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

Rapid electrochemical detection of New Delhi metallo-beta-lactamase genes to enable point of care testing of carbapenem resistant Enterobacteriaceae

*Jimmy Ming-Yuan Huang[1, 3, *], Grace Henihan[1, *], Daniel Macdonald[1], Annette Michalowski[1], Kate Templeton[2], Alan P. Gibb[2], Holger Schulze[1], Till T. Bachmann[1, **]*

[1] Division of Infection and Pathway Medicine, College of Medicine and Veterinary Medicine , The University of Edinburgh, Chancellor's Building, 49 Little France Crescent, Edinburgh EH16 4SB, Scotland, UK.

[2] Department of Laboratory Medicine, Royal Infirmary of Edinburgh, Edinburgh EH16 4SA, Scotland, UK.

[3] Emergency Department, Mackay Memorial Hospital, Taipei, Taiwan

*Both Authors contributed equally to this work

** Corresponding author: phone: +44 131 242 9437, fax: +44 131 242 6244; email: Till.Bachmann@ed.ac.uk

Abstract

The alarming rate of antibiotic resistance 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 on antibiotic therapy and patient management. Here, we report the successful development of an electrochemical biosensor to detect *bla*$_{NDM}$, the gene encoding for the emerging New Delhi metallo-beta-lactamase, using label-free electrochemical impedance spectroscopy (EIS). The presence of this gene is of critical concern as organisms harboring *bla*$_{NDM}$ tend to be multi resistant leaving very few treatment options. For the EIS assay we used a *bla*$_{NDM}$ specific PNA probe which was designed applying a new approach of combining *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 the direct, amplification free detection from the *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.

Introduction

In the EU over 25,000 patients die annually from infection due to antibiotic resistance.[1] While until recently resistance in gram positive bacteria such as Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococcus (VRE) have been the primary focus of clinicians, the increasing prevalence of multidrug resistant gram negative pathogens in combination with the lack of antimicrobials in development 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 antibiotics used in the world and these multi-resistant gram negative strains pose a severe public health threat.[5]

The bla_{NDM} gene was first isolated from a Swedish patient previously hospitalised in India in 2008, and has disseminated to broad geographical locations[6], predominantly linked to treatment in the Indian region, though also through independent routes.[7] The presence of the gene is of pivotal concern as organisms harboring bla_{NDM} tend to be multi resistant and some are only sensitive *in vitro* to agents of uncertain efficacy such as tigecycline and colistin, leaving few treatment options.[2] Originally identified in *E. 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, 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) 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) etc. but are not suited for true point of care detection as they are based on sophisticated optical detection systems and require demanding sample preparation and preanalytics.[12-14] In contrast, electrochemical biosensors offer exciting possibilities for decentralized clinical applications due to their specificity, speed, portability, and low cost.

Electrochemical impedance spectroscopy (EIS) is a technique which can be applied to gather information on biorecognition events on functionalised electrode surfaces.[15-19] Detection of DNA hybridisation with EIS is a growing field of study and has been covered in recent review articles.[15,20] Conventionally, nucleic acid probes are immobilised on the electrode surfaces as part of an alkane thiol 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 surface during target hybridisation 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 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 is causing 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). Deionised 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 named UPS Unique Probe Selector (http://array.iis.sinica.edu.tw/ups/). The UPS algorithm considers “GC content, the secondary structure, melting temperature (Tm), 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 826bp *bla*$_{NDM-1}$ sequence (accession no. FN396876.1) was entered in FASTA format. A salt concentration of 0.33M was specified and subsequently 10 probes, 20 nucleotides in length were generated. The candidate set of probes were 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 (Tm) and the 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 mM to 330 mM but default settings remained 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 the 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 S-2).

