In situ probing of intracellular pH by fluorescence from inorganic nanoparticles

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Intracellular pH (pHi) plays a critical role in the physiological processes of cells. Nanoscale sensors based on pH-sensitive fluorescent proteins attached on nanoparticles (NPs) have been designed but inorganic NP-dependent fluorescent nanosensors have not yet been explored. Herein we describe a pH sensitive inorganic semiconductor fluorescent probe based on ultrathin 3C–SiC NPs which can effectively monitor pH in the range of 5.6–7.4 by taking advantage of the linear dependence between the fluorescence intensity ratio of the surface OH− and H+ bonding states to band-to-band recombination and pH. Detection of pHi is demonstrated in living HeLa cells. In particular, pH measurements during apoptosis confirm the validity and sensitivity of this technique in monitoring real-time changes in the intracellular environment. Toxicity assessment and confocal laser scanning microscopy indicate that the 3C–SiC NPs have low cytotoxicity and are compatible with living cells.

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1. Introduction

Owing to the large surface/volume ratio and reduced dimensionality, nanomaterials offer better sensitivity than conventional micro-scale or bulk materials and are excellent biological sensors by exploiting their electrical [1–3] and/or optical properties [4–9]. Optical signals that can be detected without direct contact is sensitive, convenient, and noninvasive and impart important intracellular information. Because nearly all intracellular processes function in a narrow pH range, intracellular pH (pHi) plays an important modulating role in many cellular events [10,11] and the structural stability and functions of proteins are strongly affected by the pHi. In addition, cell cycle progression and cell death have been linked to changes in pHi [12,13] and so precise monitoring of pH fluctuations in organelles of living cells is vital to the understanding of cellular processes. In this respect, several nanoparticle (NP)-based pH-sensitive systems utilizing surface ligands and antibodies attached to the NP surface to target over-expressed receptors have been reported [14–20]. However, these pHi measurements require the interactions between organic dyes and H+ and OH− groups which cause fluorescence changes in the dye molecules. The role of the NPs in these sensors is just a scaffold to form the NP/dye composite and the NPs do not take part in the fluorescent process and pH measurement. Very recently, uncoated InGaN-GaN fluorescent nanowires were reported to exhibit good response to pH variations [21], thus indicating the potential of inorganic nanostructured materials in direct probing of biochemical processes. Therefore, it is of both scientific and technical importance to design pHi nanosensors without dyes and having the right dimensions. One possibility is to utilize the optical signals from pure 3C–SiC NPs with diameters of several nanometers.

As a versatile inorganic semiconductor, silicon carbide (SiC) has many desirable features such as high thermal conductivity, melting point, breakdown voltage, and electron saturation velocity. It is also light in weight, strong, hard, wear and corrosion resistant, as well as inert [22]. SiC-coated stents can minimize thrombosis and the restenosis rate after coronary angioplasty [23] and SiC has little interactions with biological enzymes and fluids [22,24,25], thus making it suitable for direct intracellular signal monitoring. The combined use of confocal laser scanning microscopy and flow cytometry to detect optical fluorescence has been proposed [26,27] and our recent work has shown that 3C–SiC NPs in a water suspension produce a green fluorescent band in addition to the blue band-to-band recombination one [28]. The intensity of the green band changes with pH because water molecules only react with a Si-terminated surface at room temperature, and the additional green fluorescent band arises from surface structures induced by
H⁺ and OH⁻ dissociated from water and attached to Si dimers on the modified (001) Si-terminated portion of the NPs. The PL lifetime is several nanoseconds as revealed by photoluminescence (PL) decay measurements [25] and the fluorescent quantum yield is 17% which is larger than those of traditional silicon quantum dots and comparable to some In-Vi group semiconductor quantum dots as well as dye molecules [25]. In this paper, an inorganic semiconductor fluorescent probe made of 3C–SiC NPs is described and applied to pH determination in situ.

