Electronic Supplementary Material (ESI) for ChemComm.

An antibacterial conjugate of carbon nanohorns for NIR-light mediated peri-implantitis treatment

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Experimental section.

1. General

Chemicals and solvents were purchased from Fujifilm Wako Corporation (Japan), while CNHs were obtained from NEC Corporation (Tokyo, Japan). CNHs were produced by the ablation of a pure graphite target at room temperature in Ar at 760 Torr using a CO_2 laser.[1] The CNHs were approximately 95% pure, with the majority of the impurities consisting of graphitic spheres.[2] No metal catalyst was used in the preparation of these samples. Absorption spectra were recorded using a UV-vis spectrophotometer (Evolution 220, ThermoFisher Scientific, USA). FT-IR spectra were recorded on a JASCO FT/IR-6800typeA spectrometer equipped with diamond enabled attenuated total reflectance (ATR) sample holder (JASCO, Japan).

*2. Synthesis of **CNH-rTPA***

The amine-modified CNH (**CNH-NH_2**) was synthesized according to the procedure reported by B. Alberto and co-workers.[3] A suspension of 50 mg of as-grown CNHs (**as-CNHs**) in 50 ml of 2,2′-(ethylenedioxy)bis(ethylamine) from Sigma-Aldrich was sonicated for 10 hours at a temperature below 50 °C using a water bath with a power of 20 W and a frequency of 40 kHz. After sonication, 50 ml of methanol was added to the suspension, which was then filtered through a polytetrafluoroethylene (PTFE) membrane with a pore size of 0.45 μm. The suspension was washed several times with excess methanol and the **CNH-NH_2** film on the membrane was dried under vacuum overnight.

rTPA was synthesized and characterized according to the literature[4]. To synthesize **CNH-rTPA**, THF solution of **rTPA** was mixed with DMT-MM (2.0 eq.) and added to **CNH-NH_2** (4.00 mg) dispersion of THF. The weight ratio of CNHs to **rTPA** were 1.00:0.33, 1.00:1.00, and 1.00:2.00 (w/w) (labeled as **1:1/3**, **1:1**, or **1:2**), respectively for the three conditions of samples. The reaction solutions were sonicated for 5 min and then mixed for 2 hours. An excess amount of 2,2′-(ethylenedioxy)bis(ethylamine) was added to the solutions and mixed 1 hours. The solvent was removed by evaporation. The solid sample was dispersed in EtOH by sonication, and filtered by 0.2 μm PTFE membrane filter and washed well with EtOH to remove free **rTPA** and the reaction byproduct caused by DMT-MM or **rTPA**. In this step, free **rTPA** and the reaction byproduct caused by DMT-MM or **rTPA** were removed. The residue on the filter membrane was collected and dried under a vacuum to obtain **CNH-rTPA** (**1:1/3**, **1:1**, or **1:2**) as greenish-black powders.

3. Thermogravimetric analysis (TGA)

TGA was carried out with a TG 8120 instrument (Rigaku Corporation, Tokyo, Japan). In each trial, a 2 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.

4. Singlet oxygen assay

Sample solutions were prepared by the sonication of **rTPA** or **CNH-rTPA** powder in PBS. To fairly assess the ability of CNH to produce $^1O_2$ by the rTPA moiety, the absorbance derived from the rTPA moiety in each sample was identical. The suspension solution was added 5.0 μM singlet oxygen sensor green (SOSG, Thermo Fischer) and then irradiated by a 730 nm LED light (CL, Asahi Spectra Co., Ltd. ., Japan) (200 mW / cm) for the photosensitized $^1O_2$ generation. The fluorescence peak intensity from SOSG were recorded to determine the $^1O_2$ generation abilities.

