ChemComm

RSC Publishing

COMMUNICATION

Ultrasensitive fluorescence detection of bleomycin *via* exonuclease III-aided DNA recycling amplification†

Cite this: *Chem. Commun.*, 2013, **49**, 7561

Received 6th June 2013,
Accepted 28th June 2013

DOI: 10.1039/c3cc44275f

www.rsc.org/chemcomm

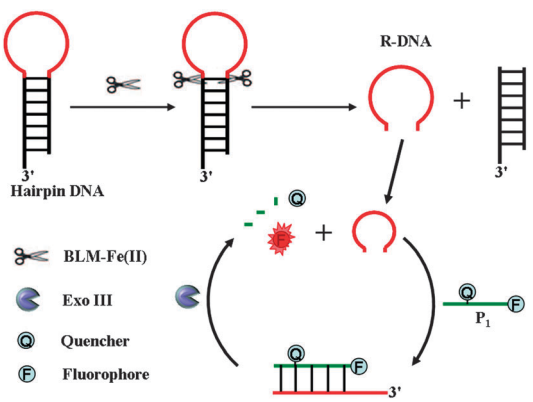
Fenglei Gao, Jianping Lei and Huangxian Ju*

A "signal on" approach for ultrasensitive detection of bleomycin was developed by bleomycin–Fe(II) induced hairpin DNA scission to release its loop, which was subsequently recycled with the aid of exonuclease III and a probe to amplify the detectable fluorescent signal.

Bleomycin (BLM) is a family of glycopeptide-derived antibiotics produced by several *Streptomyces* species, and has been used as chemotherapeutic agents in the clinical treatment of certain cancers.[1] Its molecule contains four functional domains (ESI,† Fig. S1). Its dose-limiting side effect possibly results from the renal and lung toxicity, which can cause bad pulmonary fibrosis.[2] Conventionally, to achieve the best therapeutic effect and quantitatively monitor the level of BLM, some reliable methods such as high performance liquid chromatography,[3] immunoassay,[4,5] and microbiological assay[6] have been developed. BLM–Fe(II) can selectively degradate DNA *via* C4′–H atom abstraction from deoxyribose in the minor groove of DNA.[1c,d] By using the scission function of BLM–Fe(II) at 5′-GC-3′ or 5′-GT-3′ site of the DNA strand,[7] an electrochemical method for BLM detection down to 100 pM has been proposed with ferrocene as a redox tag.[8] The BLM–Fe(II) induced DNA strand scission has also been combined with a fluorophore labeled single-stranded DNA to design a method for fluorescence detection of BLM by different affinities of graphene oxide to DNA strands with different lengths. To meet the requirements for BLM detection at the lower level, this work designed an ultrasensitive "signal on" approach by introducing for the first time a signal amplification technique into the BLM–Fe(II) induced DNA strand scission. As a proof-of-concept, a hairpin DNA with 5′-GC-3′ sequence was first used for recognizing BLM–Fe(II) and then performing a signal amplification process *via* DNA recycling in the presence of a fluorophore and its quencher co-labeled DNA probe and exonuclease III (Exo III).

DNA recycling oriented amplification, in which one DNA strand can be repeatedly utilized to trigger multiple signal probes, has been widely applied for sensitive detection of DNA,[10] RNA,[11] proteins[12] and metal ions,[13] because it successfully overcomes the inherent limitation of the target-to-signal ratio of 1 : 1 in the traditional assay. Typically, the DNA cycling procedure can be performed *via* nuclease *e.g.* polymerase,[14] endonuclease[15] and exonuclease III (Exo III).[16] Among them, Exo III is frequently used because of its easy availability, excellent cyclic efficiency and the wide applicability that does not require a specific recognition site and can catalyze the stepwise removal of mononucleotides from the blunt 3′ terminus of double-stranded DNA. Exo III-assisted DNA recycling has been applied for sensitive detection of various biomolecules in combination with molecular beacons,[17a] quantum dots[17b] and graphene oxide.[17c] This work used the product of BLM–Fe(II) induced molecular beacon scission to trigger the Exo III assisted DNA recycling, and thus led to a novel concept for ultrasensitive fluorescence detection of the scission agent, BLM–Fe(II), which is quickly formed by the *in situ* complex of BLM with Fe^{2+} and used for DNA degradation (Scheme 1).

