Analyst

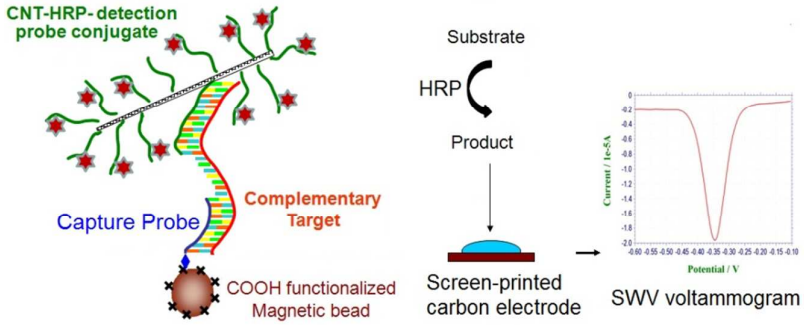
Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/analyst

A table of contents entry

An ultrasensitive electrochemical nucleic acid assay amplified by carbon nanotubes (CNTs)-based labels for the detection of leukemia oncogenes.



Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

PAPER

Electrochemical detection of leukemia oncogenes using enzyme-loaded carbon nanotube labels

Ai-Cheng Lee,[a,b] Dan Dua[a,c], Baowei Chen,[a] Chew-Kiat Heng,[d] Tit-Meng Lim[b]* and Yuehe Lin[a,c]*

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

We describe an ultrasensitive electrochemical nucleic acids assay amplified by carbon nanotubes (CNTs)-based labels for the detection of human acute lymphocytic leukemia (ALL) related p185 *BCR-ABL* fusion transcript. The carboxylated CNTs were functionalized with horseradish peroxidase (HRP) molecules and target-specific detection probes (DP) via diimide-activated amidation, and used to label and amplify target hybridization signal. The activity of captured HRP was monitored by square-wave voltammetry measuring the electroactive enzymatic product in the presence of 2-aminophenol and hydrogen peroxide substrate solution. The signal-amplified assay achieved a detection limit of 83 fM (5×10^{-18} mol in 60 µL) targets oligonuecleotides and a 4-order wide dynamic range of target concentration. The resulting assay allowed a robust discrimination between the perfect match and a three-base mismatch sequence. When subjected to the full-length (491 bp) DNA oncogene, the approach demonstrated a detection limit of 1×10^{-16} mol in 60 µL, corresponds to approximately 33 pg of the target gene. The high sensitivity and specificity of assay enabled PCR-free detection of target transcripts in as little as 65 ng of mRNA extracted from positive ALL cell lines SUP-B15, in comparison to those obtained from negative cell lines HL-60. The approach holds promise for simple, low cost and ultrasensitive electrochemical nucleic acids detection in portable devices, point-of-care and early disease diagnostic applications.

1. Introduction

The Philadelphia chromosome (Ph) is found in approximately 5 to 25 % of acute lymphoblastic leukemia (ALL) cases.[1] The Ph results from the reciprocal translocation of the long arm of chromosome 9 with the long arm of chromosome 22 producing a unique chimeric fusion gene that causes the expression of the chimeric *BCR-ABL* mRNA. In Ph-posive ALL, the unique *BCR-ABL* translocation serves as a specific marker for the detection of early relapse. There are many types of *BCR-ABL* variants.[2] The p185 *BCR-ABL* variant appears in nearly 90 % of cases in paediatric Ph-positive ALL.[1] The genetic markers are often present at ultra-low levels in the biological samples of patients with diseases in the early and remission stages. Therefore, an ultra-sensitive, simple and low cost method of detecting genetic markers is clearly essential for point-of-care (POC) and early disease diagnosis.

Target amplification method such as polymerase chain reaction (PCR) is frequently used to increase target concentration. Conventionally, real-time quantitative PCR (RQ-PCR) has been developed for both detection and quantification of a target sequence. The efficiency and variability of reverse transcription (RT) and PCR amplifications could introduce bias as any minute error introduced at the early steps can greatly influence the final yield of the amplified products. Despite its high sensitivity, RQ-PCR is costly and analytically complex, thereby limited to use in research laboratories. In the efforts to overcome the shortcoming of target amplification techniques, several signal amplification strategies have been developed in the past decades towards the PCR-free ultrasensitive genosensing. Signal amplification accomplished by multiple labelling is one of the most promising methods that could provide excellent assay sensitivity. It has enabled the detection of near single DNA molecule.[3] In general, these methods utilized platforms such as DNA dendrimers, branched DNA (bDNA), encapsulating vesicles and carbon-nanotubes (CNTs) to carry numerous signalling molecules, i.e. labels for signal amplification of individual biorecognition event.

In general, DNA dendrimers[4-5] and bDNA[6-8] have to rely on a series of DNA hybridization steps to assemble the building blocks into multilayered or highly branched constructs for high degree of labelling. Such remedies are often reagent and labor intensive, inciting higher levels of complexity and cost. A material with high loading capacity for signalling molecules would be an attractive alternate carrier platform.

Nanotechnology has enabled the high sensitivity for bioassays [6-7]. Encapsulation of numerous labels in a carrier vesicle, such as polystyrene microbeads,[9, 10] liposomes,[11] , and apoferritin[12, 13] have been employed for signal-amplified bioassays. However, the preparation of these dye-encapsulating vesicles generally

involves tedious processes; an additional dye-releasing step is also required upon target recognition. The confined volume of these micro/nano-sized vesicles could constrain the loading capacity of large labels, for examples, 1340 cadmium ions have been captured in the cavities of nano-sized apoferritin (12.5 nm),[12] whereas a 10 μm-sized polystyrene microbeads could carry a total of 5×10^{11} ferrocenecarboxaldehyde markers[9] and 7.5×10^{9} tris(2,2′-bipyridyl)ruthenium-(II) ($Ru(bpy)_3^{2+}$) species.[10]

CNTs have been explored extensively in electroanalytical applications owing to their unique properties.[14] They are inherently lightweight and exhibit high surface area coupled with a high aspect ratio and abundant surface sites for functionalization.[15] CNTs have therefore been utilized as a novel support material for carrying numerous labels, namely, nanoparticles,[16,37,38] ferrocene[17] and enzymes[18-23,39,40] to amplify the target binding signals in electrochemical bioassays. Most of these attempts focused on the enzyme tracers in which the catalytic amplification power of enzymes was further manifested by loading numerous enzymes on individual CNT carrier.[18-21] The dendrimers and bDNA platforms rely on sequential steps to achieve high degree of labeling. Contrastingly, a large number of labels could be easily loaded to individual nanotube in a single-step conjugation reaction, i.e. monolayer of 500 to 9600 molecules conjugated on a CNT (0.8-2.0 μm average length),[16, 18, 19] attributed to the high surface area-to-weight ratio of CNTs. Without the cavity constraint encountered by encapsulating vehicles, loading capacity of CNTs have been increased with a layer-by-layer (LBL) assembly of enzymes film, such as ALP,[20] glucose oxidase[23] and bienzyme of choline oxidase and horse-radish peroxidase (HRP)[22] on the CNT template. These externally conjugated labels on CNT platform are capable of generating measurable signals without the need of a releasing step as required for encapsulation technique.

Electrochemical genosensing[24, 25] has gained considerable interest because of its high sensitivity, low cost and inherent simplicity for miniaturization. Combining the signal-amplified genosensing and electrochemical analysis presents a new avenue to develop ultrasensitive nucleic acids detection technology suitable for routine analysis, POC and portable devices applications. Wang's group reported an ultrasensitive electrochemical DNA biosensor involving a dual-amplification route.[19] The approach combined the use of a CNT amplifying label (9600 ALP per SWNT) and a CNTs-modified electrode pre-concentrating the ALP-catalyzed enzymatic product for the detection of synthetic DNA down to 54 aM. Further sensitivity enhancement (5.4 aM) was demonstrated using a LBL assembly of 4-layer ALP films (total 196000 molecules) on individual SWNT (3 μm average length).[20] We reported previously a novel electrochemical bDNA assay for PCR-free detection and quantification of p185 BCR-ABL leukemia fusion transcripts in the population of mRNA extracted from cell lines. The bDNA amplifier carrying high loading of ALP tracers was used to amplify the target signal. The activity of captured ALP was monitored electrochemically with a detection limit of 1 fM of target transcripts (1×10^{-19} mol in 100 μL). Such limit corresponds to approximately 17 fg of the p185 BCR-ABL fusion transcripts.[26]

In this work, we describe an ultrasensitive electrochemical nucleic acids assay amplified by CNT-based labels carrying multiple HRP enzymes for the detection of human ALL related oncogene, namely, p185 *BCR-ABL*.

