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A G-triplex based molecular beacon for label-free fluorescence "turn-on" detection of bleomycin†

Lin Ma, Xue Han, Lian Xia, Rong-Mei Kong * and Fengli Qu *

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Since bleomycins (BLMs) play a prominent role in the clinical treatment of various cancers, the development of convenient and sensitive detection assays for BLM is of great significance in cancer therapy and related biological mechanism research. Here, taking advantage of the easily controllable and excitation of the G-triplex DNA structure, we reported a facile, label-free G-triplex based functional molecular beacon (G3MB) sensing system for fluorescence "turn-on" detection of BLM based on BLM-Fe(II) mediated DNA strand scission. In the presence of BLM, the stable hairpin structure of G3MB undergoes an irreversible cleavage in the loop region that contains a 5′-GT-3′ recognition site for BLM. The released G-tract DNA fragment self-assembles into a G-triplex–ThT complex showing a strong fluorescence. Owing to the effective locking of G-tracts in the stem of the G3MB and the specific DNA strand scission by BLM which is like a key for the release of G-tracts, the assay shows high sensitivity and selectivity with a detection limit of 0.2 nM. In addition, satisfactory results were obtained for the detection of BLM in human serum samples. Critically, the convenient "mix-and-detect" protocol, fast response and no need for modifying DNA offered a potential application of the proposed strategy for BLM assay in biomedical and clinical studies.

1. Introduction

Cancer is a generic term for a large group of diseases that can affect any part of the body. It was responsible for 8.8 million deaths (nearly 1 in 6 deaths is due to cancer) in 2015 and the deaths from cancer worldwide are projected to rise continuously, with an estimated 11 million deaths in 2030.[1] The use of antitumor drugs for cancer therapy or to considerably prolong life while improving the patient's quality of life has achieved considerable success in recent years. As a family of naturally occurring glycopeptide-derived antibiotics,[2] bleomycins (BLMs) have been employed as a chemotherapeutic agent for the clinical treatment of several types of cancers with the advantages of low myelosuppression and low immunosuppression.[3–5] The antitumor activity of BLMs has been attributed to their well-characterized sequence selective cleavage of single-stranded (ss) or double-stranded (ds) DNAs and possibly also shape-selective cleavage of some RNAs in the presence of oxygen and metal ions such as Fe(II).[3,6,7] However, the clinical application of BLMs also exhibits some serious dose-limiting side effects which include pulmonary fibrosis and pneumonitis.[8,9] In order to achieve the best therapeutic efficacy and weaken the toxicity, various methods have been developed for quantitatively monitoring the level of BLMs, such as high-performance liquid chromatography (HPLC),[10] enzyme immunoassay (EIA),[11] radioimmunoassay (RIA),[12] micro-biological assay,[13] electrochemical detection,[7,14,15] fluorescence analysis[16–20] and colorimetric assay.[21,22] Among these established methods, signal transduction based on the BLM–Fe(II) complex-induced selective degradation of DNA has been especially attractive in recent years.[7,16–19,23–25] As reported, the BLM–Fe(II) complex reacts with oxygen to generate BLM–Fe(III) OOH species which can selectively degrade DNA *via* C4′–H atom abstraction from deoxyribose in the minor groove of DNA. The selective scission mainly occurred at 5′-GC-3′ or 5′-GT-3′ recognition sites of the DNA strand.[3,26] Based on this scission function, Yin *et al.* reported a BLM electrochemical detection assay based on BLM-induced DNA strand scission with an estimated detection limit down to 100 pM.[7] However, the tedious surface modification of electrodes made electrochemical detection assays time-consuming. Therefore, developing homogeneous assays for BLM has become the current research focus, especially those assays that are based on fluorescence approaches with remarkable features of high sensitivity and facile operation. For example, based on the difference in the affinity of graphene oxide and WS_2 nanosheets for single-stranded DNA containing different numbers of bases in length, Li *et al.* reported a fluorescence analysis method for

College of Chemistry and Chemical Engineering, Qufu Normal University, Qufu Shandong 273165, P. R. China. E-mail: kongrongmei@126.com, fengliquhn@hotmail.com

