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Unveil Early-Stage Nanocytotoxicity by a Label-Free Single Cell pH Nanoprobe

Qingbo Yang¹, Alexandre Cristea¹, Charles Roberts¹, Kun Liu¹, Yang Song², Hai Xiao², Honglan Shi¹, Yinfu Ma^{1,*}

¹Department of Chemistry, and Center for Biomedical Research, Missouri University of Science and Technology, Rolla, MO 65409, USA

²Department of Electrical and Computer Engineering, Clemson University, Clemson, SC 29634, USA

Abstract

Single-cell analysis is an emerging research area that aims to reveal delicate cellular status and underlying mechanisms by conquering the intercellular heterogeneity. Current single-cell research methods, however, are highly dependent on cell-destructive protocols and cannot sequentially display the progress of cellular events. A recently developed pH nanoprobe in our lab conceptually showed its ability to detect intracellular pH (pH_i) without cell labeling or disruption. In the present study, we took the cytotoxicity of nanoparticles (NPs) as a typical example of cell heterogeneity, to testify the practicality of the pH nanoprobe in interpreting cell status. Three types of NPs (CeO_2 , TiO_2 , and SiO_2) were employed to generate varied toxic effects. Results showed that the traditional assays - including cell viability, intracellular ROS generation, and mitochondrial inner membrane depolarization - not only failed to report the nanotoxicity accurately and timely, but also drew confusing or misleading conclusions. The pH nanoprobe revealed explicit pH_i changes induced by the NPs, which corresponded well with the cell damages found by the transmission electron microscopic (TEM) imaging. Besides, our results unveiled an unexpectedly devastating effect of SiO_2 NPs on cells during the early stage NP-cell interaction. The developed novel pH nanoprobe demonstrated a rapid sensing capability at single-cell resolution with minimum invasiveness. Therefore, it may become a promising alternative for a wide range of applications in areas such as single-cell research and precision medicine.

Graphical Abstract

*Corresponding Authors: Yinfu Ma, Department of Chemistry, Center for Biomedical Research (CBR), Missouri University of Science and Technology, Rolla, MO 65409, USA, yinfu@mst.edu.

Conflicts of Interest

The authors declare no conflicts of interest in the presented study.

Electronic supplementary information (ESI) is available online.

The developed pH nanoprobe unveiled nanomaterial properties that previously unknown (e.g., devastating cytotoxicity) via real-time label-free monitoring on single cells.

Keywords

pH sensor; Fiber-optic nanoprobe; Single-cell analysis; Intercellular heterogeneity; Nanoparticle cytotoxicity

Introduction

As single-cell research plays an increasingly critical role in the development of fundamental medical science and precision medicine, the accuracy and instantaneity of single-cell analysis methods become very important, especially towards the interpretation of the delicate cell-cell heterogeneity. Existing methods cover a wide range of technology platforms, including the combined use of sequencing and mass spectrometry, etc., revealed a variety of biochemical details and processes within a single cell from the DNA,¹ RNA,² transcriptional,³ epigenetic,⁴ proteomic^{5, 6} and metabolomic^{7, 8} levels. Yet, these achievements compromised dramatically due to their mostly destructive protocols and failed to visualize the progress of cellular events sequentially. It is almost impossible to track hundreds or thousands of specific molecular beacons along with the development of target cells. However, monitoring one or several representative parameters that are broadly related to downstream cell behavior (such as deterioration or inflammation) can be useful as an accurate and cost-effective way to track cell behavior and even predict cell fate quickly.

In this regard, the intracellular pH (pH_i) is known to be stringently regulated by cells.⁹ The pH_i was found correlated with many intracellular status and processes, such as the structure of macromolecules,⁹ carcinogenesis,^{10, 11} bioenergetic homeostasis,¹² and so on. Thus, the pH_i homeostasis may serve as a universal indicator of cellular status.⁹ The pH_i was found correlated with carbonic anhydrase 9,¹¹ Smad5,¹² oxidative phosphorylation and caspase-3,¹³ histone acetylation,¹⁴ Na^+/H^+ exchanger isoform 1 (NHE-1),¹⁵ cell proliferation and apoptosis,¹⁶ etc. Thus, attempts of interpreting and predicting cell behaviors and responses using pH_i may highly be a precise and effective, and hence a promising single-cell research strategy.^{17–20}

Many pH_i monitoring methods have been developed so far, such as the widely used fluorescence-based indicators 2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF),²¹ 5-(and-6)-seminaphtharhodafleur (SNARF-1),²² 1,4-dihydroxyphthalonitrile (1,4-DHPN)²³ and trisodium 8-hydroxypyrene-1,3,6-trisulfonate (HPTS)²⁴ etc.¹⁷ In addition, pH_i indicators that take advantage of nano-structures to enter cytoplasmic

membrane via the endocytic pathway were also reported.^{20, 25–29} However, these and other methods^{30–33} mostly rely on cell up-taking mechanisms that inevitably involve the use of acetoxymethyl (AM)^{34, 35} for membrane permeability. Such methods, admittedly, enabled the cytosolic hydrolyzation and assisted the cytosolic retaining of the dye molecules, but resulted in the retention of an excessive amount of the indicators at the same time. Concerns regarding potential interruption to normal cell function³⁶ by excessive dye amount have long been raised, and questions do exist about whether it can reveal the actual status of cells. AM ester-containing dyes also tend to be enriched in cell compartments where a higher esterase activity exists, such as the lysosomes. However, the lysosomal lumen has a significantly lower pH than the cytoplasm (ranges from pH 5 to 7). An erroneously low pH could thus be measured on a whole-cell region of interest (ROI). Besides, other fundamental issues of the dye method, such as uneven cytosolic staining,³⁷ varied cell uptake rate as well as photobleaching, blinking, quenching, and so on, may add more uncertainty to the cell assay, not to mention the cell-cell heterogeneity that already exists.

A series of novel optical microprobes have recently been developed in our group for real-time, label-free pH measurements.^{38–41} Recently, we have developed a sensor with a tip diameter of about 3 μm and a sensing length of approximately 5 μm , which enabled a true intracellular detection capability.⁴² Detection sensitivity of ~ 0.038 pH unit and detection (signal equilibrium) time of ~ 20 s was successfully achieved. The intracellular sensing capability was preliminarily demonstrated on several cultured human lung cancer A549 cells.

Nanoparticles (NPs) induced cytotoxicity has drawn increasing health and environmental concerns, and it is also a challenging topic in single-cell research for largely heterogeneous and unpredictable cellular behavior that may be involved. Destructed cell membranes,^{43–45} generation of reactive oxygen species (ROS),^{46–49} compromised metabolic activities,^{50, 51} degraded genetic materials,^{52, 53} as well as interrupted pH_i ,^{41, 54} were frequently observed during the nanocytotoxicity process. Unfortunately, most commercially available assays are based on cell populations, which not only sacrifice the precision and instantaneity of the assay, but also loses most of the heterogeneous information hidden behind the cell-cell differences. A single cell-based method equipped with high accuracy and real-time detection ability would thus be needed to solve these concerns.

In this study, the newly developed pH nanoprobes⁴² was used to evaluate the cytotoxicity of three types of NPs, cerium dioxide (CeO_2), titanium dioxide (TiO_2), and silicon dioxide (SiO_2), whose toxicity is still controversial.^{44, 55–58} Conventional viability and cytotoxic evaluations are also performed in parallel, including a metabolic activity based cell viability assay, an intracellular reactive oxygen species (ROS) generation assay, and a mitochondrial inner membrane potential stability assay. Both the fluorescence-activated cell sorting (FACS) flow cytometry and the laser scanning confocal imaging (LSCM) were used as different data analyzing techniques. Finally, the single transmission electron microscopy (TEM) imaging was applied to cell samples to confirm the actual cytotoxic effects of the NPs.

Experimental Section

Chemicals:

Titanium (IV) oxide (TiO_2), cerium (IV) oxide (CeO_2), and silicon dioxide (SiO_2) nanoparticles (NPs) were purchased from Sigma-Aldrich (Saint Louis, MO) at 99.0% purity. Fetal bovine serum (FBS) was purchased from American Type Culture Collection (ATCC) (Manassas, VA). Ham's F-12K medium with added L-glutamine was purchased from Fisher Scientific (Pittsburgh, PA). Trypsin–EDTA (0.25%), and 0.1 M phosphate-buffered saline (PBS) were purchased from Life Technology Co. (Carlsbad, CA). Ultra-pure water was generated with a Milli-Q system (Millipore, Bedford, MA). The mitochondria inner membrane potential depolarization reagent, carbonyl cyanide m-chlorophenylhydrazone (CCCP), was purchased from Sigma-Aldrich (Saint Louis, MO). Chemical and reagents used in specific assays are separately described in the following sections.