2. Materials and methods

2.1. Preparation of 3C–SiC NPs

3C–SiC NPs were synthesized by chemical etching of micro-scale 3C–SiC grains [25,29,30]. Six grams of micro-scale 3C–SiC powders (purchased from Alfa Aesar) were added to a solution containing 65% nitric acid (HNO₃) (15 mL) and 40% hydrolfluoric acid (45 mL). The powders were etched at 100 °C for 1 h and afterwards, the solution was cooled and centrifuged at 8000 rpm for 5 min to remove the excess acid. The powders were washed with de-ionized water and dried at 70 °C for several hours. The Roswell Park Memorial Institute (RPMI) medium 1640 free of phenol red (purchased from Gibco-BRL Life Technologies, Inc.) was added and agitated ultrasonically for about 30 min. The ultrasonic frequency and power were 40 kHz and 150 W, respectively. The medium with the suspended 3C–SiC NPs was placed still for several hours resulting in a suspension containing the ultrathin 3C–SiC NPs.

2.2. Cell cultures

HeLa cells were purchased from the Chinese Academy of Sciences cell library. Prior to use, the cells were cultured in the NP-free medium for two weeks, followed by further culturing in RPMI 1640 with the suspended 3C–SiC NPs and 10% (v/v) fetal bovine serum at 37 °C in a humidified air with 5% CO₂ for 24 h. Before the analysis, the cells were put into a 15 mL centrifuge tube and centrifuged at 1000 rpm for 5 min. The supernatant medium was removed and the new 3C–SiC NP-free medium was added.

2.3. Fluorescent analysis for pH measurement

The RPMI medium 1640 containing 3C–SiC NPs with different pH values were obtained by adding NaOH or HCl and the added NaOH or HCl only accounted for 1/10,000 of the medium volume so that the concentration in the media was essentially the same. The fluorescent spectra were acquired on an FL9200 spectrometer at an excitation wavelength of 360 nm at room temperature. An asymmetrical fluorescent peak was obtained and Gaussian deconvolution produced two subpeaks at 442 nm (2.80 eV in the F1 spectrum) and 505 nm (2.46 eV in the F2 spectrum) according to the density functional theory (DFT) calculation. The ratio of the fluorescent intensities of the two subpeaks varied linearly with the pH value.

2.4. pH measurement by 3C–SiC NPs

To measure the pH of HeLa cells by 3C–SiC NPs, calibration curves were obtained. The HeLa cells were incubated in the RPMI medium 1640 with K⁺–rich buffers with pH values of 5.9, 6.2, 6.5, 6.8, 7.1, and 7.4 respectively in the presence of the H⁺/K⁺ ionophore nigericin to change the pH in concordance with the medium pH values. By deconvoluting the fluorescent spectra using Gaussian fittings, the intensity ratio I₂/I₁ of the F2 to F1 peaks was obtained and a calibration curve was obtained by linearly fitting the dependence of the intensity ratio on pH. The cells labeled with 3C–SiC NPs and suspended in the serum-free RPMI 1640 without the H⁺/K⁺ ionophore nigericin were analyzed at an excitation wavelength of 360 nm and the corresponding pH values of the cells were obtained.

2.5. pH measurement by 2,7'-bis-(2-carboxyethyl)-5-(and 6)-carboxy-fluorescein (BCECF)

K⁺–rich buffers containing KCl (90 mmol/L), NaCl (5 mmol/L), MgSO₄ (1 mmol/L), CaCl₂ (1 mmol/L), and N-2-Hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES; purchased from Sinopharm Chemical Reagent Co., Ltd.) (10 mmol/L) were prepared. These K⁺–rich buffers were divided into 6 parts with pH values of 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, respectively, by adding NaOH or HCl. Nigericin (purchased from Sigma–Aldrich Company) and BCECF (purchased from Sigma–Aldrich Company) were added to these buffers at concentrations of 30 and 0.2 μmol/L, respectively. The cells were washed with phosphate-buffered saline (PBS) three times and incubated in K⁺–rich buffers at a predetermined pH at 37 °C for 20 min. The BCECF fluorescence intensity was measured on a spectrofluorometer (Photon Technology, NJ) with excitation wavelengths of 505/439 nm and emission wavelength of 535 nm. The intensity ratio of the BCECF fluorescence at 535 nm under the 505/439 nm excitation is a function of pH. The cell suspensions in serum-free RPMI 1640 without K⁺–rich buffers were labeled with BCECF and incubated at 37 °C for 1 h. After washing with serum-free RPMI 1640 three times, the labeled cells were analyzed at excitation wavelengths of 505 and 439 nm, and the pH of the cells were obtained according to the intensity ratio of the fluorescence peaks.