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BLM using graphene oxide as a fluorescence quencher.[16] Based on a similar strategy, Qin *et al.* developed a WS_2 nanosheet-based fluorescence method for sensitive detection of BLM.[17] Recently, Li *et al.* reported the preparation of aggregation induced emission (AIE) dots and used them for ultrasensitive detection of BLM.[24] Although these fluorescence methods are well-established, the DNA modification enhances the cost and the complexity of experimental operation. Most recently, Liu *et al.* presented a novel label-free biosensor for fluorescence "turn off–on" detection of BLM based on the DNA scission of nitrogen-doped graphene quantum dot adsorbed ssDNA.[27] Zhang *et al.* developed a sensitive and label-free fluorescence method for quantitative detection of BLMs on the basis of BLM-initiated enzymatic polymerization-mediated synthesis of fluorescent CuNPs.[28] However, the nanomaterials required in the above-mentioned methods may need a time-consuming synthesis procedure and thus they become costly. Therefore, the development of a label-free DNA scission based fluorescence method is still imperative for BLM recognition and detection.

A guanine (G)-quadruplex is a well-known kind of four-stranded secondary nucleic acid structure which arises from G-rich DNA and RNA sequences.[29,30] With the non-covalent combination of some cations like Na^+ or K^+, small molecules or some cationic dyes, G-rich DNA sequences are able to fold into quadruplex structures of different topologies that exhibit interesting functions such as catalysis and light-up fluorescence.[31–33] Owing to their biological significance and inherent advantages, G-quadruplexes have been widely exploited as effective responsive or signal components for the construction of nucleic acid related label-free biosensing applications.[34–37] As a commercially available water-soluble fluorogenic dye, thioflavin T (ThT) can selectively induce quadruplex folding in G-rich DNA and consequently generate remarkable fluorescence light-up.[38] Due to the above-mentioned properties as well as low cost and low fluorescence background, a G-quadruplex–ThT complex has been widely used for the development of various reporter systems for sensing applications.[38–41] However, controlling and triggering G-quadruplex structures such as G-quadruplex based label-free molecular beacons (MB) are often encountered with some difficulties. For example, the requirement of a long stem sequence containing "GC" pairs of G-quadruplex based MB always results in the difficulty in designing and operating the MB opening triggered by the targets.[42] Recently, Zhou *et al.* reported a stable G-triplex (G31) which can combine with ThT and function as an efficient label-free fluorescence light-up probe for miRNA detection.[43] Compared to the traditional G-quadruplex based probe, this G-triplex based probe exhibited the advantages of being easy to control and excite which efficiently solve the challenges faced during the designing of G-rich sequence based fluorescence biosensing methods.

Hererin, a label-free and rapid G-triplex based MB (G3MB) sensing system was constructed for sensitive fluorescence "turn-on" detection of BLMs. The MB contains three G-tracts, the first one of which is locked in the MB stem, thereby inhibiting the signal generation of the G-triplex–ThT complex. Under the oxidative effect of BLMs with Fe(II) as a cofactor, G3MB undergoes an irreversible cleavage event in the loop region that contains a 5′-GT-3′ recognition site for BLMs. The released G-tract DNA fragment self-assembles into a G-triplex–ThT complex showing a strong fluorescence. Owing to the stable hairpin structure of the G3MB with locking of one G-tract in the stem, the sensing system shows a low background fluorescence and therefore high sensitivity for BLM detection. The sensing system has a good detection performance in human serum samples. Moreover, this sensing system shows distinct advantages over conventional methods in terms of its no label cost, easy preparation and rapid response, making it a promising alternative approach for the determination of trace amounts of BLMs at the point of care.

2. Experimental section

2.1 Reagents and apparatus

Bleomycin, dactinomycin D, mitomycin C, dactinomycin and daunorubicin were purchased from Melone Pharmaceutical Co., Ltd (Dalian, China). Metal salts including $FeCl_2$, $FeCl_3$, $CuCl_2$, $CaCl_2$, $CoCl_2$, $MnCl_2$, $MgCl_2$, $Pb(NO_3)_2$ and $Zn(Ac)_2$ were obtained from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). All other reagents were of analytical grade and used without further purification. A 25 mM Tris-HCl buffer containing 50 mM KCl (Tris-HCl, pH 7.4) was used in the experiments. All aqueous solutions were prepared with deionized water (resistivity >18 MΩ cm) produced using a Millipore system. DNA oligonucleotides were synthesized and purified by Takara Biotechnology Co. Ltd (Dalian, China), and the sequences are as follows:

G3 5′-TGGGAAGGGAGGG-3′
G31 5′-TGGGTAGGGCGGG-3′
G3MB 5′-TGGGAAGGGAGGGATTAATTTGTTTTTAATCCC-3′
G4MB 5′-TGGGTAGGGCGGGTTGGGATTAATTTGTTTTTA
ATCCC-3′

All fluorescence measurements were carried out on an F-7000 spectrometer (Hitachi, Japan) operated at an excitation wavelength of 430 nm, with both excitation and emission slit widths of 5.0 nm and a PMT detector voltage of 700 V. Circular dichroism (CD) measurements were performed on a MOS-500 spectropolarimeter (Bio-Logic, France) at room temperature. Three scans were accumulated and averaged under the following conditions: range from 200 to 500 nm of the scanning spectrum at 1 nm interval with a scanning speed of 200 nm min^{-1}, 0.5 s response time, 1.0 nm bandwidth and a 0.1 cm path length quartz cuvette. Denaturing PAGE analysis was performed on a BIO-RAD Powerpac Basic Gel electrophoresis apparatus at 120 V for 1.5 h in 1× TAE buffer (pH 8.0, 10 mM KCl), and imaged with a BIO-RAD GelDoc XR+ Gel imager.

2.2 Fluorescence detection of BLMs

The G3MB oligonucleotides were used as received and diluted in Tris-HCl buffer. The BLM–Fe(II) solution was prepared by

mixing BLM with $FeCl_2$ in a 1 : 1 molar ratio. Under the optimized conditions, the reaction sample was prepared by mixing 10 μL of G3MB (2 μM), 10 μL of different concentrations of BLM–Fe(II) aqueous solutions, 20 μL of ThT (60 μM) and 160 μL of Tris-HCl buffer to give a final volume of 200 μL. The solutions were mixed together and incubated at room temperature for 30 min for the oxidative cleavage of G3MB. Then the fluorescence intensity of the final solution was measured in the range from 450 nm to 600 nm with an excitation wavelength at 430 nm. For detecting the BLM in real complex samples, experiments were conducted similar to that in buffer solutions just containing diluted human serum. The human serum samples were obtained from healthy volunteers at the Hospital of Qufu Normal University. All experiments were performed in accordance with the Guidelines of the relevant institutional authorities, and approved by the ethics committee at Qufu Normal University. Informed consents were obtained from human participants of this study.

3. Results and discussion

3.1 Design principle

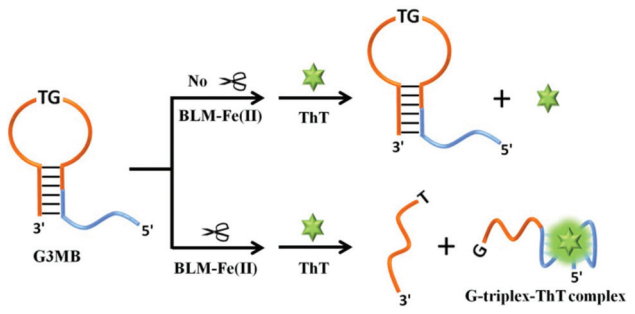
A simple and rapid G3MB sensing system was constructed for sensitive fluorescence "turn-on" detection of BLMs based on the highly specific, metal-induced activation of BLMs, which is presented schematically in Scheme 1. The hairpin structured oligonucleotide (G3MB) contains two function domains: a 5′-GT-3′ site is employed as the substrate for oxidation cleavage in the loop region; the three G-tracts with the first one locked in the MB stem, thereby inhibiting the signal generation of the G-triplex–ThT complex. In the presence of BLM, the G3MB was specifically cleaved at the scission site *via* the oxidative effect of BLM with Fe(II) as the cofactor, and the three G-tracts were then released. Thus, ThT could induce the released G-tract DNA fragment to self-assemble into a G-triplex–ThT complex with a strong fluorescence enhancement. Because the fluorescence enhancement was dependent on the amount of the released G-tract DNA fragment, which in turn is dependent on the concentration of BLM, this cleavage event can be applied to the fluorescence detection of BLMs with high sensitivity and specificity.

