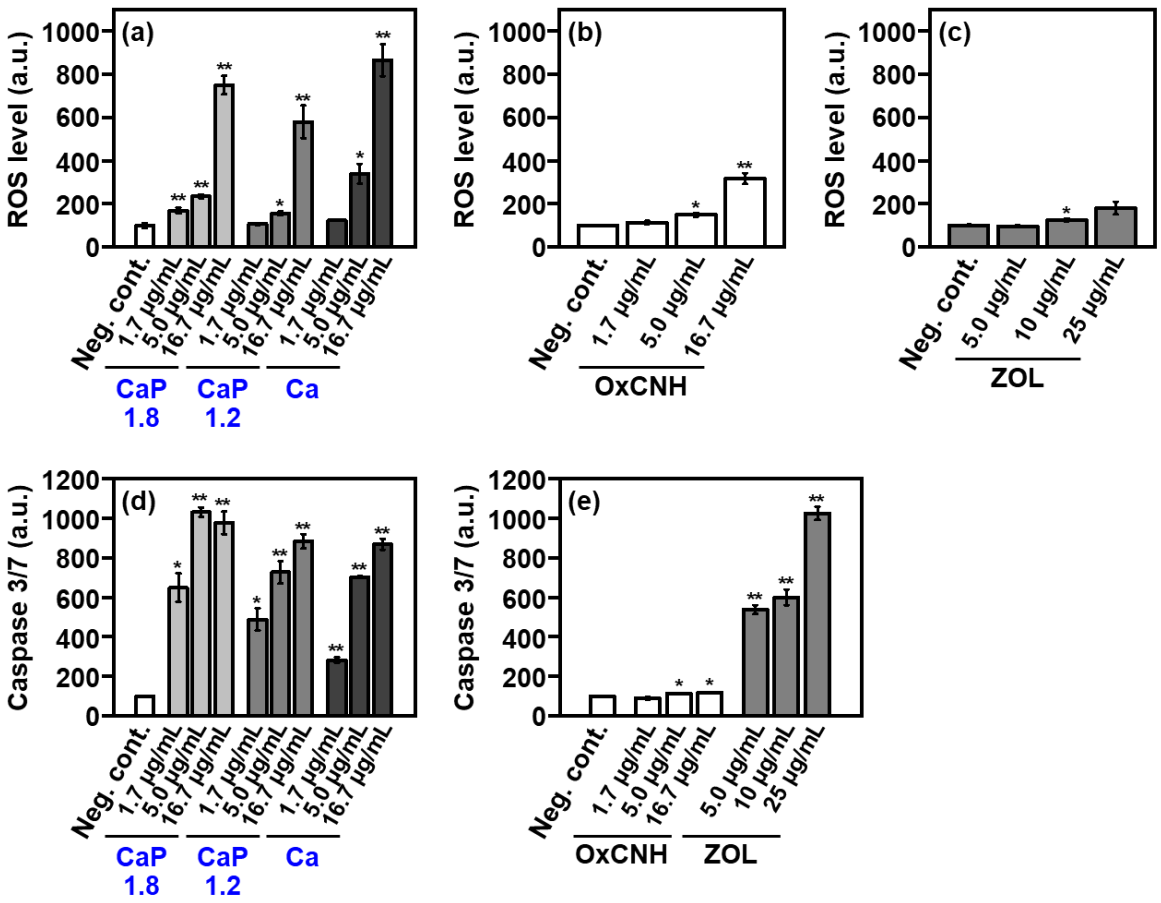

Figure S3. ROS levels (a–c) and caspase 3/7 activity (d and e) in RAW 264.7 cells exposed to **OxCNH-CaP1.8-ZOL** (CaP1.8) (a and d), **OxCNH-CaP1.2-ZOL** (CaP1.2) (a and d), **OxCNH-Ca-ZOL** (Ca) (a and d), OxCNH (b and e), or ZOL (c and e) for 24 h (average ± standard error, $n = 4$ for (a–c) or $n = 3$ for (d and e)). The estimated final OxCNH concentrations in medium containing nanocomposites were 1.7, 5.0, and 16.7 µg/mL (30-, 10-, and 3-fold dilutions, respectively). Injection water was used as a negative control. * $P < 0.05$ and ** $P < 0.01$, relative to the negative control. (b) and (c): Taken from ref. 5, and presented with some modifications.