2.6. Confocal microscopy

The cells (2 × 10⁵ cells/well) used in confocal microscope imaging were cultured in 6-well plates containing a cover glass for 24 h at 37 °C. The cells were rinsed with PBS three times and then washed with the fresh media. The cells were fixed with 4% paraformaldehyde for 30 min and washed repeatedly with PBS to remove the paraformaldehyde. Afterwards, the cells were washed and mounted with the anti fade mounting medium and examined by confocal laser scanning microscopy (LSM510 Axiovert Zeiss) using a 60 oil immersion objective.

2.7. Cell viability analysis

The cytotoxicity of 3C–SiC NPs was evaluated using the CCK-8 (DojinDo Laboratories, Kyushu, Japan). In brief, 5 × 10⁴ of HeLa cells per well were plated on a 96-well plate and incubated at 37 °C for 18 h. The cells were treated with the indicated concentrations of 3C–SiC NPs and incubated at 37 °C for additional 24 h. 10 μL of the CCK-8 solution was added to each well and the cells were incubated for 3 h. The absorbance was measured on a microplate reader (Synergy HT, Bio-Tek) using 450/650 nm as the reference wavelength. The average optical density in the control cells (which incubated without 3C–SiC NPs) was taken as 100% viability, and the results after the treatments with different concentrations of 3C–SiC NPs were expressed as a percentage of the control. The HeLa cell viability before and after UV irradiation was evaluated by the Annexin V/Propidium Iodide (PI) staining. Briefly, the cells were cultured for 24 h with 3C–SiC NPs at 37 °C. The cells were washed in cold PBS and incubated with Annexin V in binding buffer. PI was added prior to analysis on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and the data were analyzed by WinMDI (Scripps Institute, La Jolla, CA).

2.8. TEM imaging of endocytosis of 3C–SiC NPs into HeLa cells

The cells with NPs were trypsinized, centrifuged, and washed with PBS three times. The cell pellet was fixed with glutaraldehyde and formaldehyde. After 24 h, the pellet was washed with PBS to remove the fixative and dehydrated in a series of alcohol, embedded in Epon, and sliced to a thickness of 100 nm. TEM images were acquired on the HD 7000 transmission electron microscope.

3. Result and discussion

3.1. Characterization of 3C–SiC NPs

The transmission electron microscopy (TEM, JEOL JEM-4000EX at 200 kV) images reveal that the NPs are crystalline and have nearly a spherical shape with diameters between 2 and 7 nm. The histogram of the size distribution shows that the most probable NP diameter is 3.8 nm (Fig. 1), which is in good agreement with the results obtained by dynamic light scattering. It should be noted that the size of the NPs can be reduced to 3.0 nm by further chemical etching and ultrasonic treatment and higher light emission efficiency can be achieved [30,31]. As the NPs are designed for pHi measurement, they are pure 3C–SiC NPs without surface modification and the fluorescent quantum yield and excited state lifetime are also consistent with those reported previously [25].

