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# Sensitive fluorescence "turn-on" detection of bleomycin based on a superquenched perylene–DNA complex

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Bleomycins (BLMs) are widely used in the clinical treatment of various cancers. Here, taking advantage of the super-quenching effect of the cationic perylene diimide derivative (PDI) on adjacent fluorophores, we reported a PDI–DNA complex based method for fluorescence ''turn-on'' detection of BLM, based on BLM-Fe(II) mediated DNA strand scission. Owing to the very high quenching efficiency of aggregated PDI, the method shows low background fluorescence and high sensitivity with a detection limit of 0.2 nM. It also exhibits a good performance in complex biological samples. Moreover, this method has the advantages of no quencher label cost, easy preparation and rapid response, making it a promising alternative for the determination of trace amounts of BLMs in clinical samples.

## Introduction

Cancer is a major cause of death worldwide, accounting for 8.2 million deaths in 2012. The deaths from cancer worldwide are projected to continue to rise. It is expected that annual cancer cases will rise from 14 million in 2012 to 22 within the next 2 decades.[1] Effective treatment by the usage of antitumor drugs is a major protocol to cure cancer or to considerably prolong life while improving the patient's quality of life. As a family of glycopeptide-derived antibiotics isolated from *Streptomyces verticillus*, [2,3] bleomycins (BLMs) are widely employed in combination with chemotherapy for the clinical treatment of a variety of cancers, including squamous cell carcinomas, germ cell tumors, Kaposi's sarcoma, cervical cancers, and malignant lymphomas. [4-6]

The antitumor mechanism of BLM is generally believed to be a deoxyribose oxidation that resembles free radical damage of cellular DNA and possibly RNA, in the presence of $O_2$ and a redox-active metal ion such as Fe(II). [7-9] With the advantages of low myelosuppression and low immunosuppression, BLM is less toxic to the human body and of particular importance in the clinical treatment of a variety of cancers among the various antitumor drugs. [10] However, the clinical application of BLMs is featured by the occurrence of sometimes fatal side effects, such as renal and lung toxicity, and the dose-limiting side effect potential for pulmonary fibrosis. [11,12] Fever, rigors and skin toxicity may occur as well. [13] To take full advantage of therapeutic efficacy and to minimize its toxicity, it is a compelling need for developing sensitive, simple, and selective analytical methods to quantitatively determine the BLM content in both pharmaceutical analysis and clinical samples.

Over the past decades, many reliable and sensitive methods have been developed for BLM determination, such as radioimmunoassay (RIA), [14,15] enzyme immunoassay (EIA), [16,17] high-performance liquid chromatography (HPLC), [18,19] microbiological assay, [20] electrochemical assay, [9,21-23] colorimetric assay, [24,25] and fluorescent methods. [11,13,26,27] However, these methods are usually challenged with some drawbacks, such as harmful to the health for RIA, instability and sensitive to denaturalization for EIA, expensive instruments and complicated separation procedures for HPLC, time consuming experimental procedures for electrochemical assay. Among them, the fluorescent methods have attracted increasing attention owing to their remarkable features of high sensitivity, facile operation, low cost, fast response, on-site and real-time detection. [28,29] Although BLM is employed clinically at an atypically low dosage (~5 μM), the appearance of pulmonary toxicity is unpredictable. [30,31] Thus, to achieve the best therapeutic effect and to minimize the toxicity of BLM, it is still highly desirable to develop simple and reliable fluorescent methods for rapid and sensitive determination trace amounts of BLMs in clinical analysis.