The investigation took into account the difference between sense and anti-sense hybridization as well as the potential variability that may arise from immobilizing the probes at either the 5' or the 3' end of the oligonucleotide probe. In this regard, probes

were ordered with amino modification at 5’ or 3’ of the sequence for immobilisation of both orientations. Sense (the strand corresponding to mRNA sequence) and anti-sense (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 of nine deduced from the selection process to 18. Each of the 18 probes, both sense and anti-sense, 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 within supplementary materials (Table S-2).

DNA extraction and PCR

Plasmid DNA was extracted from an overnight Luria-Bertani Broth culture of an NDM-1 producing *Citrobacter freundii* clinical isolate using Qiaspin Miniprep Kit (Qiagen Crawley, UK). For PCR amplification 5µL *Citrobacter freundii* NDM-1 plasmid DNA was mixed with 4 µM *bla*$_{NDM-1}$ specific primers (sequence shown in Supporting Information), 0.1 mM dNTPs, 1x *Taq* buffer, 1x Q solution, 1.0 mM MgCl2 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. Where fluorescent PCR products were required for microarray dNTPs were substituted with 0.1 mM dATP, dGTP and dTTP were added, 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 and conditions and primers detailed in 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 at 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 amplicon was pooled and Lambda exonuclease treated.

DNA enzymatic treatments

To perform exonuclease treatment 200 ng PCR product was incubated with 15 U Lambda exonuclease (EURx, Gdansk, Poland) in 1x exonuclease buffer for 25 min at 37°C. In optimisation 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 DNase I (Promega, Mannheim, Germany) per ng plasmid in 1x DNase buffer for 1.5 min at room temperature. By adding 3mM ethylene glycol tetraacetic acid (EGTA) and incubating at 65°C for 10 min, the fragmentation reaction was stopped. For plasmid linearization, S1 nuclease 100U per µg of DNA was incubated for 20 min at 37°C, in the presence of 1X 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 the 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 hrs alongside GeneRuler 50 bp and high molecular weight ladders (Life Technologies, UK).

DNA microarray fabrication

Amino-modified oligonucleotides were spotted in 1x Schott Nexterion spot buffer (20 μM) in replicates of three within each array on Schott Nexterion Slides E (epoxy silane 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% TritonX-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 blocking 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 microliter hybridization solution, consisting of 100 nM hybridization control plus bla_{NDM-1} PCR product, 2x SSC (300 mM NaCl + 30 mM Na-citrate), were added to each of the gaskets. The printed slide which had been washed and blocked was placed array facing down on top of the hybridization solution. The sandwich slides were then sealed using a hybridization chamber and rotated in a pre-heated oven at 55ºC for 2 h. Epoxysilane slides were washed with 2x SSC + 0.1 % SDS

for 10 min, 2x SSC for 10 min, 0.2x SSC for 10 min. Each slide was then dipped in H_20 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 the Quantarray software (Perkin Elmer, 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, USA) and the mean and standard deviation of all replicates were calculated.

Electrode Preparation

Screen printed dual gold working electrode (1.75 mm X 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 sulphuric acid and functionalised with 1.5 µM thiol-modified PNA solution + 30 µM mercaptohexanol + 5 mM Tris(2-carboxyethyl)phosphine in 50 % (v/v) as described in 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 Hz – 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 to 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 resulting in improved resolution upon binding of 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 resulting from the abundant negative charges of the target hybridised at the electrode 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 nucleotides (nt) complementary to the immobilised 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 surface upon target hybridization. While the on-line 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 non-specific absorption of molecules on the electrode 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 which could specifically and sensitively detect *bla$_{NDM}$* sequences. This was achieved by *in silico* probe design and validation on glass DNA microarrays with fluorescently labelled *bla$_{NDM}$* PCR products. *bla$_{NDM}$* specific probes of 20 nt in length were designed *in silico* with an online tool named UPS Unique Probe Selector, the merits of which are reviewed by Chen et al.[35] Since the hybridisation efficiency of long PCR products with probes immobilised on solid supports is influenced by probe orientation and relative probe/target position both 3' and 5' immobilised 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 immobilised via a 12-thymine spacer and terminal amino-modification. The hybridization of fluorescence-labelled 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 (see Figure 1). Despite being selected by *in silico* analysis, many probe sequences proved 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 identification of probes which bind efficiently to long DNA targets of interest when immobilised on a solid support. The use of fluorescence-based DNA microarrays for pre-selection *of in-silico* designed probes

enabled the test of a large number of probes 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 assay performance of nucleic acid based, electrochemical *in-vitro* diagnostic tests.