NPs characterization:

Three types of NPs were firstly characterized using TEM imaging and high-resolution TEM (HRTEM), selected area electron diffraction (SAED) as well as energy-dispersive X-ray (EDX) (Tecnai F20 STEM, FEI, OR). The singlet particle sizes were measured and averaged as 20 nm, 40 nm, and 46 nm, respectively, for CeO_2 , TiO_2 , and SiO_2 NPs (Fig. S1). The HRTEM images of these NPs (Fig. S1, b, c, g, and h) show lattice fringes at 3.10 Å and 3.17 Å in CeO_2 and TiO_2 NPs, respectively, corresponding to the (111) and (110) interplanar spacing of the NPs. The crystallinity was further confirmed by SAED pattern shown as the bottom inset (Fig. S1, d and i), where the diffraction spots could be indexed to the (111) and (110) lattice planes, respectively. The result revealed that the TiO_2 NPs were in the form of rutile. SiO_2 NPs were confirmed by the TEM and SAED pattern (Fig. S1, k and l) that they are non-crystallized. Finally, EDX analysis (Fig. S1, e, j, and m) showed good purity with each NPs (the third spike on CeO_2 graph resulted from the copper grid which was used in TEM sample holding).

The zeta (ζ) potential and hydrodynamic size of NPs in serum-free cell culture medium (Ham's F-12K) were characterized using Zeta-Sizer (ZEN 3690, Malvern Instrument Ltd). Ultrasonication (FS-60H, Fisher Scientific, Pittsburg, PA, USA) was applied each time for 15 minutes right before the Zeta-Sizer tests. Three NP dosages, 5, 20, and 100 $\mu\text{g/mL}$ for ζ -potential, and 100 $\mu\text{g/mL}$ for particle size determination, were respectively used. The NPs ζ -potential was found correlated with particle dosages with CeO_2 and TiO_2 NPs, but not quite the case in SiO_2 NPs (Table 1). In general, higher ζ -potential rendered higher stabilization of the NP-suspension. The SiO_2 NP suspension was found to have the highest colloidal stability than the other two NPs (Table 1). DLS particle size measurements show that CeO_2 and TiO_2 nanoparticles have a higher degree of aggregation (Fig. S2, a and b), while SiO_2 NPs have less aggregation and the smallest hydrodynamic size (Fig. S2c).

Single-cell pH nanoprobe fabrication and validation:

The novel designed probe featuring a hexagonal 1-in-6 fiber configuration (Scheme S1b)⁴² was fabricated as described previously with minor modifications.^{39–41} Briefly, single-mode optical fiber (SMF-28, Corning Inc., NY) was used, and the probe was fabricated by using a

home-built coaxial-twisting and gravitational-stretching system.⁴⁰ Once the 1-in-6 fiber taper stem was fabricated, a uniform Au/Pd layer with an approximate thickness of 250 – 300 nm was sputter-coated, to optically isolate the probe tip from environmental influences. Then focused ion beam (FIBs, Helios Nanolab 600, FEI) milling was applied to remove the very tip (< 5 μm) Au/Pd layer. Immediately the tip was subjected to an organic-modified silicate (OrMoSiI) sol-gel coating to introduce the embedded pH-sensing HPTS dye (excitation at 488 nm and emission at 525 nm). The featured small sensing tip and the designed 1-in-6 fiber configuration ensured a high spatial resolution detection and high-efficiency emission-collection. All fabricated probes were validated through measuring a series of standard pH buffer solutions with known pH, which have been pre-confirmed by an Accumet AB15 Plus pH meter (Thermo Fisher Scientific Inc., MA).

Cell culture and NPs dosing to cells:

The human adenocarcinomic alveolar basal epithelial A549 cell line (ATCC, CCL-185) was purchased from ATCC (Manassas, VA) and used as an in vitro cytotoxicity model in this study. This cell line has been widely used in particulate matter-related pulmonary toxicity studies,^{59–61} and was used in our previous work to demonstrate irradiation-enhanced ZnO NP cytotoxicity.^{48, 49} Cells were maintained in Ham's F-12K medium that supplemented with 10% FBS, 100 units per mL penicillin, and 100 $\mu\text{g mL}^{-1}$ streptomycin. Cells were cultured at 37 °C in a 5% CO₂ humidified environment. In each test, cells were seeded and allowed to attach for 18 hours (overnight) before NPs exposure. The cell density of 1×10^5 per milliliter was used. Cells without NPs exposure were used as the control in each experiment. All NPs suspension was freshly made each time with designed dosages in serum-free F-12K culture medium and subjected to 15 minutes ultrasonication. Pre-seeded cells will be washed by PBS three times and co-cultured with NPs suspensions. Cells used as control will also be washed by PBS and continuously cultured using a serum-free F-12K medium. The use of the serum-free medium in the NP-dosing experiments was to avoid nanocytotoxicity controversy that may be caused by the formation of protein corona outside NPs.⁵⁸

Cell viability and ROS assays:

Cell viability was measured using the water-soluble tetrazolium salt (WST-1) fluorescent indicator. Intracellular generation of reactive oxygen species (ROS) was measured using the 2',7'-dichlorofluorescein (DCFH) and its diacetate form (DCFH-DA, Sigma-Aldrich, MO). The use of tetrazolium salt WST-1 can correlate the absorbance of formazan produced with cell viability. The activity of the succinate-tetrazole reductase system in the mitochondria required by this method will weaken as the cell state deteriorates. The ROS assay was based on an intracellular redox-sensitive reagent DCFH-DA to detect H₂O₂ and oxidative stress while NPs are inducing cellular destructions. Briefly, WST-1 and DCFH-DA at working concentrations were added into NP-exposed A549 cells after gentle PBS washing, respectively, incubated for 40 and 60 minutes, and tested using absorbance at 450 nm for WST-1, and fluorescence with excitation at 488 nm and emission at 520 nm for DCF. Both tests were using the Fluostar Optima microplate reader (BMG Labtech, NC).

Laser Scanning Confocal Microscopic (LSCM) Imaging of NP-treated Cells:

Cells after co-culturing with NPs were washed gently three times with PBS and stained by a nucleus dye (2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride, DAPI, Invitrogen, NY) for 5 minutes, and a mitochondria inner membrane dye (5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylimidacarbocyanine iodide, JC-1, Invitrogen, NY) for one hour, respectively. The laser scanning confocal microscopic (LSCM) images (Eclipse Ti confocal microscope, Nikon, Japan) were taken under bright field and three fluorescent channels, DAPI, FITC, and Cy5. Further image merging and fluorescence analyses were conducted using the ImageJ software (version 1.48, NIH). The fluorescent ratio of JC-1 between its monomer and J-aggregates status was based on the corrected total fluorescence intensity (CTFI), which was calculated as below:

$$CTFI = I_{\text{integd.}} - (S_{\text{target}} \times I_{\text{ave.bkg}})$$

where the $I_{\text{integd.}}$, S_{target} and $I_{\text{ave.bkg}}$ represent the integrated total fluorescent intensity of the target cell, area of the target cell, and the averaged fluorescent intensity of a background area.

Flow Cytometry Analysis:

The fluorescence-activated cell sorting (FACs)-based flow cytometry analysis was conducted using the MitoProbe JC-1 Assay kit (ThermoFisher Scientific Inc., MA). A detailed protocol can be found in the product instruction. Positive control cells were ordinarily cultured cells, while negative control cells were subjected to 5 minutes co-culturing with the cytotoxic CCCP (50 μM final concentration). JC-1 dye was added (2 μM final concentration) to each cell sample and incubated under 37°C, 5% CO_2 for 30 minutes. In the subsequent NP cytotoxic assays, cells were pre-seeded at the same concentration (1×10^5 cells per mL) in each well for varied NP-exposure. After NP treatment for a designed period, cells were stained with JC-1 dye as described above for an optimized period (30 minutes in this study). Cells were then gently washed three times with pre-warmed PBS and trypsinized (< 3 minutes), centrifuged (110 g, 5 minutes), and re-suspended in 1 mL pre-warmed serum-free medium. Cells were analyzed immediately on a flow cytometer (C6 BD Accuri, BD Biosciences, CA) with excitation (488 nm) and emission wavelength filters (525 nm and 560 nm) that appropriate for Alexa Fluor 488 dye and R-phycoerythrin. In this study, cell event gates were set as three different cellular parts as described in the supporting information, namely: 1) intact cells (P-I), 2) cell debris (P-II), 3) agglomerated NPs (P-III). In the area where intact cells are recorded, two polygonal sub-areas are also set to indicate healthy (mitochondrial polarization) and unhealthy (mitochondrial depolarization) cells.

Cellular NP-Uptaking Imaging by TEM:

To confirm NP uptaking, NP-treated cells were collected at each checkpoint and fixed for one hour at room temperature, in a 2.5% glutaraldehyde solution in 0.1 M Sorensen phosphate buffer (pH 7.3). The samples were then rinsed with Sorensen phosphate buffer (pH 7.3), and post-fixed for one hour in 1% osmium tetroxide in deionized water. Traditional dehydration/infiltration procedures (start from 70% EtOH) were used, where a lengthened

time in 95%, 100% EtOH, and propylene oxide was applied. Cells were then embedded into the EMBED 812 (Electron Microscopy Sciences) resin according to its instruction, followed by ultrathin sectioning using a diamond knife on a Leica U6 ultramicrotome. Sections were finally mounted on copper grids and poststained with uranyl acetate for 30 minutes and lead citrate for 15 minutes before being examined by the FEI Tecnai F20 STEM system.

