Sensing and Bio-Sensing Research 3 (2015) 92–97

Contents lists available at ScienceDirect

Sensing and Bio-Sensing Research

journal homepage: www.elsevier.com/locate/sbsr

Cy5 labeled single-stranded DNA-polydopamine nanoparticle conjugate-based FRET assay for reactive oxygen species detection

Lina Ma [a], Zhen Lei [a,b], Fuyao Liu [a,b], Zhenxin Wang [a,*]

[a] State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, PR China
[b] University of Chinese Academy of Sciences, No. 19A Yuquan Road, Beijing 100049, PR China

ARTICLE INFO

Keywords:
Polydopamine nanoparticles
Cy5 labeled single-stranded DNA
Reactive oxygen species
Fluorescence resonance energy transfer
Living cell

ABSTRACT

This work reports on a simple and feasible fluorescence resonance energy transfer (FRET) assay for detecting reactive oxygen species (ROS) both in solution and living cell using polydopamine nanoparticle (PDA NP) as energy acceptor and Cy5 labeled single-stranded DNA (Cy5-ssDNA) as energy donor. The Cy5-ssDNA and PDA NPs form self-assembled conjugates (Cy5-ssDNA-PDA NP conjugates) via π-stacking interactions. In the presence of ROS, the PDA NP adsorbed Cy5-ssDNAs can be effectively cleaved, resulting in the release of Cy5 molecules into solution and recovery of fluorescence emission of Cy5. In order to obtain ROS solution, the glucose oxidase-catalyzed oxidation reaction of glucose with O_2 is employed to generate hydrogen peroxide for Fenton-like reaction. The formation of ROS in Fenton-like reaction can be detected as low as glucose oxidase-catalyzed oxidation of 100 pM glucose by the Cy5-ssDNA-PDA NP conjugate-based FRET assay. The recovery ratio of Cy5 fluorescence intensity is increased linearly with logarithm of glucose concentration from 100 pM to 1 μM, demonstrating that the FRET assay has wide dynamic range. In particular, intracellular ROS has been successfully detected in chemical stimulated HepG-2 cells by the Cy5-ssDNA-PDA NP conjugate-based FRET assay with a fluorescence microscopy, indicating that this approach has great potential to monitor ROS in living cells.

1. Introduction

As biochemistry mediators of cellular pathology, reactive oxygen species (ROS) including superoxide (O_2^-), singlet oxygen (O_2^1), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), hypochlorite (ClO^-) and peroxynitrite ($ONOO^-$) play key roles in metabolic processes [8]. In normal aerobic cells, ROS level is normally adjusted by biochemical antioxidants [5]. Overaccumulation of ROS can induce oxidative stress, which is related to many diseases such as diabetes, cancer and molecular neurodegenerative disorders [2,10,24]. Several techniques/assays have been used to determinate ROS including electron spin resonance (ESR) spectroscopy, fluorescence spectroscopy, chemiluminescence assay and electrochemical biosensors [1,16,17,28,3]. Among of these methods, fluorescence-based assays can be used for real-time monitoring ROS level in cells and tissues [4,11,9].

Recently, due to the unique physicochemical properties, excellent biocompatibility and biodegradability, polydopamine nanoparticles (PDA NPs) have attracted much attention and been extensively investigated for various applications including surface modification, bio-inspired hydrogel generation, biosensor fabrication, metal deposition and drug delivery [21,18,20,31,13,32,30,26,22,14]. Especially, it was found that the fluorescence quenching capacity of PDA NPs was equivalent to that of graphene oxide [23]. For instance, PDA NP-based FRET assay has been used to detect DNA and thrombin [18,20,23]. The PDA NP-based FRET assay is fast, simple and homogeneous, and could be used for *in vivo* fluorescence imaging.

