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Competitive coordination-based CeO_2 nanowire-DNA nanosensor: fast and selective detection of hydrogen peroxide in living cells and in vivo

Wen Gao,[a] Xueping Wei,[a] Xuejun Wang,[b] Guanwei Cui,[a] Zhenhua Liu[a] and Bo Tang*[a]

[a]College of Chemistry, Chemical Engineering and Materials Science, Collaborative Innovation Center of Functionalized Probes for Chemical Imaging in Universities of Shandong, Key Laboratory of Molecular and Nano Probes, Ministry of Education, Shandong Provincial Key Laboratory of Clean Production of Fine Chemicals, Shandong Normal University, Jinan 250014, P.R. China.

[b]Shandong Center for Disease Control and Prevention, Jinan 250014, P.R. China.

Corresponding Authors

*E-mail: tangb@sdnu.edu.cn. Fax: +86 531 86180017.

Experimental procedures

Materials. DNA oligonucleotides were synthesized and purified by Sangon Biotechnology Co., Ltd (Shanghai, China). The sequences of these oligonucleotides are shown in Table S1. Cerium(III) nitrate (99.5%, $Ce(NO_3)_3 \cdot 6H_2O$) were obtained from Shanghai Siyu Chemical Technology Co. Ltd. (Shanghai, China). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), anhydrous ethanol, NaOH, NaCl were purchased from Sinopharm Chemical Reagent. Co. Ltd. (Shanghai, China). Cell culture products, unless mentioned otherwise, were purchased from GIBCO. hypochlorite (NaOCl) and Hydrogen Peroxide (H_2O_2) were delivered from 10% and 30% aqueous solutions, respectively. Hydroxyl radical ($^{\bullet}OH$) was generated by reaction of 1 mM Fe^{2+} with 200 μM H_2O_2. Singlet oxygen ($^1O_2$) was prepared by the ClO^-/H_2O_2 system. Peroxynitrite ($ONOO^-$) was used from stock solution 10 mM in 0.3 M NaOH. Superoxide ($O_2^{\cdot -}$) was delivered from KO_2 in DMSO solution. All the chemicals were of analytical grade and used without further purification. Sartorius ultrapure water (18.2 MΩ cm) was used throughout the experiments. Zebrafish larvae and tricaine methanesulfonate (MS-222) were provided by the Biology Institute of Shandong Academy of Sciences.

Characterization. High resolution transmission electron microscopy (HRTEM) was carried out on a JEM-2100 electron microscope. X-ray diffraction (XRD) analysis was carried out on a D/Max 2500 V/PC X-ray diffractometer using Cu (40 kV, 30 mA) radiation. Absorption spectra were measured on a pharmaspec UV-1700 UV-visible spectrophotometer (Shimadzu, Japan). X-ray photoelectron spectroscopy (XPS) spectra were performed with a Phobios 100 electron analyzer (SPECS GmbH) equipped 5 channeltrons, using an unmonochromated Mg Ka X-ray source (1253.6 eV). The C 1s line at 284.6 eV was used to calibrate the binding energies (BE). Raman spectrum was measured on NEXUS 670 FT-IR & Raman spectrometer. Zeta potential was measured with a Malvern Zeta Sizer Nano (Malvern Instruments). Fluorescence spectra were obtained with FLS-920 Edinburgh fluorescence spectrometer with a Xenon lamp and 2.0

mm quartz cells at the slits of 1.0/1.0 nm. All pH measurements were performed with a pH-3c digital pH-meter (Shanghai LeiCi Device Works, Shanghai, China) with a combined glasscalomel electrode. Absorbance in the MTT assay was measured in a microplate reader (RT 6000, Rayto, USA). Imaging flow cytometry analysis was performed using an ImageStream^X multispectral imaging flow cytometer (Amnis Corporation). Confocal fluorescence imaging studies were performed with a TCS SP5 confocal laser scanning microscopy (Leica Co., Ltd. Germany) with an objective lens (×40).

Preparation of nanoceria. The CeO_2 nanocubes (NCs), nanorods (NRs) and nanowires (NWs) were synthesized by a modified hydrothermal method.[1] Briefly, 5 mL of 0.8 M $Ce(NO_3)_3$ solution was added dropwise into 75 mL of 6.4 M NaOH aqueous solution. The mixture was aged at room temperature under continuous stirring for 30 min. The white slurry was then transferred into an autoclave and maintained at 100 °C, 24 h for NRs, and 180 °C, 24 h for NCs to get well defined samples, respectively. When C_{NaOH} was further increased to 9 M, highly crystallized CeO_2 NWs with a high aspect ratio were obtained. After cooling to room temperature, the precipitates were washed by deionized water and ethanol alternatively for three times and dried at 60 ºC overnight.

