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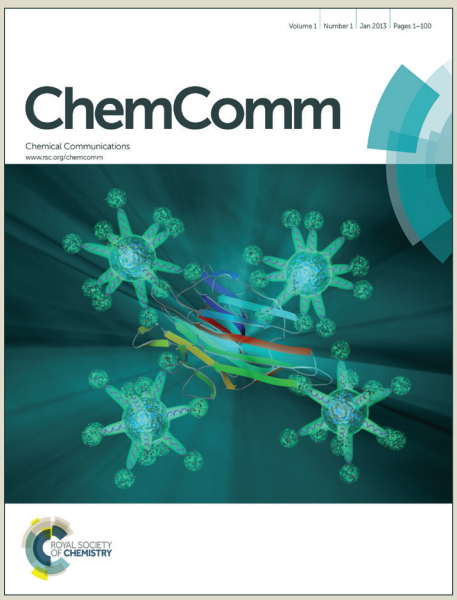

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Biomolecular Logic Gate for Analysis of the New Delhi Metallo-β-Lactamase (NDM)-Coding Gene with Concurrent Determination of Its Drug Resistance-Encoding Fragments

Cite this: DOI: 10.1039/x0xx00000x

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/x0xx00000x

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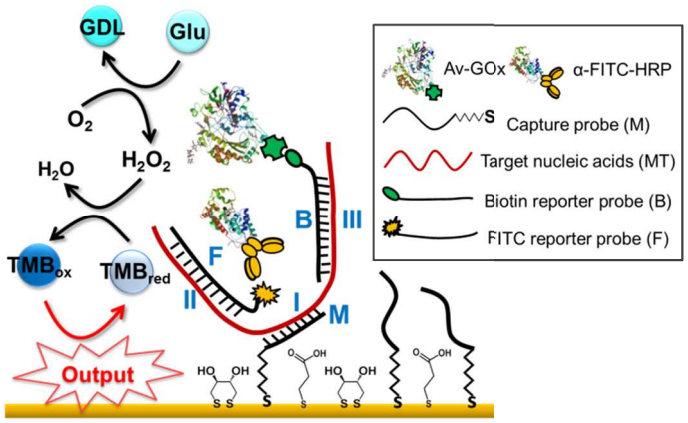
We herein exploit a newly schemed logic gate to superiorly facilitate analysis of long and highly-structured nucleic acids. This strategy uniquely realizes an identification of NDM-specific gene and concurrent screening of two active sites-encoded fragments, which is promising to evaluate microbial drug resistance.

The rapidly increasing collections of multi-drug resistant pathogens with enhanced capacity toward extended spectra of antibiotics have been posing a serious threat to the public health on a global scale[1-3]. Recently, a potent enzyme New Delhi metallo-β-lactamase (NDM), which is capable of hydrolyzing multiple β-lactams including the antibiotics of the last resort, cabapenem[4, 5], was identified[6] and has been reported to exist in various Enterobacteriaceae strains worldwide. Moreover, NDM is encoded in highly transferable genetic fragments and regularly transmitted along with variable resistance determinants of other antibiotics coded by the same plasmid[7, 8]. All these features underscore the significance of monitoring the spread of encoding gene over bacteria, which facilitates precise medical prescriptions and prevents its further dissemination. Nevertheless, to date, there are few methods[9-11] developed to fast screen the existence of NDM, and none of the aforementioned assays were capable of accurately indicating its potential catalytic activity against antibiotics.

Seeing the tremendously complex genetic code of programming algorithm in biology, genetic determination of single target was insufficient to vividly describe biological systems, necessitating simultaneous analysis of multiple genetic segments. Yet, there is limited work addressing the issue of multi-sequence identification with intelligent design which can suffice the need in biomedical applications and, most importantly, significantly simplify the process of determination and the interpretation of results[12-16]. Furthermore, of the limited endeavour, no evaluation was commenced to validate the systems towards analyzing genuine (i.e., non-synthetic) nucleic acids in which the targeted fragments frequently reside in distant positions and length of whole DNA is much longer than those exemplified in prior reports[12, 14].

Motivated by need, in this communication we describe a newly strategic scheme, as shown in Scheme 1, for identification of multiple genetic segments locating distantly in a long DNA target.



Scheme 1. The intelligent strategy which captures targeted nucleic acids in the midsection with two reporter probes located at the two ends for analysis of multiple sequences residing distantly in a long genetic fragment. The inset illustrated components utilized in this design.