In this paper, we developed a "light on" assay for detecting ROS through FRET between PDA NP (acceptor) and Cy5 labeled ssDNA (Cy5-ssDNA, donor). The Cy5-ssDNA and PDA NPs form self-assembled conjugates (Cy5-ssDNA-PDA NP conjugates) via π-stacking interactions, leading to significantly quenching of fluorescence of Cy5. The fluorescence of Cy5 can be restored by the decomposition of Cy5-ssDNA-PDA NP conjugates through the ROS cleavage of ssDNA. The capacity of the Cy5-ssDNA-PDA NP conjugate-based FRET assay has been demonstrated by detection of Fenton-like reaction generated ROS in solution and intracellular ROS of chemical stimulated HepG-2 cells. To the best of our knowledge, this is the first example of using fluorescence quenching property of PDA NPs for monitoring intracellular ROS level.

* Corresponding author. Tel./fax: +86 431 85262243.
E-mail address: wangzx@ciac.ac.cn (Z. Wang).

http://dx.doi.org/10.1016/j.sbsr.2014.12.006
2214-1804/

2. Experimental section

2.1. Materials and reagents

Dopamine hydrochloride and ammonia aqueous ($NH_4{\cdot}H_2O$, 28–30%) solution were purchased from Alfa Aesar (USA). Synthetic oligonucleotide 5′-TTTTTAGATACTATAT-Cy5-3′ was supplied by Sangon Ltd. (Shanghai, China). The concentration of DNA was determined by measurement of UV absorbance at 260 nm. Glucose, glucose oxidase (GOD) and Iron (II) sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$) were purchased from Sigma–Aldrich (USA). Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were purchased from Aladdin Industrial Corporation (Shanghai, China). HepG-2 cell line was obtained from Shanghai cell bank of Chinese Academy of Science (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from HyClone (USA). Phorbol-12-Myristate-13-Acetate (PMA) was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). All other chemicals were analytical grade. Milli-Q water (18.2 MΩ cm) was used in all experiments.

2.2. Instrumentation

Fluorescent emission spectra were recorded on a QE65 Pro fiber optic spectrometer (Ocean Optics, USA). Laser scanning confocal fluorescence microscope (ZEISS LSM 780, Germany) was used for living cell imaging. Transmission electron microscopy (TEM) micrographs were obtained on a Hitachi H-600 transmission electron microscope (Hitachi, Japan) with an accelerating voltage of 100 kV. Scanning electron microscopy (SEM) measurements were made on a XL30 ESEM FEG scanning electron microscope at an accelerating voltage of 20 kV (FEI, USA). UV–visible spectra were obtained by Mini 1240 UV–visible spectrophotometer (Shimadzu, Japan). The Fourier transform infrared (FTIR) spectra were recorded on a Bruker Vertex 70 spectrometer (Bruker, Germany). Raman spectra were recorded on a J-Y T64000 Raman spectrometer with 514.5 nm wavelength incident laser (J-Y, France). X-ray photoelectron spectra (XPS) measurements were conducted with a VG ESCALLAB MKII spectrometer (VG, UK). Dynamic light scattering (DLS) experiments were carried out on a Malvern Nano-ZS Zetasizer (Malvern, UK).

2.3. Preparation of PDA NPs

The PDA NPs were synthesized according to a previous report with slight modification [19]. Briefly, 0.5 mL $NH_4{\cdot}H_2O$ was mixed with 10 mL ethanol and 22.5 mL H_2O under mild stirring at 30 °C for 30 min. Then, a total of 0.125 g dopamine hydrochloride was dissolved in 2.5 mL H_2O and immediately injected into reaction mixture. The reaction mixture was stirred at 30 °C for another 24 h. The as-prepared PDA NPs were washed with 25 mL H_2O (3 times) and collected by centrifugation (9000 rpm).

In order to obtain pure Cy5-ssDNA-PDA NP conjugates for intracellular ROS detection, 14 μL Cy5-ssDNA (1.44 μM) with 2 μL PDA NPs (2 mg/mL) were mixed in 184 μL PB (10 mM, pH 7.0) and incubated at 30 °C for 1 h. Subsequently Cy5-ssDNA-PDA NP conjugates were purified by centrifugation (9000 rpm, 3 times) and redispersed in 200 μL PB.