3.2. Fluorescent spectra of 3C–SiC NPs in RPMI at different pH values

The pH value of the cell medium with 3C–SiC NPs is carefully changed by adding NaOH or HCl (see Materials and methods Section). Hepe is contained in RPMI 1640 medium to ensure pH stability during the measurements. Fluorescence from the medium excited by the 360 nm line of a Xe lamp is measured at a series of pH values. The fluorescent spectra are similar to those acquired from water suspensions of 3C–SiC NPs [28]. Three typical fluorescent spectra at pH values of 5.6, 6.4, and 7.4 are depicted in Fig. 2a. Each spectrum consists of a principal blue peak and green shoulder (in web version). According to the origin of the composite fluorescent peak derived theoretically (Supplementary Figs. S1 and S2), we
consider OH⁻ and H⁺ adsorption on the NP surface. Using DFT calculation, the asymmetrical fluorescent peak is divided into two subpeaks at 442 nm (2.80 eV in the F₁ spectrum) and 505 nm (2.46 eV in the F₂ spectrum). The 505 nm fluorescence originates mainly from surface OH⁻ and H⁺ bonding. Since the amounts and distribution of OH⁻ and H⁺ bonds vary on the surface of the NPs with a large size distribution, the linewidth of the 505 nm fluorescent peak is broader than that of the band-to-band recombination peak at 442 nm. Our experiments reveal that the amounts of H⁺ and OH⁻ on the Si dimers of the NPs are closely related to the pH of the medium, consequently leading to significant changes in the relative intensity (I₂/I₁) of the 505 nm to the 442 nm peaks. Accordingly, a series of fluorescent spectra is acquired from the media with different pH values and the dependence of the intensity ratio (I₂/I₁) of the F₂ to F₁ spectra on the pH of the cell medium is plotted and displayed in Fig. 2b. The I₂/I₁ versus pH relationship is linear with an error of less than 7% in the pH range between 5.6 and 7.4 and hence, this technique is expected to be suitable for pH monitoring in a weakly acidic or alkaline environment. Our recent investigation reveal that when the size of the NPs is reduced to
about 3.0 nm, the higher light emission efficiency increases the $F_1$ intensity (band-to-band recombination efficiency) significantly [30,31] and therefore, the sensitivity can be further increased to 4.5%, which is close to or even better than that observed from small molecular and polymeric probes.

When the medium is too acidic ($pH < 5.5$), the lack of OH$^-$ on the Si dimers of the NPs leads to a smaller peak at 505 nm, thereby leaving the spectral shape unchanged albeit changes in the $pH$ (Fig. 2c). This causes the intensity ratio to deviate from the linear relationship (Fig. 2b). In contrast, if the medium environment is too alkaline ($pH > 7.5$), the lack of attached H$^+$ on the Si dimers shifts the spectrum towards the low energy side due to the modified optical band gap of the 3C-SiC NPs. As a result, the blue and green peaks will not remain at 442 and 505 nm (Fig. 2d) and consequently, we cannot obtain a reasonable intensity ratio $I_{52}/I_{51}$. Hence, no data points are plotted in Fig. 2b when the pH is more than 7.5.

### 3.3. Fluorescent spectra of 3C–SiC NPs in Hela cells

To show the practicality and potential of 3C–SiC NPs as an in situ pH probe, the $pH_i$ values of Hela cells are determined experimentally in vitro. Fig. 3a shows a confocal microscopy image of the HeLa cells with 3C–SiC NPs excited by the 360 nm line of a Xe lamp. The bright green-blue spots represent fluorescence from 3C–SiC NPs and no fluorescence is observed from the cell medium. Fig. 3b depicts an overlapped confocal microscopy image of the cells excited by the 360 nm line and the bright field. 3C–SiC NPs are distributed...
in the cells and no NPs are outside the cells as suggested by lack of the fluorescence. These image and cytotoxicity tests shown in Fig. 3c show that the 3C–SiC NPs have no cytotoxicity to HeLa cells under our experimental conditions [25]. To further identify the subcellular localization of the 3C–SiC NPs, high-resolution TEM (HR-TEM) image of the HeLa cells after culturing with 3C–SiC NPs for 16 h is depicted in Fig. 4c. The NPs mainly cluster at 50–70 nm which is consistent with previous results [32]. Although some big NPs larger than 100 nm can be seen from Fig. 3b, the number of large NPs is quite small compared to the smaller ones distributed uniformly in the cells. Since the nuclei and phagosomes of the cells are dark in the confocal microscopy images of Fig. 3b. The clusters are mainly distributed in the cytosol of cells but not in the nuclei or phagosomes thereby confirming the practicality of 3C–SiC NPs as a pH probe. In addition, HR-TEM images of the HeLa cells incubated with NPs for a shorter duration (Fig. 4a and b) show that the NP clusters enter the cell via the clathrin-mediated endocytosis pathway [32]. Hence, to measure the real pH value of cells, a long culturing time is necessary as adopted in our experiments (>16 h).