With the in-depth investigation into the interaction between BLM and DNA, researchers found that the BLM-Fe(II) complex binds with oxygen to generate BLM-Fe(III)OOH which can selectively degradate DNA via C4'-H atom abstraction from deoxyribose in the minor groove of DNA. The generally observed selective scission was occurred predominantly at 5'-GC-3' or 5'-GT-3' sites of DNA strand. [32-34] Therefore, using specific DNA scission as a signal transduction induced by BLM, combined with the flexible design of DNA has becoming a

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powerful tool in BLM sensing. For example, Yin et al. developed an electrochemical detection method for BLM.[9] Li et al. used BLM, complexed with Fe(II), to restore the fluorescence of dye-labeled DNA quenched by graphene oxide.[13] Recently, Qin et al. developed a $WS_2$ nanosheet-based "turn-on" fluorescent method for sensitive detection of BLM.[35]

The water-soluble cationic perylene diimide derivative (PDI, Scheme 1) shows strong monomer fluorescence but significantly reduced fluorescence due to noncovalent selfassembly of PDI induced by nucleic acid in aqueous solution. Based such a phenomenon, Yu et al. have developed various fluorescence "turn-on" sensing systems through a reduced aggregation strategy.[36-38] Recently, aggregated cationic perylene diimide derivative was reported to be able to act as a superquencher to quench the fluorescence of the adjacent oligonucleotide-labeled fluorophore donor, with a quenching efficiency of 99.6%.[39,40] Moreover, the perylene derivative has high thermal stability and excellent chemical inertness. Different substantially from the normally used organic quenchers, and the nanomaterial based quenchers, the superquencher can be formed "in situ", which make it an attractive universal quencher for constructing various fluorescence methods.

In this work, we employ the cationic perylene diimide derivative to construct superquened PDI–DNA complex for fluorescence ''turn-on'' detection of BLM with high sensitivity and specificity. When aggregated on oligonucleotides, PDI is able to efficiently quench the adjacent conjugated anionic fluorophores. Under the oxidative effect of BLM with Fe(II) as a cofactor, the fluorescein (FAM)-labeled long ssDNA (F-ssDNA) probe undergoes an irreversible cleavage event to release the FAM-linked DNA fragment into the solution which far from the aggregated PDI, resulting in the restoration of fluorescence of the sensing system. Owing to the very high quenching efficiency on ssDNA-labeled fluorophores of aggregated PDI, the method shows low background fluorescence and therefore low detection limits. The method can be further applied in human serum for the detection of BLM with satisfactory results. Moreover, this method has the advantages of no quencher label cost, easy preparation and rapid response, making it a promising alternative for the determination of trace amounts of BLMs in clinical samples.

## Experimental section

### Reagents and apparatus

Bleomycin, dactinomycin D, mitomycin C, dactinomycin and daunorubicin were purchased from Melone Pharmaceutical Co., Ltd. (Dalian, China). The metal salts ($FeCl_2$, $FeCl_3$, $Hg(NO_3)_2$, $CoCl_2$, $MgCl_2$, $Pb(NO_3)_2$ and $Zn(Ac)_2$)were obtained from Shanghai Chemical Reagents (Shanghai, China). Perylenetetracarboxylic dianhydride and N, N-Dimethyl-1, 3-propanediamine were purchased from Alfa Aesar. Methyl iodide and other compounds were obtained from Shanghai Chemical Reagent Co. (Shanghai, China). All other reagents were of analytical grade and were used without further purification. The serum samples were kindly provided by Qufu Normal University hospital and stored at 4 °C. The buffer involved in this work was phosphate-buffered saline (PBS, 20 mM $Na_2HPO_4/NaH_2PO_4$, 50 mM NaCl, pH 7.4) buffer. The pH measurement was carried out on a Mettler-Toledo Delta 320 pH meter. Milli-Q water (resistance >18 MΩ•cm) was used in all experiments. DNA oligonucleotides (F-ssDNA: 5'-/FAM/-ATGCTATATCTACAACTAAC-3') used in this work were synthesized and purified by Takara Biotechnology Co., Ltd (Dalian, China).

All fluorescence measurements were carried out on an F-4600 spectrometer (Hitachi, Japan). The instrument settings were chosen as follows: λex = 494 nm (slit 5 nm), λem = 518 nm (slit 5 nm), PMT detector voltage = 950 V.

### Synthesis of PDI

Cationic perylene diimide derivative (PDI) was synthesized following the literature procedure.[39] The experimental details of synthesis see electronic supplementary information.