2.4. Sensing and inhibition assay of ROS

Detection of Fenton-like reaction generated ROS. Typically, 3 μL of 2 mg/mL PDA NPs solution was incubated with 20 μL of 1.44 μM Cy5-ssDNA in 147 μL PB at 30 °C for 1 h. Subsequently, varying concentrations of glucose solution, 6.3 U GOD and 30 μL of 30 μM $FeSO_4$ solution were added into the reaction mixture, respectively. The total volume of the reaction mixture was adjusted to 300 μL by PB and the solution was incubated under O_2 atmosphere at 30 °C for another 5 min. Finally, the fluorescence spectra of the mixture were recorded on a QE65 Pro fiber optic spectrometer at the excitation wavelength of 633 nm.

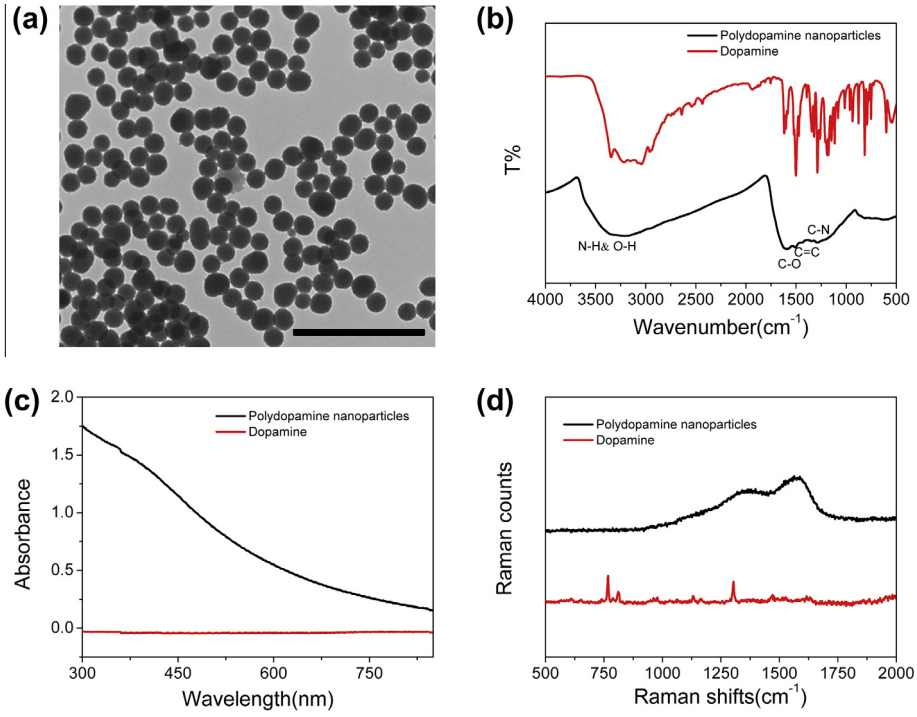
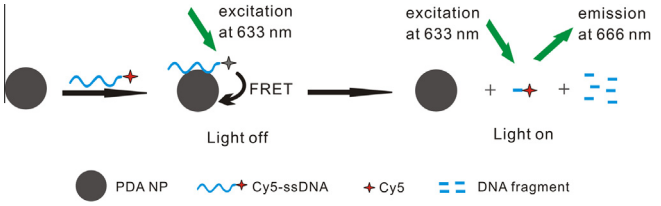


Fig. 1. (a) TEM micrograph, (b) FTIR spectrum, (c) UV–visible spectrum and (d) Raman spectrum of as-prepared PDA NPs. For comparison, the spectra of dopamine monomer have also been shown in the figure, respectively. The scale bar of (a) is 1 μm.

For the inhibition assay of ROS, 30 μL of antioxidants with desired concentrations were added into 140 μL Cy5-ssDNA-PDA NP conjugates solution (1.44 μM Cy5-ssDNA and 2 mg/mL PDA NPs in PB). After incubation at 30 °C for 1 h, 30 μL of 20 nM glucose, 6.3 U GOD and 30 μL of 30 μM Fe^{2+} were added into the reaction mixture, respectively. Then, the reaction mixtures were treated as previously described.