First, a specific hairpin DNA was designed with two 5′-GC-3′ sequences in its stem. Upon the complex of BLM with Fe(II), BLM–Fe(II) induced hairpin DNA strand scission was triggered at the 5′-GC-3′ sites of the stem, and the loop of hairpin DNA



Scheme 1 Schematic representation of fluorescence detection strategy of BLM *via* BLM–Fe(II) induced scission coupled with Exo III-aided DNA recycling amplification. F and Q are FAM and BHQ, respectively.

State Key Laboratory of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210093, P. R. China. E-mail: hxju@nju.edu.cn; Fax: +86 25 83593593; Tel: +86 25 83593593

† Electronic supplementary information (ESI) available: Experimental details, structure of bleomycin and BLM detection without Exo III. See DOI: 10.1039/c3cc44275f

(R-DNA) was released to hybridize with the probe 1 (P_1), which is modified with a carboxyfluorescein (FAM) fluorophore at its 5′ terminus and a black hole quencher (BHQ) at an internal position, to form a double-stranded structure. Subsequently, Exo III catalyzed the stepwise removal of P_1 from the blunt 3′ terminus to liberate the fluorophore and ultimately released the R-DNA. In this manner, the recycling of R-DNA was formed by the digestion of Exo III to accumulate free fluorophores. Therefore, the enhanced fluorescence signal was obtained, providing a possibility for ultrasensitive detection of BLM–Fe(II) and thus BLM.

In order to evaluate the amplification ability of the proposed assay system, the fluorescence intensity of the assay system was tested. As shown in Fig. 1, P_1 exhibited a weak fluorescence emission since the fluorescence of FAM was quenched by the BHQ (curve a), which were separated by 6 bases. Upon the addition of Exo III into P_1 solution, the fluorescence intensity slightly increased (curve b), which was attributed to the fact that Exo III also digested a small part of single-stranded P_1. After adding hairpin DNA to this system, no fluorescence change was observed (curve c), indicating P_1 could not completely hybridize with the loop part of hairpin DNA to form double-stranded DNA due to the limitation of space conformation, which led to the absence of the recognition site of Exo III. Upon incubation with BLM–Fe(II), the fluorescence sharply increased (curve d), indicating the occurrence of BLM–Fe(II) induced strand scission in the hairpin DNA to release the R-DNA and then to initiate the Exo III-added DNA recycling, which led to the separation of the FAM fluorophore from its quencher and thus an enhanced fluorescence signal. The BLM–Fe(II)-related DNA recycling produced a highly sensitive method for the detection of BLM.

Polyacrylamide gel electrophoresis (PAGE) analysis was used to investigate the viability of the assay strategy (inset in Fig. 1). Compared with one band of hairpin DNA (lane a), the mixture of hairpin DNA and BLM–Fe(II) showed two bands at longer electrophoresis distances (lane b), indicating the occurrence of BLM–Fe(II) induced strand scission to produce two shorter DNA strands, two different parts of the hairpin DNA. The faster band corresponded to the released single stranded R-DNA and the later band corresponded to the double stranded stem part of the hairpin DNA.

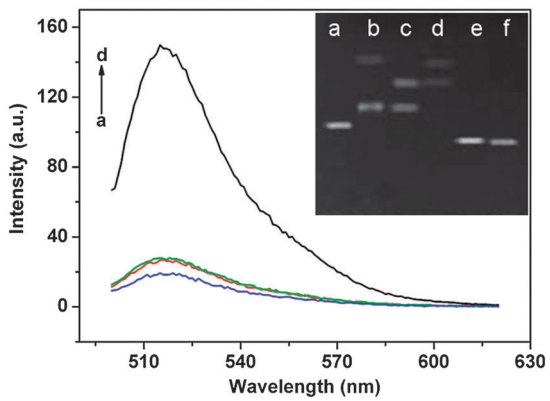


Fig. 1 Fluorescence spectra of 1.0 μmol L^{-1} P_1 (a), a + 5 U Exo III (b), b + 1.0 μmol L^{-1} hairpin DNA (c), c + 10.0 nmol L^{-1} BLM–Fe(II) for 30 min (d). Inset: PAGE analysis of 1 μmol L^{-1} hairpin DNA (a), a + 1.0 μmol L^{-1} BLM–Fe(II) (b), b + 1 μmol L^{-1} P_1 (c), c + 5 U Exo III (d), a + 1 μmol L^{-1} P_1 (e) and e + 5 U Exo III (f).