3.2. Feasibility assay

The proposed facile assay for fluorescence "turn-on" and sensitive sensing of BLM is based on the specific scission of G3MB and thus the release of the G-tract DNA fragment which can self-assemble into a G-triplex–ThT complex with a strong fluorescence emission. In order to design a suitable DNA sequence to determine BLMs, the G-triplex sequence should not contain 5′-GT/C-3′ in case it is cleaved by BLM and cannot form the G-triplex–ThT complex. Therefore, we have tried to change the G-triplex sequence G31 (5′-TGGGTAGGGCGGG-3′) that was developed by Zhou *et al.*[43] to G3 (5′-TGGGAAGGGAGGG-3′) in our work. Herein, the fluorescence of the new G3 sequence–ThT complex was first tested to investigate the design feasibility of the change. As shown in Fig. 1A, the G3–ThT complex exhibits strong fluorescence emission at 490 nm which is obviously higher than that of the G31–ThT complex, indicating the success of the change for new G3 sequence.

Then, the fluorescence emission spectra under different conditions were investigated to prove the feasibility of the G3MB sensing system for fluorescence "turn-on" detection of BLMs. As shown in Fig. 1B, the ThT exhibits very weak fluorescence in Tris-HCl buffer (curve a), and the mixture of G3MB and ThT induces almost no fluorescence signal changes (curve b), indicating that the three G-tracts are effectively locked in the MB stem and thus no G-triplex–ThT complex is generated. However, upon incubation with BLM–Fe(II), the fluorescence sharply increased (curve c), indicating the occurrence of BLM–Fe(II) induced strand scission in the loop region of G3MB to release the G-tract DNA fragment which can self-assemble into a G-triplex–ThT complex with a strong fluorescence emission. These results indicate that the G3MB based sensing system holds the promise for label-free fluorescence "turn-on" detection of BLMs.

In addition, 20% denaturing PAGE analysis was performed to verify the cleavage of the 5′-GT-3′ site in the loop region of G3MB by BLM–Fe(II). As shown in Fig. 2A, the G3MB exhibits only one DNA band in the gel (lane 1). However, the presence of BLM–Fe(II) successfully triggered the cleavage of G3MB to give two distinct DNA fragment bands (lane 2), corresponding to the scission products 5′-TGGGAAGGGAGGGATTAATTTG-3′ and 5′-TTTTTAATCCC-3′, respectively. The electrophoresis result is consistent with the mechanism (Scheme 1), indicating the high efficiency of the DNA scission based G3MB sensing



Scheme 1 Schematic illustration of the G3MB sensing system for BLM detection.

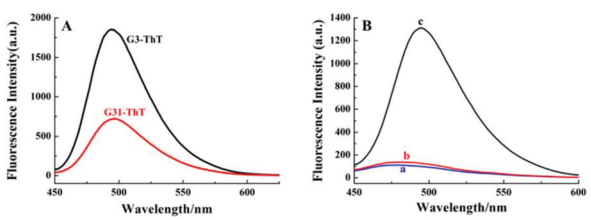


Fig. 1 (A) Fluorescence emission spectra of ThT (6 μM) in the presence of G3 (100 nM) and G31 (100 nM). (B) Fluorescence emission spectra of the G3MB sensing system under different conditions: (a) ThT, (b) G3MB + ThT, and (c) G3MB + BLM–Fe(II) + ThT. The concentrations of ThT, G3MB and BLM–Fe(II) are 6 μM, 100 nM, and 5 μM, respectively.

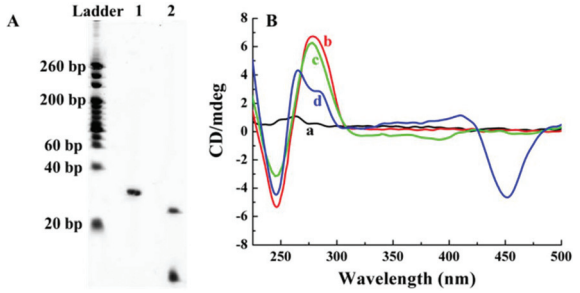


Fig. 2 (A) 20% denaturing PAGE analysis of G3MB and G3MB is disposed by BLM Fe(II). Lane 1: G3MB; lane 2: G3MB added BLM–Fe(II); the concentrations of G3MB and BLM–Fe(II) are about 3.0 μM and 50 μM, respectively. (B) CD spectra monitoring the conformational transition of G3MB in Tris-HCl buffer: (a) ThT, (b) G3MB, (c) G3MB + ThT, and (d) G3MB + BLM–Fe(II) + ThT. The concentrations of ThT, G3MB and BLM–Fe(II) are 100 μM, 10 μM, and 50 μM, respectively.