2.5. Cell culture and MTT assay

HepG-2 cell line was cultured in DMEM supplemented with 10% FBS and 100 U/mL penicillin–streptomycin at 37 °C in humidified air with 5% CO_2. The cytotoxicity of PDA NPs and Cy5-ssDNA-PDA NP conjugates were evaluated by a traditional [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay. Generally, HepG-2 cells were seeded at a density of 8×10^3 cells per well in 96-well plate and incubated for 12 h. The culture medium was removed and the cells were washed by 100 μL PBS (10 mM PB supplemented with 0.137 M NaCl, pH 7.4, 3 times). Then, the cells were incubated in fresh culture medium containing different concentrations of PDA NPs and Cy5-ssDNA-PDA NP conjugates for another 24 h. Subsequently, the cells were washed with 100 μL PBS (3 times) and treated with 10 μL of MTT (5 mg/mL) for 4 h. Finally, the supernatant was discharged, and purple formazan product was dissolved by DMSO (100 μL per well) with gentle shaking for 10 min. The absorbance of each well was read on a Power Wave XS 2 Microplate Spectrophotometer at 510 nm. The relative cell viabilities (%) were calculated by using the optical densities with respect to the control value. HepG-2 cells cultured without NPs were used as control sample.



Scheme 1. The schematic representation of the FRET assay with Cy5-ssDNA and PDA NPs as the energy donor–acceptor pair for ROS detection. The illustration is not drawn to scale.

2.6. Intracellular imaging

The cells were seeded at a density of 1.5×10^5 cells per well in six-well culture plate with 22 mm × 22 mm glass coverslip at the bottom of well and grown in fresh culture medium for 24 h. Then, supernatant was discharged and fresh culture medium containing 20 μg/mL Cy5-ssDNA-PDA NP conjugates was introduced into the wells and incubated for another 24 h. Finally, the cells were washed and imaged by ZEISS LSM 780 Laser scanning confocal fluorescence microscope. For PMA stimulation, the cells were incubated with 3 μM PMA in 1.5 mL culture medium for another 30 min before fluorescence imaging.