Single Cell pH Measurement:

The A549 cell line was cultured and exposed to NPs as described above but in a controlled environmental chamber that mounted on an Olympus IX51 inverted fluorescent microscope to enable long-term cell observation, manipulation, and measurement. When conducting single-cell probing, an appropriate number of cultured cells (50 – 100) were trypsinized to achieve single cells suspended in a fresh medium. Each time, a randomly selected cell is gently picked using the cell holding probe through applying –1 to –5 mbar negative pressure (as schematically demonstrated in Fig. 1b) followed by intracellular insertion of the pH nanoprobe at a 180° angle opposite to the holding probe. The probe was kept inside the cytosol for approximately 10 – 15 seconds for pH equilibration. Then excitation laser was turned on for 3 – 5 seconds to acquire sufficient signal accumulation, followed by gentle retreating of the probe out of cell membrane.⁴²

SP-ICP-MS Assay:

A549 cells were cultured and exposed to CeO₂ NPs, as described above. Single particle-inductively coupled plasma mass spectrometry (SP-ICP-MS) (NexION 300, Perkin Elmer Inc., MA) was used to ascertain the NPs deep interaction with the cell membrane. TiO₂ and SiO₂ NPs were not tested in this study mainly because of the potential system error caused by their composition. Titanium has a similar atomic mass as the carrier gas Argon, and SiO₂ may highly be influenced by the sample inducing tube made from quartz. The cells were exposed to 5, 20, and 100 µg/mL CeO₂ NPs for 48 hours, followed by gentle washing by PBS ten times to thoroughly remove most of the loosely attached NPs on the cell surface. Cells were then fully digested using concentrated nitric acid and an Enviro Mac™ Hot Block. Finally, digested samples were analyzed via ICP-MS (for detailed parameters, please refer to Table S2).

Statistical Analysis:

All cell population-based experiments were conducted in triplicate for statistical validation purposes. However, single-cell probing was not limited under this category, to avoid multiple insertions induced damages to the cell. Also, it was unnecessary to do so due to the intrinsically high heterogeneity between cells. When appropriate, experimental data were normalized against the corresponding control group and expressed as the mean ± standard deviation (SD). Statistical analyses were performed using Prism 5 (Graph-Pad Software, CA), including a one-way analysis of variance test (ANOVA) followed by a post hoc Tukey test to determine the statistical significance. Differences were established to have statistical significance at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). Minitab™ software (State College, Pennsylvania) was also used partially for statistical analysis, as shown in Fig. S3.

Results and Discussion

Establishment of the Fiber-Optic pH Nanoprobe Sensing System

A fiber-optic pH nano-probing system for single-cell sensing was established as described in our recent work,^{40, 42} where the 1-in-6 fiber configuration was still adopted (Scheme S1a). Briefly, the whole probe includes five hierarchies (Scheme S1b), which are: (1) a core fiber for excitation laser delivery; (2) peripheral six fibers for fluorescence emission collection; (3) a sputter-coated gold (Au)/palladium (Pd) thin layer (~300 nm) for optical isolation of the probe stem from ambient light; (4) a 5 μm Au/Pd removal area at the tip to expose the inner fiber; and (5) a pH-sensitive HPTS-embedded OrMoSil layer that coated at the exposed area depicted in (4). Detailed fabrication procedures can be found in our previous works.^{39–42}

High-Resolution pH Sensing Calibration and Single Cell Sensing Validation

Probe calibration was conducted using a series of standard buffer solutions that cross a pH range from 6.17 to 8.11. The fluorescence spectrum was collected, and the peak area from 495 nm to 640 nm was plotted (Fig. 1a, inset). A linear correlation ($R^2 = 0.9827$) between peak areas and applied buffer pHs was found (Fig. 1a), and the sensitivity, as defined as the peak area change per pH unit ($\Delta \text{Spec.}/U_{\text{pH}}$), was found to be 14,867 a.u. Thus, a pH resolution was calculated as 0.06 pH unit on average, and slightly higher near the neutral pHs (~pH7.0 – pH 7.3), at approximately 0.02 pH unit.^{40, 42}

Single-cell sensing is schematically illustrated in Fig. 1b. Briefly, a hollow cell-holding probe was firstly used to mildly clamp a suspended single cell by applying mild negative pressure. Then the pH nanoprobe was inserted for pH_i detection. An exemplary single A549 cell probing is shown in Fig. 1, c–e. The bright field view shows the probe insertion at the targeted cytoplasmic region (Fig. 1c). A combined image from two channels, DAPI and FITC, shows the cell nucleus (blue) and the shining nanoprobe (green) during laser excitation (Fig. 1d, yellow dash line illustrated the cell boundary). An expanded image (Fig. 1e) highlights the fluorescent probe tip under the FITC channel. The laser-excited fluorescence illuminates an elliptic region of the cytoplasm with a height of 610 nm and a width of 930 nm, indicating a nanoscale detection volume of 0.28 fL. An ideal cell with a spheroidal shape has a typical diameter of 10 – 15 μm , which corresponding to a volume range from 4.2 pL to 14.1 pL. Thus, our nanoprobe only illuminates about 0.2 % – 0.6 % of the total volume of a single cell. This is a record-breaking nanoscale sensing resolution of the local subcellular environment. For data collection, 10 – 15 seconds is used to equilibrate the probe, and 3 – 5 seconds is used to switch on and off the laser to record the signal at its plateau level. Admittedly, cell insertion in this study does draw concerns regarding cell viability. Previously established single-cell manipulation guidelines, such as those in the *in vitro* fertilization (IVF), provided that an acceptable cell cleft should be smaller than 5 μm in diameter. In our case, the probe tip diameter that contributes to the opening cleft was at most 1 – 1.5 μm , and sometimes at nanoscale levels, which is considerably below the announced threshold. Cell deterioration after probe insertion does exist sometimes, but below 10% of all inserted cells. Finally, the multi-cell continuous measurement was also conducted to validate probe robustness. Fifteen A549 cells that were cultured within the same Petri dish were

randomly selected for this test (Fig. S3). The measured pH_i values fell into a range of traditionally agreed values from pH 6.8 to pH 7.6.^{62, 63} Thus, cell-cell heterogeneity does exist, indicating an individually maintained pH_i homeostasis.

Establishment of a preliminary nanotoxicity model within 48 hours

Three types of widely used NPs, CeO_2 , TiO_2 , and SiO_2 , were employed to create typical nanotoxicity events, and 48 hours was selected as the preliminary evaluation range. NPs were characterized using transmission electron microscopy (TEM), energy-dispersive X-ray spectroscopy (EDX), and dynamic light scattering (DLS). Results showed varied crystallization and ζ -potential values, as described in the Experimental section, and illustrated in Fig. S1 and S2. The human lung cancer A549 cell line was used for NP-exposure and cytotoxic evaluations. 48-hour cell viability and intracellular ROS generation assays were firstly conducted (Fig. 2, a–f). Three NP concentrations of 5, 20, and 100 $\mu\text{g/mL}$ were used to produce varied cytotoxic effects. NPs were freshly suspended and immediately administrated to pre-cultured cells at time T0 (as detailed in the Experimental section).

Results from Fig. 2 demonstrated that cell viability and intracellular ROS generation were correlated with both NP concentrations and administrated times, but the effects were different among different types of NPs. After 12 hours of exposure, even though the cell viability decreased in all NP-treated groups, only the TiO_2 NP-treated group showed statistical significance in all NP concentrations ($p < 0.05$) (Fig. 2b). After 24 hours of exposure, all NP-treated groups showed apparent reductions in cell viability. However, the cell viability of the 100 $\mu\text{g/mL}$ SiO_2 NP-treated group decreased radically ($p < 0.001$) after 12 hours, which further decreased down to merely 6.3% of the control group's level after 48 hours (Fig. 2c). Meanwhile, the ROS generations of both CeO_2 and TiO_2 NP-treated groups increased in 48 hours and were proportional to the applied NP concentrations (Fig. 2, d and e). A steep ROS increase was already observed after 24 hours in the high concentration (100 $\mu\text{g/mL}$) TiO_2 group. However, in the SiO_2 NP-treated groups, no higher ROS generation was observed statistically than the control. Instead, a decreased ROS production was found in the 100 $\mu\text{g/mL}$ SiO_2 NP concentration group (Fig. 2f). Such a decrease caused doubt about the feasibility of this method for nanotoxicity assessment, especially for certain types of NPs, such as SiO_2 . This phenomenon will be further studied.