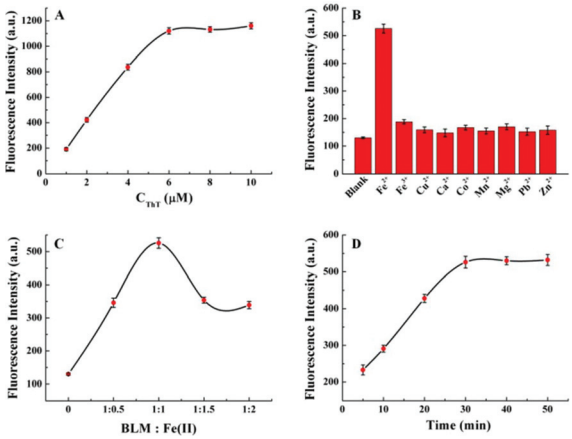


Fig. 3 Effects of (A) ThT concentration, (B) metal ions, (C) BLM and Fe(II) molar ratios and (D) reaction time on the fluorescence intensity at 490 nm. Error bars were estimated from three replicate measurements.

system for the label-free detection of BLMs. Circular dichroism (CD) spectroscopy was further conducted to demonstrate the structural changes of G3MB in the absence and presence of BLM–Fe(II). As shown in Fig. 2B, the CD spectrum of G3MB exhibits a positive peak at 278 nm and a negative peak at 240 nm, which are the characteristic CD spectrum signals of single stranded or double stranded DNA.[44] There is no obvious change in the CD spectrum after the addition of ThT into the solution of G3MB, indicating the effective locking of the G-tracts in G3MB and no G-triplex can be generated by the inducement of ThT. However, with the addition of BLM–Fe(II) into the mixture solution of G3MB and ThT, the CD spectrum shows an obvious positive peak at approximately 265 nm and a ThT binding band at about 425 nm. The result suggests that a parallel G-triplex is formed due to the release of the G-tract DNA fragment by the BLM–Fe(II) induced effective oxidation scission for the G3MB strand.[43]

3.3. Optimization of assay conditions

To achieve the best analytical performance, several important experimental conditions were optimized before the standard BLM assay. First, we optimized the concentration of ThT by fixing the concentration of the BLM–Fe(II) complex in a 1 : 1 molar ratio at 2 μM and the reaction time at 30 min while varying the concentration of ThT. As shown in Fig. 3A, the fluorescence signal is enhanced significantly as the ThT concentration is increased, and little change is observed along with the concentration of ThT exceeding 6 μM. Hence, 6 μM was chosen as the optimal ThT concentration in the following experiments. It is well known that the DNA lesions by BLMs which are responsible for tumor necrosis require a reduced transition metal and oxygen. Therefore, the effect of different metal ion coordination environments for BLM-mediated DNA cleavage was investigated. The BLM samples were prepared by mixing 500 nM BLMs with different metal ions in a 1 : 1 molar ratio. Fig. 3B shows the fluorescence changes of the G3MB sensing system upon the treatment with different BLM–metal ion (Fe^{2+}, Fe^{3+}, Cu^{2+}, Ca^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+}, Pb^{2+} and Zn^{2+}) mixtures, respectively. As expected, the BLM–Fe(II) combination which is significantly less toxic than other BLM–metal ion combinations at therapeutically effective concentrations *in vivo*[45] induces the best performance of a significant fluorescence enhancement. Meanwhile, the effect of Fe(II) alone on the G3MB sensing system was also investigated as a control experiment. A negligible fluorescence change can be observed with the introduction of Fe(II) even at a high concentration of 10 μM (Fig. S1†), indicating the necessity of BLM for the DNA scission. Moreover, the effects of BLM (500 nM) and Fe(II) ratios and the reaction time on the detection performance were also investigated. The BLM and Fe(II) molar ratio of 1 : 1 gives the largest fluorescence response (Fig. 3C), and the maximum fluorescence response to BLM can be achieved at 30 min and then reached a plateau (Fig. 3D). Therefore, the BLM and Fe(II) ratio of 1 : 1 and the reaction time of 30 min were selected in subsequent experiments.

