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Rapid Electrochemical Detection of New Delhi Metallo-beta-lactamase Genes To Enable Point-of-Care Testing of Carbapenem-Resistant Enterobacteriaceae

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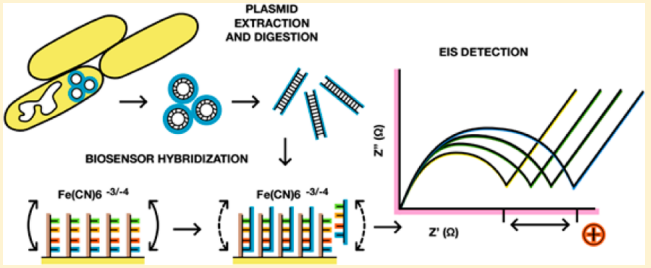
Supporting Information

ABSTRACT: The alarming rate at which antibiotic resistance is occurring in human pathogens causes a pressing need for improved diagnostic technologies aimed at rapid detection and point-of-care testing to support quick decision making regarding antibiotic therapy and patient management. Here, we report the successful development of an electrochemical biosensor to detect bla_{NDM}, the gene encoding the emerging New Delhi metallo-beta-lactamase, using label-free electrochemical impedance spectroscopy (EIS). The presence of this gene is of critical concern because organisms harboring bla_{NDM} tend to be multiresistant, leaving very few treatment options. For the EIS assay, we used a bla_{NDM}-specific PNA probe that was designed by applying a new approach that combines *in silico* probe design and fluorescence-based DNA microarray validation with electrochemical testing on gold screen-printed electrodes. The assay was successfully demonstrated for synthetic targets (LOD = 10 nM), PCR products (LOD = 100 pM), and direct, amplification-free detection from a bla_{NDM}-harboring plasmid. The biosensor's specificity, preanalytical requirements, and performance under ambient conditions were demonstrated and successfully proved its suitability for further point-of-care test development.



In the EU, over 25 000 patients die annually from infection due to antibiotic resistance.[1] Although, until recently, resistance in Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococcus (VRE) has been the primary focus of clinicians, the increasing prevalence of multidrug resistant Gram-negative pathogens in combination with the lack of antimicrobials in development has become the biggest and most pressing concern in human healthcare.[2−4] Beta-lactam antibiotics conventionally used to target such infections at present make up more than half of the antibiotics used in the world, and these multiresistant Gram-negative strains pose a severe public health threat.[5]

The bla_{NDM} gene was first isolated from a Swedish patient previously hospitalized in India in 2008 and has disseminated to broad geographical locations,[6] predominantly linked to treatment in the Indian region, although it has also disseminated through independent routes.[7] The presence of the gene is of pivotal concern because organisms harboring bla_{NDM} tend to be multiresistant and some are sensitive only *in vitro* to agents of uncertain efficacy such as tigecycline and colistin, leaving few treatment options.[2] Originally identified in *Escherichia coli* and *Klebsiella* strains, the gene is now found in many Enterobacteriaceae responsible for a spectrum of infections such as sepsis and urosepsis. The bla_{NDM} gene is carried on plasmids of various sizes and is easily transferred laterally between Gram-negative genera. As a result, prompt identification of NDM strains is of paramount importance to prevent transmission and dissemination of resistant strains.

Many diagnostic methods currently in use for detection of extended-spectrum beta-lactamase (ESBL) and carbapenemase producing bacteria are culture-based, with a time-to-result (TTR) that is incompatible with a rapid treatment decision (e.g., ChromID ESBL, Etest ESBL, Vitek (bioMerieux), etc.).[8−11] Some molecular diagnostic approaches for detection of beta-lactam resistance in Gram-negative bacteria already exist in the market, such Check-MDR (Checkpoints), Evigene (AdvanDx), Hyplex SuperBug ID (Amplex), and others, but they are not suited for true point-of-care detection because they are based on sophisticated optical detection systems and require demanding sample preparation and preanalytics.[12−14] In contrast, electrochemical biosensors offer exciting possibil-

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ities for decentralized clinical applications due to their specificity, speed, portability, and low cost.