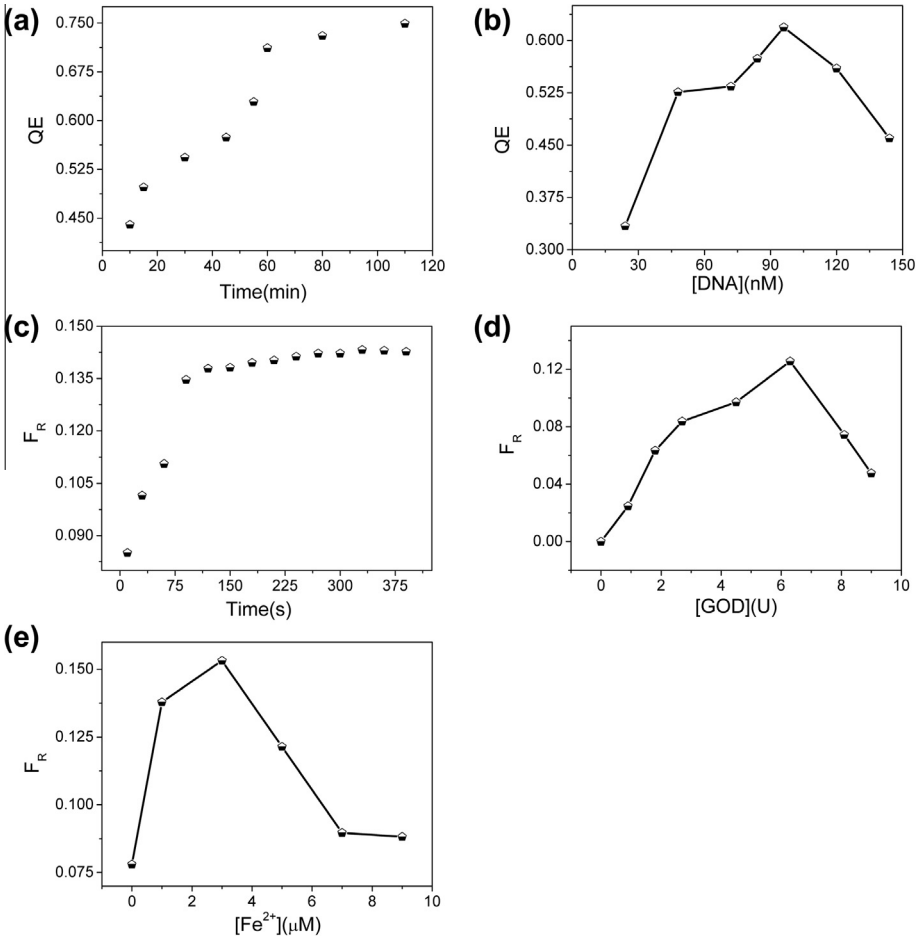


Fig. 2. Effects of (a) quenching time and (b) concentration of Cy5-ssDNA on quenching efficiency in the presence of 20 μg/mL PDA NPs. Effects of (c) cleavage/reaction time, (d) GOD concentration and (e) Fe^{2+} concentration on the recovery ratio of Cy5 fluorescence intensity. The concentration of glucose in (d) and (e) is 2 nM. All experiments were conducted in PB under excitation at 633 nm.

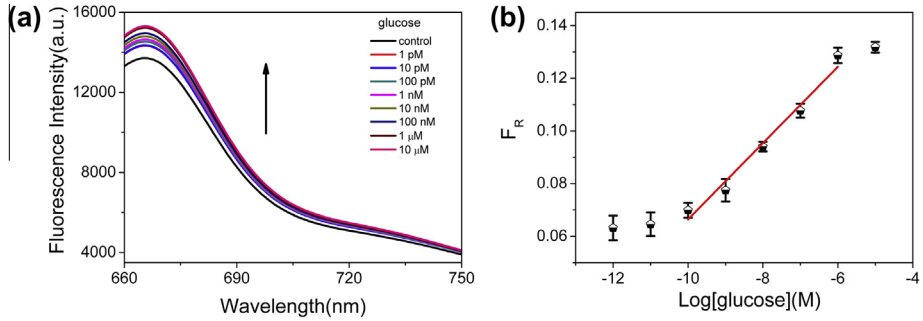


Fig. 3. (a) The fluorescence spectra of Cy5 in the presence of various concentrations of glucose. (b) The calibration curve of recovery ratio of Cy5 fluorescence intensity versus logarithmic concentration of glucose. The Error bars mean standard deviations ($n = 3$). All experiments were conducted in PB under excitation at 633 nm.