DNA Adsorption Assays. DNA adsorption capacity on nanoceria is dependent on salt concentration and pH.[2] To optimize salt concentration in adsorption buffer, DNA (R1, 100 nM) was dissolved in HEPES buffer (pH 7.4, 10 mM) containing varying concentrations of NaCl (50, 100, 150 and 200 mM). After the absorbance of free DNA was measured by UV/Vis spectroscopy at 260 nm, a small volume of nanoceria (final concentration = 20 μg/mL) were added to induce DNA adsorption. After 30 min incubation, the supernatant was collected by centrifuging the mixture at 14000 rpm for 20 min. The DNA loading capacity as a function of salt was calculated as follows: initial absorbance − supernatant absorbance/initial absorbance. The optimize concentrations of NaCl for CeO_2 NCs and NRs is 100 mM, and for NWs is 150 mM (Fig. S1a). For

optimizing pH value of adsorption buffer, R1 (100 nM) was dissolved in buffer with just 100 mM NaCl. The DNA loading capacity as a function of pH was measured according to the method mentioned above. Although pH 4.6 shows a slightly higher DNA adsorption, the optimal value should be 7.4, which is close to the values under physiological conditions (Fig. S1b). Then, CeO_2 NCs, NRs and NWs (20 μg/mL) were incubated with the different concentration R1 (0 nM, 10 nM, 20 nM, 40 nM, 60 nM, 80 nM, 100 nM, 150 nM and 200 nM) in optimized absorption buffer for 30 min under 37 °C, respectively. The effect of surface charge on their DNA absorption capacities were compared by monitoring the changes in zeta-potential.[3]

Fluorescence Quenching Assay. FAM-R_{15} (100 nM) was mixed with different volumes of CeO_2 NWs (0, 1, 2, 5, 10, 20, 50 μg/mL) in 1 mL absorption buffer (150 mM NaCl, 10 mM HEPES, pH 7.4). The fluorescence intensities were recorded at 15 min after CeO_2 NWs added with excitation and emission wavelength of 494 nm and 518 nm, respectively. For the quenching kinetic assay, the concentration of CeO_2 NWs in final absorption buffer was adjusted to 20 μg/mL, and 100 nM of FAM labeled-R_{15}, R_{30} and R_{45} was added.

Preparation of CeO_2 NWs-DNA nanosensor. FAM-R_{15} was added to a solution of CeO_2 NWs (20 μg/mL) with a final concentration of 200 nM. Then, the mixture was allowed to react under stirring for 1 h in darkness at room temperature, followed by centrifugation (14000 rpm, 20 min, 4 °C) to remove excess FAM-R_{15}. The purified product, CeO_2 NW-DNA (CNWD) nanosensor, was diluted to a concentration of ~1 mg/mL with ultrapure water and stored at 4 °C for future use. The supernatant and all the washings solutions were collected and the amount of FAM-R_{15} washed was measured using fluorescent spectroscopy (λex=494 nm, λem=518 nm). The fluorescence was converted to molar concentrations of FAM-R_{15} by interpolation from a standard linear calibration curve that was prepared with known concentrations of FAM-R_{15} with identical buffer pH, ionic strength and HEPES concentrations.[4] The loading of FAM-R_{15}

(5.95 nM/μg CeO_2 NWs) was calculated with the difference between the original and the washed amount of FAM-R_{15}.

Fluorescence response to H_2O_2. Aliquots of 1.0 mL volume of prepared CNWD nanosensor (20 μg/mL) were added to 2 mL microcentrifuge tubes, and then different concentrations (0, 1, 4, 8, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 μM and 1mM) of H_2O_2 solution were added. The fluorescence of the samples was measured immediately at λex/λem = 494/518 nm. In selectivity experiment, In selectivity experiment, reactive oxygen/nitrogen species (OCl^-, $^1O_2$, $O_2^{\bullet-}$, $ONOO^-$, $^\bullet OH$), amino acids (lysine, glycine, L-cystine, glutamic acid), sugars (sucrose, lactose), metal ions (Fe^{3+}, Fe^{2+}, Cu^{2+}, Cu^+, Mg^{2+}, Zn^{2+}), and as well as oxidative-stress-associated redox chemicals, including glutathione (GSH) and ascorbate acid (AA), were examined. The concentration is 100 μM for each. The measuring procedure was the same as above.

