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荧光过氧化氢酶纳米传感器的制备及性能

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Ratiometric Fluorescent Enzymatic Nanosensors for Intracellular Hydrogen Peroxide

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Abstract: Hydrogen peroxide(H_2O_2) is the main marker of reactive oxygen species and closely related to various diseases such as neurodegenerative diseases. In this work, we presented an enzymatic nanosensor for fluorescence sensing of intracellular hydrogen peroxide. The nanosensor consisted of a biocompatible shell of poly-l-lysine and horseradish peroxidase(HRP), and an oxygen sensing core from a porous polymeric matrix containing oxygen probes. H_2O_2 was catalyzed by HRP to yield the product of oxygen, which was reported by the sensing core in the form of quenched fluorescence. The enzymatic nanosensors had a hydrodynamic size of about 270 nm, a zeta potential of −18 mV and good biocompatibility. Their fluorescence were highly sensitive to H_2O_2 in both ratiometric and the time-resolved fluorescence. Moreover, the nanosensors can be efficiently internalized by live cells, thereby intracellular H_2O_2 was sensitively detected with TRF modality. These results suggest that our enzymatic nanosensors may be used for monitoring H_2O_2-related cellular events, such as oxidative stress.

Key words: H_2O_2; nanosensor; time-resolved fluorescence(TRF); ratiometric fluorescence; oxidative stress
CLC number: TP212 **Document code**: A **DOI**: 10.37188/fgxb20204107.0826

荧光过氧化氢酶纳米传感器的制备及性能

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摘要: 过氧化氢(H_2O_2)是活性氧类的主要标志物,它与多种疾病如神经退行性疾病密切相关。本文设计了一种检测细胞内过氧化氢的荧光过氧化氢酶纳米传感器。这种纳米传感器由含多聚赖氨酸、辣根过氧化物酶的生物相容性外壳和含有氧探针的多孔聚合物基质的氧传感核组成。辣根过氧化物酶(HRP)催化 H_2O_2 生成氧,进而通过荧光氧气探针进行探测。该酶纳米传感器的流体动力学尺寸约为 270 nm,zeta 电位为 −18 mV,具有良好的生物相容性。它的荧光比率和时间分辨荧光均对 H_2O_2 高度敏感。此外,该纳米传感器可以被活细胞有效地摄取,从而可以用 TRF 方式灵敏地检测细胞内的 H_2O_2 浓度。结果表明,本实验制备的酶纳米传感器有望进一步用于监测 H_2O_2 相关的细胞生化反应,如氧化应激。

关 键 词: 过氧化氢; 纳米传感器; 时间分辨荧光; 比率荧光; 氧化应激

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1 Introduction

Hydrogen peroxide (H_2O_2) is the signaling[1-3] and homeostasis molecules[4-5] to maintain the fundamental biochemical processes in living organism. Moreover, H_2O_2 is the most important molecular and marker of reactive oxygen species (ROS)[6-8]. The overbalancing intracellular ROS concentration, known as oxidative stress, is mainly pernicious to DNA[9], lipids and proteins[4-5], which leads to many diseases, such as cancer[6], cardiovascular diseases[7] and neurodegenerative diseases[8]. Therefore, sensitive detection of intracellular H_2O_2 is helpful not only to understand fundamental pathological processes but also to diagnose related diseases.

Up to now, various approaches have been developed to detect H_2O_2 including titration[10], chromotography[11] and electrochemical sensors[12]. In terms of *in vitro* and *in vivo* sensing, optical detection methods are widely used owing to the merits of sensitivity, noninvasiveness and accuracy[13]. Generally there are two strategies utilized to sense H_2O_2, *i. e.* chemical reaction stimulated chemiluminescence[14-16] and fluorescent probe based photoluminescence[17]. In particular, fluorescence modality are more popular and a plenty of fluorescent probes have been constructed for intracellular H_2O_2, such as lipophilic cationic molecular probes[18], genetic encoded fluorescent proteins[19], cobalt/carbon nanotube hybrid nanocomplex[20], and graphitic carbon nitride and silver oxide nanocomposite[21]. Since most fluorescent probes need to react with H_2O_2 to change their emissions, their selectivity remains questionable as other ROS also are highly reactive. It is known that horseradish peroxidase (HRP) specifically converts H_2O_2 into oxygen. Hence detection of oxygen can real-time report the concentration of H_2O_2 indirectly. Previously, we have reported a series of fluorescent oxygen nanosensors[22]. Thus it is occurred to us that the combination of HRP and oxygen nanosensors may result in a highly sensitive enzymatic H_2O_2 nanosenros.