Supplementary Information VIII. Effect of three types of nanocomposites, OxCNH, and ZOL on RANKL-induced osteoclast differentiation from RAW264.7 cells (treated on Day 0).

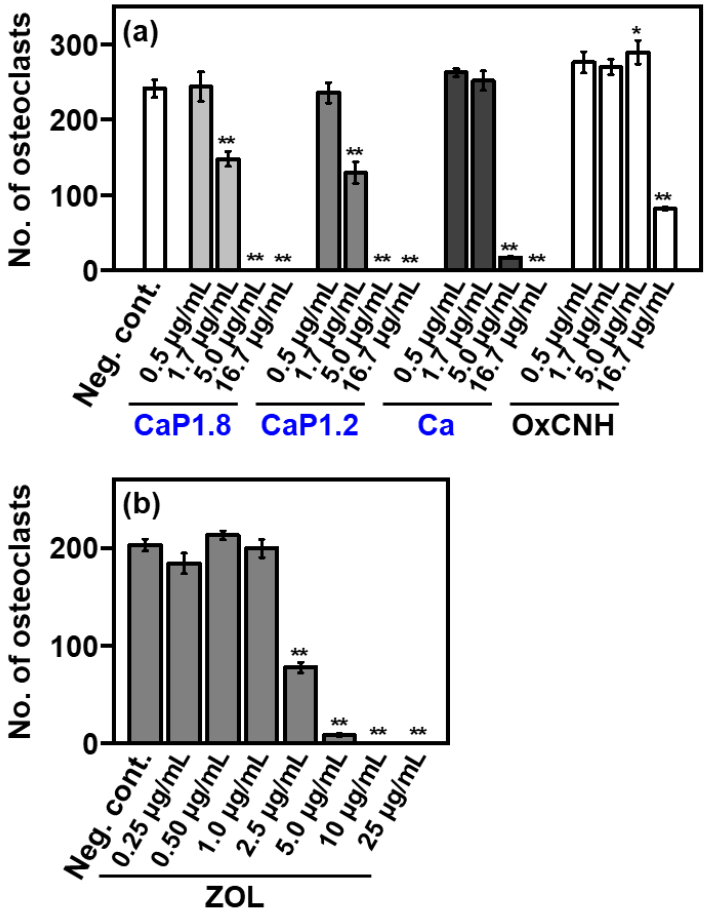

Figure S4. Number of TRAP-positive multinucleated osteoclasts per well (average ± standard error, $n = 4$). RAW 264.7 cells were incubated with RANKL (Day 0), followed by addition of injection water (as a negative control), **OxCNH-CaP1.8-ZOL** (CaP1.8), **OxCNH-CaP1.2-ZOL** (CaP1.2), **OxCNH-Ca-ZOL** (Ca), or OxCNH (a), or ZOL (b) on the same day (Day 0). After incubation for 4 d, cells were stained to detect TRAP expression (Day 4). * $P < 0.05$ and ** $P < 0.01$, relative to the negative control. (a): The estimated final OxCNH concentrations in medium containing nanocomposites were 0.5, 1.7, 5.0, and 16.7 μg/mL (100-, 30-, 10-, and 3-fold dilutions, respectively). TRAP-positive multinucleated osteoclasts in each well are shown in Figure S5.