Intracellular Stability Assay. To evaluate the nuclease stability, two groups of 20 μg/mL CNWD in detection buffer (150 mM NaCl, 10 mM HEPES, pH 7.4) were placed in a 96-well fluorescence microplate at 37 ºC. After allowing the samples to equilibrate (10 min), 1.3 μL of DNase I in assay buffer (2 U/L) was added to one group. The fluorescence of these samples was monitored for 1 h and was collected at 10 min intervals during this period. Then 100 μM H_2O_2 were paralleled added into the two samples, the fluorescence of FAM was measured immediately at λex/λem = 494/518 nm, respectively. To evaluate pH stability, 20 μg/mL CNWD were dispersed in 10 mM HEPES buffer (150 mM NaCl) with various pH values (4.6, 5.5, 6.8, 7.4, 8.3). The fluorescence of FAM was measured as mentioned above.

Cell Culture and Cytotoxicity Assays. RAW 264.7 macrophage cells were obtained from the Committee on Type Culture Collection of Chinese Academy of Sciences. Cells were cultured in Dulbecco's modified Eagles medium (DMEM) with 10% fetal bovine serum and 100 U/mL of 1% antibiotics penicillin/streptomycin and maintained at 37 °C in a 100% humidified atmosphere containing 5% CO_2.

Cytotoxicity was measured by using the MTT assay in the logarithmic phase of cell growth. RAW 264.7 macrophage cells were seeded at a density of 5×10^4 cells/well in a 96 well-plate and incubated for 24 h before adding the test substance. After the original medium was removed, the cells were incubated with CNWD (20 and 40 μg/mL) for 6, 12 and 24 h, respectively. Then the cells were washed three times with HEPES. Subsequently, 150 μL of MTT solution (0.5 mg/mL) was added to each well. After 4 h, the remaining MTT solution was removed, and 150 μL of DMSO was added to each well to dissolve the formazan crystals. The absorbance at 490 nm was monitored using an RT 6000 microplate reader. Viability was calculated based on the recorded data.

Imaging of H_2O_2 in Living Cells by Imaging Flow Cytometry. RAW 264.7 macrophages were seeded at a density of 5×10^6 cells/well in a 6 well-plates and incubated for 24 h. Cells were first incubated in the medium containing CNWD (20 μg/mL) for 30 min at 37 °C and then washed twice with HEPES to remove the excess probe. Then fresh medium containing PMA (1 μg/mL) were added to the dish and treated for 0, 2, 5 and 10 min at 37 °C, respectively. As a control, CNWD-loaded cells were incubated with both PMA (1 μg/mL) and catalase (100 μM) for another 10 min. Afterwards, all the treated cells were trypsinized, fixed with 4% formaldehyde in HEPES and centrifuged (1000 *g*) to obtain a pellet of about 10^6 cells in 50 μL. Cell images were acquired using the ImageStream^X multispectral imaging flow cytometer, collecting 20,000 events per sample at 40× magnification. A 488 nm wavelength laser was used to excite FAM. The fluorescence images were collected using the 500-560 nm spectral detection channels. For stained cells, unstained controls were used to compensate fluorescence between channel images on a pixel-bypixel basis. Cell images were analyzed using IDEAS® image-analysis software (Amnis).

Imaging of H_2O_2 in Zebrafish. Three-day-old zebrafish larvae were fed with the CNWD (0.1 mg/mL) in water for 20 min, and then anaesthetized with 100 mg/L MS-222 for wounding and imaging experiments.[5] All media were sterile filtered. Blades were

treated with 70% ethanol before use. Confocal images were captured at appropriate time intervals using a LTE confocal laser scanning microscope (CLSM, Leica Co. LTD., Germany).

Reference

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2. Y. Song, K. Qu, C. Zhao, J. Ren and X. Qu, *Adv. Mater.*, 2010, **22**, 2206.
3. R. Pautler, E. Y. Kelly, P. J. Huang, J. Cao, B. Liu and J. Liu, *ACS Appl. Mater. Interfaces*, 2013, **5**, 6820.
4. N. Li, Z. Yu, W. Pan, Y. Han, T. Zhang and B. Tang, *Adv. Funct. Mater.*, 2013, **23**, 2255.
5. K. Xu, M. Qiang, W. Gao, R. Su, N. Li, Y. Gao, Y. Xie, F. Kong and B. Tang, *Chemical Science*, 2013, **4**, 1079.