In this work, HRP modified poly-l-lysine (HRP-PLL) was firstly synthesized, assisted by which H_2O_2 nanosenros were then facilely prepared *via* a modified reprecipitation-encapsulation method[23]. Herein, Pt(Ⅱ)-meso-tetra(pentafluorophenyl)porphine (PtTFPP) and coumarin 6 (C6) were doped into the sensing particle core as oxygen probe and reference dye, respectively. As is expected, the as-prepared fluorescent nanosensors were highly sensitive to H_2O_2. Moreover, the nanosensor can detect H_2O_2 level with two robust approaches of ratiometric fluorescence and the time-resolved fluorescence (TRF).

2 Materials and Methods

2.1 Materials

Coumarin 6 (C6), Pt(Ⅱ)-meso-tetra(pentafluorophenyl) porphine (PtTFPP), 4-morpholinoethanesulfonic acid (MES), polystyrene (PS, M_w 280 ku), poly-l-lysine (PLL, M_w 30 – 70 ku), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and phosphate-buffered saline (PBS) of pH 7.4 were purchased from Sigma-Aldrich. 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and dimethylsulfoxide (DMSO) were purchased from MP biomedicals and Ameresco, respectively. Dodecyltrimethoxysilane (DTS) was obtained from Jiaxing Sicheng Chemicals Co. Ltd (Jiaxing, China). Tetrahydrofurane (THF) and mineral oil (pure) were obtained from J&K (Beijing, China). All reagents were used as received without further purification. Ultra pure water (UP) was used in all experiments. Standard cell culture 96-well plates were from Corning Inc., and black 96-well plates were from WHB (Shanghai, China). Fetal bovine serum (FBS) was obtained from Zhejiang Tianhang Bio-Technology Co. Ltd (Huzhou, China).

2.2 Modification of PLL with HRP (HRP-PLL)

Modification of PLL with HRP (HRP-PLL) was realized by the conjugation between the carboxylates of HRP and amines of PLL. Specifically, PLL was dissolved at a concentration of 10 mg · mL^{-1} in 0.1 mol · L^{-1} MES buffer. Then HRP was dissolved in 0.1 mol · L^{-1} MES buffer at a concentration of 10 mg · mL^{-1}. We added HRP solution to the PLL solution,

and kept the mixture at less 10-fold molar excess of HRP to the PLL. Then EDC was added into the above mixture to obtain at least a 10-fold molar excess of EDC to the HRP. After reacting overnight using rotary platform in a dark place, the samples were purified by centrifugal ultrafiltration to remove the unreacted molecules and the potential self-polymerization.

2.3 Synthesis of HRP Coated NPs(HRP-NPs)

HRP-NPs were prepared by the modified reprecipitation-encapsulation method assisted by HRP-PLL molecules. Briefly, C6, PtTFPP, PS and DTS were dissolved in THF at 2∶1∶48∶50 weight ratios. The total concentration was 200×10^{-6}. 500 μL of the above solution was rapidly added into 8 mL 60 μg·mL^{-1} PLL-HRP solution (pH 9, adjusted by ammonia) under ultrasound surrounding. The sample was stood for 2 h and then dialyzed against water for 24 h.

2.4 Cell Culture and Staining with HRP-NPs

Pheochromocytoma cells (PC12) were cultured in high glucose Dulbecco's Modified Eagle medium (H-DMEM) containing 10% fetal bovine serum (FBS) in CO_2 incubator containing 5% CO_2 at 37 ℃. The cells were then incubated in the medium dispersed with a 10 μg · mL^{-1} of HRP-NPs for 24 h. After that, the stained cells were washed twice with PBS to eliminate the outside nanosensors.