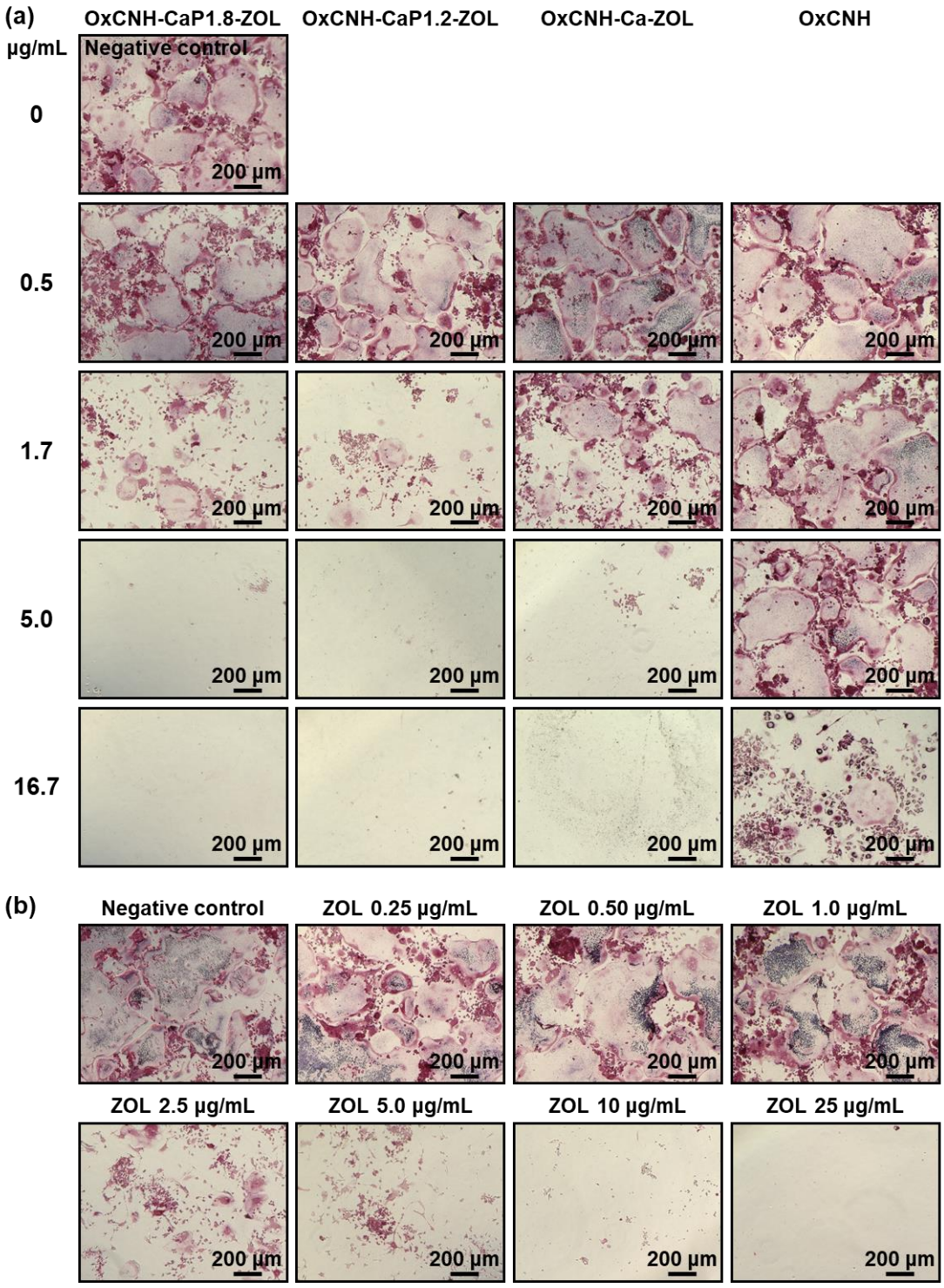

Figure S5. Optical micrographs of osteoclasts. RAW 264.7 cells were incubated with RANKL (Day 0), followed by addition of injection water (as a negative control), **OxCNH-CaP1.8-ZOL**, **OxCNH-CaP1.2-ZOL**, **OxCNH-Ca-ZOL**, or OxCNH (a), or ZOL (b) on the same day (Day 0). After incubation for 4 d, cells were stained to detect TRAP expression (Day 4). (a): The estimated final OxCNH concentrations in medium containing nanocomposites were 0.5, 1.7, 5.0, and 16.7 μg/mL (100-, 30-, 10-, and 3-fold dilutions, respectively).

Supplementary Information IX. Effect of three types of nanocomposites, OxCNH, and ZOL on RANKL-induced osteoclast differentiation from RAW264.7 cells (treated on Day 3).