Supporting Table:

Table. S1 DNA sequences employed in this work.

Oligonucleotide	Sequence
R_1: random sequence	5'-CATACGATTCCAACA-3'
FAM-R_{15}: FAM-tagged 15 bp DNA	5'-FAM-CAGAGGCAGTAACCA-3'
FAM-R_{30}: FAM-tagged 30 bp DNA	5'-FAM-CTACTATGTCCAATTTCAAGGACAGTTCAG-3'
FAM-R_{45}: FAM-tagged 45 bp DNA	5'-FAM-ATTTCACTACAGTTCAAACAAACTCCAACGA CTAACTACTGCCAA-3'

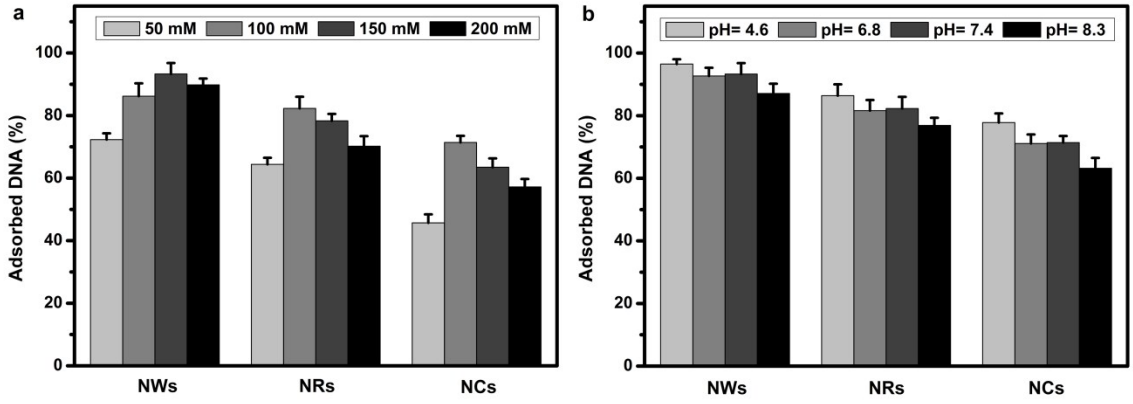

Fig. S1 Changes in DNA adsorption capacity on CeO_2 NWs, NRs and NCs as a function of (a) NaCl concentrations and (b) pH. The optimize concentrations of NaCl for CeO_2 NCs and NRs is 100 mM, and for NWs is 150 mM. The optimal value for CeO_2 NWs, NCs and NRs is 7.4, which is close to the values under physiological conditions.