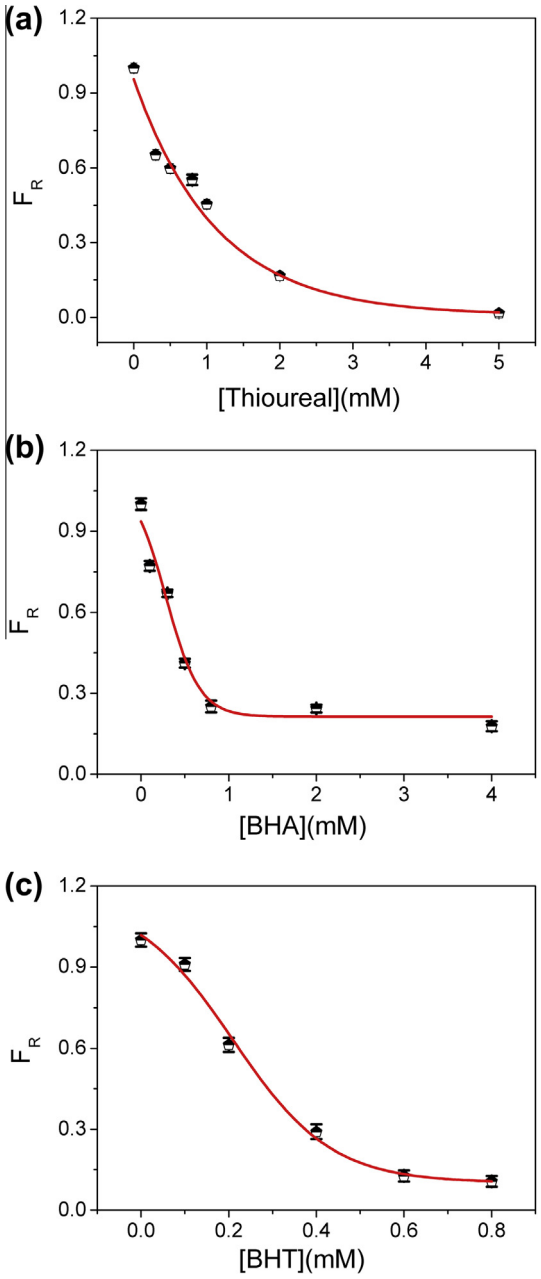


Fig. 4. Recovery ratio of Cy5 fluorescence intensity plotted as the function of concentration of (a) thiourea, (b) BHA and (c) BHT, respectively. The Error bars mean standard deviations ($n = 3$). All experiments were conducted in PB under excitation at 633 nm.

3. Results and discussion

3.1. Characterization of the as-prepared PDA NPs

The PDA NPs are synthesized by literature reported strategy with slight modification [19]. The average size of the as-prepared PDA NPs is 160 nm in diameter (as shown in Fig. 1a and Fig. S1), respectively. The characteristic bands of several groups including C=C, N–H and C–N are clearly observed in the FTIR spectrum of PDA NPs (as shown in Fig. 1b). The FTIR experimental result indicates that PDA NP is a π-rich and nitrogen-containing polymer [29]. Therefore, the PDA NP can strongly adsorb ssDNA on its surface via π–π stacking interactions between unpaired DNA bases and PDA NP. The UV–visible spectrum of PDA NPs exhibits broad band absorption which leads to a spectral overlap with the fluorescence emission spectra of many fluorophores (as shown in Fig. 1c). The phenomenon suggests that PDA NP can be used as a broad spectrum of FRET acceptor. In addition, Raman and XPS measurements give a further confirmation of successful preparation of PDA NPs (as shown in Fig. 1d, Fig. S2) [19].

The as-prepared PDA NPs can be re-dispersed in different media (e.g., H_2O, PB, DMEM supplemented with 10% FBS and human serum) and stored under chamber conditions for 24 h without significant aggregation (as shown in Fig. S3 and Table S1). The experiment results indicate that the as-prepared PDA NPs have good colloidal stabilities. After incubated with Cy5-ssDNA, the hydrodynamic size of PDA NPs is slightly increased (as shown in Table S1). The experimental result indicates that Cy5-ssDNA have been successfully conjugated on the PDA NP surface.

3.2. Principle of the FRET assay

Scheme 1 outlines the principle of the "light on" FRET assay for detecting ROS. The Cy5-ssDNA and PDA NPs form Cy5-ssDNA-PDA NP conjugates via π-stacking interactions. Upon binding with PDA NP, the fluorescence emission of Cy5-ssDNA decreases, consistent with the energy transfer from Cy5 to PDA NP. In the presence of ROS, the ssDNAs take place irreversible self-cleavage, leading to decompose Cy5-ssDNA-PDA NP conjugates. Following decomposition of Cy5-ssDNA-PDA NP conjugates, Cy5 molecules are released from PDA NP surfaces and the fluorescence emission of Cy5 is recovered.