Electrochemical impedance spectroscopy (EIS) is a technique that can be applied to gather information on biorecognition events on functionalized electrode surfaces.[15−19] Detection of DNA hybridization with EIS is a growing field of study and has been covered in recent review articles.[15,20] Conventionally, nucleic acid probes are immobilized on the electrode's surfaces as part of an alkanethiol mixed self-assembled monolayer (SAM).[21,22] Upon EIS measurements in the presence of a redox mediator such as potassium ferri−ferrocyanide, negative charges accumulate on the electrode's surface during target hybridization, and an increase in resistance to charge transfer is detected. This may result from the combined effects of increases due to repulsion of anionic redox mediators at the electrode surface and blocking of SAM pinholes by a bound target slowing electron transfer.[23] The method lends itself well to multiplex detection within an electrode array, whereby multiple markers of infection and resistance may be interrogated in parallel, as a feasible extension of the dual working electrode setup employed herein.[16] Using EIS, we previously demonstrated detection of PCR products derived from the gene target *mecA*, which causes antibiotic resistance in methicillin-resistant *S. aureus* (MRSA).[23]

Here, we report the development of an EIS assay for the direct, label-free detection of the multidrug resistance causing bla_{NDM} gene. A bla_{NDM}-specific probe was designed *in silico* and selected from 36 candidate probes using fluorescence-based microarrays. With this probe immobilized on gold screen-printed electrodes, we developed a label-free EIS assay for the detection of bla_{NDM} PCR products and also for direct, label- and amplification-free detection of bla_{NDM} plasmid DNA.

MATERIALS AND METHODS

Reagents. DNA oligonucleotides were purchased from Metabion (Martinsried, Germany). PNA oligonucleotides were ordered via Cambridge Research Biochemicals (Cleveland, UK) from Panagene (Daejeon, South Korea). PCR kit and Qiaspin miniprep kits were purchased from Qiagen (Crawley, UK). Potassium ferricyanide, potassium ferrocyanide, phosphate buffered saline, monosodium phosphate, disodium phosphate, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Poole, UK). Lambda exonuclease was sourced from EURx (Gdansk, Poland). Deionized water was used throughout the study (>18 MΩ cm). DNase RQ1 was obtained from Promega (Mannheim, Germany). The clinical isolate used for all investigations was a NDM-1 producing *Citrobacter freundii* strain isolated at the Edinburgh Royal Infirmary.

***In Silico* NDM-1 Probe Design.** bla_{NDM}-specific probes of 20 nt in length were designed *in silico* with an online tool, UPS Unique Probe Selector (http://array.iis.sinica.edu.tw/ups/). The UPS algorithm considers GC content, the secondary structure, melting temperature (T_m), the stability of the probe−target duplex estimated by the thermodynamic model, sequence complexity, similarity of probes to non-target sequences, and other empirical parameters used in the laboratory when selecting probes.

The option to select probes at a pangenomic level was selected, and the 826 bp bla_{NDM-1} sequence (accession no. FN396876.1) was entered in FASTA format. A salt concentration of 0.33 M was specified, and subsequently 10 probes of 20 nt in length were generated. The candidate set of probes was further scrutinized as follows, and as a result of analysis, seven of 10 probes were selected for further investigation. Using OligoAnalyzer 3.1 (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/), the %GC content, melting temperatures (T_m), and free energy (ΔG) values of possible secondary structures, such as self-dimer and hairpin structures, were all considered. The sodium ion concentration was adjusted from 50 to 330 mM, but default settings were used otherwise. The results were used to refine the probe selection upon recommendation. All probe candidates were subjected to a further specificity test using BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Probecheck (http://131.130.66.200/cgi-bin/probecheck/content.pl?id=home).[24] The candidate sequences were entered in FASTA format to both servers and checked against the nr/nt database within BLASTN and both the 16S/18S rRNA and 23S/28S rRNA databases within Probecheck. Default settings were used in both cases. The complete probe set chosen for further experiments consisted of two previously identified primers (probes numbered 29−32[25] and 32−36[26]) and seven UPS-generated probe sequences (Table S2).

The investigation took into account the difference between sense and antisense hybridization as well as the potential variability that may arise from immobilizing the probes at either the 5′ or 3′ end of the oligonucleotide probe. In this regard, probes were ordered with amino modification at the 5′ or 3′ end of the sequence for immobilization in both orientations. Sense (the strand corresponding to mRNA sequence) and antisense (the strand complementary to the mRNA sequence) sequences were also investigated, as variability in hybridization efficiency also occurs in this respect. This doubled the size of the probe set from nine deduced through the selection process to 18. Each of the 18 probes, both sense and antisense, was designed with a 12-thymine spacer and amino modification at either the 5′ or 3′ end. This concluded the bla_{NDM-1}-specific probe selection process with a total of 36 probes deduced from the initial pool of nine probes. Sequences of each probe are shown in the Supporting Information (Table S2).