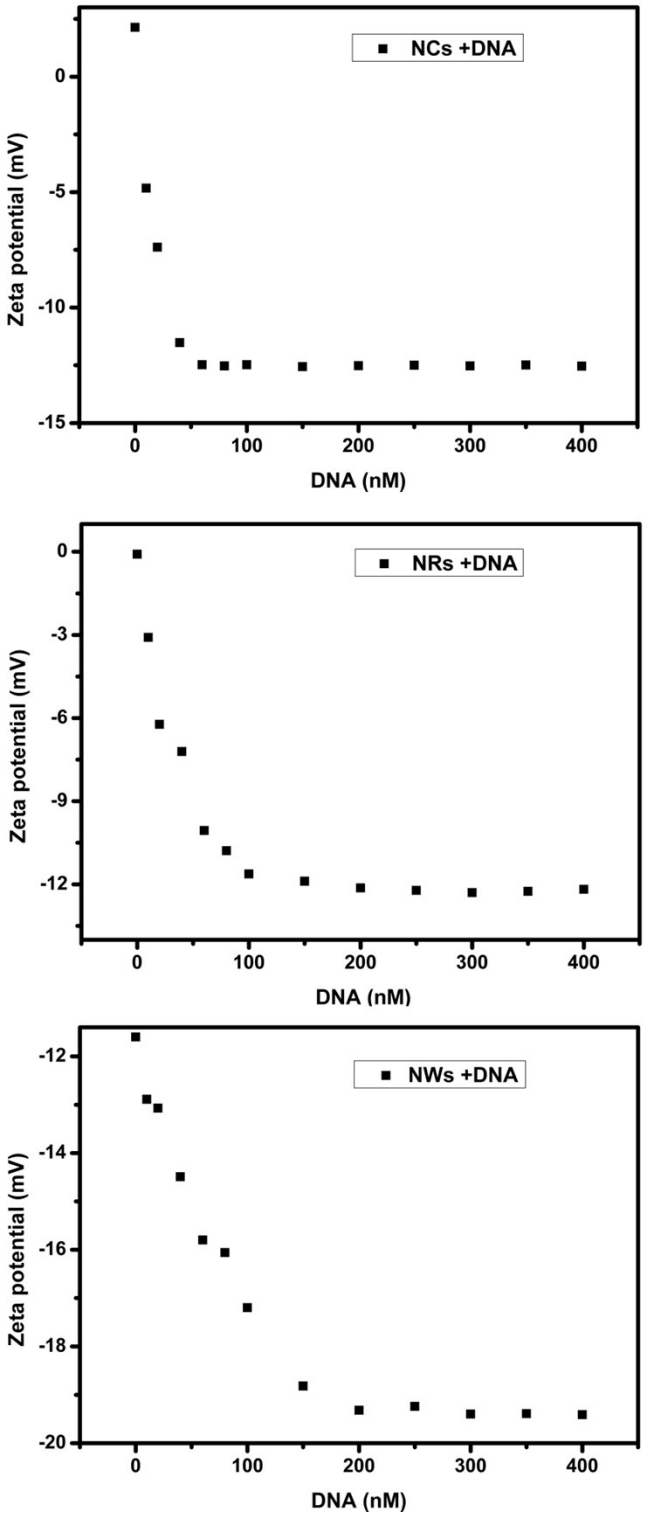

Fig. S2 DNA absorption capacities of CeO_2 NWs, NRs and NCs. The zeta potential of nanoceria becomes more negatively as adding increasing concentrations of DNA.

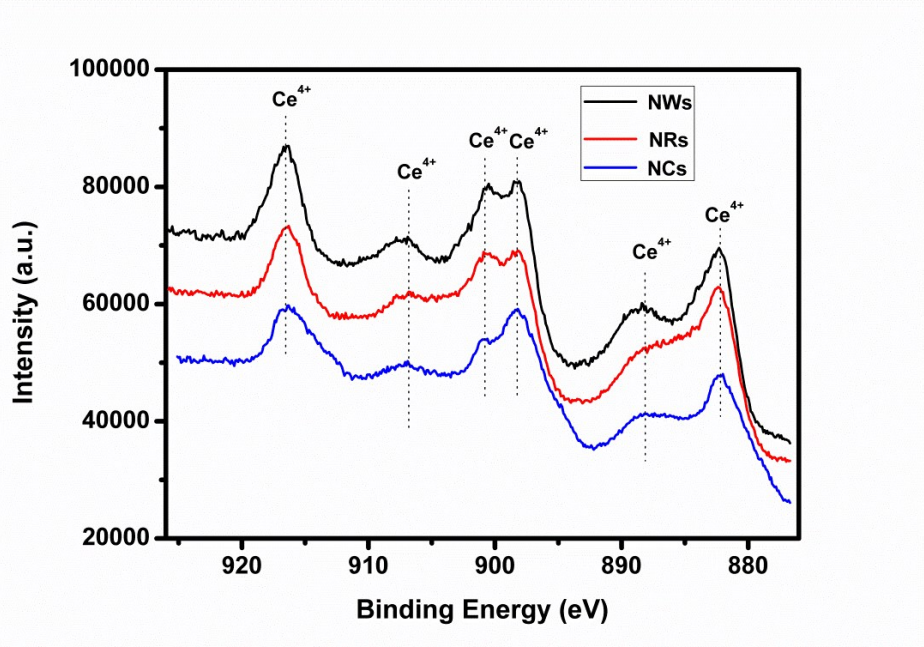

Fig. S3 Ce 3d XPS spectra of as-prepared CeO_2 NWs, NRs and NCs. The peaks between 875 and 895 eV belong to the Ce 3d5/2 while peaks between 895-910 eV correspond to the Ce 3d3/2 levels. The peak at 916 eV is a characteristic satellite peak of Ce^{4+}.