The probes were also tested for their cross reactivity towards other relevant antibiotic resistance gene specific PCR products, including genes encoding for the beta-lactamases TEM, SHV and *Klebsiella pneumoniae* carbapenemase (KPC), and a *P. aeruginosa* 16S rDNA PCR product.[38-41] As can be seen in Figure S-3, 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 a short (32 nt) 3' and long (571 nt) 5' overhang. The long overhang was expected to be facing towards 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 numbers 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 ten subsequent EIS spectra, each plotted as Nyquist Plots for capture of charge transfer resistance values (Rct). As an example, the Rct value increased by more than 1600 % (dRcT = 17) after 50 min incubation with 500 nM short, synthetic target. Figure 2 shows the standard curve which was established based on the signal increase ratio (dRct) 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 immobilised PNA probes defined as the buffer negative control signal plus three times the standard deviation was determined to be 10 nM ($10x10^{-9}$M).

EIS detection of PCR product

PCR products were generated using plasmid DNA isolated from an NDM-1 producing *Citrobacter freundii* strain isolated at the Edinburgh Royal Infirmary and published primers. Initially, investigations into hybridization of double stranded PCR products on PNA probe 7 functionalised electrode chips were carried out. However, the Rct change yielded upon hybridization of 10 nM bla_{NDM-1} PCR product (620 bp, dsDNA) was in the range of non-specific absorption observed on the PNA-negative control electrode and upon hybridization of non-complementary DNA (see Figure 3). We were expecting higher signals caused by an invasion of PNA into the dsDNA helix displacing the complementary DNA strand or forming a stable triplex structures.[42] On the other hand, the ability of immobilised 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 generation of ssDNA target was applied to improve target accessibility. Lambda exonuclease, which degrades one strand of double-stranded DNA in 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 altered migration in the gel (above the upper marker at >1500bp), since ssDNA tends to fold into secondary structures (Figure S-1). The hybridization of 10 nM of 620 bp long ssDNA *bla*$_{NDM}$ PCR product produced a specific, significant Rct 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. Optimisation of enzyme digestion time over a range of 5-25 min allowed a further enhancement of this detection, with a 20% increase in sensitivity when 25 min incubation time was applied (see Figure 3). This optimized Lambda exonuclease treatment protocol applied to 10 nM *bla*$_{NDM}$ PCR product generated a Rct value increase of 170% (dRct 2.7) after 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 set up used 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 x 10^{-9}M and an EIS standard curve was constructed using Rct data derived from Nyquist plots of baseline EIS spectra (before target addition) and after ten consecutive EIS measurements following sample addition which corresponds to a hybridization time of 52 min (Figure 4). A LOD of 100 pM ($0.1x10^{-9}$M; 0.05 ng/µL) was 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 surface repelling anionic redox mediators. These results correlate well with data previously received by us for the EIS based *mecA* detection, where we observed a LOD of 10 nM ($10x10^{-9}$M) for an artificial target and even an 1,000-times lower 10 pM ($10x10^{-12}$M) LOD for *mecA* PCR products.[23]