The cell viability and ROS production were assessed by using WST-1 and DCFH-DA reagents, which are widely used cytotoxicity evaluations. The effectiveness of these assays relies on the internalized dye molecules and their reaction with cytoplasmic enzymatic complex. However, the cytoplasmic levels of succinate-tetrazolium reductase and redox species may well be influenced by intracellular NPs. In addition, the WST-1 assay was reported to be flawed, and it can only yield relatively quantitative results.^{64, 65} Balaraman et al. also warned the limitation of DCFH-DA, especially when redox-active metals exist within a niche environment.⁶⁶ Our previous study on ZnO NPs also suggested that metal oxide NPs may cast negative effects on ROS generation. For example, the quenching effect of high ionic strength and the consumption of hydroxyl radicals by NPs.⁴⁹ These issues may also be responsible for the problems observed in the above two tests. For example, in the TiO_2 NP-treated cell group, there was no significant difference in ROS production in all

groups at 12 hours; meanwhile, the cell viability value was already reduced significantly. Besides, SiO₂ NP-treated cells showed severely reduced levels of cell viability, but even lower ROS production was observed than the control cells, which are totally contradictory.

To overcome the above issues and better evaluate the cytotoxic effects of NPs, a mitochondrial dye JC-1 was used to stain the cells after NP-exposure, and the LSCM was used for direct imaging. By quantifying the ratio of total red to green fluorescence intensity (CTFI, as described in the Experimental section) after background correction, the mitochondrial depolarization, and hence the state of cell health, can be compared. The results from Fig. 2g showed that after 24 hours of NP-exposures, the TiO₂ and SiO₂ NPs-treated groups exhibited significant depletions ($p < 0.05$) of the mitochondrial potential (0.74 ± 0.09 and 0.40 ± 0.06 of the control group level, respectively). However, after 48 hours of exposure, the mitochondrial potential of CeO₂ and TiO₂ NPs-treated groups reached the same level ($0.46 \pm 0.05 \sim 0.48 \pm 0.05$ of the control group level) as those of SiO₂ NP-treated group at 24-hour exposure. This result confirmed the negative effects of NPs on A549 cells at different toxic levels. It also showed the potential issues existing in the cell viability and ROS generation assays that may lead to false conclusions. In summary, based on the results of the decline in cell viability, mitochondrial depolarization, and ROS generation, the toxicity levels of the three types of NPs can be estimated as SiO₂ > TiO₂ > CeO₂. Cell deterioration can be confirmed at about 24 hours, while some high concentration NP-treated groups (such as TiO₂ (Fig. 2b) as well as the SiO₂ NP-treated groups (Fig. 2c)) showed toxic effects at even earlier exposure time. Therefore, we decided to track the early nanotoxicity within the first 12 hours.

Detail assessment of nanotoxicity within the first 12-hour exposure

To avoid further erroneous conclusions caused by the discrepancy between cell viability and ROS measurements, we decided to use FAC flow cytometry combined with JC-1 dye to assess the state of cells in the early stage of nanotoxicity (first 12 hours). At the same time, the pH nanoprobe we developed was also used to verify the hypothesis that the real-time cellular pH_i monitoring can be correlated and used to track the early deterioration of cells.

First, the BD Accuri C6 flow cytometer system was calibrated using an eight-peak Rainbow fluorescence kit (Fig. S4). Cellular states can be detected by a colorimetric method based on the mitochondrial inner membrane polarization (red fluorescence, propidium iodide (PI channel)) or depolarization (green fluorescence (FITC channel)). Flow cytometry can also separate different cellular components by the side laser scattering characteristics (SSC) vs. forward laser scattering characteristics (FSC) of the particulate materials (SSC vs. FSC). Before the nanotoxicology experiments, healthy A549 cells and a known cytotoxic proton carrier CCCP (50 μ M, treated cells for 30 minutes) were used to determine the positive and negative mitochondrial function thresholds (Fig. S4, d and e). The results showed that most of the healthy cells were located in the upper part of the PI-FITC diagram (red polygon), while the mitochondrial ψ_m damaged cells were located in the lower part (green polygon). The SSC vs. FSC diagram was also divided into several parts, including intact cells (PI, black), cell debris (P-II, blue), and NPs and agglomerates (P-III, purple) (Fig. S4f). All three areas were extended to the top of the SSC axis to count in all particles or cell components

that may have high surface roughness. Meanwhile, serum-free cell culture medium and freshly prepared suspensions of three NPs in the medium were also subjected to flow cytometry (Fig. S4, g–j). No particles were detected in the medium (Fig. S4g), and the distribution of the three NPs in the P-III regions was slightly different in the SSC direction. CeO₂ NPs mainly distributed in the lower part of SSC, indicating its low granularity and relatively smooth particle surface (Fig. S4h). TiO₂ NPs, with tetragonal crystal facets, are mostly distributed in the high SSC region, indicating a high granularity status (Fig. S4i). Attributed to the amorphous shape, SiO₂ NPs distributed in the lower-left corner of the SSC-FSC diagram indicates the smallest particle radius and the smoothest surface (Fig. S4j). These results are consistent with the TEM scan and ZetaSizer analysis (Experimental section, Fig. S1, and S2), TiO₂ NPs have the most angular facets, while SiO₂ NPs have the smallest hydrodynamic particle size.

Then, the cell conditions within the first 12 hours after NPs administration were analyzed. Four-time points (0, 3, 6, and 12 hours after NPs administration) were selected for conducting the flow cytometry assay (Fig. 3). The bar graph on the left showed the ratio of polarization and depolarization of the cell mitochondria, and the bar graph on the right showed the percentage of the detected cell components, including those located in the pre-designated area (PI – PIII) and those detected outside this range (untracked, gray). Finally, the SSC-FSC diagram on the right showed the actual distribution of cells or particles in the measured area.

After three hours of NP administration, the mitochondrial depolarization rate (green) in all three NP-treated groups was already higher than that in the control group (Fig. 3, d, g, and j). The depolarization rate in the TiO₂ group has even exceeded 60% of the total cells. Many untracked cell parts (gray) were shown in the cell composition histograms of the CeO₂ and TiO₂ groups (Fig. 3, e and h). Meanwhile, the normal SSC vs. FSC graphs of these two groups lost their intact cell components, which should be distributed at the bottom along the FSC axis. Instead, a large number of data points in the high SSC direction were observed (Fig. 3, f and i, 3 hours), especially the CeO₂ group (Fig. 3f). Such wide bandwidth distribution of these data points on the entire SSC-FSC graph may indicate that during the interaction of the first three hours between NP and cells, many particles were formed with high granularity and different sizes. The number of data points in the SSC-FSC chart of the TiO₂ group was way smaller than the control. This loss may correspond to a high percentage of untracked cells in its histogram. A similar situation also appeared in the CeO₂ group. These cells were not tracked because they were beyond the range of particle attributes included in the SSC-FSC diagram. There were no high granularity particles in the SSC-FSC diagram of the SiO₂ NP group. However, many depolarized cellular components (green dots) of this group overlap with the cell debris (blue dots) (Fig. 3l). Such a phenomenon was also reflected in the percentage of cell debris (blue) in the histogram, which was higher than the other groups (Fig. 3k). The sizes of the mitochondria-containing particles here were smaller than that of healthy cells, indicating that the integrity of the cells might have been destroyed. However, the mitochondrial depolarization rate of the SiO₂ group was slightly higher than that of the CeO₂ group but far lower than the TiO₂ group. Thus, it is unreasonable to see large numbers of cell debris in the SiO₂ group at this moment. This

result and the mysterious large number of untracked cells in the CeO₂ and TiO₂ groups questioned the reliability of the assay.

The percentage of depolarized cells even decreased slightly (Fig. 3, a–c). In the CeO₂ and TiO₂ groups, the number of both depolarized and untracked cells decreased. In the SSC-FSC graph, denser data points were observed. From 3 to 12 hours, the main body of these data points gradually moves down to the lower SSC region (Fig. 3, d–i). Together they make the overall coloration more reddish (Fig. 3, f and i), indicating that more cells were not depolarized, as reflected in the increased percentage of the red column on the left histogram (Fig. 3, d and g). Interestingly, an increased percentage of polarized cells and decreased cell debris were also observed in the SiO₂ group (Fig. 3, j and k). No data points exceeded the SSC limits, and no significant untracked cells were shown in the early hours (Fig. 3l). Thus, there should be no cells fall back into the SSC-FSC graph and increase the overall healthy cell ratio.

In the CeO₂ and TiO₂ groups, both the broad bandwidth data points and large numbers of untracked cells were found in the SSC-FSC diagram and the histograms, respectively. We inferred that this was due to the highly roughened cell membranes by the NP-cell interaction, which caused the increase of SSC value that exceeded the pre-selected PI-PIII limits, and thus resulted in many untracked cells (see the hypothetical Fig. S5). After 3 hours, NPs were either excreted by the cells or entangled in the inner layer of the cells, resulting in smoother cell membranes and lower SSC values, and thus, more data points falling back within the limits of the SSC-FSC diagram. We use SP-ICP-MS (NexION 300, Perkin Elmer Inc.)^{67, 68} to verify this inference. A549 cells were co-cultured with CeO₂ NPs (5, 20, and 100 ppm) for 24 hours, washed thoroughly with PBS buffer, and immediately analyzed by SP-ICP-MS to quantify the intracellular NPs (Table S1, S2). Results showed that the NPs were absorbed by the cells, with contents that were proportional to the initially applied NP concentrations (e.g., 0.51% absorbed with an initial dose of 100 ppm). This result explained that the large number of data points observed in the upper region of the SSC-FSC diagram were surface roughened cells and debris (Fig. 3f). Although TiO₂ and SiO₂ NPs were not tested here, the absolute values of their zeta potential were higher than that of CeO₂ NPs (Table 1), resulting in their smaller hydrodynamic particle size and easier access to cells.