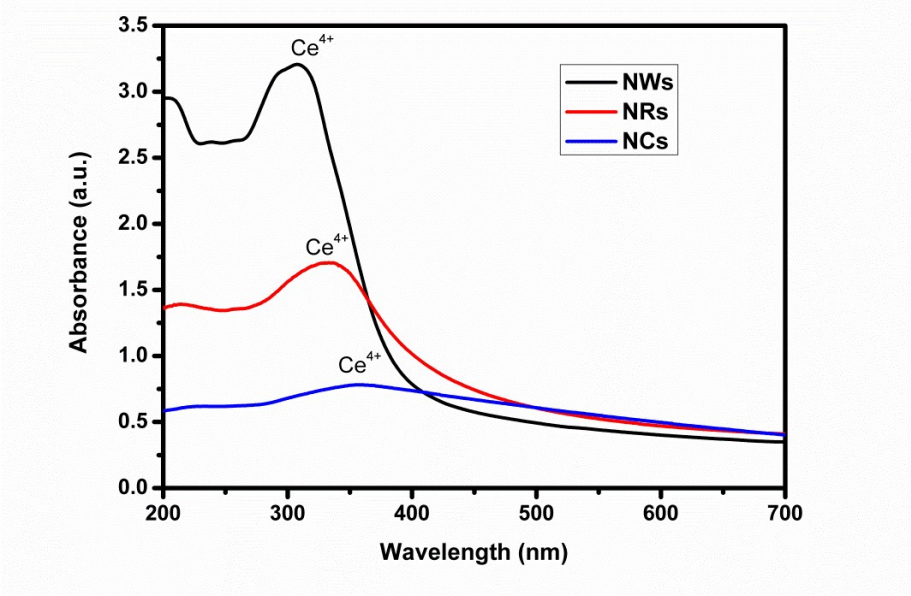

Fig. S4 UV-vis absorption spectra of CeO_2 NWs, NRs and NCs. The 300-400 nm spectral range, which corresponds to the absorbance of Ce^{4+}, show a corresponding increase in Ce^{4+} concentration of CeO_2 NWs.

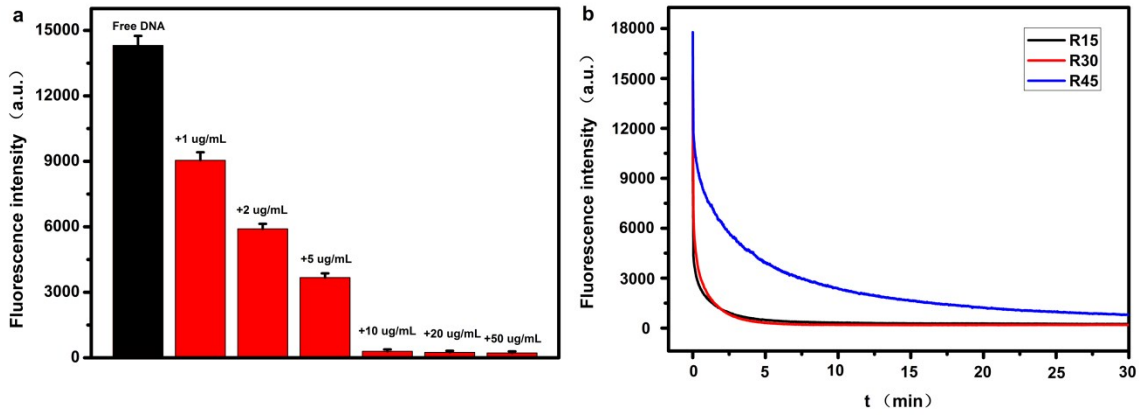

Fig. S5 (a) Fluorescence quenching of FAM-DNA of 100 nM in the absence (black) and presence of CeO_2 NWs with a series of concentrations (left to right: 1, 2, 5, 10, 20, 50 μg/mL). (b) Kinetic study for the fluorescence change of the FAM-DNA (100 nM) with different lengths in the presence of CeO_2 NWs: 15 bp (black); 30 bp (red); 45 bp (blue).

