Sensors and Actuators B 238 (2017) 318–324

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

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

A bicyclo-hairpin probe mediated strand displacement amplification strategy for label-free and sensitive detection of bleomycin

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ARTICLE INFO

Article history:
Received 27 May 2016
Received in revised form 6 July 2016
Accepted 13 July 2016
Available online 15 July 2016

Keywords:
Bicyclo-hairpin probe
Strand displacement amplification
Label-free
Bleomycin

ABSTRACT

Monitoring of bleomycin concentration is critical in clinical therapy. Here, a bicyclo-hairpin probe mediated strand displacement amplification strategy was developed for bleomycin detection. Firstly, bleomycin cleaved bicyclo-hairpin probe to release a trigger sequence. Then the trigger sequence initiated strand displacement amplification reaction to produce numerous G-quadruplex forming sequences. Finally, G-quadruplexes bound with *N*-methyl mesoporphyrin IX molecules and an enhanced fluorescent signal could be detected. In this strategy, a bicyclo-hairpin probe was ingeniously designed for bleomycin detection, which could silence the trigger sequence to reduce non-specific hybridization of detection method. In addition, based on bleomycin-induced DNA scission, a label-free fluorescent sensor for bleomycin detection was established. Our strategy could determine bleomycin sensitively with a detection limit of 0.34 nM. Fluorescent response of detection system toward four different anti-tumor drugs proved that this strategy had admirable selectivity. Satisfactory recoveries in human serum samples revealed that our method had a great potential in real sample assay.

1. Introduction

Bleomycin (BLM) is a family of glycopeptide-derived antibiotics produced by *streptomyces verticillus* [1,2]. It has been used as chemotherapeutic agents in clinical treatment of certain cancers and its antitumor activity is generally believed to be related to destroying the single-stranded or double-stranded DNA structure of carcinoma cells [3–5]. The clinical application of BLM is featured by the dose-limiting toxicity such as renal toxicity, lung toxicity and pulmonary fibrosis [6,7]. Thus, in order to quantitatively monitor the BLM level, weaken the toxicity and achieve the best therapeutic effect, various methods such as high-performance liquid chromatography (HPLC) [8,9], enzyme immunoassay [10], radioimmunoassay (RIA) [11,12] and micro-biological assay [13] have been developed for BLM detection. But these methods are generally time-consuming, labor-intensive, harmful to health, or cost expensive. For example, usage of radioactive isotope in RIA caused radioactive hazards as well as increased detection cost. HPLC is not only labor intensive and time-consuming, but also harmful to health due to usage of corrosively strong acid or a big volume of organic solvent as mobile phase. Therefore, it is still imperative to develop a sensitive and selective strategy that can provide a simple and convenient route to determine trace amount of BLM in clinically biological sample analysis.

As reported, BLM can cleave DNA mainly at a recognition site of 5′-GC-3′ or 5′-GT-3′ by oxidizing the deoxyribose moiety in the presence of oxygen and a redox-active metal ion [14,15]. Based on this BLM-induced DNA scission, many strategies have been developed for simple and sensitive detection of BLM such as electrochemical methods [16,17] and fluorescent methods [18–20]. For construction of these strategies, a crucial and indispensable aspect is to design a DNA molecular probe to recognize BLM. Currently, there are two types of such DNA molecule probe for BLM detection. One is a single-stranded DNA or hairpin DNA labeled or combined with signal molecules, which can be released after probe cleavage by BLM to produce corresponding signal response [16–19]. However, this DNA molecule probe shows a weak ability to produce detectable signal, because one probe cleavage event only creates one signal output event. To solve this problem, another DNA molecule probe named functional stem-loop probe that has a trigger sequence has been developed [20]. When recognized and cut by BLM, this probe releases a trigger sequence and the trigger sequence can be recycled with the aid of signal amplification reaction. In this way, one cleavage event can be translated into repeated

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http://dx.doi.org/10.1016/j.snb.2016.07.055
0925-4005/

signal generating events. Thus, this DNA molecule probe exhibits more excellent signal output ability. Gao et al. designed such a functional stem-loop probe with 5′-GC-3′ recognition sites in stem and a trigger sequence in loop for highly sensitive determination of BLM [20]. However, for the trigger sequence is exposed in the loop, non-specific hybridization of uncut DNA molecule probe with the follow-up reaction strand can result in relatively high background. Therefore, development of a new DNA molecular probe that can silence a trigger sequence to reduce non-specific hybridization is still imperative for BLM recognition and detection.