3.3. Sensing and inhibition assay of ROS

Several parameters, including the quenching time, the concentrations of Cy5-ssDNA, GOD and Fe^{2+}, and reaction/cleavage time were investigated systematically to establish optimized conditions for the ROS detection by monitoring the quenching efficiency (QE) and recovery ratio of Cy5 fluorescence intensity (F_R). Here,

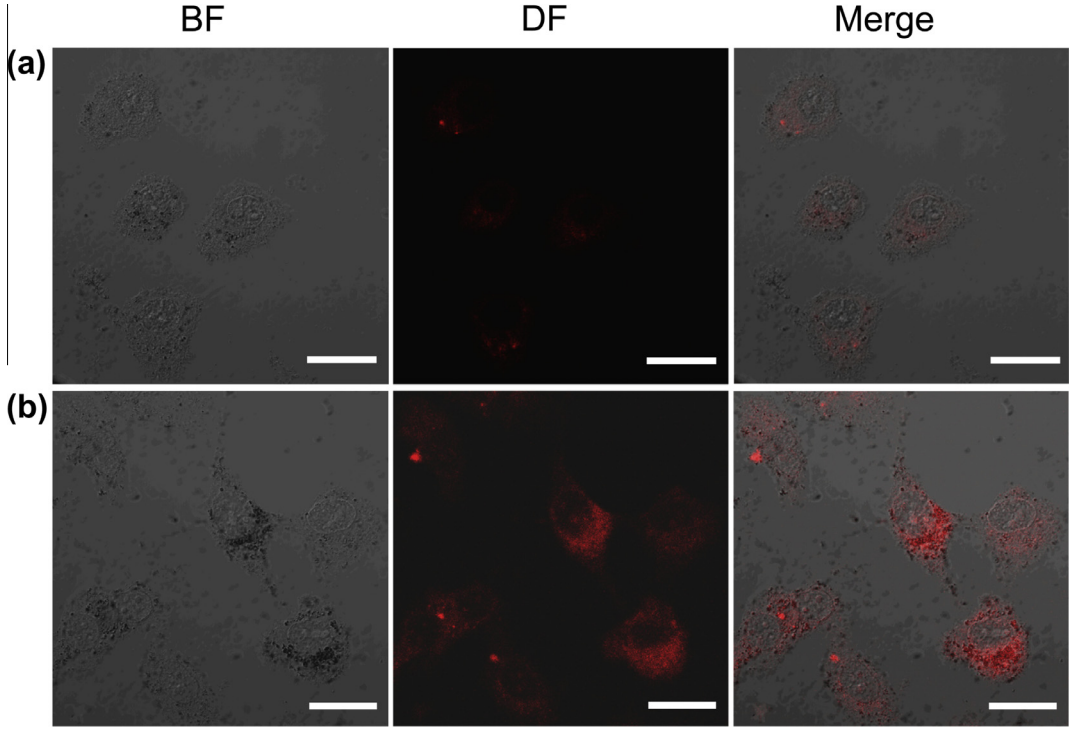


Fig. 5. Confocal fluorescence microscopy images (bright field (BF, left), dark field (DF, middle) and merging (merge, right) images) of Cy5-ssDNA-PDA NP conjugate-stained HepG-2 cells (a) before and (b) after PMA simulation, respectively. The scale bars are 20 μm.

QE = $(1 - F_0/F_I) \times 100\%$ and $F_R = (F - F_0)/F_0$. F_0 and F_I are fluorescence intensities of Cy5-ssDNA with or without PDA NPs, and F represents solution fluorescence intensity in the presence of ROS, respectively. The QE is increased with increasing the concentration of PDA NPs (as shown in Fig. S4). However, both of the PDA NPs and Cy5-ssDNA-PDA NP conjugates show moderate cytotoxicity while their concentration is more than 40 μg/mL (as shown in Fig. S5). Therefore, 20 μg/mL PDA NP is used in the FRET assay. As shown in Fig. 2, the experimental results show that the best quenching time, Cy5-ssDNA concentration, Fe^{2+} concentration, GOD concentration and reaction/cleavage time are 60 min, 96 nM, 3 μM, 6.3 U and 5 min for this FRET assay, respectively. In the following study, all the experiments are carried out under the optimized conditions.

The analytical performance of the FRET assay was tested by detection of Fenton-like reaction generated ROS (corresponding reaction process is shown in Fig. S6) [7]. In this case, the ROS level in solution is strongly dependent on the glucose concentration since hydrogen peroxide is formed by glucose oxidase-catalyzed oxidation reaction of glucose. As expected, the fluorescence intensity is increased with increasing the concentration of glucose. The increase of F_R is linear with the logarithm of glucose concentration in the range from 100 pM to 1 μM ($F_R = 0.0144\ \lg C_{glucose} + 0.2113$, $R^2 = 0.97$; as shown in Fig. 3). The experimental result indicates that the proposed FRET assay has relatively high sensitivity and wide dynamic range for ROS detection.