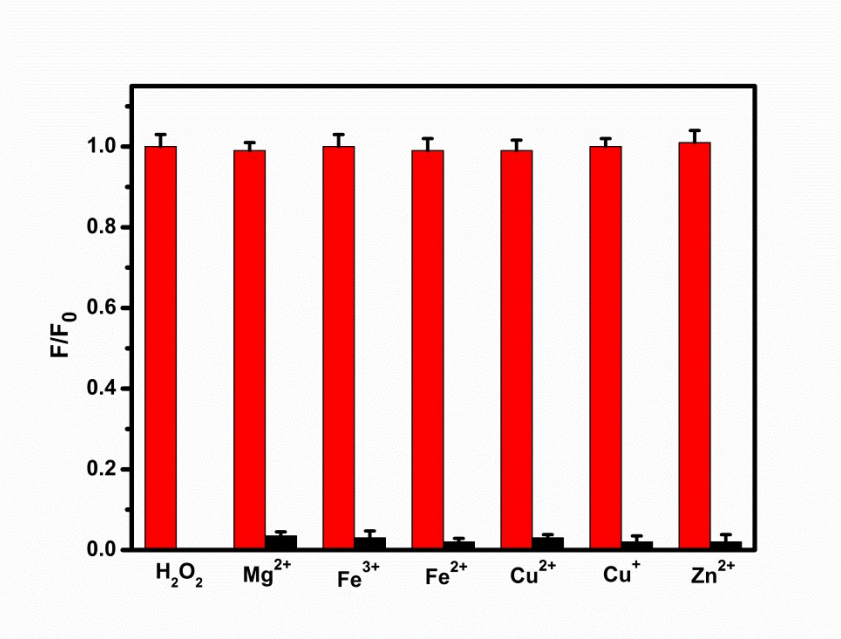

Fig. S6 Fluorescence responses of CNWD after adding various metal ions (100 μM for each). Black bars show the addition of one of these metal ions to a 20 μg/mL CNWD solution. The red bars represent the addition of both H_2O_2 and one metal ions to the sensor solution.

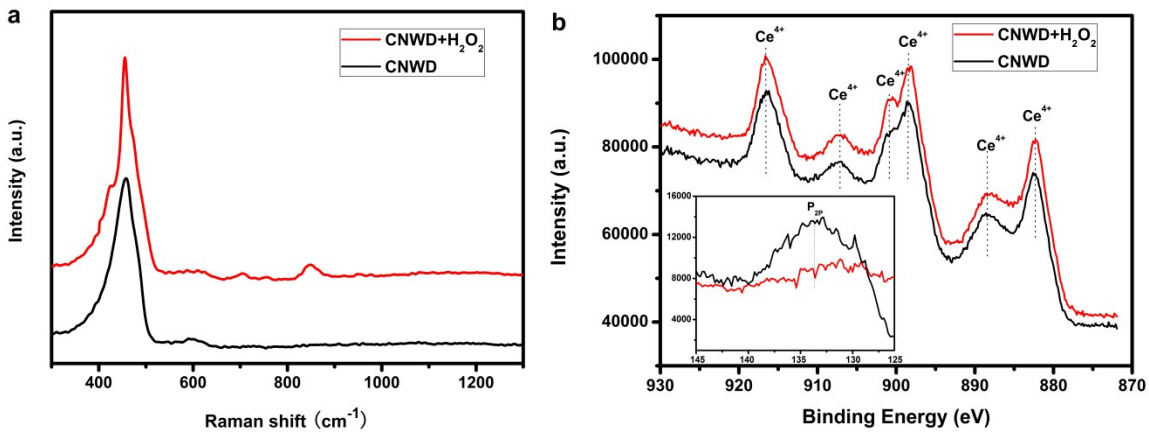

Fig. S7 (a) Raman spectra and (b) selective high-resolution XPS spectra of CNWD before and after the reaction with H_2O_2. Cerium's 3d orbital spectrum with prominent Ce^{4+} peaks at 882.1, 888.3, 898.0, 900.9, 906.4 and 916.5 eV. Inset: P 2p3/2 spectrum with a peak position at 133.3 eV.

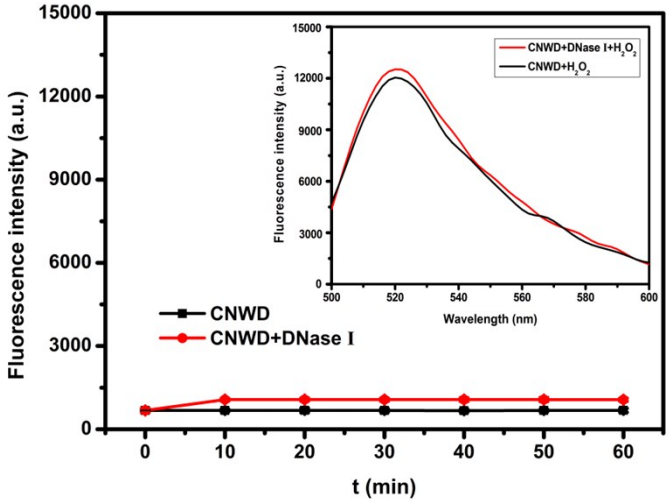

Fig. S8 Nuclease stability of CNWD in the presence or absence of DNase I. Fluorescence curves of the nanosensor (20 μg/mL) in HEPES(10 mM) without DNase I (-■-), in the presence of DNase I (-●-). Insets: fluorescence spectra of the nanosensor after incubation with H_2O_2 in the presence (red) and absence (black) of DNase I. Data are shown as mean ± S.D. of three independent experiments.