RAW264.7 cells (1.0×10^3 cells; 0.1 mL/well, n =4) were seeded into 96-well plates and precultured at 37°C for 24 h. The culture medium was then exchanged for 0.1 mL of medium supplemented with 200 ng/mL RANKL (Day 0). After incubation for 3 d, 10 μL of test specimen solution (Supplementary Information III-1) was added (Day 3). After incubation for one more day, cells were washed with PBS, fixed with 4% paraformaldehyde phosphate buffer solution (FUJIFILM Wako Pure Chemical Corporation) for 10 min on ice, and stained with a TRAP/ALP stain kit (FUJIFILM Wako Pure Chemical Corporation) (Day 4). The stained cells were observed under an optical microscope (BZ-810, Keyence Corporation, Japan), and TRAP-positive multinucleated cells containing more than three nuclei were counted as osteoclasts.

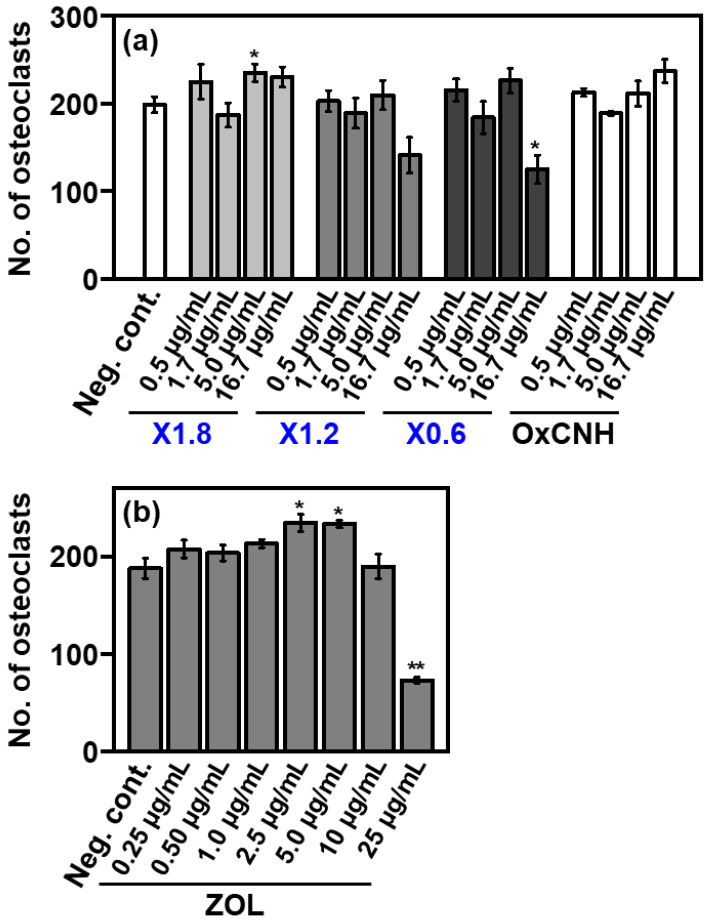

Figure S6. Number of TRAP-positive multinucleated osteoclasts per well (average ± standard error, $n = 4$). RAW 264.7 cells were incubated with RANKL for 3 d (from Day 0), followed by addition of injection water (as a negative control), **OxCNH-CaP1.8-ZOL** (CaP1.8), **OxCNH-CaP1.2-ZOL** (CaP1.2), **OxCNH-Ca-ZOL** (Ca), or OxCNH (a), or ZOL (b) (Day 3). After incubation for one more day, cells were stained to detect TRAP expression (Day 4). * $P < 0.05$, relative to the negative control. (a): The estimated final OxCNH concentrations in medium containing nanocomposites were 0.5, 1.7, 5.0, and 16.7 μg/mL (100-, 30-, 10-, and 3-fold dilutions, respectively). TRAP-positive multinucleated osteoclasts in each well are shown in Figure S7.