Distinct from the conventional sandwich configuration (designated as Terminus) in which nucleic acids are often captured at the terminus of the thread, the capture probe (**M**) is herein designed to hybridize with one of the targeted sequences residing at the midsection (**I**) of the analyte (**MT**). The other two targeted segments are (**II** & **III**) harboured at two sides of the captured position. Upon the two hybridization events of **II** & **III** with the FITC- and biotin-tagged reporter probes (**F** & **B**) respectively, the tagged molecules are recognized by enzyme complexes, horseradish peroxidase conjugated with anti-FITC antibody (α-FITC-HRP) and glucose oxidase linked with avidin (av-GOx). As a consequence, an enzymatic cascade reaction occurs upon addition of the substrate solution containing D-(+)-glucose (Glu) and 3,3',5,5'-Tetramethylbenzidine (TMB(red)). Specifically, the bound GOx catalyzes the oxidation of glucose to generate H_2O_2, which can subsequently be reduced by HRP to produce oxidized TMB (TMB(ox)). This scheme configures an equivalent circuit composing of a parallel identity and AND gate, in connection to a series of AND gate (Fig. S1). When challenging the schemed system with a long DNA strand (383 nt), the amperometric signal transduced with this new architecture (Midsection) was much greater than one from a

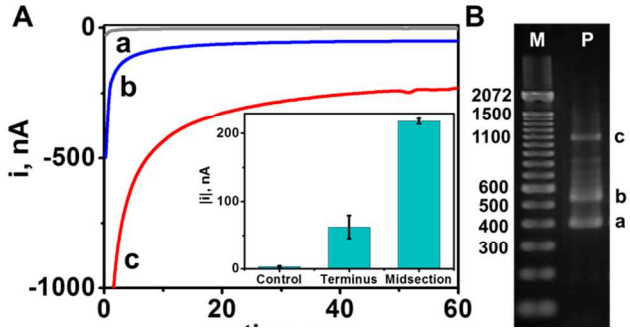


Fig. 1 (A) The chronoamperogram derived from the control group treated with buffer (a) and sensing scheme captured target DNA at the midsection (b) or the terminus (c) treated with unpurified product from asymmetric polymerase chain reaction (aPCR). Inset: the amperometric signals recorded at the 60th second for each group. (B) The result of agarose gel electrophoresis of the product from aPCR. The sample in the left lane (M) is DNA ladders as a marker and the right (P) is the amplified products.

conventional sandwich arrangement (Terminus) (Fig. 1A). This amplified magnitude presumably resulted from the spatial proximity of the two bound enzymes which enabled facile redox cascade reaction to expedite the intelligent detection of the extended DNA analyte. In contrast, in the scenario where the long strand was captured at the terminus, owing to the extensive distance between the two targeted sequences recognized by reporter probes, the bound enzymes were separated far away from each other and thus the cascaded enzymatic reaction was substantially retarded. As can be anticipated, the Midsection strategy did not exhibit the amplification in the detection of short synthetic DNA (72 nt). The electrochemical signals derived from the two designs showed minimal difference in the presence of varied concentrations of oligonucleotides (Fig. 2). This feature was explained by the short separation between II and III, which ultimately leads to an approximate level of proximity between the redox enzymes in both architectures of recognition. Therefore, exploiting the strategy which captured in the midsection of DNA significantly enhanced the utility of the intelligent design for analysis of extended nucleic acids without deterioration of the performance in detection of short oligonucleotides, facilitating sensitive determination of practical genetic fragments.

By feature of a distant separation (276 bp) of the two specific active site-encoded fragments in NDM gene (813 bp), utilizing the Midsection strategy to analyze the drug-resistant activity becomes

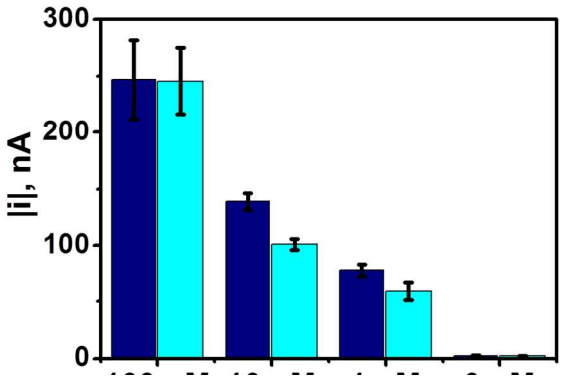


Fig. 2 The amperometric signals recorded at the 60th second for treatment of varied concentrations of a short (72 nts) DNA target. The blue bars represent currents obtained from the sensing scheme capturing target DNA in the midsection, whereas the cyan bars are those from a conventional sandwich design.