2.5 Characterization

The TEM images were obtained by Hitachi H-800 at 120 kV acceleration voltage. The NPs aqueous dispersion was placed on the TEM specimen support(cuprum). Zeta potentials and hydrodynamic sizes were determined by photon correlation spectroscopy and by dynamic light scattering (DLS) using a Zetasizer Nano instrument (Malvern Instruments, www.malvern.com). UV-Vis absorption was recorded on a UV-3101PC spectrophotometer (Shimadzu). Steadystate photoluminescent spectra were read by an F-4600 fluorescence spectrophotometer (Hitachi).

Time-resolved fluorescence(TRF) was recorded by a commercial plate reader(Victor ×4, Perkin-Elmer). Firstly, cells were incubated on microplates with HRP-NPs aqueous solution(10 μg · mL^{-1}) for 24 h(in separate wells). Then, the treated cells were washed once and measured in PBS using the plate reader. Rapid lifetime determination (RLD)[22] was performed by time-resolved fluorescence(TRF) model[24]. Specifically, two TRF intensities were recorded under delay times of 30 μs (t_1) and 70 μs (t_2), with a gating time of 100 μs (λ_{ex} = 340 nm, 642 nm emission filters). Afterwards, the two TRF intensity signals were converted into lifetime (τ) values through the following equation: $\tau = (t_1 - t_2)/\ln(F_1/F_2)$, where F_1 and F_2 correspond to the integrated signals over 100 μs obtained at delay times t_1 and t_2, respectively.

Intracellular fluorescencei maging was performed with a confocal laser scanning microscope (A1Rsi, Nikon). PC12 cells were cultured at a 1×10^5 cells density for 24 h in 35-mm confocal culture. The cells were cultured with HRP-NPs solution (10 μg · mL^{-1}) for 1 d, and then measured using confocal laser scanning microscope. For HRP-NPs under 405 nm excitation, the emission from C6 was collected between 480 nm and 520 nm.

2.6 Cytotoxicity of HRP-NPs

The MTT colorimetric assay was used to assess the cytotoxicity of HRP-NPs. The cells were cultured in 96-well micotiter plate at 1×10^5 cells in per well for 1 d. Then, 0, 20, 30, 40, 50 μg · mL^{-1} of HRP-NPs solution were added into the culture medium at 37 ℃. After 1 day later, 10 μL MTT (5 mg · mL^{-1} in PBS solution) solution was added into the treated wells and incubated for 4 h at 37 ℃. Afterwards, 100 μL of dimethylsulfoxide (DMSO) was added into above wells. Optical density (OD) was read in an ELISA measurer (BioTek, EL ×800). The value of Cell viability (%) was calculated by the following equation: Cell viability = OD(test)/OD(control) ×100%. When the dosages were below 20 μg · mL^{-1}, the cells exhibit high viability (cell viability >90%).

3 Results and Discussion

3.1 Synthesis and Characterization of Hydrogen Peroxide Nanosensors

As a prerequisite to prepare the enzymatic

H_2O_2 nanosensors, HRP modified poly-l-lysine (HRP-PLL) was firstly synthesized. This conjugation is based on a EDC-catalyzed carbodiimide reaction between the carboxylic acids of HRP and amines of PLL, as depicted in Fig. 1(a). The conjugation of HRP to PLL can be demonstrated by their absorption spectra (Fig. 1(c)), wherein PLL and HRP-PLL have the same absorption band peaking at 400 nm. With the assistance of HRP-PLL, the H_2O_2 nanosensors were prepared by a modified reprecipitation-encapsulation method[23]. Briefly, a THF solution of PtTFPP, C6, PS and DTS were rapidly injected into water solution containing PLL-HRP under ultrasonication. These hydrophobic molecules aggregated to form particles due to the quick change of environmental polarity, and HRP-PLL molecules were adsorbed onto the surface of particle by electrostatic forces between amino and silanol groups, resulting in a core-shell structured H_2O_2 nanosensors (HRP-NPs).