After adding P_1 with the same amount as hairpin DNA into the mixture of BLM–Fe(II) and hairpin DNA, P_1 hybridized R-DNA to form dsDNA, which lowered the electrophoresis rate of R-DNA and thus showed a band of the dsDNA at a shorter distance (lane c). Upon addition of Exo III to the mixture, the band corresponding to the double stranded stem disappeared and a new band corresponding to the single stranded stem was observed at a longer distance, while the band corresponding to R-DNA occurred again (lane d). These appearances resulted from the degradation of one strand in the dsDNA by Exo III. Thus the high scission efficiency of Exo III to the blunt 3′ terminus of duplex DNAs for DNA recycling was confirmed. On the other hand, the mixture of hairpin DNA and P_1 showed one band (lane e) at a position shorter than the hairpin DNA, indicating P_1 could hybridize with the loop of the hairpin DNA in spite of the unfavourable conformation. After Exo III was added to the mixture of hairpin DNA and P_1, the band observed in lane e did not change (land f), indicating that the formed double-stranded DNA did not contain Exo III recognising blunt 3′ termini. Thus the hybridization of P_1 to the loop part of the hairpin DNA was incomplete.

To obtain the good performance of the detection system, the amount of Exo III and the time for both BLM–Fe(II) induced scission and Exo III assisted DNA recycling were optimized at 1.0 nmol L^{-1} BLM–Fe(II). At a reaction time of 1 h, the ratio of signal to background increased significantly with the increasing Exo III concentration and reached a maximum value at 5 U (Fig. 2A). Therefore, 5 U was chosen as the optimum concentration of Exo III, at which the ratio of signal to background increased with the increasing reaction time up to 30 min (Fig. 2B). Therefore, 30 min was used for simultaneous scission of hairpin DNA and DNA recycling.

Under the optimal assay conditions, the fluorescence intensity increased dramatically with the increasing concentration of BLM–Fe(II) from 0.1 pmol L^{-1} to 100 nmol L^{-1} (Fig. 3), suggesting that the DNA probes were effective for fluorescence "signal-on" detection of BLM. The fluorescent intensity change ($F - F_0$) of the detection system varied linearly with the logarithm value of BLM–Fe(II) concentration from 1 pmol L^{-1} to 10 nmol L^{-1} (inset in Fig. 3). The detection limit was estimated to be 0.38 pmol L^{-1} at 3σ. Here, F_0 and F are the fluorescence intensity detected in the absence and presence of BLM–Fe(II), respectively. The detection limit was lower than those of electrochemical (100 pmol L^{-1})[8] and fluorescence (200 pmol L^{-1}) methods.[9]

To confirm the cause of the high sensitivity, a control experiment in the absence of Exo III was carried out by using

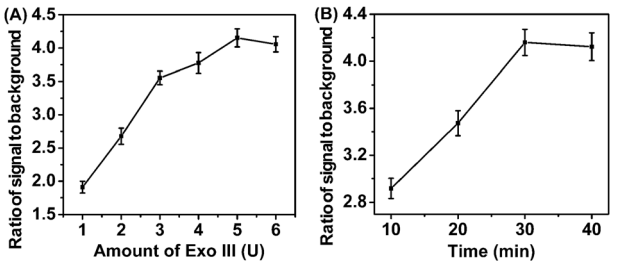


Fig. 2 Dependence of the ratio of signal to background for 1 nmol L^{-1} BLM–Fe(II) on (A) the amount of Exo III and (B) the reaction time. When one parameter changes the others are under their optimal conditions.

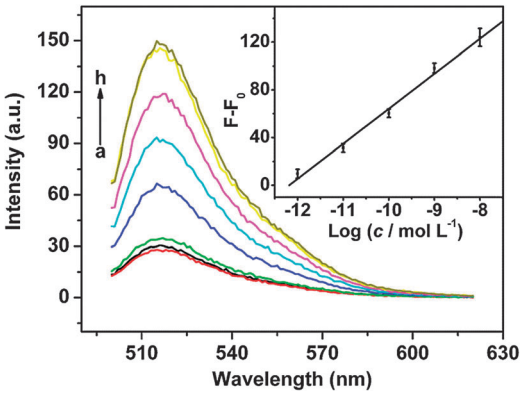


Fig. 3 Fluorescence spectra for 0, 10^{-13}, 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8} and 10^{-7} mol L^{-1} target BLM-Fe(II) (from a to h) *via* BLM–Fe(II) induced scission coupled with Exo III-aided DNA recycling amplification. Inset: linear calibration of $F - F_0$ *vs.* logarithm of BLM–Fe(II) concentration.