DNA Extraction and PCR. Plasmid DNA was extracted from an overnight Luria−Bertani Broth culture of an NDM-1 producing *C. freundii* clinical isolate using a Qiaspin miniprep kit (Qiagen, Crawley, UK). For PCR amplification, 5 μL of *C. freundii* NDM-1 plasmid DNA was mixed with 4 μM bla_{NDM-1}-specific primers (sequences are shown in the Supporting Information), 0.1 mM dNTPs, 1× *Taq* buffer, 1× Q solution, 1.0 mM $MgCl_2$, and 0.1 U HotStar *Taq* polymerase (Qiagen, Hilden, Germany) in a final volume of 25 μL. The bla_{NDM-1}-specific forward primer was 5′-phosphate-modified for lambda exonuclease treatment to produce single-stranded products. For fluorescent PCR products required for microarray, dNTPs were substituted with 0.1 mM dATP, dGTP, and dTTP in combination with 0.6 mM dCTP and 0.4 mM dCTP-Cy3 (GE Healthcare, Buckinghamshire, UK). PCR products used in microarray work, TEM, SHV, KPC, and 16S PCR were produced using the same reagent concentrations, conditions, and primers detailed in the Supporting Information. The amplification was performed in a Techne TC-512 thermal cycler (Bibby Scientific Limited, Stone, UK) using the following protocol: 95 °C for 15 min; 30 cycles of 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min; followed by a final elongation at 72 °C for 10 min. Upon completion of the reaction, the amplicon was pooled and lambda exonuclease treated.

DNA Enzymatic Treatments. To perform exonuclease treatment, 200 ng of PCR product was incubated with 15 U of

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lambda exonuclease (EURx, Gdansk, Poland) in 1× exonuclease buffer for 25 min at 37 °C. In optimization experiments, incubation times of 5, 15, and 25 min were applied at 37 °C. The enzyme was then inactivated at 95 °C for 5 min, followed by cooling on ice. DNase-treated plasmid was prepared by incubating the DNA with 0.8 mU of DNase I (Promega, Mannheim, Germany) per ng plasmid in 1× DNase buffer for 1.5 min at room temperature. By adding 3 mM ethylene glycol tetraacetic acid (EGTA) and incubating at 65 °C for 10 min, the fragmentation reaction was stopped. For plasmid linearization, DNA was incubated with S1 nuclease (100 U per μg of DNA) for 20 min at 37 °C in the presence of 1× nuclease buffer. The reaction was stopped with 16 mM EDTA and heating at 70 °C, as recommended by the manufacturer.

DNA Electrophoresis. Following enzymatic treatments, DNA molecular weight was confirmed by standard electrophoresis or capillary electrophoresis. Capillary electrophoresis was performed using a Bioanalyzer 2100 (Agilent, UK) according to the manufacturer's instructions. In order to fractionate high molecular weight plasmid DNA, samples were electrophoresed on 2% SybrSafe (Life Technologies, UK), 1% agarose (Sigma-Aldrich, UK) gel at 20 V for 8 h alongside GeneRuler 50 bp and high molecular weight ladders (Life Technologies, UK).

DNA Microarray Fabrication. Amino-modified oligonucleotides were spotted in 1× Schott Nexterion spot buffer (20 μM) in replicates of three within each array on Schott Nexterion slides E (epoxysilane modified surface; Schott, Jena, Germany) with four 200 μm (diameter) split pins and a MicroGrid II (BioRobotics, Cambridge, UK) at 40−50% relative humidity at room temperature. Epoxysilane slides were immediately immobilized at a relative humidity of 75% at room temperature for 1 h followed by storage overnight at room temperature under dry conditions. This generated spots with a diameter of approximately 200 μm. Each oligonucleotide was equipped with a 12-thymidine spacer and an amino modification at the 5′ end. The slides were then washed with 0.1% Triton X-100 solution under constant mixing for 5 min at RT, with 1 mM HCl solution for 4 min, with 100 mM KCl solution for 10 min, and with deionized water for 1 min. The slides were blocked with 50 mM ethanolamine + 0.1% sodium dodecyl sulfate (SDS) in 0.1 M Tris buffer (pH 9) for 15 min at 50 °C. After they were blocked, the slides were washed in deionized water for 1 min and then dried by centrifugation (2 min at 800*g*).

Microarray Hybridization and Data Acquisition. Hybridization was done using Agilent 8 gasket slides and hybridization chambers (Agilent Technologies, Stockport, UK). Fifty microliters of hybridization solution, consisting of 100 nM hybridization control plus bla_{NDM-1} PCR product, 2× SSC (300 mM NaCl + 30 mM Na-citrate), was added to each of the gaskets. The printed slide, which had been washed and blocked, was placed with the array facing down on top of the hybridization solution. The sandwich slides were then sealed using a hybridization chamber and rotated in a preheated oven at 55 °C for 2 h. Epoxysilane slides were washed with 2× SSC + 0.1% SDS for 10 min, 2× SSC for 10 min, and 0.2× SSC for 10 min. Each slide was then dipped in H_2O for a few seconds and dried by centrifugation for 2 min at 800*g*.