The synthesis of HRP-NPs is schematically displayed in Fig. 1(b). The as-prepared HRP-NPs have an average hydrodynamic size of 237 nm, which is slightly larger than that obtained for TEM image (Fig. 1(d)), and a zetal potential of −18.1 mV. In order to confirm the presence of HRP on nanosensors, NPs with PLL only (without HRP) were prepared as a control. It is found that the PLL-NPs have a smaller hydrodynamic size of 180 nm, and more positive zetal potential of 49.2 mV. Obviously

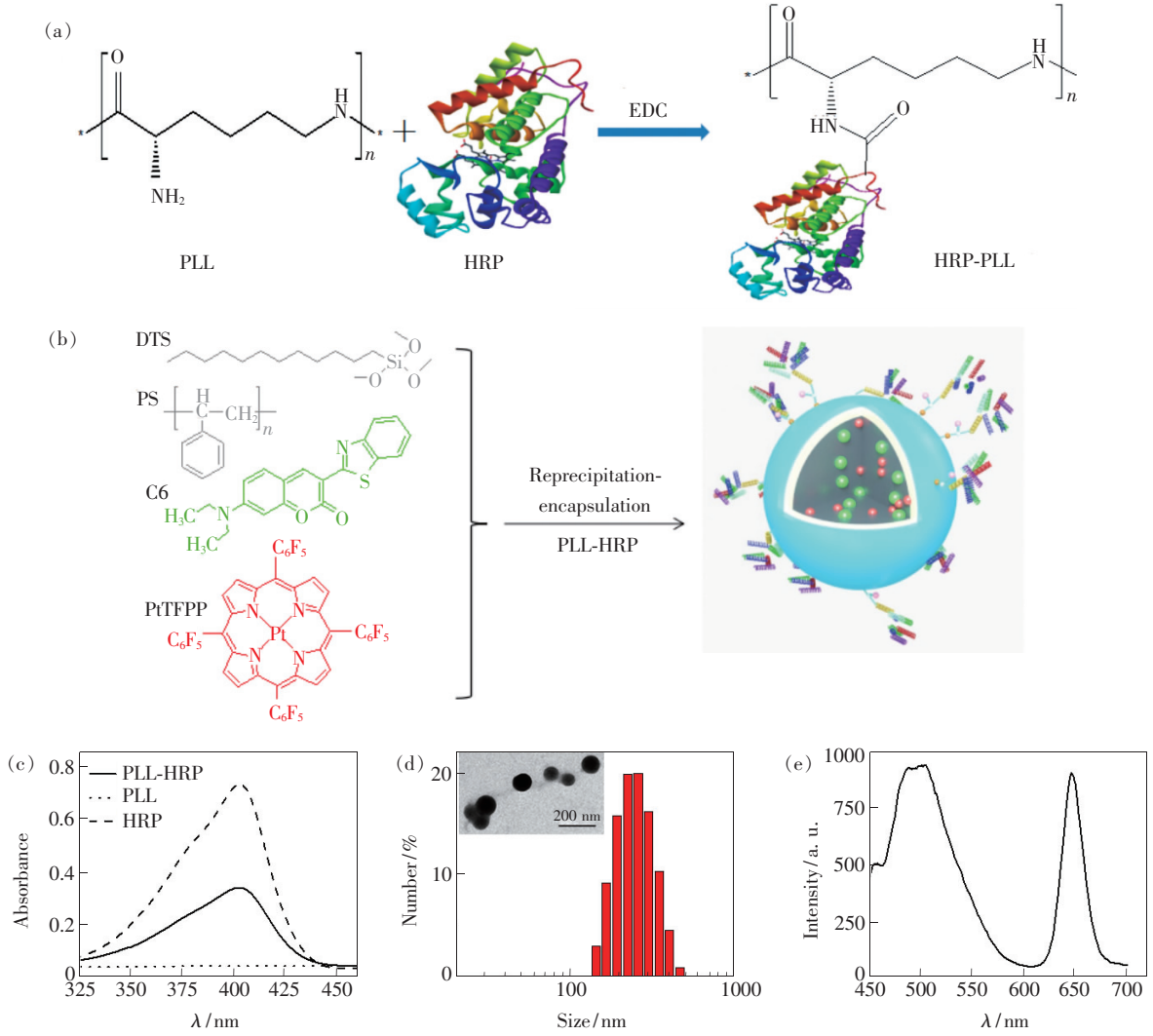

Fig. 1　Schematic illustration of the synthesis of PLL-HRP(a) and HRP-NPs(b). (c) UV-Vis absorption of HRP, PLL and HRP-PLL in water. (d) DLS data and TEM(inset) of HRP-NPs. (e) Emission spectrum of HRP-NPs suspended in water(λ_{ex} = 393 nm).