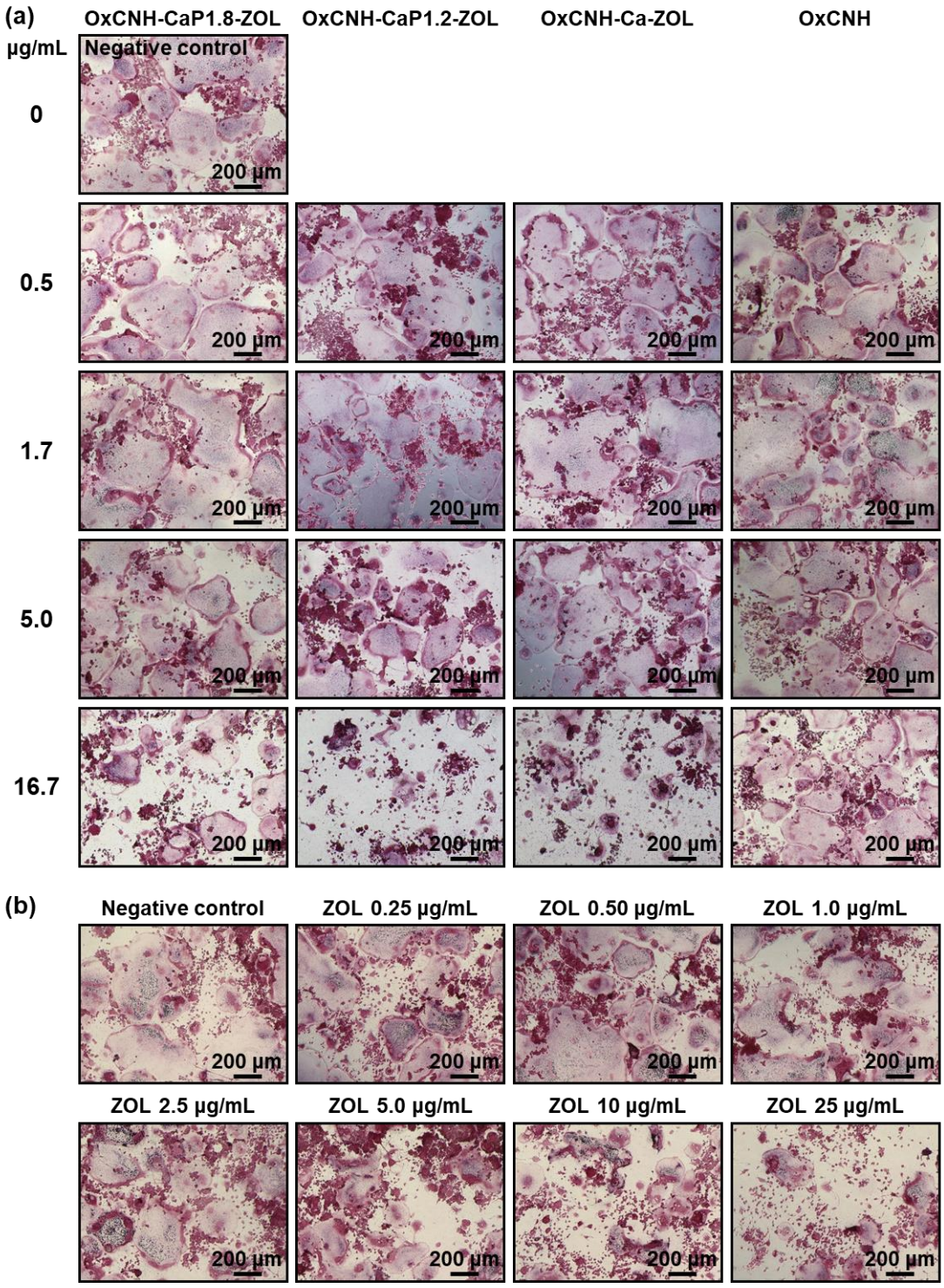

Figure S7. Optical micrographs of osteoclasts. RAW 264.7 cells were incubated with RANKL for 3 d (from Day 0), followed by addition of injection water (as a negative control), **OxCNH-CaP1.8-ZOL**, **OxCNH-CaP1.2-ZOL**, **OxCNH-Ca-ZOL**, or OxCNH (a), or ZOL (b) (Day 3). After incubation for one more day, cells were stained to detect TRAP expression (Day 4). (a): The estimated final OxCNH concentrations in medium containing nanocomposites were 0.5, 1.7, 5.0, and 16.7 μg/mL (100-, 30-, 10-, and 3-fold dilutions, respectively).

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