As for the doubts caused by SiO₂ NPs, we noticed that the FACs test for the samples from this group took much longer than other groups to reach the same total cell count. Given that all groups started with the same quantity of cultured cells, longer analysis time in FACs means a significant cell loss. Further tracking found that during the centrifugation of trypsinized cells, the cell pellet could not be re-suspended with a pipette but left a sticky intertwined clump (Fig. S6). It seemed that only small amounts, relatively healthy cells, were finally re-suspended and analyzed by the flow cytometry process, which could be the reason for the longer and abnormal FACs analysis duration of the SiO₂ group. Thus, the increased red/green fluorescence ratio and reduced cell debris after 3 hours could not simply be interpreted as an improvement of the overall cell status.

To avoid damage to the cells at making flow cytometry samples, which would affect the final result, we still used the JC-1 plus the DAPI dyes to stain the NP-treated cells directly and performed the LSCM imaging at the same checkpoints (Fig. 4). The results showed that mitochondrial depolarization occurred in the TiO_2 and SiO_2 treated groups after 6 hours of NP-administration. However, the CeO_2 treated group kept unchanged (Fig. 4a). Also, at 3 hours, the TiO_2 treated group already showed significantly higher nuclear staining than the control. After 6 hours, all three NP treated groups showed statistical differences with the control, indicating the potential occurrence of chromatin condensation and nuclear destruction (Fig. 4b).^{69, 70}

In summary, the above results are distracting (such as untracked cells in the CeO_2 and TiO_2 treated groups, massive cell loss in the SiO_2 treated group, etc.) and lack of certainty. The change of mitochondrial depolarization rate after 3 hours seems to be opposite in the results of FACs and LSCM. Therefore, these traditional methods may not be able to reflect the actual viability of cells under the influence of NPs.

Diagnose nanotoxicity via single-cell pH monitoring

To dispel any doubts caused by the above results, we used the developed pH nanoprobe to randomly inspect the NP-treated single cells (Fig. 4, c – f). Unlike the cell population-based assays, single-cell monitoring maximized the ability to reveal the heterogeneity presents in the cell population. Therefore, by conducting low cell number tests with our probe, an actual cell situation can be observed at an unprecedented single-cell scale. Single-cell pH_i was tested after 0, 0.5, 1, 3, 6 and 12 hours of NPs treatment, and the same 10 – 13 cells were repeatedly tested each time (blue lines). The red line and error bars represent the mean $\pm$ standard deviation of all blue lines. To avoid abnormal readout caused by the repeated insertion of the probe, at each time point, three randomly selected cells were given a one-time probing (yellow polygon covered range). By doing so, the overlap of the red line and the yellow polygon can be interpreted that the measurements are unbiased.

Results showed that the pH_i of the cells in the control group remained stable within 12 hours (Fig. 4c), with the averaged value (red line) fluctuated from pH 7.50 to 7.46. Whereas rapid pH_i drops were observed in all three NP treated groups (Fig. 4, d – f). The strongest downward trend was found in the first hour of NP administration, then gradually attenuated in the following hours. This is a clear sign of NPs interfering with the pH_i homeostasis in the early stages.

Interestingly, the blue line in the control and CeO_2 treated group is more convergent than those in the TiO_2 and SiO_2 treated cell groups. Meanwhile, the averaged pH_i value (red line) overlaps well with the one-time measured pH_i range (yellow polygon) in the control and the CeO_2 treated groups (Fig. 4, c and d), indicating that the CeO_2 NPs may have less or no effect on the cells. Several individual pH_i also recorded a reversing trend in the CeO_2 treated group, meaning the cells can still actively adjust their pH_i after being affected by the CeO_2 NPs. On the contrary, the decrease levels of pH_i and the fluctuation magnitudes between individual cells (blue lines) in the latter two groups, especially TiO_2 , are both more significant than that of the CeO_2 treated group (Fig. 4e), which may suggest that the TiO_2 and SiO_2 NPs stimulated larger heterogenic responses of cells. Moreover, the overlap

between the average pH_i value (red line) and the one-time measured pH_i range (yellow polygon) was also not as good as the control and the CeO_2 groups (Fig. 4, e and f). Thus, cells may become more fragile and unstable when subjected to multiple probing. In summary, all NPs caused the pH_i reduction in the first 12 hours and resulted in more than 0.2 (CeO_2) and 0.4 (TiO_2 and SiO_2) pH unit decrease, which is an order of magnitude difference than that of the control group. Such levels of decrease indicate that the applied NPs have impaired the pH_i homeostatic system.

The result above was the first real-time single-cell pH_i detection to explore the early-stage NP-cell interactions. These results revealed a unique series of cellular heterogeneity that impossible to be observed with any cell-population-based assays. By measuring the pH_i homeostasis, the probe may convey the health status of the cell as well as interpret possible influences on cell viability. However, due to its specificity in pH, the probe will not respond to cytotoxic effects that are not triggering pH_i variation, nor to a few NPs that only induced adverse effect locally and subcellularly. The key state we want to focus on is the global cytotoxic effect, during which, once triggered, pH_i variation will be one of the several leading events that would mostly occur.

It is known that cancer cells actively transport OH^- groups into cells through ion pumps on the plasma membrane to control acidic extracellular pH (pH_e) and slightly higher pH_i . This controlled extracellular acidic environment can protect cancer cells from immune system attacks are also the basis of their malignancy and metastasis. Disrupted membrane functions by NPs may threaten the maintenance of the transmembrane pHs and thus compromise the pH_i homeostasis. Our results revealed the disrupted pH_i of the lung cancer 549 cells by NP administration, which may potentially be correlated with changes in cell viability.

We extended the experiment to reach a total duration of 48 hours for a full-spectrum view of the nanotoxicity trend (Fig. S7). The results showed that after 12 hours of NP-administration, the decreasing trend of pH_i in all NP groups was considerably eased (Fig. S7, a – d). Therefore, the interaction between NPs and cells mainly occurs in the early stage of their contact. We again noticed that the averaged result (red line) showed a deviation from the range that established by the one-time measured cells (yellow polygon) after 24 hours, which was even observed in the control group. The one-way ANOVA method was thus used to determine whether this deviation was statistically meaningful (Fig. S7e). The results showed that there was no significant difference between single and multiple probed cells in the control and all three NP treated groups ($p > 0.05$). This result proves that the cells can withstand multiple probe insertions without being significantly affected. Therefore, the experimentally obtained pH_i data is reliable.

Nanotoxicity confirmation via single-cell TEM imaging

To confirm the NP-induced damage to cells, we collected cells at 0, 3, 6, and 12 hours after NP administration. Cells were immediately embedded in resin and were subjected to ultra-thin sections and TEM imaging (Fig. 5). Intact cell structures were observed in all groups at 0 hours. After 3 hours of NP-administration, most CeO_2 NPs are clustered (> 100 nm) and hover around the cell border. TiO_2 NPs have entered the cytoplasmic membrane in smaller agglomerates and even approached the nuclear membrane. SiO_2 NPs, while maintaining a

highly dispersed low-aggregated state, already occupied most of the cytoplasmic space and adhered to the boundary of the cell nucleus. These unexpected results indicated that NPs (e.g., TiO_2 and SiO_2 NPs) could deeply interact with cells in merely 3 hours. After 6 hours, CeO_2 NPs remained mostly outside the cells and were only taken up sporadically by the membrane-derived vesicles. TiO_2 NPs not only “invaded” the cytoplasm but also formed huge voids around the nucleus. SiO_2 NPs largely entered the cytoplasm and destroyed the integrity of the nuclear membrane already. After 12 hours, although a small amount of CeO_2 NPs entered the cytoplasm, the cell integrity remains intact. TiO_2 NPs agglomerates entered the cytoplasm in large portions and might have attacked the nucleus, but the cell boundary still exists. Finally, highly dispersed SiO_2 NPs caused extensive dissociation of the intracellular contents and the cell membranes.