The performance of the FRET assay was also tested by monitoring the changes of F_R with the addition of potential interferences (the ions and molecules (e.g., Ca^{2+}, Fe^{3+}, Zn^{2+} and amino acids) may coexist with ROS in practical samples) because the DNA cleavage efficiency of ROS could be disturbed by these interferences. As expect, the F_R is decreased by adding interference except Fe^{3+} (as shown in Fig. S7). The phenomenon may caused by Fe^{3+} acceleration of Fenton-like reaction, leading to generate additional ROS. The experimental results indicate that the Cy5-ssDNA-PDA NP conjugate-based FRET assay can be used to detect ROS in the presence of high concentration of interferences.

The interaction of Cy5-ssDNA with ROS has also been investigated by circular dichroism (CD) spectroscopy because CD spectrum can accurately characterize the DNA structure [12,27,25]. The absorption at 278 nm is decreased dramatically when Cy5-ssDNA is co-incubated with GOD, Fe^{2+} and glucose (as shown in Fig. S8). The experimental result demonstrates that Cy5-ssDNA is cleaved by Fenton-like reaction generated ROS.

In order to further verify the utility of the FRET assay, the efficiencies of three potential antioxidants (thiourea, BHA and BHT) have been measured. The antioxidants can inhibit the activity of ROS and disrupt the ROS-ssDNA interactions. Fig. 4 shows the inhibitory curves of the antioxidants. As expected, the F_R is decreased with increasing the concentration of antioxidant. The half maximal inhibitory concentrations (IC_{50}) of antioxidants follow the order of BHT (0.26 mM) < BHA (0.34 mM) < thiourea (1.03 mM), which is consistent with the results of literature reports [15]. The results suggest that our method has the potential to screen antioxidants and yield quantitative data on the antioxidant capacities of different chemicals.

3.4. Living cell imaging of ROS

The intracellular ROS level has also been examined by the Cy5-ssDNA-PDA NP conjugate-based FRET assay for proving practical application of this assay in biological samples. As a well known stimulant, PMA has been used to stimulate HepG-2 cells in this experiment. PMA can stimulate cell immune system to produce large amount of ROS [4,33,6]. In this case, HepG-2 cells were first incubated with Cy5-ssDNA-PDA NP conjugates in serum-supplemented cell culture medium at 37 °C for 24 h. The Cy5-ssDNA-PDA NP conjugate-stained cells show low levels of intracellular fluorescence (as shown in Fig. 5a). Upon stimulation of Cy5-ssDNA-PDA NP conjugate-stained cells with PMA, the intracellular fluorescence intensity is increased significantly (as shown in Fig. 5b). The experimental result indicates that the FRET assay has the great potential for monitoring the ROS fluctuation in living cell.

4. Conclusions

In summary, a simple and feasible Cy5-ssDNA-PDA NP conjugate-based FRET assay has been successfully developed for detecting ROS through FRET between PDA NPs and Cy5-ssDNA. The Cy5-ssDNA-PDA NP conjugates provide an excellent example for detecting biologically active substances with relatively low limit of detection (LOD) in the bioanalytical/biomedical applications of PDA NPs. Although PMA simulated HepG-2 cell and Fenton-like reaction generated ROS are chosen here to establish this new PDA NP-based FRET assay by proof of principle experiment, our approach is readily transferable to real analytical problems such as monitoring ROS level in cells or body fluids. Furthermore, our approach could be extended to test the clinical efficacies of natural products or drugs in terms of their antioxidant content.

Conflict of interest

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Acknowledgment

The authors would like to thank the National Natural Science Foundation of China (Grant No. 21127010) and Jilin Provincial Science and Technology Department (Grant No. 20100701) for financial supports.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.sbsr.2014.12.006.

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