As the cell interior is a complex environment, autofluorescence is difficult to avoid. In intracellular sensing, blue and green emission may have different background contributions. In order to avoid their influence on the accuracy of quantification, a new calibration curve for 3C–SiC NPs in the measurement of pH in HeLa cells is obtained by linearly fitting the dependence of the intensity ratio on pH (Fig. 3d, see Materials and methods Section). Fig. 3e shows the fluorescent spectra acquired from the 3C–SiC NPs endocytosed in normal HeLa cells without H⁺/K⁺ ionophore nigericin. A strong fluorescence signal can be observed in the range of 380–600 nm (note that the weak signal in the range of 600 and 700 nm arises from autofluorescence of the cells). By dividing the fluorescent spectrum, the pH value of normal HeLa cells is determined to be 7.16 (labeled by ★ in Fig. 3d).

3.4. Parallel pH measurements using BCECF in HeLa cells

In order to show the effectiveness of our method, parallel experiments by using BCECF are also performed [33]. The calibration curve is shown in Fig. 5f and the pH value of normal HeLa cells is 7.21. The values obtained by the two methods are very close. Hence, the influence of autofluorescence is within the experimental error of the pH measurements and this technique is demonstrated to be suitable for pH detection.

3.5. Real-time pH measurement during apoptosis of HeLa cells

Our previous work has shown that ultraviolet irradiation has no influence on the structure and fluorescent properties of solid 3C–SiC NPs [28,30]. To detect the pH changes in cells caused by external stimulations, real-time pH measurements are carried out during the process of apoptosis arising from ultraviolet irradiation. HeLa cells are used to endocytose 3C–SiC NPs and similar experimental steps are adopted. The fluorescent spectra are obtained at intervals of 5 min. The relationship between the pH and irradiation in Fig. 5a reveals that as the ultraviolet irradiation time increases, apoptosis occurs gradually and is accompanied by changes in the

Fig. 4. TEM images of different stages of the cellular uptake process. a) TEM image of HeLa cells incubated with 3C–SiC NPs for 4 h. Some of the NPs are attached at the surface of cells while some have internalized into the cell. b) TEM image of HeLa cells incubated with 3C–SiC NPs for 8 h. More NPs are enclosed in the phagosomes but some NPs have entered the cytosol of cell. c) TEM image of HeLa cells incubated with 3C–SiC NPs for 16 h. Most of the NPs have entered the cytosol of cell and none are in the nuclei or phagosomes.
pH toward lower (more acidic) values [34]. In the initial stage (region B), almost all the cells are alive but after irradiation for 75 min (region A), more than 90% of the cells are dead when the pH becomes acidic at 6.33. The inset in Fig. 5a shows the percentages of living cells in regions B and A assessed from the data of the cell viabilities with Annexin V/PI apoptosis detection kit by flow cytometry in Fig. 5c and d, indicating that the cells indeed undergo apoptosis under ultraviolet irradiation. Since the measurement duration of each fluorescent spectrum is only 30 s, which is far shorter than the duration of the initial stage (region B), the influence of excitation light on the cells is negligible. Moreover, the process of apoptosis is measured by using BCECF under the same conditions and the corresponding results are shown in Fig. 5b. Obviously, the values measured by the two methods are very close, demonstrating that the 3C–SiC NPs has good real-time fluorescent sensitivity to pH.

4. Conclusion

Compared to traditional organic probes and NP-based nano-sensing platforms, the use of this more robust and non-toxic inorganic NP fluorescent probe to determine pH is experimentally simpler and cheaper. This is the first pure optical semiconductor nanosensor specifically designed for pH measurements without requiring pH-sensitive fluorescent proteins to attach onto the NP surface. The small size and effectiveness of this semiconductor nanosensor enable sensitive, real-time detection of intracellular signals in the intracellular environment. It can be applied to cellular detection, disease prevention, in vivo diagnostics, as well as multifunctional drug delivery systems.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2013.08.023.

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