Fluorescence images were generated with a Tecan LS reloaded fluorescence scanner (Tecan, Maennedorf, Switzerland) with excitation at 532 nm and emission at 575 nm. Quantification of fluorescence signal intensities was performed with Quantarray software (PerkinElmer, Waltham, MA) using the histogram quantification method. For further analysis, the mean signal intensity minus local background intensity was processed with Excel (Microsoft Corp., Redmond, WA, USA), and the mean and standard deviation of all replicates were calculated.

Electrode Preparation. Screen-printed dual gold working electrode (1.75 mm × 4 mm, low temperature curing gold ink) sensors with an integrated Ag/AgCl reference electrode (Dropsens, Oviedo, Spain) were cleaned using cyclic voltammetry in 100 mM aqueous sulfuric acid and functionalized with 1.5 μM thiol-modified PNA solution + 30 μM mercaptohexanol + 5 mM Tris(2-carboxyethyl)phosphine in 50% (v/v), as described in a previous publication from this laboratory.[23]

EIS Measurement. Following functionalization, EIS measurements were performed on screen-printed sensors connected to an Autolab PGSTAT12 potentiostat (Metrohm Autolab, Herisau, Switzerland) controlled by Nova software at open circuit potential at an amplitude of 10 mV rms at 15 frequencies in the range 100 000−0.1 Hz.[23] Hybridization and measurement were performed in 0.1 mM $K_4[Fe(CN)_6]$ + 0.1 mM $K_3[Fe(CN)_6]$ + 10 mM phosphate buffer + 20 mM NaCl, pH 7. In the case of DNase-treated plasmid DNA, sample was heated at 95 °C for 5 min and transferred to ice for 2 min prior to measurement. PCR products and synthetic DNA were measured without preheating.

RESULTS AND DISCUSSION

EIS Assay Design. In order to develop a sensitive and specific bla_{NDM} assay, parameters pertaining to assay design, measurement conditions, and target state were considered. Implementation of EIS to achieve sensitive detection of nucleic acids has been explored in many assay formats.[27−30] Here, uncharged PNA probes were used in place of polyanionic DNA, which allow a low background signal to be achieved and result in improved resolution upon binding of a charged DNA target, as described previously.[31−33]

Probe density within an alkane-thiol monolayer of 5% mole fraction was employed for optimal sensitivity, in keeping with unpublished data from our laboratory and literature under similar conditions.[34] When dealing with long DNA targets, a trade-off exists between the theoretical EIS signal enhancement and the accessibility of the target sequence. Enhanced EIS signal transduction is expected, which results from the abundant negative charges of the target hybridized at the electrode's surface, causing a higher degree of repulsion of anionic redox mediators. However, a large DNA target may result in a lower accessibility of the 20 nt complementary to the immobilized probe. In this regard, achieving an optimal probe density and target length are key components of sensitive detection.

The use of low ionic strength solution during EIS measurement allows greater sensitivity to be achieved resulting from the increase in the electrostatic barrier for the redox mediator to reach the electrode's surface upon target hybridization. While the kinetic assay format demonstrated herein implies that both DNA−PNA hybridization and measurement take place in one solution, optimal ionic strength reported by Keighley et al. of 10 mM electrolyte was successfully implemented.[34] Within each dual working electrode EIS assay, a PNA-free working electrode was included for SAM quality control and background normalization of

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nonspecific absorption of molecules on the electrode's surface, which contributed to specificity of the assay.

***In Silico* Design and Microarray Validation of bla_{NDM-1} Probe Sequences.** To facilitate EIS investigations for development of a bla_{NDM} biosensor, it was necessary to develop probe sequences that could specifically and sensitively detect bla_{NDM} sequences. This was achieved by *in silico* probe design and validation on glass DNA microarrays with fluorescently labeled bla_{NDM} PCR products. bla_{NDM}-specific probes of 20 nt in length were designed *in silico* with an online tool, UPS Unique Probe Selector, the merits of which are reviewed by Chen et al.[35] Since the hybridization efficiency of long PCR products with probes immobilized on solid supports is influenced by probe orientation and relative probe/target position, both 3′- and 5′-immobilized sense and antisense probes were tested with regard to their affinity for the bla_{NDM} PCR product.[36,37] Probes were contact-printed on glass epoxy-coated slides immobilized via a 12-thymine spacer and terminal amino modification. The hybridization of fluorescence-labeled PCR products, which were generated using published bla_{NDM}-specific primers, resulted in the highest fluorescence intensity for probe 7, which was consequently selected for the further EIS assay development (Figure 1). Despite being selected by *in silico* analysis, many probe sequences proved to be unsuitable for hybridization of bla_{NDM} PCR products. These observations showed that experimental validation of *in silico* designed probes using DNA microarrays is essential for the identification of probes that bind efficiently to long DNA targets of interest when they are immobilized on a solid support. The use of fluorescence-based DNA microarrays for preselection of *in silico* designed probes enabled a large number of probes to be tested in parallel, which could not have been done on the electrochemical platform in the same time frame. This new approach of combining fluorescence-based DNA microarrays with electrochemical detection platforms for assay development has the potential to significantly enhance the performance of assays for nucleic acid-based, electrochemical *in vitro* diagnostic tests.