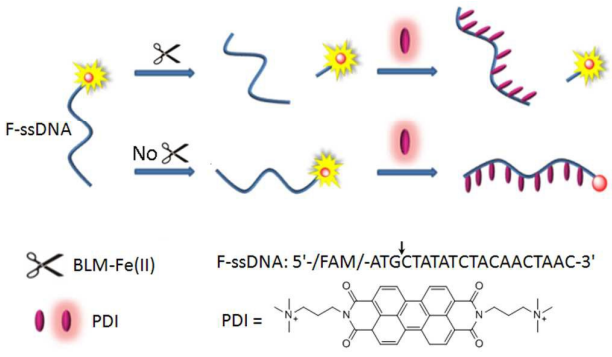
### Fluorescent Detection of BLMs

The BLM smples were prepared by mixing BLM with $FeCl_2$ in 1:1 molar ratio. Then, 1 μL F-ssDNA (3.2 μM), 67 μL PBS and 2 μL different concentrations of BLM-Fe(II) aqueous solutions were mixed together for the oxidative cleavage of ssDNA. After incubation for 30 min at 25 °C, 10 μL PDI (4 μM) was added into the reaction mixture to give a final volume of 80 μL. The fluorescence intensity was measured after incubating the mixture at 25 °C for 10 min. For BLM detection in real complex samples, the experiments were conducted similar to that in buffer solution just containing 1% diluted human serum.

## Results and discussion

### Design principle

Scheme 1 depicts DNA – perylene complex based superquencher probe for BLM detection *via* DNA strand scission. A FAM-labeled 20-mer oligonucleotide (F – ssDNA) containing a 5'-GC-3' site was employed as the substrate for oxidation cleavage. F – ssDNA could bind a large amount of PDI to form a DNA–perylene complex, and efficiently quench the fluorescence of the FAM moiety. Meanwhile, the F–ssDNA




**Scheme 1** Schematic illustration of PDI–DNA complex based fluorescent method for the detection of BLM via DNA strand scission.

induced noncovalent self-assembly of PDI could also eliminate its fluorescence emission. In the presence of BLM-Fe(II), the F-ssDNA underwent an irreversible strand scission at the 5'-GC-3' scission site. As a result, a 3-mer FAM-linked oligonucleotide fragment was generated after the oxidation cleavage. The short fragment could bind only a few molecules of PDI, which induced a weak quenching effect on the FAM's fluorescence. Thus, the fluorescence signal of the sensing system was restored. The fluorescence intensity gradually increases with the addition of increasing concentrations of BLM-Fe(II).

**Feasibility of the method for BLM detection**

Fluorescence experiments under different conditions were carried out to verify the feasibility of our above method. As shown in Fig. 1, either the F-ssDNA (curves a) or the PDI (curves b) existing alone could emit strong fluorescence. However, the fluorescence of both disappeared when they were mixed together (curves c). The significant fluorescence quenching of PDI should be ascribed to the strong perylene derivative aggregation induced by the ssDNA, which can further serve as a superquencher for quenching the fluorescence of the adjacent FAM-labeled F-ssDNA. However, upon the addition of BLM-Fe(II), the fluorescence of the FAM moiety was obviously restored (curves d). These results indicate that our DNA–perylene complex based method holds the promise for fluorescence turn on detection of BLM.

**Optimization of assay conditions**

In order to obtain optimal assay conditions to achieve a high signal-to-bacground ratio (SBR), the quenching ability of PDI on F-ssDNA was first investigated. The fluorescence signal changes were recorded after incubation of F-ssDNA (40 nM) with different concentration of PDI in PBS buffer (20 mM $Na_2HPO_4/NaH_2PO_4$, 50 mM NaCl, pH 7.4) at 25 °C for 10 min. As shown in Fig. 2, the fluorescence intensity of F-ssDNA decreased notably with the addition of increasing concentrations of PDI. In the presence of 0.5 μM of PDI, the fluorescence quenching of F-ssDNA reached equilibrium with 95% quenching efficiency (Fig. S1). A further increase in the concentration of PDI might result in excessive quenching effect