5. Bacteria culture

The stock solution was prepared by dispersing **CNH-rTPA(1:1)** in dimethyl sulfoxide (DMSO) at 5 mg/ml and diluted with saline to a concentration of 0.5 mg/mL. 20 μL of **CNH-rTPA(1:1)** solution and 180 μL bacterial suspensions of *Aggregatibacter actinomycetemcomitans* (*A.a.*: ATCC 2952) or *Streptococcus mutans* (*S.m.*: ATCC 55677) to a final concentration of 2.0 x 10^4 Colony Forming Units (CFU). were mixed. The mixture was irradiated by the LED light (730 nm, 400 mW /cm^2). After 5 min of the LED irradiation, the bacterial suspensions were diluted 10-fold with a brain heart infusion (BHI; BD, Franklin Lakes, USA) medium. 100 uL of the diluted bacterial suspensions were added to Brain Heart Infusion (BHI) agar (Becton Dickinson Labware, Franklin lakes, NJ, USA) and cultured at 37°C under microaerophilic conditions in a candle jar for 48 hours. CFU and turbidity were measured. To confirm the effect of LED irradiation on the control, *A.a.* was incubated with LED irradiation (730 nm, 400 mW /cm^2) for 10 minutes and CFU was counted using the method described above. The experiment was repeated three times

6. Cell culture

Mouse embryonic fibroblasts cell line (NIH/3T3)[5] were used to investigate cytotoxicity. The cells were seeded to the 96 well plate (IWAKI 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, Japan) and 1% penicillin/streptomycin (Thermo Fischer Scientific, Gibco, Waltham, MA) at 50 U/mL penicillin and 50 mg/mL streptomycin. After 5 hours, the medium was changed to the dispersion of the **CNH-rTPA(1:1)** in the medium at 225 μg/well. An equal concentration of DMSO was used as control. After changing medium, the well was illuminated by the LED for 10 minutes and further cultured for 24 hours at 37°C with a 5% CO_2 atmosphere. The relative number of viable cells was determined by Cell Proliferation

Reagent WST-1 (Roche, Indianapolis, IN, USA). Assays were performed in triplicate. And the viabilities were calculated by the following equation (eq 1).

$$Cell\ viability\ (\%) = \frac{OD_{PAA} - OD_{blank}}{OD_{control} - OD_{blank}} \quad eq\ (1)$$

7. Statistical analysis

All data are presented ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad software and one-way ANOVA followed by Dunnett's multiple comparisons test. All p values <0.05 were considered significant.

8. Reference

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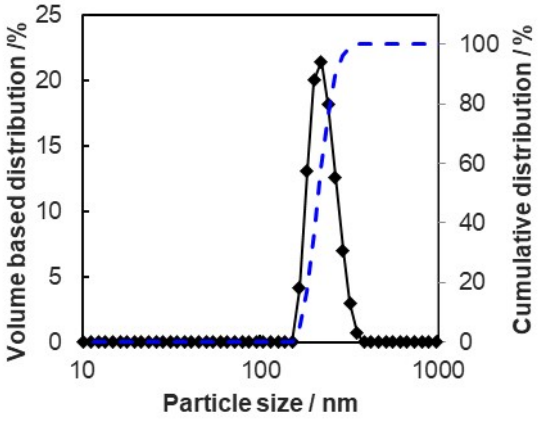

Fig. S1. Volume-based DLS profile of **CNH-rTPA(1:1)** in 10 mM HEPES solution of H_2O. Histogram average value is 228 ± 38 nm, and polydispersity index (PDI) is 0.053.