3.4. Analytical performance for the detection of BLM

The quantitative analysis capacity of the developed G3MB sensing system was investigated under optimal conditions. Fig. 4A shows the fluorescence emission spectra corresponding to different BLM–Fe(II) concentrations. It can be clearly seen that the fluorescence intensity augmented significantly with the increase of BLM concentration ranging from 0 to 10 μM, which can be ascribed to the fact that more BLM–Fe(II)-induced DNA strand scission impelled the generation of more G-triplex-ThT complexes, thus showing enhanced fluorescence. However, an abnormal phenomenon is observed that the fluorescence intensity decreases along with the concentration of BLM–Fe(II) exceeding 10 μM (data not shown). It is probably due to that the accumulation of Fe(III) which is the oxidation product of Fe(II) by oxygen potentially inhibits the DNA scission.[16] Fig. 4B shows that the fluorescence intensity of the sensing system varies linearly with the BLM concentration in the range from 0.5 nM to 1000 nM with the linear regression

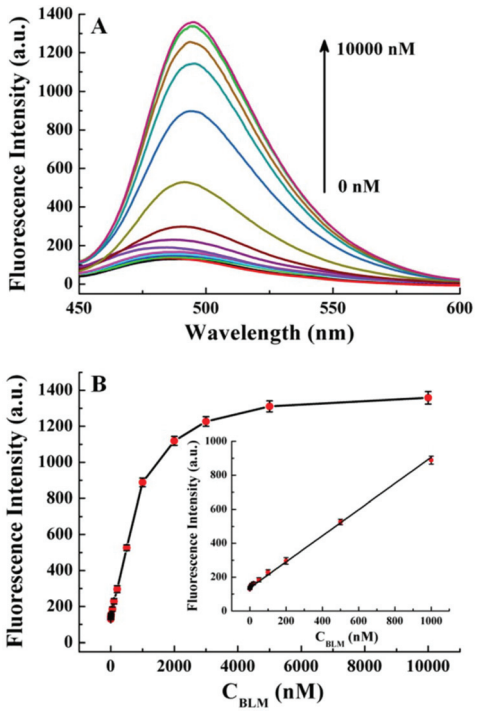


Fig. 4 (A) The fluorescence spectra of the G3MB sensing system for the detection of 0, 0.5, 1, 5, 10, 20, 50, 100, 200, 500, 1000, 2000, 3000, 5000 and 10 000 nM BLM. (B) The relationship between BLM concentration and fluorescence intensity at 490 nm. Inset: The linear region at low BLM concentrations. Error bars were estimated from three replicate measurements.

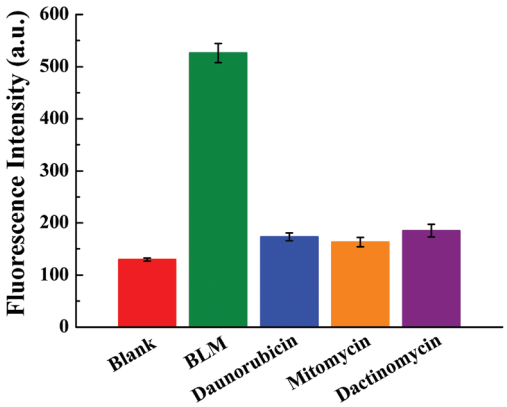


Fig. 5 Selectivity of the G3MB sensing system for BLM detection. The concentration of BLM is 500 nM, and the concentrations of the interfering substances are all 5 μM. Error bars were estimated from three replicate measurements.

equation $F = 131.6 + 0.7693C_{BLM}$ (nM) ($R^2 = 0.9907$), where F is the fluorescence intensity at 490 nm of the G3MB sensing system. The detection limit can be reached as low as 0.2 nM estimated based on 3σ/slope. Based on the same design principle, a G-quadruplex based MB (G4MB) sensing system for BLM detection was investigated. As shown in Fig. S2,† the G4MB sensing system exhibits a very high fluorescence background while low fluorescence responses for BLM detection, indicating the poor analysis performance of this G4MB sensing system. Moreover, when comparing the analysis performance (including assay time, detection limit and linear detection range) with other recently reported methods (Table S1†), this G3MB sensing strategy is fast and its sensitivity is superior or comparable to most reported strategies. More importantly, our strategy is label-free and fluorescence "turn-on" for BLM detection which endows this strategy with many advantages such as low cost, simple operation and thus more practical compared to that of labeled DNA.