An association may exist between the level of NPs agglomeration and the formation of intracellular cavities. After 3 hours of exposure (Fig. 6), CeO_2 NPs condensed into large agglomerates (200 – 500 nm) around the periphery of the cells and entered the cell membrane relatively late without causing intracellular voids (Fig. 6, a and d, yellow arrows). However, TiO_2 NPs formed smaller aggregates, entered the cells early, and formed large voids (300–700 nm) in the cytoplasm (Fig. 6, b and e, blue arrows). Surprisingly, SiO_2 NPs hardly aggregate, spread throughout the cytosolic portion, and formed many tiny (50–200 nm) cavities (red arrows) at the early stage (Fig. 6, c and f). Besides, the cell nucleus is mostly disappeared in the SiO_2 NP-treated group, and only scattered clusters of organelles and nuclear residues can be roughly identified (Fig. 6c, green arrows). Thus, we speculate that the aggregate size of NPs and the ability to enter cells and form voids are correlated with their ζ potential. CeO_2 NP has a ζ potential of -9.5 mV (Table 1), which is easy to aggregate in an intracellular environment with high ionic strength. TiO_2 and SiO_2 NPs with relatively high absolute values of ζ potential (-12.7 mV and -25.8 mV, respectively) embrace better colloidal stability and high protein affinity.^{58, 71–74} Thus, these NPs are not easy to agglomerate and are easily absorbed by cells, and therefore are more likely to cause severe intracellular damages.

These TEM images revealed different cytotoxic details of these NPs. First, images showed a large number of NP agglomerates attached to the cell membranes in CeO_2 and TiO_2 groups (Fig. 5, a and d). The severely roughened cell surface explains the large number of data points that appeared in the high SSC area in the flow cytometry results (Fig. 3, f and i), including those untracked data points (grey) with SSC values that exceeded the upper limit of the SSC-FSC graph (Fig. 3, e and h). Second, even though the cell surface roughness caused by the TiO_2 NPs was similar to CeO_2 NPs, it entered the cell and damaged the cytoplasm. Third, with the prolonged NP-administration time, the damage of TiO_2 NPs to cells continued to increase rather than decrease. Although these results are partially reflected in the cell viability and ROS generation assays, the subsequent trend of increased viability (Fig. 2b) and unchanged oxidative stress (Fig. 2e) is inconsistent with the facts.

The TEM results illustrated a strong cell-damaging effect of the SiO_2 NPs, as shown in the early hour images where large quantities of them flooded into the cytosolic region. Thus, the aforesaid cell pellet that unable to be aspirated (Fig. S6) may highly be the entangled residues of these NP-destroyed cells. These images also supported the increase of cellular

debris in Fig. 3k, the sharp decrease in cell viability in Fig. 2c, and the depolarization of mitochondrial in Fig. 2g. However, previous test results also gave some controversial results, such as the decreases in ROS generation (Fig. 2f) and mitochondrial depolarization (Fig. 3j). Here, the TEM images refuted the non-toxic conclusions that may be drawn from these results and confirmed the NP-induced cellular damage. For these confusing experiments (e.g., Fig. 2f and Fig. 3j, etc.), the cells may be damaged too fast to absorb indicator molecules and show their damage conditions. Meanwhile, those cells that were not invaded by the NPs in the initial stage showed the illusion of increased overall cell viability in the subsequent period.

The experimental results demonstrated that the findings based on our pH nanoprobe (Fig. 4) were consistent with the TEM imaging results (Fig. 5), where a rapid NP-cell interaction was observed during the first three hours. The applied NPs showed different influences on the pH_i , which corresponded to their varied destructive effect on the cytosol from light to heavy ($\text{CeO}_2 < \text{TiO}_2 < \text{SiO}_2$). Both the pH_i decrease and cellular damages were irreversibly accumulated.

Further Discussion

Cell viability and cytotoxicity have long been studied based on well-established methods and principles, such as the colorimetric methods using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT).^{75, 76} Cell viability can thus be reflected by the formation of formazan crystallization via active oxidoreductase enzymes in metabolically intact cells. Similarly, the use of DCFH-DA or DMPO for the detection of intracellular oxidative stress were also widely used for cytotoxic indication.^{77–79} However, these methods are only applicable to a population of cells, due to their lack of sufficient detection limits at the low cell number or single-cell levels. Meanwhile, by assuming that cells would behave similarly under the same culture or treatment conditions, the cell-cell heterogeneity is ignored as well. On the contrary, our newly developed pH nanoprobe provides a single cell level measurement, real-time sensing ability, as well as pH resolutions up to 0.02 unit. It revealed the existence of cell-cell heterogeneity that hiding behind the cell population-based behavior. The probe illustrated the pH_i variations in the early NP-administration period on an unprecedented single-cell scale (Fig. 4). The fluctuation of single-cell pH_i vividly detailed the cell's active control over pH_i while under NPs' attack.

Previous reports about the toxicity of CeO_2 NPs are contradictory: some concluded as toxic,⁸⁰ others are not.^{81–83} Our pH nanoprobe and TEM results showed its little effect on cell integrity. Results of the CeO_2 group that differed from control groups were also seen in this study's viability and ROS assays. These misleading results may be caused by the interaction of the NP with the cell membrane and the leakage of a small amount of cytoplasmic material. An unexpected result of this study is the strong toxic effect of the SiO_2 NPs that exhibited in its interaction with cells at an extremely early stage. SiO_2 NP has been reported as cytotoxic,^{55, 84, 85} however, these NPs are still widely used in products such as cosmetics,⁸⁶ sunscreen lotions,⁸⁷ drug delivery,⁸⁸ etc., and deemed as low-⁸⁹ or non-toxic.⁵⁶ Confusing results were also reported in this study, such as the reduction of ROS generation (Fig. 2f), decrease of mitochondrial depolarization rate (Fig. 3j), reduction of cell debris (Fig. 3k), and

non-changed fluorescence level at early hours (Fig. 4a). Nonetheless, our pH nanoprobe detected the substantial pH_i changes induced by SiO_2 NPs, and successfully correlated it with the cytotoxic evidence shown in TEM images. To sum up, our developed single-cell pH_i nanoprobe revealed the existence of intercellular heterogeneity, discovered the previously unexpected strong cytotoxicity of SiO_2 NPs, and verified the hypothesis that pH_i could effectively interpret the health condition of the cell.

Conclusion

In this study, a novel fiber-optic pH nanoprobe was applied to interpret cell health conditions and nanotoxicity via monitoring of the cell pH_i . Meanwhile, the results were compared in parallel with several conventional methods: cell viability, ROS generation, FACs flow cytometry, LSCM imaging and TEM imaging. A good correlation was found between the nanoprobe's results and the TEM imaging. Three types of NPs were applied and exhibited quick interaction with cells at the early stage of administration with varied cytotoxic effects. Unexpected fast cell-uptaking and high cytotoxicity were found in the SiO_2 NPs. This study validated that the current pH nanoprobe can serve as a novel label-free method for single-cell monitoring in real time with high-accuracy and minimum invasiveness. Our work also indicates that cellular pH_i may serve as a broad-spectrum biomarker of cell viability and cytotoxicity, for delicate monitoring of cellular events that with high heterogeneity and low occurrence rates, such as nano-drug delivery, carcinogenesis, and stem cell differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Gawad C, Koh W and Quake SR, Nature Reviews Genetics, 2016, 17, 175–188.
2. Björklund ÅK, Forkel M, Picelli S, Konya V, Theorell J, Friberg D, Sandberg R and Mjösberg J, Nature immunology, 2016, 17, 451–460. [PubMed: 26878113]
3. Tasic B, Menon V, Nguyen TN, Kim TK, Jarsky T, Yao Z, Levi B, Gray LT, Sorensen SA and Dolbeare T, Nature neuroscience, 2016, 19, 335–346. [PubMed: 26727548]
4. Angermueller C, Clark SJ, Lee HJ, Macaulay IC, Teng MJ, Hu TX, Krueger F, Smallwood SA, Ponting CP and Voet T, Nature methods, 2016, 13, 229–232. [PubMed: 26752769]
5. Lombard - Banek C, Moody SA and Nemes P, Angewandte Chemie International Edition, 2016, 55, 2454–2458. [PubMed: 26756663]
6. Poovathingal SK, Kravchenko-Balasha N, Shin YS, Levine RD and Heath JR, small, 2016, 12, 1425–1431. [PubMed: 26780498]