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 hybridisation can detect NDM-1 genes together with other resistance genes in 2.5 – 4 h with a LOD of 9.3 x 10^3 gene copies/mL. While growth based methods such as chromID ESBL have lower limits of detection[46] but 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 bla_{NDM} PCR product caused EIS response to 550 bp *mecA* ssPCR product[23] on PNA probe 7 functionalised electrodes (all 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 non-complementary DNA (*mecA*) causing Rct increases higher than for the complementary target (bla_{NDM}), as shown in Figure 5. This low specificity was not unexpected considering the fact that the hybridization was performed under highly non-stringent 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 non-specific 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 pre-analytics would be advantageous in terms of rapid point of care diagnosis to aid informed treatment decisions. An amplification-free detection of nucleic acid targets avoiding 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 *Citrobacter 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 S-2).[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 re-hybridisation may take place after a preanalytical denaturation step of the sample. Therefore, the effect of DNase treatment on detection of hybridisation was investigated. DNase treatment is routinely

used in hybridisation 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 S-2. These DNase treated plasmid samples could be detected directly by EIS at ambient conditions at the low nanomolar range (Figure 6). Data represented in Figure 6 show the specific signal increase normalised to Rct change on a negative control PNA functionalised electrode (sequence detailed in Table S-1). This is to the best of our knowledge the first example of direct, electrochemical plasmid DNA detection in an amplification- and label-free manner.

Conclusion

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 which allowed sensitive and specific target detection when 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 functionality of the online 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 report we demonstrate an online EIS assay, in continued development, for rapid

bla$_{NDM}$ detection which can be applied to targets of various levels of complexity and which could be implemented as a rapid, cost effective test suitable for point of care use.

Acknowledgements

GH was funded by a grant from the Scottish Infection Research Network (SIRN) "Towards rapid molecular detection of NDM-1 producing multidrug bacteria at point of care".

Supporting information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

Reference List

1. *Ejhp Practice*, The bacterial challenge - time to react **2009,** *15*, 15.

2. Kumarasamy, K. K.; Toleman, M. A.; Walsh, T. R.; Bagaria, J.; Butt, F.; Balakrishnan, R.; Chaudhary, U.; Doumith, M.; Giske, C. G.; Irfan, S.; Krishnan, P.; Kumar, A. V.; Maharjan, S.; Mushtaq, S.; Noorie, T.; Paterson, D. L.; Pearson, A.; Perry, C.; Pike, R.; Rao, B.; Ray, U.; Sarma, J. B.; Sharma, M.; Sheridan, E.; Thirunarayan, M. A.; Turton, J.; Upadhyay, S.; Warner, M.; Welfare, W.; Livermore, D. M.; Woodford, N. *Lancet Infectious Diseases* **2010,** *10*, 597-602.

3. Huttner, A.; Harbarth, S.; Carlet, J.; Cosgrove, S.; Goossens, H.; Holmes, A.; Jarlier, V.; Voss, A.; Pittet, D. *Antimicrob. Resist. Infect. Control* **2013,** *2*, 31.

4. Carlet, J.; Jarlier, V.; Harbarth, S.; Voss, A.; Goossens, H.; Pittet, D. *Antimicrob. Resist. Infect. Control* **2012,** *1*, 11.

5. Elander R.P. *Appl Microbiol Biotechnol* **2003,** 385-392.

6. Poirel, L.; Revathi, G.; Bernabeu, S.; Nordmann, P. *Antimicrobial Agents and Chemotherapy* **2011,** *55*, 934-936.

7. Arpin, C.; Noury, P.; Boraud, D.; Coulange, L.; Manetti, A.; Andre, C.; M'Zali, F.; Quentin, C. *Antimicrobial Agents and Chemotherapy* **2012,** *56*, 3432-3434.

8. Reglier-Poupet, H.; Naas, T.; Carrer, A.; Cady, A.; Adam, J. M.; Fortineau, N.; Poyart, C.; Nordmann, P. *Journal of Medical Microbiology* **2008,** *57*, 310-315.