promising. As a flowchart illustrated in Fig. 3, we retrieved NDM-1 in protein data bank (PDB) to acquire the deposited crystal structures of NDM-1. Among the 21 structure records, nine crystal structures are in complex with seven beta-lactam antibiotics (Table S2). We compiled R scripts (detailed in ESI) to identify the potential active sites surrounding the beta-lactam antibiotics. Briefly we defined a spherical space, centered on center of the ligand with a radius in 1.5-fold ligand molecular size. Amino acid residues (in NDM) whose alpha-carbon are inclusive in this defined spherical space were selected. Amino acid residues (27 a.a.) which appeared in every NDM structures were summarized (Table S3). Considering serial amino acid fragments (≥ 4 a.a. residues) and excluding less specific sequence resulted in GCGCATCAGGACAAG and CTCGGCAATCTCGGTGATGCC as the characteristic sequences

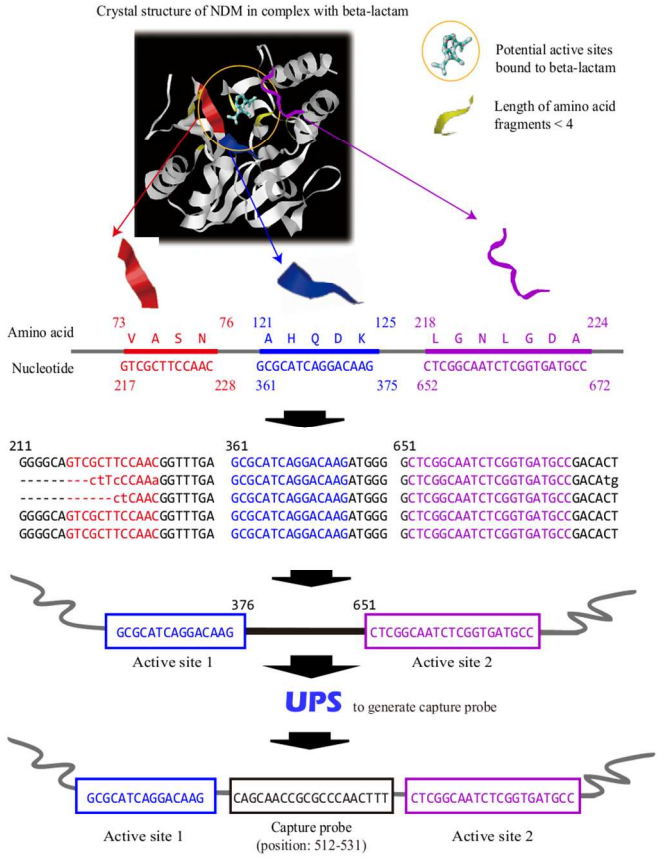


Fig. 3 The flowchart for selection of nucleic acid sequences specific for NDM and characteristic of its catalytic site.

for the active site (detailed in ESI). In addition, a unique 20 bp capture probe (CAGCAACCGCGCCCAACTTT) sequence was selected for capture probe based on its pangenomic level and the non-redundant NCBI nucleotide database using unique probe selector (UPS)[17].

Employing the designed **M**, **F**, and **B** complementary to these specific sequences for the 'Midsection' sensing configuration has allowed a successful detection of asymmetric polymerase chain reaction (aPCR) products given using NDM gene as a template (detailed in ESI). A certain fragment in NDM gene from *Klebsiella pneumoniae* clinical isolate 303K (GenBank accession AVAN00000000.1)[18] were amplified in a single-stranded form to give an expected length of 383 nt and exist in substantial secondary structures (Fig. S2). As the responding outputs which have been displayed in Fig. 1A, the strategy which arrests target DNA in the midsection remarkably outperformed the conventional sandwich configuration; in particular, it was capable of detecting long and highly structured DNA generated from genuine genetic compositions.

To ensure that the amperometric signals were intrinsically derived from the desired target, the aPCR products were cautiously characterized to exclude a possible false-positive. Three distinct bands appeared on the agarose electrophoresis gel (Fig. 1B). Nucleic acid in each band was purified and sequenced. The results indicated that the constituent in band **a** was the expected double-stranded DNA yielded from aPCR. Fig. S3-4 displays its sequence and length, recognized using both forward and reverse primers, which exactly matches with the desired amplicon. Sequencing results for band **b** and **c** suggested a single-stranded product consisting of the desired 383 nucleotides which fold in tertiary structures, since only the reverse primer activated a successful sequencing with a resulted sequence identical to one shown in band **a** (Fig. S5-8). This verified utility of the measured amperometric signal to reliably reflect the existence of desired long target DNA, exclusive of nonspecific DNA segments.