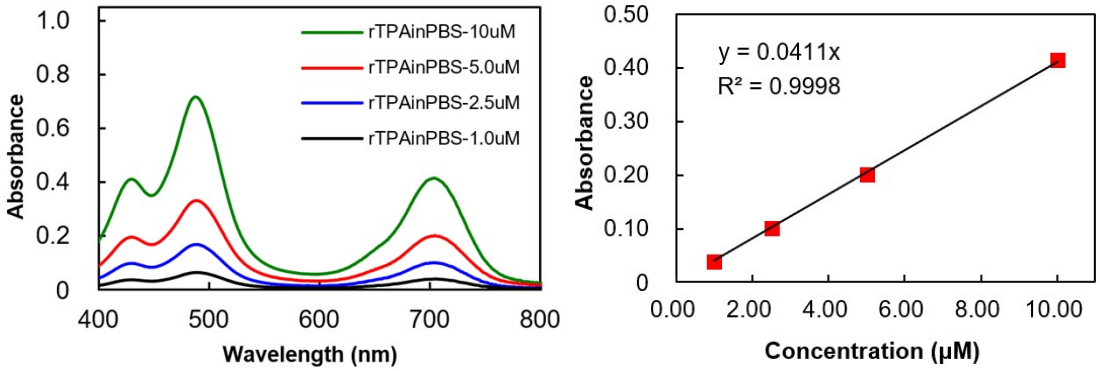

Fig. S2. (Left) Absorption spectra of various concentrations of rTPA in PBS, and (right) concentration versus peak absorbance (at 704 nm) plot. Absorption coefficients is determined to be 4.11 × 10^3 mol / L cm in PBS.

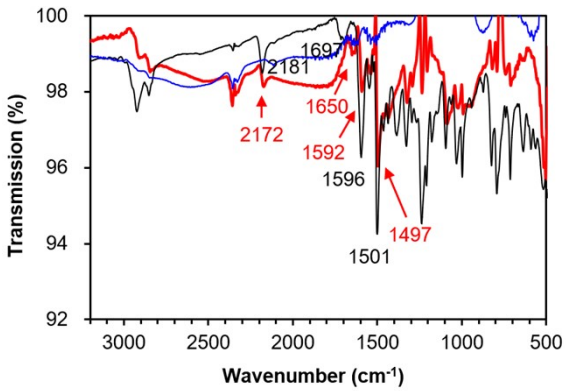

Fig. S3. Infrared (IR) spectra of (black line) **rTPA**, (blue line) **CNH-NH_2**, and (red line) **CNH-rTPA(1:1)**.

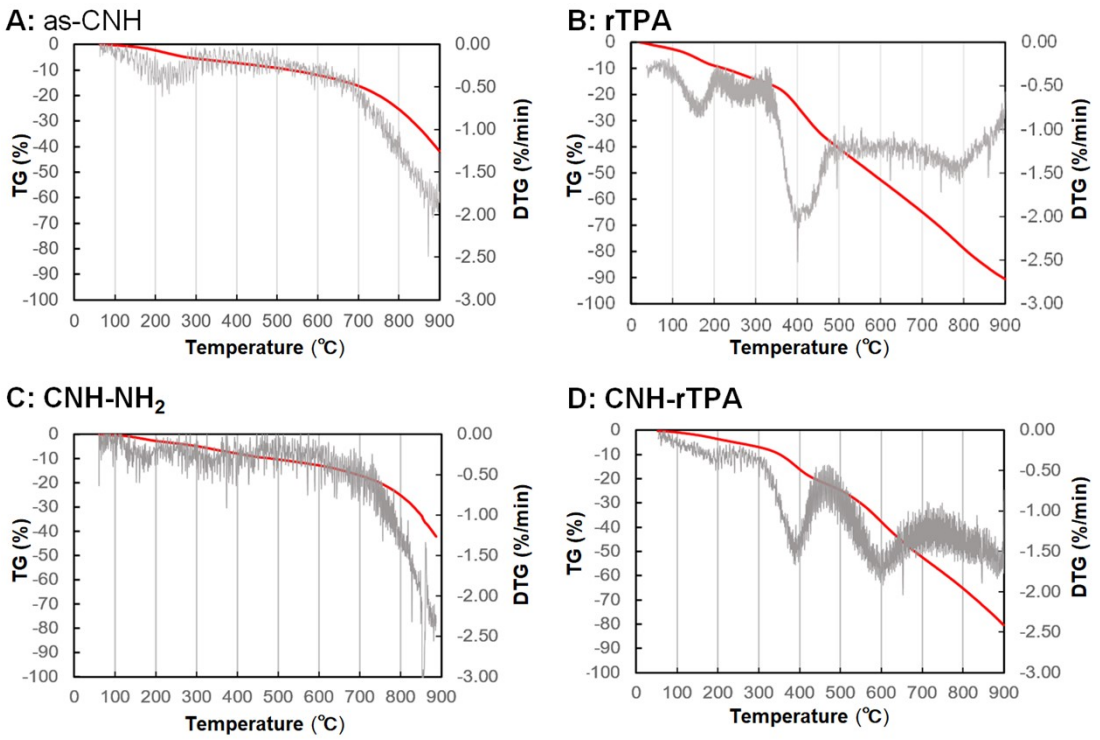

Fig. S4. Thermogravimetric (TG) and differential TG (DTG) data for (A) **as-CNH**, (B) **rTPA**, (C) **CNH-NH$_2$**, and (D) **CNH-rTPA(1:1)** specimens. Increase rate of 10 °C/min under a 100 mL/min N2 flow