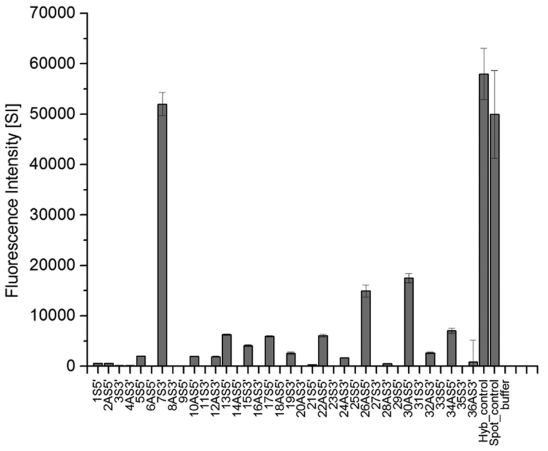

Figure 1. Fluorescence microarray data showing mean fluorescence for each probe developed *in silico* upon hybridization of 4 ng/μL fluorescently labeled bla_{NDM-1} PCR product; $n = 5$. Excluding controls, bla_{NDM-1} probe 7S3′ yields the highest detection sensitivity. The nomenclature signifies the probe number followed by sense (S)/antisense (AS) sequence output by UPS software, with either the 5′ or 3′ end immobilized on the microarray.

The probes were also tested for their cross-reactivity toward other relevant antibiotic resistance gene-specific PCR products, including genes encoding the beta-lactamases TEM, SHV, and *Klebsiella pneumoniae* carbapenemase (KPC) and a *Pseudomonas aeruginosa* 16S rDNA PCR product.[38−41] As can be seen in Figure S3, there was virtually no cross-hybridization of any of the tested probes to *P. aeruginosa* 16S rDNA PCR product. Similar results were obtained with bla_{TEM}, bla_{SHV}, and bla_{KPC} PCR products (data not shown). A thiol-terminated PNA probe of equivalent sequence to probe 7 was obtained for EIS biosensor development. PNA probe P7 was designed to bind the bla_{NDM} PCR product target with short (32 nt) 3′ and long (571 nt) 5′ overhangs. The long overhang was expected to face toward the bulk solution, which suits EIS application in terms of accessibility and signal transduction. Alignment of published sequences of all six current bla_{NDM} variants (GenBank accession nos. JF826285.1, JF703135.1, JQ734687.1, JQ348841.1, JN104597.1, JQ235754.1) using alignment software (CodonCode Aligner, CodonCode Corporation, USA) showed that this probe sequence targeted a conserved region within the bla_{NDM} gene.

EIS Detection of Synthetic DNA Target. The bla_{NDM}-specific PNA probe 7 was first tested with a short artificial target directly complementary to the probe sequence. The applied EIS setup enabled the direct, label-free detection of target hybridization to the immobilized probe. EIS measurements prior to and after target addition allowed the hybridization to be monitored over the course of 10 subsequent EIS spectra, each plotted as Nyquist plots to capture charge transfer resistance values (R_{ct}). As an example, the R_{ct} value increased by more than 1600% ($dR_{ct} = 17$) after a 50 min incubation with 500 nM short, synthetic target. Figure 2 shows the standard curve, which was established based on the signal increase ratio (dR_{ct}) after 60 min (52 min after target addition, respectively, the tenth consecutive EIS measurement after target addition). The limit of detection (LOD) of hybridization of a synthetic oligonucleotide to immobilized PNA probes, defined as the buffer negative control signal plus 3 times the standard deviation, was determined to be 10 nM (10×10^{-9} M).

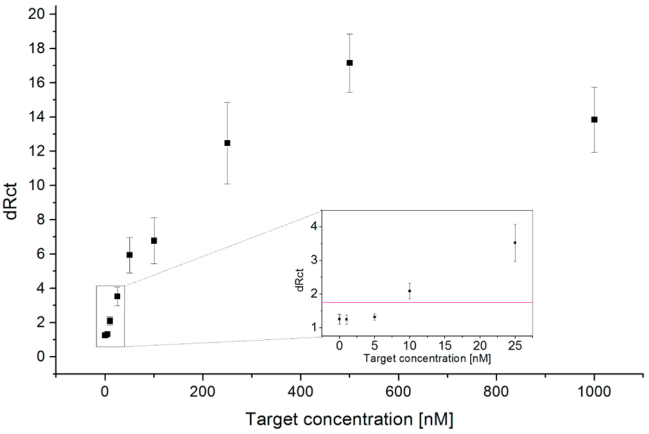

Figure 2. Dose−response curve of EIS detection of short synthetic oligonucleotide using PNA probe P7 constructed using the R_{ct} value at 60 min (52 min post sample addition) normalized to baseline R_{ct} values.