As genetic mutation can be a cause to induce alteration of microbial drug resistance, we interrogated the developed analytical system with mutated target DNA in which the substituted nucleotides would result in variations of corresponding amino acids, leading to a substantial loss of NDM catalytic activity. Mutations of asparagines and glutamine (i.e., N220 and Q123) which cause dramatic activity loss have been investigated[19-21]. These residues suited in proximity to the substrates, and were included in our selected specific sequences. This also verifies the integrity of our bioinformatics works (Fig. 3). Amperometric signals derived from the wild-type and mutated targets have been evaluated (Fig. 4A). The wild-type target yielded a greater current amplitude (77 nA) and conspicuously discriminated from the other three mutated targets with alternated nucleotides on either **II** (4 nA in Q123D) or **III** (6 nA in N220A), or both hybridized sites (4 nA). Notably the point mutation occurred in N220A is considered to barely affect the binding affinity between mutated target **N** with **B**, owing to minimal alteration of the change of Gibbs free energy (ΔG=-34.2 kcal/mol for wild type and -27.4 kcal/mol for mutant, see SI for details). A melting curve measurement underscored this feature (Fig. 4B) wherein melting curves of **B** hybridized with mutant N220A and wild-type target (**MT**) showed trivial difference. Corresponding melting temperature of both duplex species were obtained to be 66 and 78 °C (for **N** with **B** and **MT** with **B**, respectively, from the derivative fluorescence intensity (Fig. 4C)), which were much higher than the operating temperature (~27 °C) of the analytical system. This suggested that formation of **N**-**B** complex was still thermodynamically favoured (at ~27 °C) and thus discrimination for

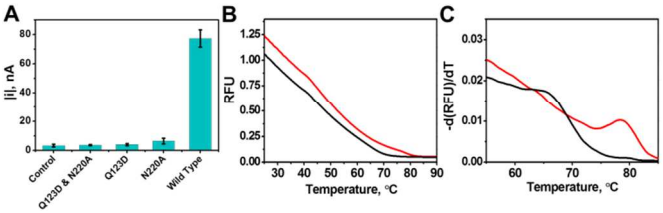


Fig. 4 (A) The amperometric signals recorded at the 60th second for the wild-type target (MT) and mutants with nucleotide alterations at the site II (Q), III (N) or both (QN) indicated in Scheme 1. (B) The melting curves for the hybridization of the biotin-tagged probe with MT (red) or N (black). (C) The curve of derivatives from melting curves in (B) with peaks indicating melting temperatures of different duplex species.

single nucleotide mutation in target was theoretically challenging. Interestingly, in our intelligent strategy, the signal to noise ratio (signal obtained with **MT** divided by the one with **N**) was substantially elevated from 1.2 (in the SYBR green assay) to 12, demonstrating enhanced specificity using the approach processed via biomolecular logic gate. We attributed the high specificity and amplification to the serial bridging from biomolecular recognition to biocatalytic reactions. The trivial mis-match in the hybridization event between the probes and target DNA was relayed to the reduced binding events of the enzyme conjugates, subsequently leading to mitigated catalysis and inefficient generation of the redox products. With diminished efficiency in each step, transduction of the whole successive process was significantly deteriorated with the existence of few mismatched sites in the target sequence. Thereby mutated NDM with diminished hydrolytic activity against their substrates can be unambiguously recognized and discriminated from the wild type, which is deemed highly beneficial to a surveillance and accurate prescription without antibiotics abuse.

Despite the system has been demonstrated to simultaneously detect multiple genetic segments in a long and highly structured nucleic acid, particular attention should be paid on the sensitivity which is highly dependent on density of capture probe and geometry of formed DNA complex. As length of genuine genetic sequence can be varied diversely, the system is potentially applied to versatile applications upon appropriately envisaging architecture of DNA complex in connection to varied separation (of any two target segments) and whole length. For a concern in practice, the present analytical chip, with built-in biomolecular logic gates, allows for rapid, sequence-specific identification of unpurified PCR products without light sources and optical detector; as a consequence, can be embodiments of simple procedure and minimal labors. Such the configuration is particularly desirable to in-field measurement, rather than a sophisticated platform (e.g., qPCR) competent to mass screening process in central laboratory.

In summary, we herein demonstrated an intelligent approach for identification of the NDM-encoding gene (blaNDM-1) and concurrently to screen, by a tailor-designed biomolecular logic gate, two genetic fragments encoding NDM active sites bound to carbapenem. Most importantly, a new geometry of recognition was proposed and proved to greatly enhance the utility, compared to the conventional sandwich architecture, for analysis of long and highly structured nucleic acids from a genuine genetic sequence. This design also exhibited high specificity for differentiation between mutated and wild-type DNA, accurately reflecting the potential loss of drug resistance in NDM and its host bacteria. Pursuant to its simple, reliable, and powerful features, this strategy exploits great promise to be a sophisticated molecular approach for surveillance and diagnostics of microbial resistance.

This work is funded by National Science Council Taiwan under contracts 102-2113-M-029-003-MY2 and 100-2113-M-029-009-MY2. The authors thank Miss Chieh-Hua Lin (in NHRI) for data analysis.

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