Herein, to overcome the above problem, a DNA molecule probe named bicyclo-hairpin probe (BHP) was constructed to mediate signal amplification strategy for BLM detection. This BHP integrated two function domains: a 5′ −GTGC-3′ recognition site for BLM and a trigger sequence for signal amplification reaction (Fig. 1). Unlike the above-mentioned functional stem-loop probe, to reduce non-specific hybridization, the trigger sequence was designed semi-closed in BHP. Most of the trigger sequence was sequestrated in bilateral arms of stem-bulge-loop and only a small part of it containing several nucleotides was exposed in the stem-bulge-loop. The design of semi-closed trigger sequence in BHP was masterly. On the one hand, since the intra-molecule hybridization of BHP was powerful, the trigger sequence could be silenced in stem to avoid non-specific hybridization. On the other hand, for the presence of stem-bulge-loop, the trigger sequence could be easily released after cleavage of BHP and then annealed with follow-up reaction strand to form a stable double helix with sufficient base-pairs [21], initiating subsequently amplified reaction. To realize sensitive and label-free detection of BLM, strand displacement amplification (SDA) strategy [22,23] and G-quadruplex/*N*-methyl mesoporphyrin IX (NMM) complex which could emit much stronger fluorescence than free NMM [24–31], were introduced into detection system. Firstly, BLM induced cleavage of bicyclo-hairpin probe and a length of trigger sequence was released. Next, strand displacement amplification reaction was touched off and a large number of replicated strands came into being. Finally, with the help of K^+, replicated strand formed G-quadruplex structure that could bind with NMM, and an enhanced fluorescent signal was achieved. Through adoption of SDA reaction to produce single strands and the ability of G-quadruplex/NMM complex to emit strong fluorescence, a label-free fluorescent sensor for BLM detection based on BLM-induced DNA scission was developed. Besides these single-strand products, G-quadruplex forming sequence in unfolded signal probe (SP, shown in Fig. 1) could also bound with NMM to emit fluorescence. This strategy was sensitive for BLM determination with a detection limit of 0.34 nM. Fluorescent response of detection system toward four different anti-tumor drugs proved that this strategy had admirable selectivity. Furthermore, recovery tests in human serum were also performed, suggesting that this strategy had a great potential to be applied in clinical sample analysis.

2. Experimental section

2.1. Reagents and apparatus

HPLC-purified BHP and SP used in this work were synthesized and purified by Sangon Biotechnology Co., Ltd. (Shanghai, China). Sequences of all of the oligonucleotides were listed in Table S1. BLM A_5, mitomycin and daunorubicin were purchased from National Institutes for Food and Drug Control. Dactinomycin was purchased from Melone Pharmaceutical Co., Ltd. (Dalian, China). Iron chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$) was obtained from Tianjin Guangfu Fine Chemical Research Institute. Klenow fragment (KF) (3′ −5′ exo$^-$) polymerase and nicking enzyme Nt.AlwI (recognition sequence: 5′-GGATCNNNN↓N-3′) [32,33] were obtained from New England Biolabs Ltd. (Beijing, China). The deoxynucleotide triphosphates (dNTPs) were purchased from Thermo Fisher Scientific Ltd. (China). NMM was purchased from Frontier Scientific Inc. (Utah, USA). The physiological buffer saline (PBS, 15 mM) was consisted of 13.4 mM Na_2HPO_4, 1.6 mM NaH_2PO_4 and 50 mM NaCl (pH = 8.0). Human serum samples were obtained from the Hospital of Shandong University, China. All other chemicals involved in the work were of analytical grade. Ultrapure water (resistance >18.25 MΩ·cm) was used throughout.

Hitachi F-2500 spectrofluorophotometer (Hitachi, Japan) was used to acquire fluorescence emission spectra. The excitation wavelength was fixed at 399 nm and the scanning range of emission spectra was from 550 nm to 680 nm. Both of excitation and emission slit widths were set at 10 nm. The photomultiplier tube voltage was 700 V.

2.2. The activation of BLM and oxidative cleavage of BHP

BLM was mixed with fresh $FeCl_2$ solution at the same concentration [16] to acquire activated BLM. Meanwhile, BHP and SP were heated at 95 °C for 5 min in 15 mM PBS (pH = 8.0, 0.05 M NaCl), respectively, and then cooled slowly to the room temperature to ensure that the desirable structures could take shape before use. Subsequently, the activated BLM and 5 μL BHP were mixed together and incubated at 37 °C for 30 min, so that cleavage of BHP could happen sufficiently.

2.3. SDA reaction

After cleavage of BHP, 1 μL 2 mM dNTPs, 1 U KF polymerase, 4 U Nt.AlwI, 3 μL SP, 2 μL 10 × NEBuffer 2 and some ultrapure water were added to the above reaction solution and the final volume was designed to be 20 μL. Then the mixture was incubated at 37 °C for 40 min.

2.4. Fluorescence measurement

Afterward, 1.2 μL NMM, 4.8 μL KCl and 4 μL 1 × NEBuffer 2 (10 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, 1.0 mM dithiothreitol, pH 7.9) were added to the resulting solution to make the final volume 30 μL and then incubated at 37 °C for 30 min. Then fluorescent emission spectra was recorded on the Hitachi F-2500 spectrofluorophotometer with the parameters described above.

2.5. Gel electrophoresis

To investigate the feasibility of the strategy and whether BHP could functioned as designed, the cleavage of BHP and SDA reaction was verified by 10% nondenaturing polyacrylamide gel electrophoresis. After staining in ethidium bromide (EB) solution for 5 min, gels were photographed under UV imaging system (Bio-RAD Laboratories Inc., USA).

3. Results and discussion

3.1. The principle of BHP mediated SDA strategy

The design of BHP and principle of this BHP mediated SDA strategy for BLM determination were shown in Fig. 1. The BHP with a special loop named stem-bulge-loop integrated a 5′-GTGC-3′ site for BLM recognition and a trigger sequence for signal amplification. Owning to powerful intra-molecular hybridization, 3′-terminal and 5′-terminal of BHP could form a duplex structure (this structure had been simulated with the help of DNA folding software, mfold [34]) and this structure was stable enough to silence trigger sequence.