EIS Detection of PCR Product. PCR products were generated using plasmid DNA isolated from an NDM-1

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producing *C. freundii* strain isolated at the Edinburgh Royal Infirmary and published primers. Initially, investigations into hybridization of double-stranded PCR products on PNA probe 7 functionalized electrode chips were carried out. However, the R_{ct} change yielded upon hybridization of 10 nM bla_{NDM-1} PCR product (620 bp, dsDNA) was in the range of nonspecific absorption observed on the PNA-negative control electrode and upon hybridization of noncomplementary DNA (Figure 3).

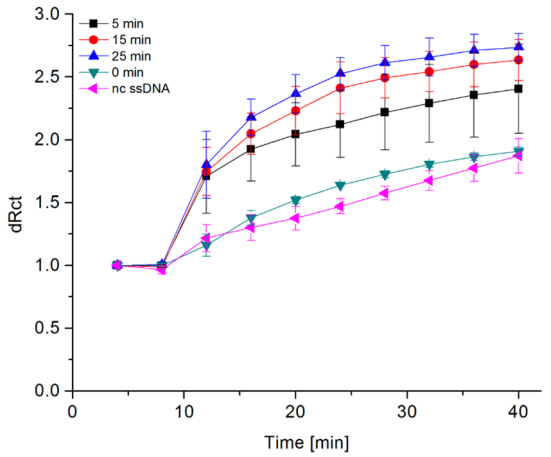

Figure 3. Kinetic EIS detection assay plot showing R_{ct} changes on P7 functionalized electrodes over time post addition of 10 nM PCR product treated with lambda exonuclease for different incubation times (0−25 min) normalized to baseline R_{ct} values. A synthetic ssDNA oligonucleotide noncomplementary to the probe was included as a negative control (nc ssDNA), showing response comparable to that of dsDNA without lambda exonuclease treatment (0 min). A 20% enhancement in EIS R_{ct} increase was achieved by increasing the lambda exonuclease incubation time from 5 to 25 min.

We were expecting higher signals caused by an invasion of PNA into the dsDNA helix, which would displace the complementary DNA strand or form stable triplex structures.[42] On the other hand, the ability of immobilized PNA to infiltrate the dsDNA target may be hindered by the restricted accessibility of the immobilized PNA probe, the length of the dsDNA target, and the ionic strength of the solution.[43] Consequently, a protocol for generating a ssDNA target was applied to improve target accessibility. Lambda exonuclease, which degrades one strand of double-stranded DNA in the 5′−3′ direction when a phosphate is present at the 5′ position, was employed for this purpose.[44] The use of lambda exonuclease to enhance fluorescence-based DNA microarray and EIS assay performance has been reported previously by our group.[23,36] Upon digestion, PCR products were analyzed by capillary electrophoresis. The ssDNA state of digested product was identified by its altered migration in the gel (above the upper marker at >1500 bp), since ssDNA tends to fold into secondary structures (Figure S1). The hybridization of 10 nM of 620 bp long ssDNA bla_{NDM} PCR product produced a specific, significant R_{ct} signal increase. Figure 3 shows the signal change over time caused by target binding to the probe under ambient conditions without mixing of the solution. Optimization of the enzyme digestion time over a range of 5−25 min allowed this detection to be further enhanced, with a 20% increase in sensitivity when a 25 min incubation time was applied (Figure 3). This optimized lambda exonuclease treatment protocol applied to 10 nM bla_{NDM} PCR product generated a R_{ct} value increase of 170% (dR_{ct} 2.7) after a 28 min incubation. Figure 3 shows also that there is already a significant signal change within the first 10 min after target addition; however, measurement was continued for 45 min. Under the laboratory setup used, the time-to-result across a linear range has not been optimized and there is scope for improvement in this regard in terms of system miniaturization and use of an enclosed flow cell or microfluidic apparatus.[45]

The 620 bp bla_{NDM} ssPCR product was tested over a concentration range from 0.1 to 50 × 10^{-9} M, and an EIS standard curve was constructed using R_{ct} data derived from Nyquist plots of baseline EIS spectra (before target addition) and after 10 consecutive EIS measurements following sample addition, which corresponds to a hybridization time of 52 min (Figure 4). A LOD of 100 pM (0.1 × 10^{-9} M; 0.05 ng/μL) was