9. Carrer, A.; Fortineau, N.; Nordmann, P. *Journal of Clinical Microbiology* **2010,** *48*, 1913-1914.

10. Farber, J.; Moder, K. A.; Layer, F.; Tammer, I.; Konig, W.; Konig, B. *Journal of Clinical Microbiology* **2008,** *46*, 3721-3727.

11. Linscott, A. J.; Brown, W. J. *Journal of Clinical Microbiology* **2005,** *43*, 1081-1085.

12. Kaase, M.; Szabados, F.; Wassill, L.; Gatermann, S. G. *Journal of Clinical Microbiology* **2012,** *50*, 3115-3118.

13. Fosheim, G. E.; Carey, R. B.; Limbago, B. M. *Journal of Clinical Microbiology* **2007,** *45*, 1611-1613.

14. Naas, T.; Cuzon, G.; Bogaerts, P.; Glupczynski, Y.; Nordmann, P. *Journal of Clinical Microbiology* **2011,** *49*, 1608-1613.

15. Lisdat, F.; Schafer, D. *Analytical and Bioanalytical Chemistry* **2008,** *391*, 1555-1567.

16. Ciani, I.; Schulze, H.; Corrigan, D. K.; Henihan, G.; Giraud, G.; Terry, J. G.; Walton, A. J.; Pethig, R.; Ghazal, P.; Crain, J.; Campbell, C. J.; Bachmann, T. T.; Mount, A. R. *Biosensors & Bioelectronics* **2012,** *31*, 413-418.

17. Gebala, M.; Stoica, L.; Neugebauer, S.; Schuhmann, W. *Electroanalysis* **2009,** *21*, 325-331.

18. Kafka, J.; Panke, O.; Abendroth, B.; Lisdat, F. *Electrochimica Acta* **2008,** *53*, 7467-7474.

19. Witte, C.; Lisdat, F. *Electroanalysis* **2011,** *23*, 339-346.

20. Park, J. Y.; Park, S. M. *Sensors* **2009,** *9*, 9513-9532.

21. Herne, T. M.; Tarlov, M. J. *Journal of the American Chemical Society* **1997,** *119*, 8916-8920.

22. Gebala, M.; Schuhmann, W. *ChemPhysChem* **2010,** *11*, 2887-2895.

23. Corrigan, D. K.; Schulze, H.; Henihan, G.; Ciani, I.; Giraud, G.; Terry, J. G.; Walton, A. J.; Pethig, R.; Ghazal, P.; Crain, J.; Campbell, C. J.; Mount, A. R.; Bachmann, T. T. *Biosensors & Bioelectronics* **2012,** *34*, 178-184.

24. Loy, A.; Arnold, R.; Tischler, P.; Rattei, T.; Wagner, M.; Horn, M. *Environmental Microbiology* **2008,** *10*, 2894-2898.

25. Zarfel, G.; Hoenigl, M.; Wurstl, B.; Leitner, E.; Salzer, H. J. F.; Valentin, T.; Posch, J.; Krause, R.; Grisold, A. J. *Clinical Microbiology and Infection* **2011,** *17*, E5-E8.

26. Chihara, S.; Okuzumi, K.; Yamamoto, Y.; Oikawa, S.; Hishinuma, A. *Clinical Infectious Diseases* **2011,** *52*, 153-U170.

27. Baur, J.; Gondran, C.; Holzinger, M.; Defrancq, E.; Perrot, H.; Cosnier, S. *Analytical Chemistry* **2010,** *82*, 1066-1072.

28. Bonanni, A.; Pividori, M. I.; Campoy, S.; Barbe, J.; del Valle, M. *Analyst* **2009,** *134*, 602-608.

29. Zhou, N.; Yang, T.; Jiang, C.; Du, M.; Jiao, K. *Talanta* **2009,** *77*, 1021-1026.

30. Zhu, N. N.; Gao, H.; Xua, Q.; Lin, Y. Q.; Su, L.; Mao, L. Q. *Biosensors & Bioelectronics* **2010,** *25*, 1498-1503.