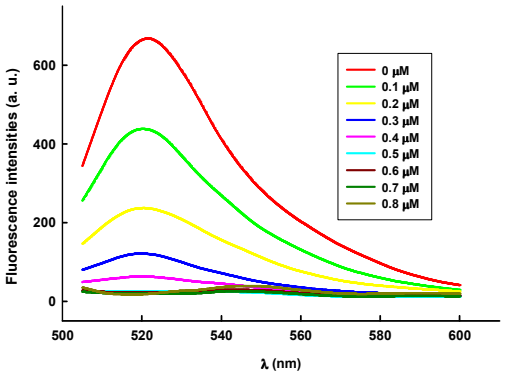



**Fig. 2** Fluorescence emission spectra of F-ssDNA upon the addition of different concentration of PDI.

on the cleavage-induced short FAM-labeled oligonucleotide. Therefore, 0.5 μM of PDI was chosen as the superquencher for further experiments.

It is well-known that the DNA scission process by BLM require a transitional metal ion, an experiment was carried out to investigate the effect of different metal ion coordination environments for BLM-mediated DNA cleavage. The BLM samples were prepared by mixing 500 nM BLM with different metal ions at a molar ratio of 1 : 1. Fig. 3 shows the fluorescence changes of F-ssDNA upon treatment with a mixture of metal ion ($Hg^{2+}$, $Co^{2+}$, $Mg^{2+}$, $Pb^{2+}$, $Zn^{2+}$, $Fe^{3+}$, or $Fe^{2+}$) and BLMs, respectively. Obviously, the combination of BLM-Fe(II) gave the best performance of a significant fluorescence restoration. Moreover, it is reported that BLM-Fe(II) is significantly less toxic than other metal ions coordinated BLM complexes at therapeutically effective concentrations in vivo. [41] A control experiment upon the effect of Fe(II) alone on DNA–perylene complex was also investigated. The experiment result indicated that the introduction of Fe(II) only had a negligible effect on the fluorescence intensity even in high concentration of 10 μM, demonstrating the necessity of BLM for the DNA scission.

To get the best performance, we also investigated the effect of different ratios of BLM and Fe(II) on the DNA scission

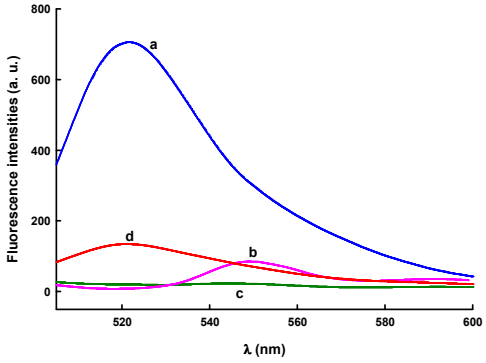



**Fig. 1** Fluorescence emission spectra of (a) 40 nM FP; (b) 500 nM PDI; (c) 40 nM FP + 500 nM PDI; (d) 40 nM FP + 500 nM PDI+ 500 nM BLM-Fe(II).

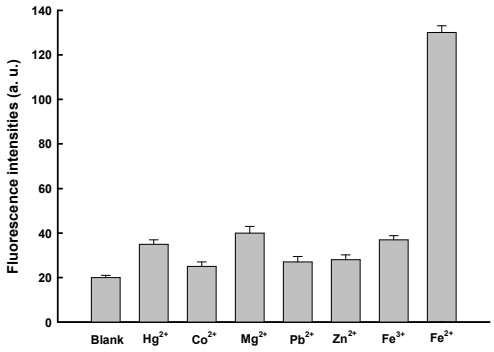



**Fig. 3** Fluorescence enhancement of PDI–DNA complex incubated with the mixture of BLM and different metal ions. The concentrations of BLM and metal ions were all kept at 500 nM. Error bars represent standard deviations from three replicate measurements.