HRP molecules are attached to the surface of nanosensors, which not only enlarge the diameter of NPs, but also consume the positively charged amine groups of PLL. Since the sensing core is doped with both oxygen probe PtTFPP and the reference dye C6, the as-prepared H_2O_2 nanosensors give a two-wavelength emission under a single excitation (Fig. 1(e)). The 650 nm red emission is attributed to PtTFPP, while the 500 nm green emission to C6, which endows the enzymatic nanosensors with the capacity of ratiometric fluorescence detection. It is instructive to point out that PtTFFP is more photostable than unfluorinated conuterparts, and thus is widely used as fluorescent oxygen probe.

3.2 Hydrogen Peroxide Detection Based on Ratiometric Fluorescence

Firstly, fluorescence response of HRP-NPs towards H_2O_2(0.1 mmol · L^{-1}) was tested by measuring their time-evolved emissions. It can be seen clearly from Fig. 1(b) that, after the addition of H_2O_2, the 650 nm emission of PtTFPP decreases gradually whilst the 500 nm emission of C6 keeps rather constant. It is evidently that our enzymatic fluorescent nanosensors work as being designed: HRP catalyzed H_2O_2 to produce oxygen, thereby fluorescence of oxygen probe is quenched by elevated oxygen concentration; as the reference dye, fluorescence of C6 is barely affected by oxygen.

The kinetic fluorescence response of HRP-NPs was then recorded against different concentrations of H_2O_2(0.1, 0.5, 1.5, 10, 20, 50, 100 μmol · L^{-1}). By defining R as the intensity ratio of 650 nm emission to 500 nm emission, a ratiometric sensing modality is thus available for H_2O_2 detection. Since single-intensity based sensing is affected by the concentration of probes, the ratiometric method gives more robust signals due to the built-in calibration nature. It should be noted that R vaies with the reaction time, and the generation rate of oxygen is proportional to the concentration of H_2O_2. So a series of calibration plots can be obtained with different determination times. Fig. 2(b) displays a characteristic calibration plot with 16 min determination time. The Y-axis is defined as R_0/R, where R and R_0 denote the intensity ratio with and without the presence of H_2O_2 at certain concentrations, respectively. The experimental data can be fitted by the Allometric function, which is consistent with the enzymatic catalytic mechanism[25]. It needs to note that oxygen-quenching based calibration plot usually follows a Stern-Volmer equation. Herein the power-function type procession against H_2O_2 indicates the enzymatic nature of our nanosensors. From the figure it can be deduced that the ratiometric nanosensors are very sensitive to H_2O_2 in the range of 0 – 5 μmol · L^{-1}, but become insensitive in 5 – 20 μmol · L^{-1}. Limit of detection (LOD) of HRP-NPs was determined by linear regression method, *i. e.* LOD = $3S_a/b$, where S_a is the standard deviation of y-intercept and b is the slope of the calibration curve. According to the fitting results as displayed in the inset to Fig. 2(b), LOD is calculated to be 48 nmol · L^{-1}.