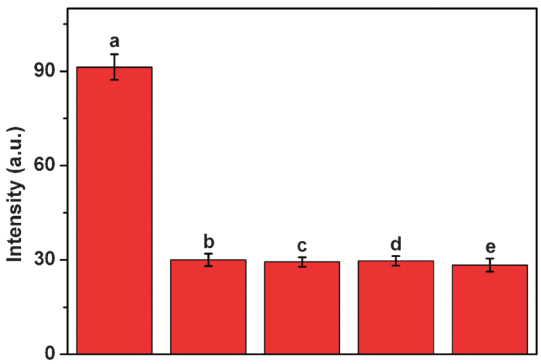


Fig. 4 Histograms of fluorescence intensity for 0.1 nmol L^{-1} BLM (a), daunorubicin (b), mitomycin (c), dactinomycin (d) and blank (e).

probe 2 (P_2) instead of both hairpin DNA and P_1. P_2 included 5′-GC sequence as the recognition site for BLM scission to restore the fluorescence of FAM. As shown in Fig. S2 (ESI†), the fluorescent intensity changed linearly increased with the increasing logarithm value of BLM–Fe(II) concentration in the range from 0.1 nmol L^{-1} to 1 μmol L^{-1} with a detection limit of 23 pmol L^{-1}, which was about 60 times higher than that obtained in the presence of Exo III. At the same target concentrations of 0.1 and 1.0 nmol L^{-1}, the fluorescent intensity in the presence of Exo III was 3.2 and 2.5 times higher than that in the absence of Exo III, respectively. Therefore, the high sensitivity was attributed to the Exo III-aided R-DNA recycling.

The selectivity of the designed method was evaluated by the detection of three antitumor drugs (daunorubicin, mitomycin and dactinomycin) at the same concentration. The peak intensity at 517 nm for BLM, daunorubicin, mitomycin and dactinomycin was about 3.22, 1.06, 1.04, and 1.05 times higher than the background signal, respectively (Fig. 4). The results showed that only BLM induced a significant fluorescence signal increase, indicating that this method has good selectivity for discriminating BLM against other antitumor drugs. To test the generality of our proposed assay in the clinical sample, recovery testing was carried out by spiking BLM–Fe(II) solution into human serum. At the concentration of 10^{-11} and 10^{-9} mol L^{-1}, the recoveries were 97.0% and 104% (n = 3), indicating that the proposed fluorescent strategy for BLM could be used in real sample analysis.

In conclusion, this work presented a novel concept of Exo III-aided DNA recycling amplification triggered by the BLM–Fe(II) induced scission product for ultrasensitive fluorescence detection of the scission agent. The scission and signal amplification strategy could be completed in 30 min. This strategy showed a detection limit down to the sub-picomolar level, which was about 2 orders of magnitude lower than that of the conventional fluorescent analysis. The proposed assay exhibited high selectivity for BLM against other three important antitumor antibiotics, and satisfactory performance in trace BLM determination in serum samples. This designed method offered an interesting alternative approach for the determination of trace amounts of BLM in clinical diagnosis.

This work was funded by National Basic Research Program of China (2010CB732400), National Natural Science Foundation of China (21121091, 21135002), and Excellent Talents in Chinese Universities (NCET100479).