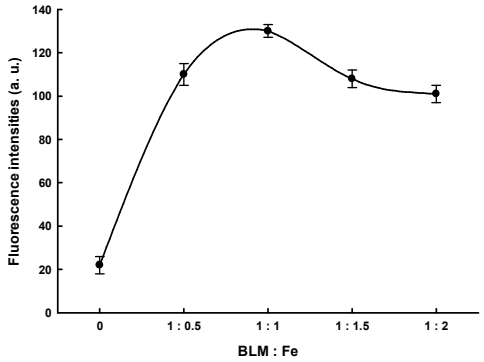



**Fig. 4** Fluorescence enhancement of PDI–DNA complex upon the treatment of different concentration ratios o f BLM and Fe(II). The concentrations of F-ssDNA and PDI were 40 nM and 500 nM, respectively. Error bars represent standard deviations from three replicate measurements.

efficiency. Fig. 4 shows the fluorescence changes of PDI–DNA complex upon treatment with a mixture of BLM (kept at 500 nM) and Fe(II) at different concentration ratios, respectively. The results indicated that an increasing ratios of BLM and Fe(II) result in the increase of fluorescence signal, and the ratio of 1 : 1 could give the largest SBR, which were chosen as the optimal ratio for further investigations.

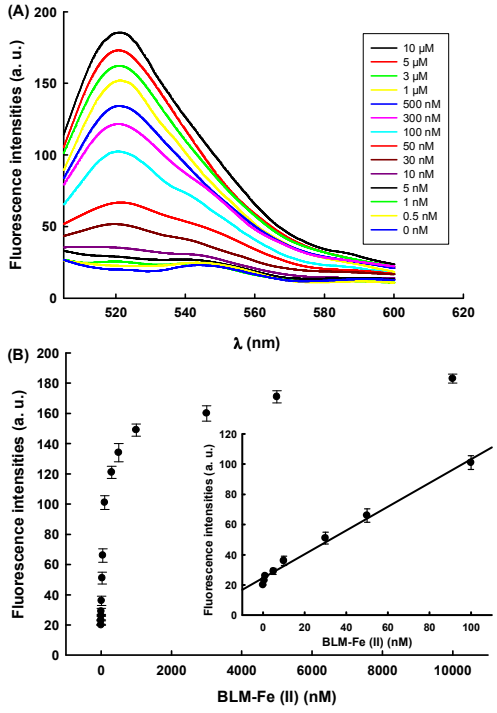



**Fig. 5** (A) Fluorescence emission spectra of PDI–DNA complex upon the addition of BLM with different concentrations (from bottom: 0, 0.5, 1, 5, 10, 30, 50, 100, 300, 500, 1000, 3000, 5000 and 10000 nM). (B) The relationship between fluorescence intensity at 518 nm versus BLM concentrations. Inset is a linear region at low BLM concentrations. Error bars were estimated from three replicate measurements.

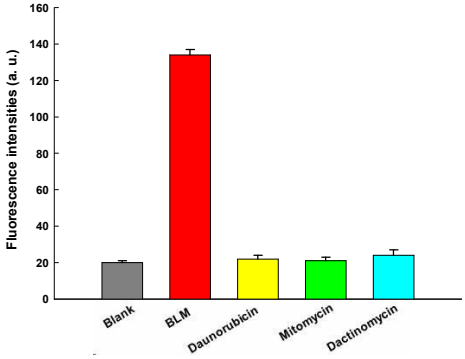



**Fig. 6** Selectivity of the PDI–DNA complex based method for BLM detection. The concentration of BLM was 500 nM, and the concentrations of other antitumor drugs were 1 mM. Error bars were estimated from three replicate measurements.

### Analytical performance for BLM detection

The performance of the method for quantitative analysis of BLM was investigated under the optimized condition. Fig. 5A depicts the fluorescence emission spectra the PDI–DNA complex based method to BLM-Fe(II) at different concentrations. The fluorescence intensity increases dramatically with increasing the concentration of BLM-Fe(II) from 0 to 10 μM, suggesting that the PDI–DNA complex is effective for fluorescence ''turn-on'' detection of BLM *via* BLM-Fe(II)-induced DNA strand scission. Fig. 5B shows that dynamically increased fluorescence intensity at 518 nm upon the addition of BLM-Fe(II) at concentration ranging from 0 to 10 μM. The inset in Figure 5B exhibits a linear relationship to the BLM concentration in the range from 0.5 nM to 100 nM with a correlation coefficient R= 0.9925. The detection limit was estimated to be 0.2 nM (based on 3σ/slope), which is much lower than those of traditional methods. [14, 18] We also compared the BLM assay performance with that of other recently reported methods (Table S1). The data indicated that the present method is fast, and the detection limit is superior or comparable to that of the recently reported methods. The results demonstrated that the present method can be successfully applied to detect the BLM.