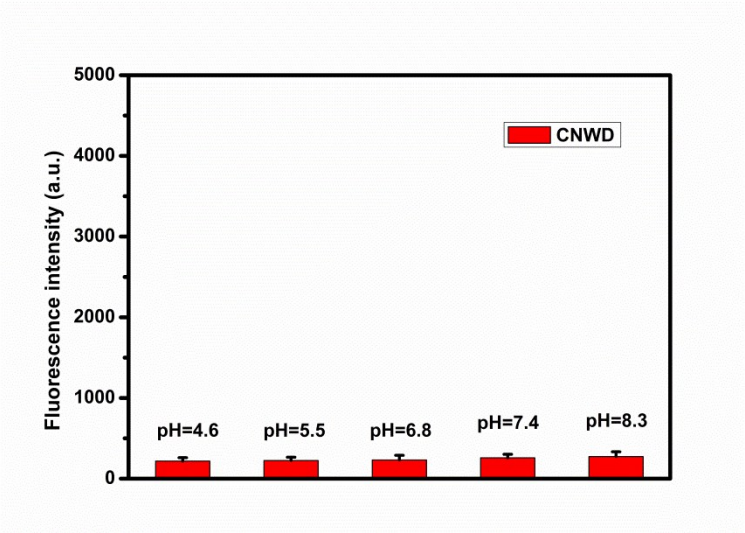

Fig. S9 Fluorescence intensity of CNWD (20 μg/mL) in 10 mM HEPES buffer with different pHs.

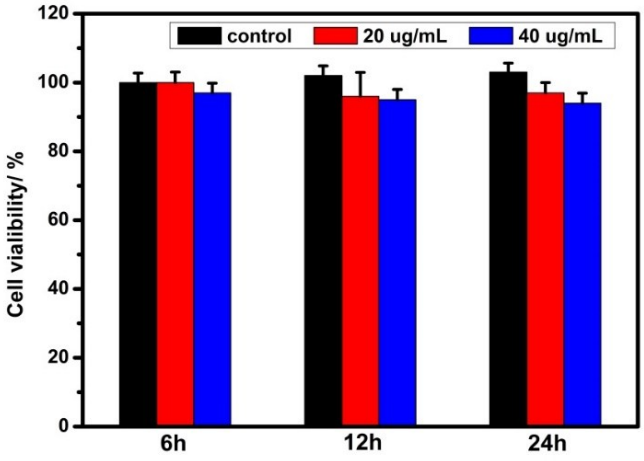

Fig. S10 Cell viability of RAW 264.7 macrophages incubated with different amounts of nanosensor (20 μg/mL and 40 μg/mL) for different times (6 h, 12 h and 24 h). Data are shown as mean ± S.D. of three independent experiments.

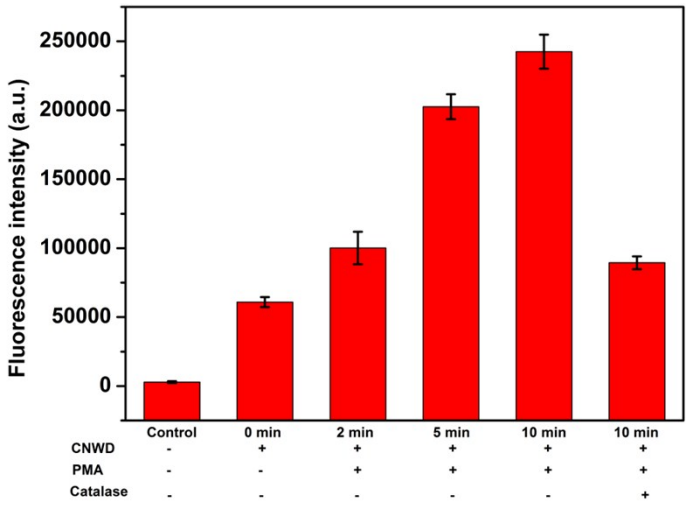

Fig. S11 Mean fluorescence intensity of RAW 264.7 macrophages undergoing each of above treatments. 20 000 cells were measured in each flow cytometry experiment, 5000 cells acquired, and only focused cells were analyzed in each imaging flow cytometry experiment. Data are shown as mean ± S.D. of three independent experiments.