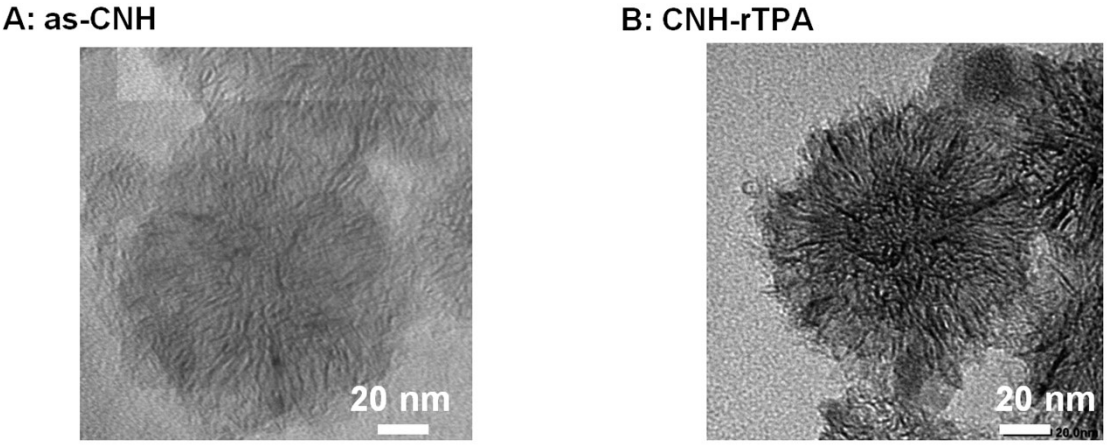

Fig. S5. TEM images of (A) **as-CNH** and (B) **CNH-rTPA(1:1)** (HAADF-STEM 80 kV).

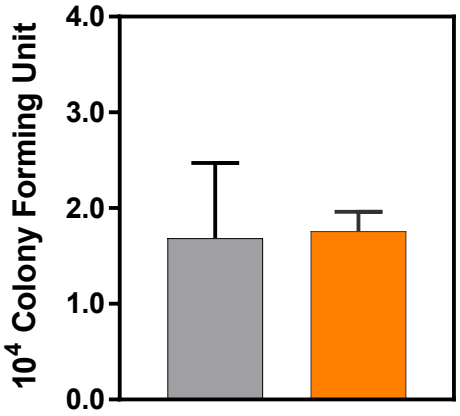

Fig. S6. NIR photo-induced bactericidal evaluation on *Aggregatibacter actinomycetemcomitans (A.a.)* in the absence of CNHs, (left) without and (right) with illumination by a 730 nm LED (120 J, equal to 400 mW cm^{-2}, 5 min). No significant difference was observed between the two conditions.

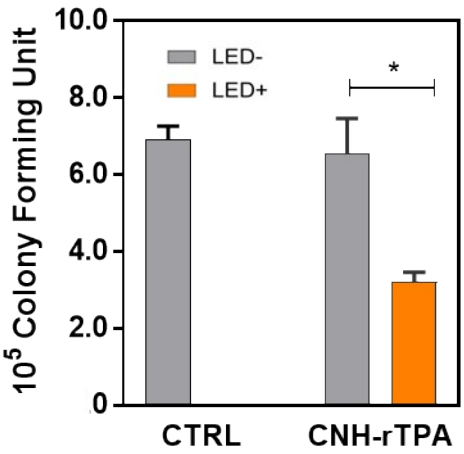

Fig. S7. NIR photo-induced bactericidal effect on *Streptococcus mutans (S.m.)* (left) without and (right) with illumination by a 730 nm LED (120 J, equal to 400 mW cm^{-2}, 5 min).