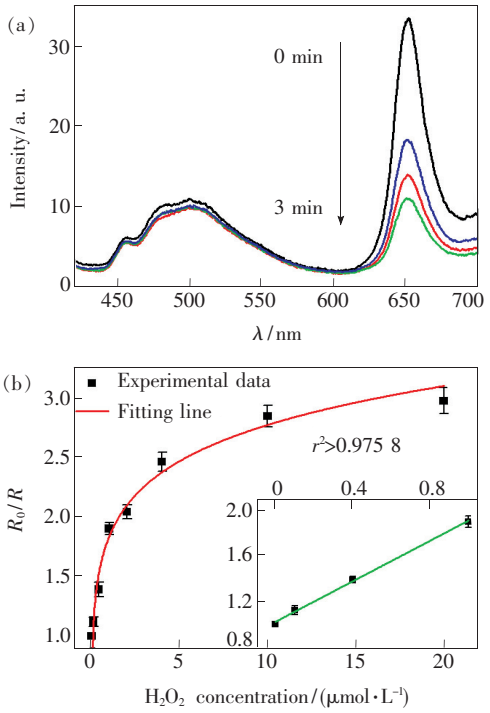

Fig. 2 (a) Time-dependent emission spectra of NPs-HRP in response to hydrogen peroxide (1 mmol · L^{-1}). The spectra were recorded at the time of 0, 1, 2, 3 min after the addition of H_2O_2 (λ_{ex} = 393 nm). (b) Ratiometric fluorescence intensity-based calibration plot. The fitting function is $y = a + bX^c$. The inset to (b) is a linear fitting to obtain LOD, in which the slop and standard deviation of y-intercept is 0.014 and 0.89, respectively.

This value is comparable to the LOD of most reported fluorescent H_2O_2 sensors, which ranges from 10 to 100 nmol · L^{-1} [18,20-21].

3.3 Hydrogen Peroxide Calibrations Based on Time-resolved Fluorescence

Lifetime-based measurement is another robust means in that it is free of not only fluctuation of probe concentration but also of photobleaching. In this work, lifetime of HRP-NPs was measured by monitoring the luminescence of PtTFPP using a time-resolved fluorescence microplate reader. Fig. 3(a) shows life-time based response of HRP-NPs against the concentration of H_2O_2. It can be observed clearly that lifetime decreases with the evolution of reaction time as well as with the increase of H_2O_2. The results are similar to the case of ratiometric fluorescence, *i. e.* elevation of dissolved oxygen quenches (shortens) the fluorescence (lifetime) of oxygen probes. Since the evolution of lifetime is dependent on both response time and H_2O_2 concentration, various calibration plots can be drawn. Considering the sensitivity of our nanosensor, lifetime data with 10 min determination time were utilized for the calibration plot, as displayed in Fig. 3(b). The *Y*-axis is defined as τ_0/τ, where τ and τ_0 denote the lifetime ratio with and without the presence of H_2O_2 at certain concentrations, respectively. The experimental data are well fitted by the Allometric function, which give a dynamic range of 0 – 5 μmol·L^{-1}, similar to that in ratiometric calibration.

Prior to the detection of intracellular H_2O_2, cytotoxicity of HRP-NPs was studied in advance with MTT assay. The MTT assay is a colorimetric method that measures the products of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) reduced by mitochondrial succinate dehydrogenase. As displayed in Fig. 4(a), a dose of <40 μg/mL gives a low inhibition of cells. Then the cellular uptake of HRP-NPs was evaluated by confocal fluorescence imaging(Fig. 4(b)). It can be seen from the figure that HRP-NPs are efficiently swallowed by PC12 cells.

To PC12 cells loaded with HRP-NPs, lifetime was monitored by TRF mode. Fig. 5 displays the

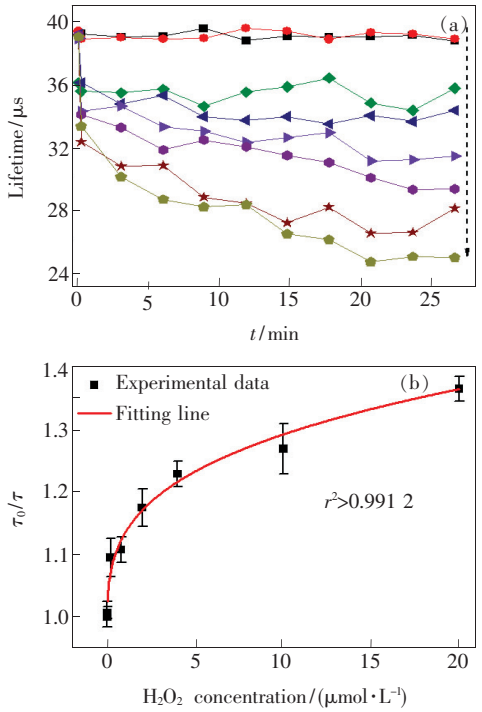

Fig. 3　(a) Kinetic response of HRP-NPs to H_2O_2 in lifetime. As indicated by the arrow, concentration of H_2O_2 increases from 0 to 0.02, 0.2, 0.8, 2, 4, 10, 20 μmol · L^{-1}. (b) Lifetime-based calibration plot of HRP-NPs. The data are obtained from Fig. 3(a) with the determination time of 10 min.