31. Degefa, T. H.; Kwak, J. *Journal of Electroanalytical Chemistry* **2008,** *612*, 37-41.

32. Liu, J. Y.; Tian, S. J.; Nielsen, P. E.; Knoll, W. *Chemical Communications* **2005,** 2969-2971.

33. Steichen, M.; Decrem, Y.; Godfroid, E.; Buess-Herman, C. *Biosensors & Bioelectronics* **2007,** *22*, 2237-2243.

34. Keighley, S. D.; Estrela, P.; Li, P.; Mighorato, P. *Biosensors & Bioelectronics* **2008,** *24*, 906-911.

35. Chen, S. H.; Lo, C. Z.; Su, S. Y.; Kuo, B. H.; Hsiung, C. A.; Lin, C. Y. *Bmc Genomics* **2010,** *11*.

36. Brinker, A.; Schulze, H.; Bachmann, T.; Moller, R. *Biosensors & Bioelectronics* **2010,** *26*, 898-902.

37. Peytavi, R.; Tang, L. Y.; Raymond, F. R.; Boissinot, K.; Bissonnette, L.; Boissinot, M.; Picard, F. J.; Huletsky, A.; Ouellette, M.; Bergeron, M. G. *Biotechniques* **2005,** *39*, 89-96.

38. Peter, H.; Berggrav, K.; Thomas, P.; Pfeifer, Y.; Witte, W.; Templeton, K.; Bachmann, T. T. *J. Clin. Microbiol.* **2012,** *50*, 3990-3998.

39. Leinberger, D. M.; Grimm, V.; Rubtsova, M.; Weile, J.; Schroppel, K.; Wichelhaus, T. A.; Knabbe, C.; Schmid, R. D.; Bachmann, T. T. *Journal of Clinical Microbiology* **2010,** *48*, 460-471.

40. Malic, S.; Hill, K. E.; Hayes, A.; Percival, S. L.; Thomas, D. W.; Williams, D. W. *Microbiology-Sgm* **2009,** *155*, 2603-2611.

41. Perry-O'Keefe, H.; Rigby, S.; Oliveira, K.; Sorensen, D.; Slender, H.; Coull, J.; Hyldig-Nielsen, J. J. *Journal of Microbiological Methods* **2001,** *47*, 281-292.

42. Shakeel, S.; Karim, S.; Ali, A. *Journal of Chemical Technology and Biotechnology* **2006,** *81*, 892-899.

43. Kurakin, A.; Larsen, H. J.; Nielsen, P. E. *Chemistry & Biology* **1998,** *5*, 81-89.

44. Higuchi, R. G.; Ochman, H. *Nucleic Acids Research* **1989,** *17*, 5865.

45. Chiriaco, M. S.; Primiceri, E.; D'Amone, E.; Ionescu, R. E.; Rinaldi, R.; Maruccio, G. *Lab on A Chip* **2011,** *11*, 658-663.

46. Gazin, M.; Paasch, F.; Goossens, H.; Malhotra-Kumar, S. *Journal of Clinical Microbiology* **2012,** *50*, 1140-1146.