7. Emara S, Amer S, Ali A, Abouleila Y, Oga A and Masujima T, in *Metabolomics: From Fundamentals to Clinical Applications*, Springer, 2017, pp. 323–343.
8. Chen X, Love JC, Navin NE, Pachter L, Stubbington MJ, Svensson V, Sweedler JV and Teichmann SA, *Nature biotechnology*, 2016, 34, 1111–1118.
9. Casey JR, Grinstein S and Orlowski J, *Nature reviews Molecular cell biology*, 2010, 11, 50–61. [PubMed: 19997129]
10. Webb BA, Chimenti M, Jacobson MP and Barber DL, *Nature Reviews Cancer*, 2011, 11, 671–677. [PubMed: 21833026]
11. Swietach P, Vaughan-Jones RD and Harris AL, *Cancer and Metastasis Reviews*, 2007, 26, 299–310. [PubMed: 17415526]
12. Fang Y, Liu Z, Chen Z, Xu X, Xiao M, Yu Y, Zhang Y, Zhang X, Du Y and Jiang C, *Cell Research*, 2017, 27, 1083–1099. [PubMed: 28675158]
13. Sergeeva TF, Shirmanova MV, Zlobovskaya OA, Gavrina AI, Dudenkova VV, Lukina MM, Lukyanov KA and Zagaynova EV, *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 2017, 1864, 604–611. [PubMed: 28063999]
14. McBrien MA, Behbahan IS, Ferrari R, Su T, Huang T-W, Li K, Hong CS, Christofk HR, Vogelauer M and Seligson DB, *Molecular cell*, 2013, 49, 310–321. [PubMed: 23201122]
15. Wang P, Li L, Zhang Z, Kan Q, Gao F and Chen S, *Molecular medicine reports*, 2016, 13, 4443–4450. [PubMed: 27035646]
16. Shrode LD, Tapper H and Grinstein S, *Journal of bioenergetics and biomembranes*, 1997, 29, 393–399. [PubMed: 9387100]
17. Han J and Burgess K, *Chemical reviews*, 2009, 110, 2709–2728.
18. Srivastava J, Barber DL and Jacobson MP, *Physiology*, 2007, 22, 30–39. [PubMed: 17289928]
19. Medintz IL, Stewart MH, Trammell SA, Susumu K, Delehanty JB, Mei BC, Melinger JS, Blanco-Canosa JB, Dawson PE and Mattoussi H, *Nature materials*, 2010, 9, 676. [PubMed: 20651808]
20. He C, Lu K and Lin W, *Journal of the American Chemical Society*, 2014, 136, 12253–12256. [PubMed: 25136764]
21. James-Kracke MR, *Journal of cellular physiology*, 1992, 151, 596–603. [PubMed: 1295907]
22. Buckler K and Vaughan-Jones R, *Pflügers Archiv European Journal of Physiology*, 1990, 417, 234–239. [PubMed: 2084617]
23. Kurtz I and Balaban RS, *Biophysical journal*, 1985, 48, 499–508. [PubMed: 4041540]
24. Willoughby D, Thomas RC and Schwiening CJ, *Pflügers Archiv European Journal of Physiology*, 1998, 436, 615–622. [PubMed: 9683736]
25. Bao Y, De Keersmaecker H, Corneillie S, Yu F, Mizuno H, Zhang G, Hofkens J, Mendrek B, Kowalczyk A and Smet M, *Chemistry of Materials*, 2015, 27, 3450–3455.
26. Du F, Ming Y, Zeng F, Yu C and Wu S, *Nanotechnology*, 2013, 24, 365101. [PubMed: 23942146]
27. Näreoja T, Deguchi T, Christ S, Peltomaa R, Prabhakar N, Fazeli E, Perälä N, Rosenholm JM, Arppe R and Soukka T, *Analytical chemistry*, 2017, 89, 1501–1508. [PubMed: 27977142]
28. Dowd A, Pissuwan D and Cortie MB, *Trends in biotechnology*, 2014, 32, 571–577. [PubMed: 25277768]
29. Korzeniowska B, Woolley R, DeCoursey J, Wencel D, Loscher C and McDonagh C, *Journal of biomedical nanotechnology*, 2014, 10, 1336–1345. [PubMed: 24804554]
30. Ding C and Tian Y, *Biosensors and Bioelectronics*, 2015, 65, 183–190. [PubMed: 25461156]
31. Han X, Wang H, Ou X and Zhang X, *ACS applied materials & interfaces*, 2013, 5, 5811–5814. [PubMed: 23725318]
32. Orte A, Alvarez-Pez JM and Ruedas-Rama MJ, *ACS nano*, 2013, 7, 6387–6395. [PubMed: 23808971]
33. Chu B, Song B, Ji X, Su Y, Wang H and He Y, *Analytical Chemistry*, 2017.
34. Verma A, Uzun O, Hu Y, Han H-S, Watson N, Chen S, Irvine DJ and Stellacci F, *Nature materials*, 2008, 7, 588–595. [PubMed: 18500347]
35. Wang X.-d., Stolwijk JA, Lang T, Sperber M, Meier RJ, Wegener J and Wolfbeis OS, *Journal of the American Chemical Society*, 2012, 134, 17011–17014. [PubMed: 23017056]

36. Alford R, Simpson HM, Duberman J, Hill GC, Ogawa M, Regino C, Kobayashi H and Choyke PL, Molecular imaging, 2009, 8, 7290.2009. 00031.
37. Hamada S and Fujita S, Histochemistry and Cell Biology, 1983, 79, 219–226.
38. Lan X, Cheng B, Yang Q, Huang J, Wang H, Ma Y, Shi H and Xiao H, Sensors and Actuators B: Chemical, 2014, 193, 95–99.
39. Yang Q, Wang H, Lan X, Cheng B, Chen S, Shi H, Xiao H and Ma Y, Sensors and Actuators B: Chemical, 2015, 207, 571–580.
40. Yang Q, Wang H, Chen S, Lan X, Xiao H, Shi H and Ma Y, Analytical chemistry, 2015, 87, 7171–7179. [PubMed: 26118725]
41. Chen S, Yang Q, Xiao H, Shi H and Ma Y, Sensors and Actuators B: Chemical, 2017, 241, 398–405.
42. Yang Q, Zhang X, Song Y, Li K, Shi H, Xiao H and Ma Y, Medical Devices & Sensors, e10079.
43. Lewinski N, Colvin V and Drezek R, small, 2008, 4, 26–49. [PubMed: 18165959]
44. Lin W, Huang Y.-w., Zhou X-D and Ma Y, International journal of toxicology, 2006, 25, 451–457. [PubMed: 17132603]
45. Mathelié-Guinlet M, Béven L, Moroté F, Moynet D, Grauby-Heywang C, Gammoudi I, Delville M-H and Cohen-Bouhacina T, Advances in Colloid and Interface Science, 2017.
46. Sharma V, Anderson D and Dhawan A, Apoptosis, 2012, 17, 852–870. [PubMed: 22395444]
47. Lipovsky A, Nitzan Y, Gedanken A and Lubart R, Nanotechnology, 2011, 22, 105101. [PubMed: 21289395]
48. Yang Q and Ma Y, International journal of toxicology, 2014, 33, 187–203. [PubMed: 24700570]
49. Yang Q, Lin T-S, Burton C, Park S-H and Ma Y, Toxicology Research, 2016, 5, 482–491. [PubMed: 30090362]
50. Przybytkowski E, Behrendt M, Dubois D and Maysinger D, The FEBS journal, 2009, 276, 6204–6217. [PubMed: 19780838]
51. Petters C, Irrsack E, Koch M and Dringen R, Neurochemical research, 2014, 39, 1648–1660. [PubMed: 25011394]
52. Trouiller B, Reliene R, Westbrook A, Solaimani P and Schiestl RH, Cancer research, 2009, 69, 8784–8789. [PubMed: 19887611]
53. Shukla RK, Sharma V, Pandey AK, Singh S, Sultana S and Dhawan A, Toxicology in vitro, 2011, 25, 231–241. [PubMed: 21092754]
54. Mittal S, Sharma PK, Tiwari R, Rayavarapu RG, Shankar J, Chauhan LKS and Pandey AK, Particle and fibre toxicology, 2017, 14, 15. [PubMed: 28454554]
55. Lin W, Huang Y.-w., Zhou X-D and Ma Y, Toxicology and applied pharmacology, 2006, 217, 252–259. [PubMed: 17112558]
56. García-Saucedo C, Field JA, Otero-Gonzalez L and Sierra-Álvarez R, Journal of hazardous materials, 2011, 192, 1572–1579. [PubMed: 21782338]
57. Hoecke KV, Quik JT, Mankiewicz-Boczek J, Schamphelaere KAD, Elsaesser A, Meeren P. V. d., Barnes C, McKerr G, Howard CV and Meent DVD, Environmental science & technology, 2009, 43, 4537–4546. [PubMed: 19603674]
58. Mortensen NP, Hurst GB, Wang W, Foster CM, Nallathamby PD and Retterer ST, Nanoscale, 2013, 5, 6372–6380. [PubMed: 23736871]
59. Oberdörster G, Philosophical Transactions of the Royal Society of London A: Mathematical, Physical and Engineering Sciences, 2000, 358, 2719–2740.
60. Oberdörster G, Oberdörster E and Oberdörster J, Environmental health perspectives, 2005, 113, 823. [PubMed: 16002369]
61. Ramakrishna G and Ghosh HN, Langmuir, 2003, 19, 3006–3012.
62. Rupprecht C, Wingen M, Potzkei J, Gensch T, Jaeger K-E and Drepper T, Journal of Biotechnology, 2017.
63. Chen S, Hong Y, Liu Y, Liu J, Leung CW, Li M, Kwok RT, Zhao E, Lam JW and Yu Y, Journal of the American Chemical Society, 2013, 135, 4926–4929. [PubMed: 23506236]