A high selectivity is another important parameter for a new designed sensing system with potential applications in practical samples. Thus, we evaluated the selectivity of the designed sensing system by detecting the fluorescence signal changes of other antitumor drugs, including daunorubicin, mitomycin and dactinomycin. The obtained fluorescence intensities by different drugs were compared with BLM, and the results was shown in Fig. 6. Obviously, in contrast to the significant increase of fluorescence intensity induced by BLM at 500 nM, the addition of control drugs at even 1 mM trigger negligible fluorescence changes of the sensing system. These results indicated that the proposed fluorescence sensing system has a good selectivity for BLM assay.

Since the clinical application of BLMs is featured by the occurrence of sometimes fatal side effects, it is very important to quantitatively determine the BLM content in clinical samples. Therefore, in order to evaluate the practicability of



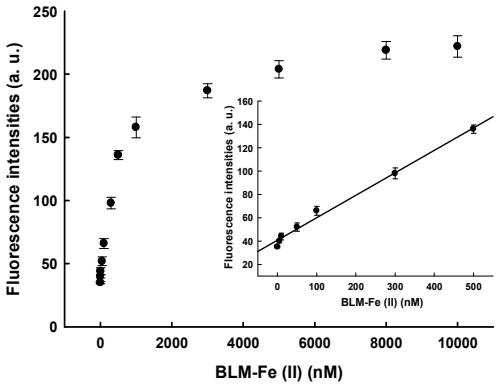



**Fig. 7** The relationship of the fluorescence enhancement with the BLM concentration in diluted human serum samples (1%). Inset is a linear region at low BLM concentrations. Error bars were estimated from three replicate measurements.

the method in complex biological samples, we conducted the detection of BLM in diluted serum samples (1%). As shown in Fig. 7, under these conditions, the background fluorescence increased which may be due to the partially F-ssDNA conformation interference by proteins and other contaminants contained in serum. Fortunately, the target-induced signal enhancement is much larger than the background, and the fluorescence intensity was increased upon the addition of BLM-Fe(II) at concentration ranging from 0 to 10 μM. The inset exhibits a linear relationship to the BLM concentration in the range from 5 nM to 500 nM with a correlation coefficient R= 0.9930. The detection limit was estimated to be 2 nM (based on 3σ/slope). Although the detection limit slightly increased, the calibration curve in the serum samples is similar to that in the buffer solution. Generally, the BLMs are introduced clinically at an atypically low dosage (~5 μM). [42,43] Therefore, the experimental result indicated that the proposed method possesses the promising applications for practical BLM assay in complicated samples.

## Conclusions

In summary, we report a perylene derivative as superquencher constructed PDI–DNA complex method for fluorescence ''turn-on'' detection of BLM, based on BLM-Fe(II) mediated DNA strand scission. The design of the method is based on the remarkable difference in quenching effect of PDI on an oligonucleotide-linked fluorophore containing different number of bases. PDI could be aggregated on F-ssDNA, and efficiently quench the fluorescence of the adjacent conjugated FAM. Upon the addition of BLM-Fe(II) complex, F-ssDNA undergoes an irreversible cleavage event to release the short FAM-linked DNA fragment into the solution which far from the aggregated PDI, resulting in the restoration of fluorescence of the sensing system. Compared with traditional methods, the developed sensing strategy was simple, rapid and easy to implement without tedious procedures. In addition, this method possesses a high sensitivity with a detection limit of 0.2 nM for BLM. Moreover, it also exhibits a high selectivity, and satisfactory performance in complex biological samples was achieved. Given the significance of BLM in cancer therapy and related mechanism research, the proposed strategy offered a potential application of BLM assay in biomedical and clinical study.

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