47. Strommenger, B.; Kettlitz, C.; Werner, G.; Witte, W. *Journal of Clinical Microbiology* **2003,** *41*, 4089-4094.

48. Tullman, J.; Guntas, G.; Dumont, M.; Ostermeier, M. *Biotechnology and Bioengineering* **2011,** *108*, 2535-2543.

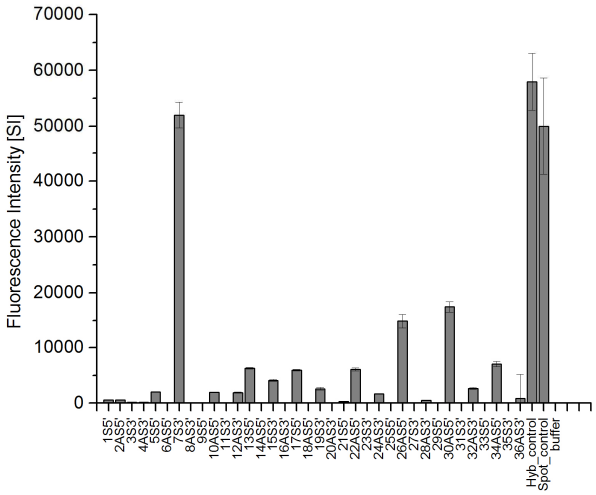

Figure 1: Fluorescence microarray data showing mean fluorescence for each probe developed *in silico,* upon hybridization of 4ng/µL fluorescently labelled bla_{NDM-1} PCR product; n=5. Excluding controls bla_{NDM-1} probe 7S3' yields highest detection sensitivity. The nomenclature signifies probe number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

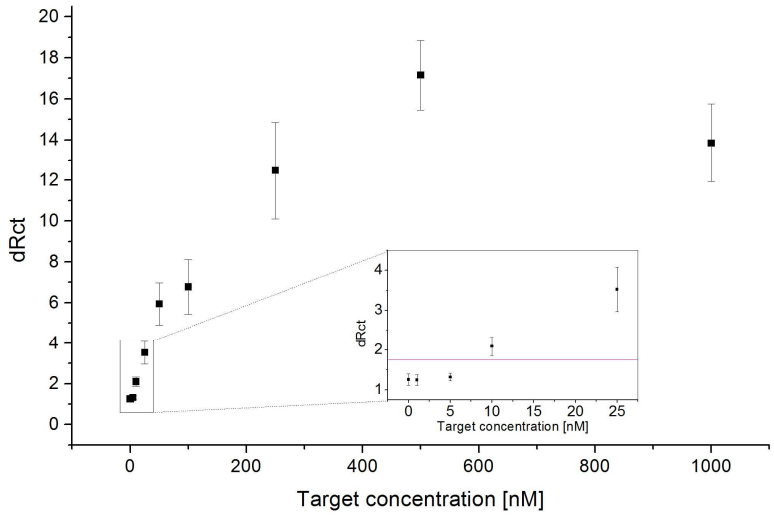

Figure 2: Dose-response curve of EIS detection of short synthetic oligonucleotide using PNA probe P7 constructed using Rct value at 60 min (52 min post sample addition) normalized to baseline Rct values.

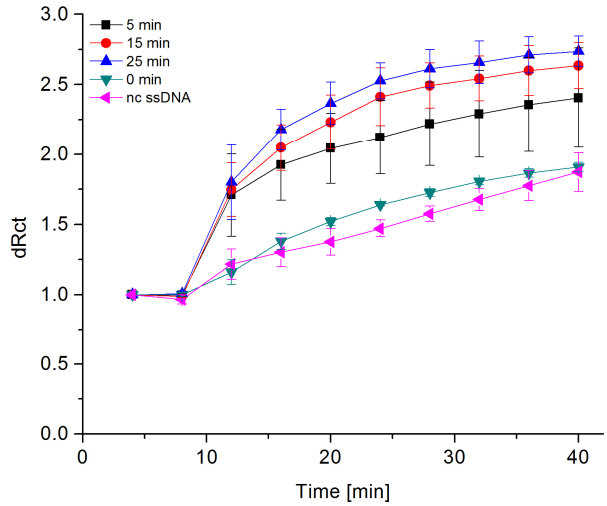

Figure 3: Online EIS detection assay plot showing Rct changes on P7 functionalised electrodes over time post addition of 10 nM PCR product treated with Lambda exonuclease for different incubation times (0-25 min) normalized to baseline Rct values. A synthetic ssDNA oligonucleotide non-complementary to 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 Rct increase was achieved by increasing the Lambda exonuclease incubation time from 5 to 25 min.