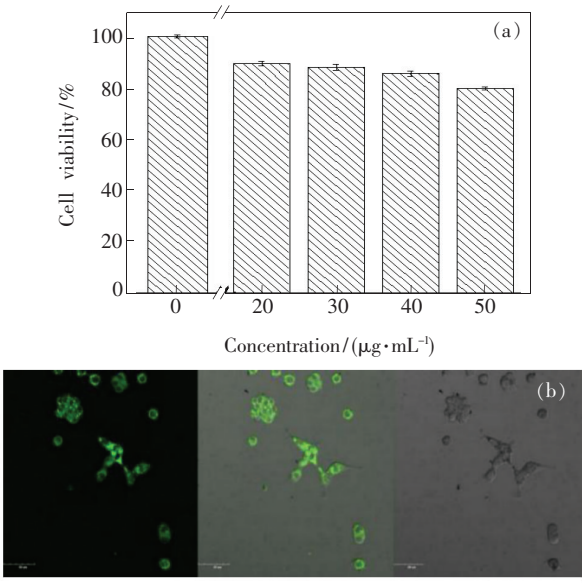

Fig. 4　(a) Viability of PC12 cells treated with different concentrations of HRP-NPs. (b) Confocal fluorescence images of PC12 cells stained with HRP-NPs. The central image is overly of left(fluorescence) and right (DIC) images. The green fluorescence (C6) was collected at 480 – 520 nm under the excitation of 405 nm light.

lifetime evolution of HRP-NPs against intracellular H_2O_2. It can be observed that lifetimes only slightly fluctuate with the absence of H_2O_2 ($t<8$ min), but after the addition of H_2O_2 ($t>9$ min), decrease differently proportional to respective concentrations. The higher concentration of H_2O_2, the faster the decrease of lifetime. The results indicate that our HRP-NPs are sensitive to intracellular H_2O_2, which are very promising for detection of ROS-related cellular events such as oxidative stress.

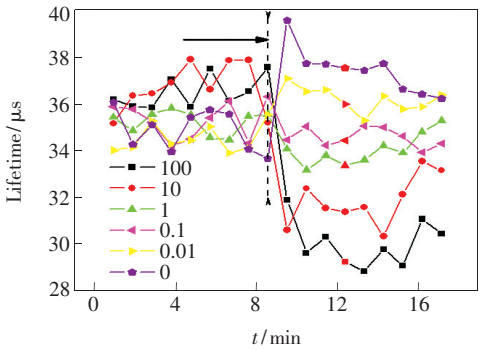

Fig. 5 Lifetime-based kinetic response of intracellular HRP-NPs treated with different concentrations of H_2O_2 (0, 0.01, 0.1, 1, 10, 100 μmol · L^{-1}). The cells were equilibrated for 8 min before the addition of respective H_2O_2 solutions (time is indicated by the arrow).

4 Conclusion

In summary, we prepared enzymatic fluorescent H_2O_2 nanosensors HRP-NPs with a facile reprecipitation-encapsulation method. HRP-PLL macromolecules were firstly synthesized, and then adopted to assist the formation of core-shell structured HRP-NPs. The as-prepared HRP-NPs had an average hydrodynamic size of 237 nm and a zetal potential of −18.1 mV. Under a single wavelength excitation, our nanosensors gave two emissions: green emission peaking at 500 nm from the reference dye C6 and red emission peaking at 650 nm from the oxygen probe PtTFPP. The red emission was sensitive to H_2O_2 in both ratiometric fluorescence and time-resolved fluorescence, decreased with the increase of H_2O_2. Accordingly calibration plots were constructed and fitted with the Allometric function, yielding a dynamic range of 0 − 5 μmol · L^{-1} and a LOD of 48 nmol · L^{-1}. Intraceullular H_2O_2 was further detected using HRP-NPs based on RTF mode, and shortened lifetime was observed. Although sensitivity of our H_2O_2 nanosensor is not so high, they possess the merits of good reversibility based on oxygen-quenched-fluorescence which enables real-time monitoring and good selectivity originated from enzymatic nature.

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