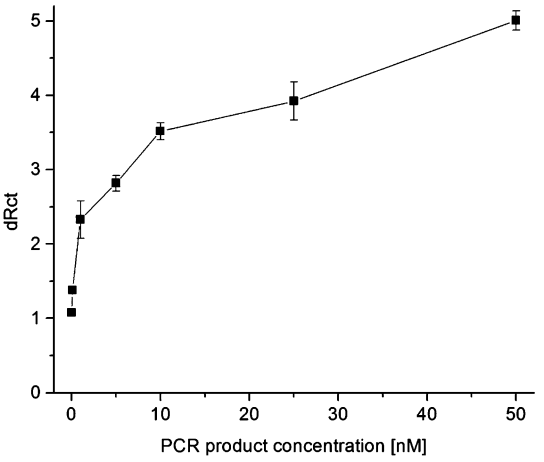

Figure 4. Dose−response curve for 620 bp ssDNA bla_{NDM-1} PCR product detected with the kinetic EIS assay applying PNA probe P7 using R_{ct} values at 60 min (52 min post sample addition) normalized to baseline R_{ct} values (LOD of 100 pM, n = 3).

achieved. This very sensitive detection of long single-stranded PCR products and a 100-time lower LOD compared to the detection of a short complementary target was attributed to the enhancement of EIS signal transduction resulting from the large number of negative charges at the electrode's surface that repelled anionic redox mediators. These results correlate well with data previously demonstrated by us for EIS-based *mecA* detection, where we observed a LOD of 10 nM (10 × 10^{-9} M) for an artificial target and even an 1000-times lower 10 pM (10 × 10^{-12} M) LOD for *mecA* PCR products.[23]

bla_{NDM} PCR products of 100 pM can be generated from 10^3 gene copies/mL, as determined by colony counting. This LOD of 10^3 gene copies/mL is similar to the LOD described by a commercial molecular assay for ESBL and carbapenem resistances in Gram negative bacteria. The hyplex SuperBug ID test (Amplex Biosystems, Gars, Germany; http://www.hyplex.de/index.php?id=126&L=2), which is based on PCR amplification and reverse hybridization, can detect NDM-1 genes together with other resistance genes in 2.5−4 h with a LOD of 9.3 × 10^3 gene copies/mL. Although growth-based methods such as chromID ESBL have lower limits of detection,[46] the drawbacks of a much longer time-to-result and *in vitro* growth conditions are limiting.

The specificity of direct EIS detection of ssPCR products under ambient conditions was investigated by comparison of the bla_{NDM} PCR product caused EIS response to 550 bp *mecA* ssPCR product[23] on PNA probe 7 functionalized electrodes (all

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lambda exonuclease treated). *MecA* PCR product was generated using published primers.[47] Initial experiments performed in the absence of formamide yielded poor discrimination, with the noncomplementary DNA (*mecA*) causing R_{ct} increases higher than those for the complementary target (bla_{NDM}), as shown in Figure 5. This low specificity was not unexpected considering

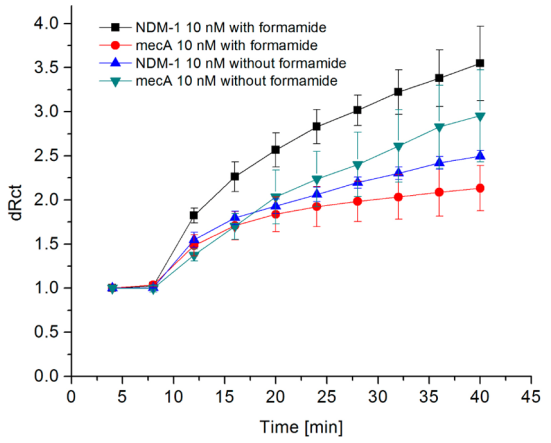

Figure 5. R_{ct} changes over time upon hybridization with 10 nM ssDNA bla_{NDM-1} PCR products and noncomplementary *mecA* PCR products (negative control) under ambient conditions in the presence and absence of 50% formamide in the EIS buffer normalized to baseline R_{ct} values (target addition after 8 min). The figure shows the enhancement of specificity of bla_{NDM-1} PCR product detection by addition of formamide during hybridization.

the fact that the hybridization was performed under highly nonstringent conditions working at room temperature without mixing of the sample solution. However, as these conditions were desirable for a potential application of the bla_{NDM} assay at the point-of-care, we investigated alternative ways to enhance the stringency. Here, the incorporation of 50% formamide during hybridization (still at room temperature) resulted in a 66% enhanced discrimination between the specific hybridization of the bla_{NDM} ssDNA and the nonspecific binding of the *mecA* ssDNA to PNA probe 7, as seen in Figure 5.