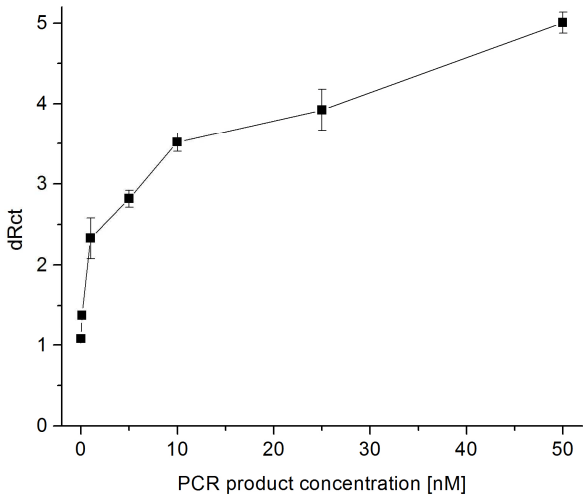

Figure 4: Dose response curve for 620 bp ssDNA *bla*$_{NDM-1}$ PCR product detected with the online EIS assay applying PNA probe P7 using Rct values at 60 min (52 min post sample addition) normalized to baseline Rct values (LOD of 100 pM, n=3).

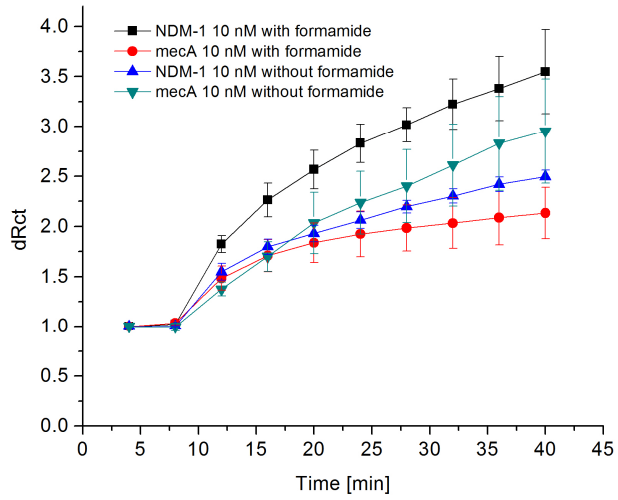

Figure 5: Rct changes over time upon hybridization with 10 nM ssDNA *bla*$_{NDM-1}$ PCR products and non-complementary *mecA* PCR products (negative control) under ambient conditions in the presence and absence of 50% formamide in the EIS buffer normalized to baseline Rct values (target addition after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

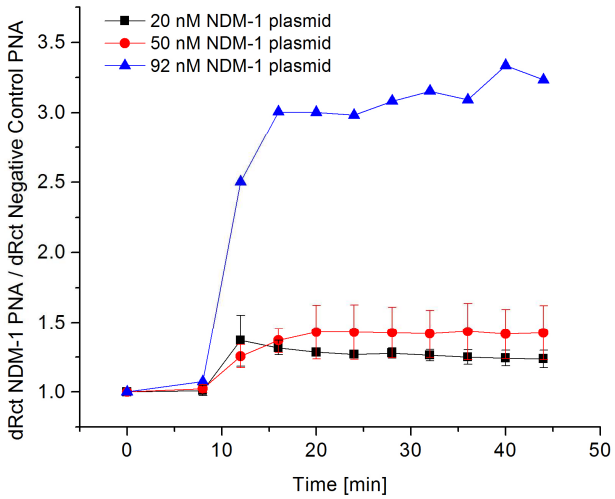

Figure 6: Direct detection of NDM-1 plasmid DNA. Online EIS detection assay plot showing Rct change on *bla*$_{NDM-1}$ specific PNA P7 functionalised electrodes normalised to Rct change on negative control PNA functionalised electrode over time (n≥2).

For Table of Contents Only: