Electronic Supplementary Material (ESI) for Nanoscale.

Near-infrared Light-boosted Antimicrobial Activity of Minocycline/Hyaluronan/Carbon Nanohorn Composite toward Peri-implantitis Treatments

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Experimental section

Preparation of minocycline, hyaluronan, and carbon nanohorn (MC/HA/CNH) composites

A stock solution of MC (5.0 mg/mL) was prepared by dissolving MC in deionized water. HA was dissolved in deionized water at 1.0 mg/mL, and the mixture was heated at 80 °C for 60 min to prepare a solution. Dispersions of 1.0-mg/mL CNHs [consisting of as-grown CNHs (as-CNHs), CNHs oxidized at 550 °C (CNH550), or CNHs oxidized at 575 °C (CNH575)] were prepared by suspension of the indicated reagent in deionized water.

HA/CNH composites were prepared by adding 0.10 mL of HA solution (0.1 mg) to 1.0 mL of CNH dispersion (1.0 mg/mL) that had been irradiated with a light-emitting diode (LED; 738 nm, 200 mW/cm^2, CL-1501, UD1746, Asahi Spectra Co., Ltd., Tokyo, Japan). The resulting mixture was shaken at room temperature for 30 min, then centrifuged (10,000 × g, 10 min, room temperature) to pellet the composites.

MC/HA/CNH composites were prepared by adding 1.0 mL of MC solution (5.0 mg/mL) to 1.0 mL of HA/CNH composites. The resulting mixtures were irradiated (using an LED as above) with shaking at room temperature for 10 min, followed by centrifugation (as above) to pellet the composites. For use in bacterial or cell culture experiments, the composites were suspended in saline (9 g/L NaCl, pH=4.5–8.0, Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan). Each dispersion incorporating CNHs prepared at the three different oxidation temperatures (indicated above) was sonicated for 15 min, and photographs were taken of the suspensions at 0, 1, 3 and 24 h after sonication (High-speed switching oscillation between 24 and 31 kHz, W113MK-2, Honda Electronics Co., Ltd., Toyohashi, Japan).

Thermogravimetric analysis

Prior to thermogravimetric analysis (TGA), the MC/HA/CNH dispersions were dried using a VD-400 F lyophilizer (TAITEC, Koshigaya, Japan) to obtain powders (which were black in color). TGA was conducted using a TG 8120 instrument (Rigaku Corporation, Tokyo, Japan). In each trial, a 2.0-mg sample was placed on a platinum pan, and the sample temperature was increased from ambient to 900 °C at a rate of 10 °C /min under a 100 mL/min N_2 flow.

Quantification of released MC

The amount of MC released from each MC/HA/CNH specimen was calculated by assessing the supernatant obtained by centrifugation during the MC/HA/CNH sample preparation process; the measured parameters included the weight of the supernatant and its absorbance at 350 nm using a ultraviolet-visible light (UV-vis) spectrophotometer (Nanodrop, Thermo™ INSIGHT, Thermo Fisher Scientific, Waltham, MA, USA). Specifically, the MC/HA/CNH dispersion was dispensed into the insert dish (Nunc™ Polycarbonate Cell Culture Inserts in Multi-Well Plates; pore size, 0.4 μm, Thermo Fisher Scientific), and the dish was exposed to light while shaking at 20 °C for 10 min. The MC/HA/CNH sample that accumulated outside of the insert then was assessed for weight and absorbance, and the resulting data were used to calculate the amount of MC released.

Bacterial culture

A stock suspension was prepared by dispersing MC/HA/CNHs at 1.0 mg/mL in saline. For both CNH575 and MC/HA/CNH575, an aliquot (180 μL) of the suspension was combined with 20 μL of a bacterial suspension of *Aggregatibacter actinomycetemcomitans* (*A.a.*: ATCC 29522) to yield a final density of 2.0×10^4 colony-forming units (CFU). The resulting mixtures were irradiated with an NIR-LED light (738 nm, 400 mW/cm^2) for 10 min.

Solutions contained MC at the initial preparation concentration or at the actual loaded MC concentration. The samples were also incubated with bacterial stock solutions described above, respectively.

Photostatic assay

The bacterial suspensions were diluted 10-fold with a brain heart infusion (BHI; BD, Franklin Lakes, NJ, USA) medium. A total of 100 μL of the diluted bacterial suspensions was added to BHI agar (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and cultured at 37°C under microaerophilic conditions in a candle jar for 48 h. CFU were measured by Image J Fiji[4]. The experiment was repeated four times.

Cell culture

A mouse embryonic fibroblast cell line (NIH/3T3)[5] was used to investigate cytotoxicity. The cells were seeded in a 96-well plate (IWAKI, AGC Techno Glass, Shizuoka, Japan) at 1×10^4 cells/well and

cultured in Dulbecco's Modified Eagle's Medium-high glucose (Sigma-Aldrich, St. Louis, MO, USA) containing 10% calf serum (Funakoshi, Tokyo, Japan) and 1% penicillin/streptomycin (Thermo Fischer Scientific, Gibco, Waltham, MA, USA), at 50 U/mL penicillin and 50 mg/mL streptomycin. After changing medium, the well was illuminated by the LED for 10 min and further cultured for 24 h at 37 °C with a 5% CO_2 atmosphere. After a 1 d cell culture, the DNA concentration of each sample was measured. Prior to this process, each well was washed once with PBS and 50 μL of a cell suspension containing 0.2% IGEPAL CA630 (Sigma-Aldrich), 10 mM tris-HCl and 1 mM $MgCl_2$ (pH 7.4) was added to each sample. The samples were subsequently frozen, thawed and homogenized, and each sample solution was added to 50 μL of 4 M NaCl in a 0.1 M phosphate buffer (pH 7.4) and then centrifuged for DNA analysis.

Photocytotoxicity assay

Picogreen (Molecular Probes, Leiden, Netherlands) was used to determine the DNA content in conjunction with a microplate reader (infinite F200 PRO, TECAN, Männedorf, Swizerland) with the excitation filter set at 365 nm and the emission filter at 450 nm.

References

1 T. Azami, D. Kasuya, R. Yuge, M. Yudasaka, S. Iijima, T. Yoshitake and Y. Kubo, *J. Phys. Chem. C*, 2008, **112**, 1330–1334.

2 J. Fan, M. Yudasaka, D. Kasuya, T. Azami, R. Yuge, H. Imai, Y. Kubo and S. Iijima, *J. Phys. Chem. B*, 2005, **109**, 10756–10759.

3 J. Fan, M. Yudasaka, J. Miyawaki, K. Ajima, K. Murata and S. Iijima, *J. Phys. Chem. B*, 2006, **110**, 1587–1591.

4 J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J. Y. Tinevez, D. J. White, V. Hartenstein, K. Eliceiri, P. Tomancak and A. Cardona, *Nat. Methods*, 2012, **9**, 676–682.

5 G. J. Todaro and H. Green, *J. Cell Biol.*, 1963, **17**, 299–313.

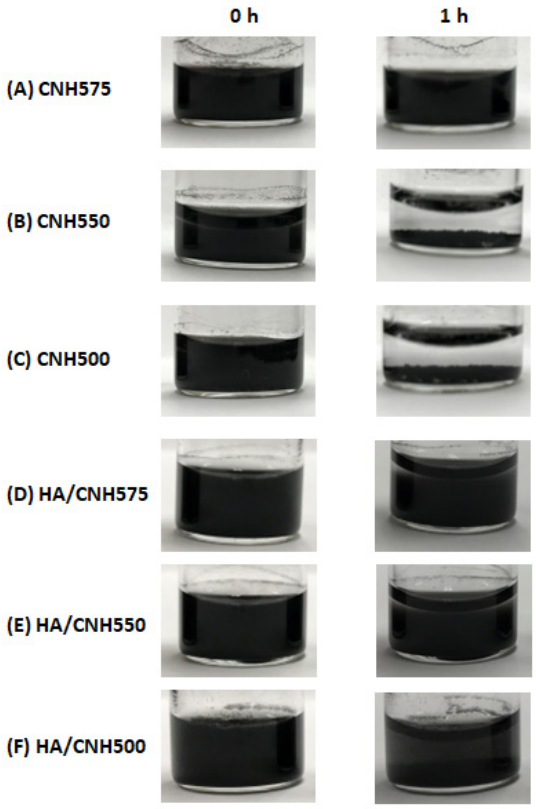

Fig. S1. Photographic images of suspensions of (A) CNH575, (B) CNH550, (C) CNH500 (carbon nanohorns oxidized at 575, 550, and 500 °C, respectively), (D) HA/CNH575, (E) HA/CNH550, and (F) HA/CNH500 specimens (hyaluronan coated CNH575, CNH550, and CNH500, respectively) at 0 and 1 h after dispersion.

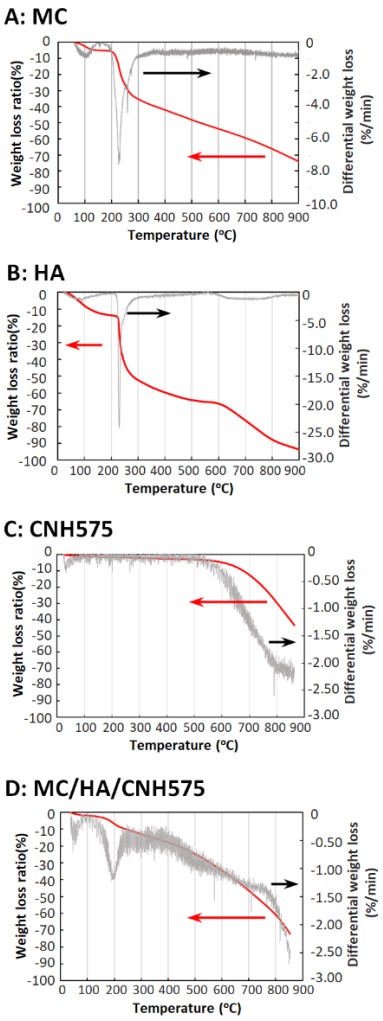

Fig. S2. Thermogravimetric (TG; red lines) and differential TG (DTG; gray lines) data were obtained for (A) MC alone, (B) HA, (C) CNH575, and (D) MC/HA/CNH575 specimens. Temperatures were increased at a rate of 10 °C/min for samples maintained under a 100 mL/min flow of N_2.

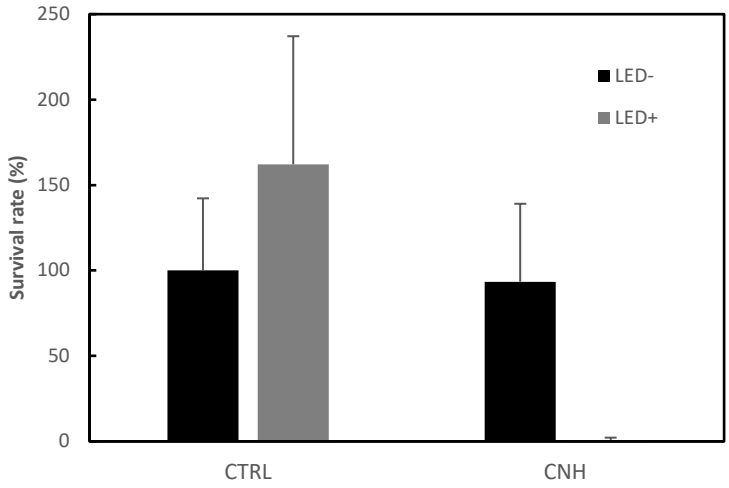

Fig. S3. Survival rates of *Aggregatibacter actinomycetemcomitans* (*A.a.*; initial density, 2.0×10^4 CFU/mL) following exposure to CNH575 (CNH) or saline (Control; CTRL) followed by irradiation (LED+; black bars) or lack thereof (LED-; gray bars) with an NIR-LED (738 nm, 400 mW/cm^2) for 10 min. Exposure and irradiation were performed on bacterial suspensions, which then were plated on growth agar and incubated at 37 °C for 48 h before counting of colonies. Data are presented as mean + SD (n = 3).

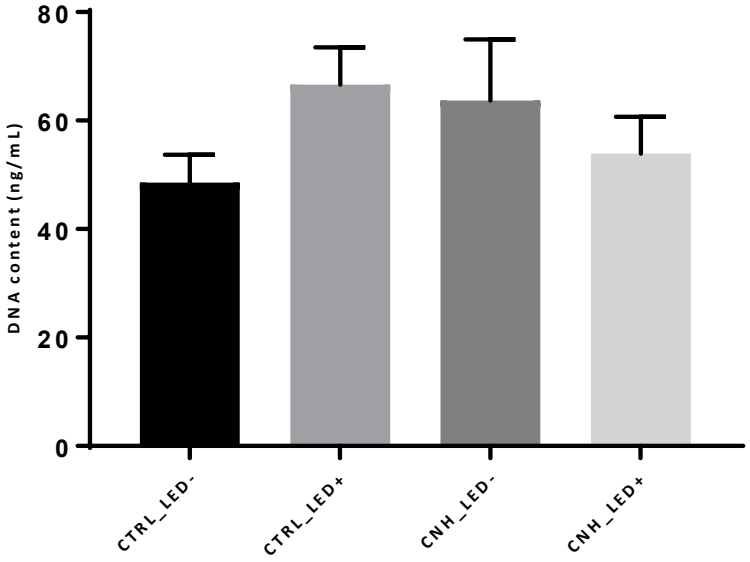

Fig. S4. Cell viability as assessed by DNA content of NIH/3T3 cells. Carbon nanohorns (CNH, 0.01 mg/mL) were added to the "CNH" cultures. "LED+" cultures were subjected to irradiation with an NIR-LED light (738 nm, 400 mW/cm^2, 10 min). "LED-" cultures were left in the dark for the equivalent interval. Data are presented as mean + SD (n = 4).