Amplification-Free EIS Detection of bla_{NDM} Plasmid DNA. Sample preparation has become a bottleneck for molecular diagnostics, and a shift toward simpler preanalytics would be advantageous in terms of rapid point-of-care diagnosis to aid informed treatment decisions. An amplification-free detection of nucleic acid targets that avoids PCR and thermal cycling requirements would significantly reduce the time-to-result and the instrumental requirements of the test. Consequently, we investigated the direct detection of the target gene containing plasmid. The bla_{NDM-1} gene of the *C. freundii* clinical isolate detected in this study was encoded on an approximately 3.5 kb plasmid. The size of the plasmid was determined by S1 nuclease treatment and gel electrophoresis (see Figure S2).[48]

When using long DNA targets, an enhancement in EIS signal transduction was expected; however, the large target size may result in a lower accessibility of the target sequence. Initial attempts to detect the intact plasmid and S1 treated linearized plasmids failed (data not shown). This may be attributed to the above-mentioned reduced accessibility of this large target sequence. In addition, a plasmid target may also contain some supercoiled regions if the plasmid is not completely linearized or a level of rehybridization may take place after a preanalytical denaturation step of the sample. Therefore, the effect of DNase treatment on the detection of hybridization was investigated. DNase treatment is routinely used in hybridization assays to improve accessibility of target sequences to probes. Success of DNase treatment in EIS depends on generating a target of a length with which the balance between accessibility and signal enhancement is optimum. The incubation of the plasmid DNA for 1.5 min with 0.8 U/ng DNase resulted in 25−50 bp long fragments, as can be seen in Figure S2. These DNase-treated plasmid samples could be detected directly by EIS under ambient conditions at the low nanomolar range (Figure 6).

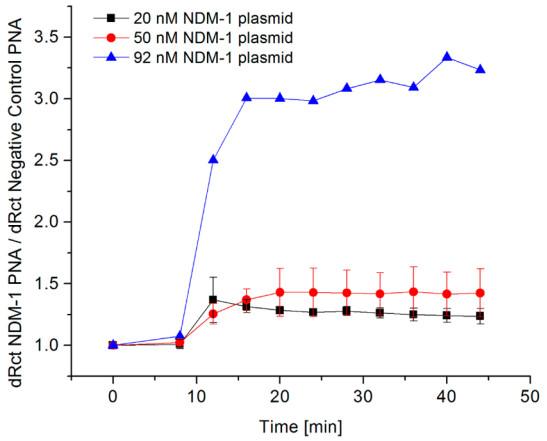

Figure 6. Direct detection of NDM-1 plasmid DNA. Kinetic EIS detection assay plot showing R_{ct} change on bla_{NDM-1} specific PNA P7 functionalized electrodes normalized to R_{ct} change on negative control PNA functionalized electrode over time ($n \geq 2$).

Data represented in Figure 6 show the specific signal increase normalized to R_{ct} change on a negative control PNA functionalized electrode (sequence detailed in Table S1). This is, to the best of our knowledge, the first example of direct, electrochemical plasmid DNA detection in an amplification- and label-free manner.

CONCLUSIONS

Rapid detection of bla_{NDM} producing strains is of paramount importance to prevent transmission and dissemination of resistant strains. Herein, a sensitive and specific biosensor for the label-free detection of bla_{NDM} from clinical isolates was developed. By implementing fluorescent microarray methods, an optimal probe was identified that allowed sensitive and specific target detection when it was transferred to the EIS platform. Use of PNA probes and low ionic strength conditions allowed excellent sensitivity to be achieved. Using a synthetic oligonucleotide complement to the selected probe, standard curves were established, demonstrating the functionality of the kinetic EIS assay design. Following optimal enzymatic digestion, single-stranded PCR product could be detected directly without the need to label the target. Specificity relative to a negative control PCR product was significantly enhanced by addition of formamide to the measurement buffer. The potential for direct PCR-free detection of plasmid DNA was shown to be feasible. In this article, we demonstrate an kinetic EIS assay, in continued development, for rapid bla_{NDM} detection that can be applied to targets of various levels of complexity and that could be implemented as a rapid, cost-effective test suitable for point-of-care use.

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■ ASSOCIATED CONTENT

Supporting Information

Figure S1: Capillary gel electrophoresis of lambda exonuclease digested NDM-1 PCR products. Figure S2: Agarose gel electrophoresis image showing the effect of DNase digestion on plasmid DNA. Figure S3: Example of specificity check on bla_{NDM} probes on fluorescence DNA microarray. Table S1: PNA probe and primer sequences. Table S2: Microarray DNA probe sequences. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b01270.

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Notes

The authors declare no competing financial interest.

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