Selectivity is another important parameter for a newly developed detection method, so evaluating the specificity of the proposed biosensor is an indispensable part of the assay. The fluorescence changes as induced by other antitumor drugs were investigated. Three antitumor drugs including mitomycin, daunorubicin and dactinomycin, which are often used in combination with the application of BLM in clinical practice, were selected for experiment of selectivity. As shown in Fig. 5, compared to a significant increase of the fluorescence intensity of the sensing system upon the addition of BLM (500 nM), negligible changes in fluorescence intensity are observed when other antitumor drugs were added into the G3MB system, even though at the concentration 10 times as that of BLM. The high selectivity of this G3MB sensing system for BLM detection might be owing to the following reasons. On one hand, the G3MB design is relying on the specific DNA scission at a recognition site of 5′-GT-3′ by the BLM-Fe(II) complex.[3,26] On the other hand, the mechanisms of the other three tested antitumor drugs are different from BLM. Daunorubicin can selectively intercalate in the small groove of the DNA double helix, thus hindering the function of RNA polymerase and inhibiting the synthesis of RNA, especially the synthesis of mRNA;[46] mitomycin forms crosslinks with DNA strands and functions as a alkylating agent to inhibit DNA replication;[47] dactinomycin is a DNA-binding small molecule which leads to the disorder of the DNA spatial structure, thus inhibiting DNA and DNA-dependent RNA synthesis.[48]

3.5. Detection of BLM in human serum samples

In order to evaluate the potential practical application, we utilized the designed fluorescent system to measure BLM in human serum recognized as a complex biological fluid. The potential interfering proteins in serum were eliminated by adding acetonitrile to serum samples and centrifuged at 8000 rpm for 5 min before the BLM assay. The recovery experiments were performed by spiking BLM–Fe(II) with different concentrations into 20% human serum samples according to the same detection procedure as that in Tris-HCl buffer. As shown in Table 1, the measured values of BLM concentrations are kept consistent with the added amounts, with the recoveries in the range of 95.5–104.7%, implying no severe interference in human serum samples. Moreover, the relative standard deviations (RSDs) are all below 4.3%, demonstrating the validity of the developed method. These results are highly encouraging as they demonstrate the great potential of this method for

Table 1 Detection of BLM spiked in human serum samples

Sample	Added (nM)	Found (nM)	Recovery (%)	RSD (n = 3, %)
Serum I	20.0	20.5	102.5	4.3
	100.0	103.6	103.6	3.9
	500.0	496.6	99.3	2.8
Serum II	20.0	19.1	95.5	4.2
	100.0	104.7	104.7	3.7
	500.0	513.4	102.7	4.2

specific detection of BLM in complicated samples. Moreover, as the amount of BLM present in the real sample is unknown, the detection of BLM spiked in human serum samples with one single Fe(II) concentration (100 nM) was performed to verify the suitability of the G3MB sensing system. As shown in Table S2,† the BLM recovery results of less than or equal to 100 nM are consistent with those of the optimal BLM and Fe(II) molar ratio of 1 : 1. However, the obtained amount of BLM for a spiked high concentration (500 nM) is similar to that of BLM at 100 nM, indicating that the excess Fe(II) has less impact on the detection results, while the deficiency of Fe(II) would cause lower recovery. Therefore, in practical applications, a little bit more Fe(II) can be added in the real samples to ensure the complete detection of BLM.

4. Conclusions

In summary, we demonstrated a facile, label-free G-triplex based functional molecular beacon (G3MB) sensing system for fluorescence "turn-on" detection of BLMs based on BLM–Fe(II) mediated DNA strand scission. The unique feature of the G-triplex that it is easy to control and excite "light-up" by specific binding to the small molecule ThT made this novel assay not only sensitive and reliable but also general to be adopted for the analysis of other DNA related biological targets. The G3MB presented here offered a convenient "mix-and-detect" protocol for homogeneous and rapid detection within 30 min, while possessing a high sensitivity with a detection limit of 0.2 nM for BLMs. Moreover, the application of the sensing system to the determination of BLMs in diluted human serum also showed satisfactory performance due its high selectivity. Given the advantages including simple in design, fast in operation and high sensitivity and selectivity, the proposed strategy offered a potential application of the BLM assay in biomedical and clinical studies.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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