64. Chang S, Hamajima H, Amin I, Yanagita T and Baharuldin M, International Food Research Journal, 2014, 21.
65. Elgqvist J, Timmermand OV, Larsson E and Strand S-E, Anticancer research, 2016, 36, 103–109. [PubMed: 26722033]
66. Kalyanaraman B, Darley-USmar V, Davies KJ, Dennery PA, Forman HJ, Grisham MB, Mann GE, Moore K, Roberts LJ and Ischiropoulos H, Free Radical Biology and Medicine, 2012, 52, 1–6. [PubMed: 22027063]
67. Dan Y, Ma X, Zhang W, Liu K, Stephan C and Shi H, Analytical and bioanalytical chemistry, 2016, 408, 5157–5167. [PubMed: 27129977]
68. Zhang W, Dan Y, Shi H and Ma X, Journal of Environmental Chemical Engineering, 2017, 5, 572–577.
69. Hendzel MJ, Wei Y, Mancini MA, Van Hooser A, Ranalli T, Brinkley B, Bazett-Jones DP and Allis CD, Chromosoma, 1997, 106, 348–360. [PubMed: 9362543]
70. Alarifi S, Ali D, Al Omar Suliman Y, Ahamed M, Siddiqui MA and Al-Khedhairy AA, International journal of nanomedicine, 2013, 8, 189. [PubMed: 23326189]
71. Cedervall T, Lynch I, Lindman S, Berggård T, Thulin E, Nilsson H, Dawson KA and Linse S, Proceedings of the National Academy of Sciences, 2007, 104, 2050–2055.
72. Ji Z, Jin X, George S, Xia T, Meng H, Wang X, Suarez E, Zhang H, Hoek EM and Godwin H, Environmental science & technology, 2010, 44, 7309–7314. [PubMed: 20536146]
73. Lesniak A, Fenaroli F, Monopoli MP, Åberg C, Dawson KA and Salvati A, ACS nano, 2012, 6, 5845–5857. [PubMed: 22721453]
74. Lee Y-G, Jeong J, Raftis J and Cho W-S, Journal of Toxicology and Environmental Health, Part A, 2015, 78, 185–195. [PubMed: 25506634]
75. Marks DC, Belov L, Davey MW, Davey RA and Kidman AD, Leukemia research, 1992, 16, 1165–1173. [PubMed: 1361210]
76. Barltrop JA, Owen TC, Cory AH and Cory JG, Bioorganic & Medicinal Chemistry Letters, 1991, 1, 611–614.
77. Aranda A, Sequedo L, Tolosa L, Quintas G, Burello E, Castell J and Gombau L, Toxicology in Vitro, 2013, 27, 954–963. [PubMed: 23357416]
78. Dugan LL, Lin T-S, He Y, Hsu CY and Choi DW, Free radical research, 1995, 23, 27–32. [PubMed: 7647917]
79. Matuszak Z, Reszka KJ and Chignell CF, Free Radical Biology and Medicine, 1997, 23, 367–372. [PubMed: 9214572]
80. Srinivas A, Rao PJ, Selvam G, Murthy PB and Reddy PN, Toxicology letters, 2011, 205, 105–115. [PubMed: 21624445]
81. Alaraby M, Hernández A, Annangi B, Demir E, Bach J, Rubio L, Creus A and Marcos R, Nanotoxicology, 2015, 9, 749–759. [PubMed: 25358738]
82. García-Saucedo C, Field JA, Otero-Gonzalez L and Sierra-Álvarez R, Journal of hazardous materials, 2011, 192, 1572–1579. [PubMed: 21782338]
83. Sendra M, Yeste P, Moreno-Garrido I, Gatica JM and Blasco J, Science of The Total Environment, 2017, 590, 304–315.
84. Eom H-J and Choi J, Environmental health and toxicology, 2011, 26.
85. Panas A, Marquardt C, Nalcaci O, Bockhorn H, Baumann W, Paur H-R, Mülhopt S, Diabaté S and Weiss C, Nanotoxicology, 2012, 7, 259–273. [PubMed: 22276741]
86. Swain B, Park JR, Park K-S and Lee CG, Materials Science and Engineering: C, 2019, 95, 95–103. [PubMed: 30573275]
87. Popov AP, Priezzhev AV, Lademann J and Myllylä R, 2008.
88. Manzano M and Vallet-Regí M, Advanced Functional Materials, 2019, 1902634.
89. Pellen-Mussi P, Tricot-Doleux S, Neaime C, Nerambourg N, Cabello-Hurtado F, Cordier S, Grasset F and Jeanne S, Journal of nanoscience and nanotechnology, 2018, 18, 3148–3157. [PubMed: 29442814]

Figure 1. pH probing standard curve establishment and single-cell probing. (a) Linear correlation between standardized buffer pHs and the signal peak-area that covers a wavelength that ranged from 495 nm to 640 nm. The inset plot shows one of the original fluorescence spectra and its covered area that used for peak area calculation. (b) A schematic view of how to conduct single-cell probing via assistance from a cell holding probe. An exemplary process of a single A549 cell capturing and intracellular probing under (c) bright-field, and (d) merged DAPI and FITC fluorescence channels. (e) Zoom-in view of the probe tip lightening spot and correlated geometry measurements.

Figure 2.

The 48 hour-exposure results of cell viability, ROS generation, and LSCM imaging of the CeO₂, TiO₂, and SiO₂ NPs treated groups in comparison with control. (a - c) cell viability results of the three NPs treated cell groups. (d - f), Intracellular ROS generation results of the three NPs treated cell groups. All graphs from (a) to (f) were plotted as the percentage of control after normalization. Experiments were repeated three times, and results are presented as the average $\pm$ standard deviation (error bar). (g) LSCM imaging result of NP-treated cells in 48 hours, followed by analysis by ImageJ software as detailed in the Method section. Each data in the (a) bar graph represent averaged values out of at least 30 single-cell measurements using the CTFI method (see Experimental Section). Error bars represent the standard deviations.

Figure 3.

FACS flow cytometry on control and three types of NPs (TiO_2 , CeO_2 and SiO_2) co-cultured cells for the first 12 hours. (a, d, g and j) Fluorescence ratio of data set from two channels (PI vs. FITC). Cells were separated according to polarized (red) or depolarized (green) mitochondrial inner membrane potential. All cells have been pre-screened using the P-I gating as designated of “intact cells”. All data has been normalized by control. (b, e, h and k) Percentage of four separate cell parts sorted on the flow cytometry, namely: P-I, intact cells (black), P-II, cell debris (blue), P-III, agglomerated NPs (purple), and P-IV, untracked cell parts (gray) (Fig. S4). (c - l) Consecutive 12-hour FACS records of four sample groups cell behavior on flow cytometry. Plots were established based on SSC (side scattering) vs. FSC (Forward scattering). Error bars in bar graphs represent standard deviations out of three separate experiment results. Each data point in the SSC-FSC plots represents an experimentally single-cell event.

Figure 4.

LSCM imaging analysis for the first 12 hours for JC-1 and DAPI stained cells, respectively (a and b), and pH_i measurements results on TiO₂, CeO₂, and SiO₂ NPs co-cultured single cells for the first 12 hours (c - f). Both (a) and (b) are derived from averaged values out of at least 30 single-cell measurements using the CTFI method. Error bars represent the standard deviations. Each red line spots in (c - f) represent averaged values out of at least 11 single cells probing with corresponding error bars that were also derived from standard deviation. Yellow polygons in each plot stand for a data coverage of at least three separately measured single-cell events (one-time probed cell).

Figure 5.

TEM cell images of cells after CeO_2 , TiO_2 and SiO_2 NP-administration for 0, 3, 6, and 12 hours. The small picture on the upper left shows one or two representative cells of the group. The large picture on the lower right is an enlarged view of the area marked by the yellow box in the small picture. Cell parts were stained in light gray, except that the nuclei were slightly darker. All NPs were stained deeper than the cell parts. Abbreviation: CN = cell nucleus, CP = cytoplasm; CM = cell membrane. Image scales can be found at the bottom-left corner of each zoom-in figure.

Figure 6.

Zoom-in TEM images of a few NP-agglomerates of (a, d) CeO_2 , (b, e) TiO_2 and (c, f) SiO_2 that interacting with cells after 3 hours of NP-administration. (d) Extended image of the yellow box in (a). Sphere-like CeO_2 NP agglomerates (yellow arrows) with smooth outside surface were observed without creating intracellular cavity; (e) Extended image of the blue box in (b). TiO_2 NPs aggregated into smaller agglomerates with rougher edges, creating several large intracellular cavities (blue arrows); (f) Extended image of the red box in (c). SiO_2 NPs barely aggregated but spread through the whole intracellular space, creating many small cavities (red arrows). Green arrows in (c) indicated intracellular organelles or dissociated nucleus components. CN = cell nucleus, CP = cytoplasm; CM = cell membrane. Scale bar: ac, 2 μm ; d-f, 200 nm.

Table 1

NPs' ζ -potential measurement under different NP concentrations in a pH buffered (pH = 7.4) serum-free F-12K cell culture medium (details see Experimental Section).

NPs	ζ -potential (mV)		
	5 $\mu\text{g/mL}$	20 $\mu\text{g/mL}$	100 $\mu\text{g/mL}$
CeO ₂	−18.4	−14.6	−9.5
TiO ₂	−19.5	−16.9	−12.7
SiO ₂	−25.1	−29.0	−25.8