Notes and references

1 (*a*) U. Galm, M. H. Hager, S. G. Van Lanen, J. Ju, J. S. Thorson and B. Shen, *Chem. Rev.*, 2005, **105**, 739–758; (*b*) Z. Q. Yu, R. M. Schmaltz, T. C. Bozeman, R. Paul, M. J. Rishel, K. S. Tsosie and S. M. Hecht, *J. Am. Chem. Soc.*, 2013, **135**, 2883–2886; (*c*) Y. Akiyama, Q. Ma, E. Edgar, A. Laikhter and S. M. Hecht, *J. Am. Chem. Soc.*, 2008, **130**, 9650–9651; (*d*) R. A. Giroux and S. M. Hecht, *J. Am. Chem. Soc.*, 2010, **132**, 16987–16996.
2 J. Liu, Z. Liu, X. Hu, L. Kong and S. Liu, *Luminescence*, 2008, **23**, 1–6.
3 R. P. Klett and J. P. Chovan, *J. Chromatogr.*, 1985, **337**, 182–186.
4 S. T. Crooke, F. Luft, A. Broughton, J. Strong, K. Casson and L. Einhorn, *Cancer*, 1977, **39**, 1430–1434.
5 K. Fujiwara, M. Isobe, H. Saikusa, H. Nakamura, T. Kitagawa and S. Takahashi, *Cancer Treat. Rep.*, 1983, **67**, 363–369.
6 T. Onuma, J. F. Holland, H. Masuda, J. A. Waligunda and G. A. Goldberg, *Cancer*, 1974, **33**, 1230–1238.
7 Q. Ma, Y. Akiyama, Z. Xu, K. Konishi and S. M. Hecht, *J. Am. Chem. Soc.*, 2009, **131**, 2013–2022.
8 B. C. Yin, D. Wu and B. C. Ye, *Anal. Chem.*, 2010, **82**, 8272–8277.
9 F. Li, Y. Feng, C. Zhao, P. Li and B. Tang, *Chem. Commun.*, 2012, **48**, 127–129.
10 (*a*) Y. Chen, Q. Wang, J. Xu, Y. Xiang, R. Yuan and Y. Q. Chai, *Chem. Commun.*, 2013, **49**, 2052–2054; (*b*) W. Shen, H. M. Deng and Z. Q. Gao, *J. Am. Chem. Soc.*, 2012, **134**, 14678–14681; (*c*) H. Q. Zhang, F. Li, B. Dever, X. F. Li and X. C. Le, *Chem. Rev.*, 2013, **113**, 2812–2841.
11 (*a*) H. X. Jia, Z. P. Li, C. H. Liu and Y. Q. Cheng, *Angew. Chem., Int. Ed.*, 2010, **49**, 5498–5501; (*b*) R. X. Duan, X. L. Zuo, S. T. Wang, X. Y. Quan, D. L. Chen, Z. F. Chen, L. Jiang, C. H. Fan and F. Xia, *J. Am. Chem. Soc.*, 2013, **135**, 4604–4607.
12 (*a*) Z. Z. Zhang and C. Y. Zhang, *Anal. Chem.*, 2012, **84**, 1623–1629; (*b*) X. H. Zhao, L. Gong, X. B. Zhang, B. Yang, T. Fu, R. Hu, W. H. Tan and R. Q. Yu, *Anal. Chem.*, 2013, **85**, 3614–3620.
13 (*a*) L. Qi, Y. Zhao, H. Yuan, K. Bai, Y. Zhao, F. Chen, Y. H. Dong and Y. Y. Wu, *Analyst*, 2012, **137**, 2799–2805; (*b*) Y. X. Zhao, L. Qi, W. J. Yang, S. Wei and Y. L. Wang, *Chin. J. Anal. Chem.*, 2012, **40**, 1236–1240.
14 (*a*) Q. P. Guo, X. H. Yang, K. M. Wang, W. H. Tan, W. Li, H. X. Tang and H. M. Li, *Nucleic Acids Res.*, 2009, **37**, e20; (*b*) R. Ren, C. C. Leng and S. S. Zhang, *Chem. Commun.*, 2010, **46**, 5758–5760.
15 W. Xu, X. J. Xue, T. H. Li, H. Q. Zeng and X. G. Liu, *Angew. Chem., Int. Ed.*, 2009, **48**, 6849–6852.
16 (*a*) F. Xuan, X. T. Luo and I. M. Hsing, *Anal. Chem.*, 2012, **84**, 5216–5220; (*b*) S. F. Liu, C. F. Wang, C. X. Zhang, Y. Wang and B. Tang, *Anal. Chem.*, 2013, **85**, 2282–2288; (*c*) C. H. Lu, F. Wang and I. Willner, *Chem. Sci.*, 2012, **3**, 2616–2622.
17 (*a*) X. L. Zuo, F. Xia, Y. Xiao and K. W. Plaxco, *J. Am. Chem. Soc.*, 2010, **132**, 1816–1868; (*b*) R. Freeman, X. Liu and I. Willner, *Nano Lett.*, 2011, **11**, 4456–4461; (*c*) P. Hu, C. Z. Zhu, L. H. Jin and S. J. Dong, *Biosens. Bioelectron.*, 2012, **34**, 83–87.