2. Experimental

2.1 Reagents

The carboxylated SWNTs (P3-SWNT) were supplied by Carbon Solution, Inc. (Riverside, CA). RPN3005 ECL™ Direct Nucleic Acid Labelling System was purchased from Amersham Bioscience (Pittsburgh, PA). The kit includes Gold Hybridization Buffer, blocking reagent, glutaraldehyde and HRP as labeling reagent. Spectra/Por® CE dialysis membrane was obtained from Spectrum Laboratories, Inc. (Rancho Dominguez, CA). Microcon YM-30 Centrifugal filter units was obtained from Millipore Corp. (Bedford, MA). The p185 *BCR-ABL*-positive human leukemic cell line SUP-B15 and negative cell line HL-60, fetal bovine serum (FBS) and Iscove's Modified Dulbecco's Medium (IMDM) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). Oligotex Direct mRNA extraction kit was obtained from Qiagen, Inc. (Valencia, CA). BioMag® Plus Streptavidin Particles was purchased from Polysciences, Inc. (Warrington, PA). Dynabead® M-270 Carboxylic Acid beads, Superscript First-strand Synthesis and Quant-iT dsDNA HS kits were purchased from Invitrogen Corp. (Carlsbad, CA). Power SYBR® Green PCR Master Mix was obtained from Applied Biosystems (Foster City, CA). The phenol:chloroform solution was purchased from Pierce Biotechnology, Inc. (Rockford, IL). Phosphate buffer saline (0.01 M PBS pH 7.4), Tris-HCl buffer, bovine serum albumin (BSA), sodium azide (NaN_3), 2-(N-Morpholino)ethanesulfonic acid (MES), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), Tween-20 and *o*-AP were purchased from Sigma-Aldrich. All other chemicals and reagents are of analytical grade. All stock solutions were prepared using ultrapure water (18 MΩ.cm) purified with the Nanopure System (Kirkland, WA). A pH 5.7 Britton-Robinson (BR) buffer was prepared from an acidic solution containing 0.04 M each of H_3PO_4, HOAc and H_3BO_3. The stock substrate solution of *o*-AP was prepared daily or prior to use in the BR buffer at a final concentration of 0.01 M. The *o*-AP working substrate solution containing 0.1 mM of *o*-AP and 0.2 mM of H_2O_2 (denotes hereafter as *o*-AP-H_2O_2) was prepared from the stock solution prior to use. The oligonucleotides (TG, DP, CP and NTG) were synthesized by Integrated DNA Technologies (Coralville, IA). Primer pair (FP and RP) used for RT-PCR and general PCR amplification was synthesized by Sigma-Genosys (Sigma-Aldrich). The sequences are listed as following:

Target [TG] (leukemia oncogene sequence):
5'-GATCTGGCCCAACGATGGCGAGGGCGCCTTCCATGG AG ACGCAGAA-3'

Non-complementary target [NTG] (negative control sequence):
5'-AGTTCCACCTCGTATGTTACTCCCAAGGACTTTGAAA GCTTAAAGG -3'

Detection probe [DP]:
5'-NH_2-TTCTGCGTCTCCATGGAA-3'

Capture probe [CP]:
5'-CCATCGTTGGGCCAGATC-NH_2-3'

Forward primer [FP]:
5'-GCATGATGGAAGGGGAGGGCAAG-3'

Reverse primer [RP]:
5'-biotin-CTTGGAGTTCCAACGAGCGGCT TCA-3'

2.2 Preparation of CNT-based labels

The recommended procedure of RPN3005 ECL™ Direct Nucleic Acid Labelling System was modified to pre-label the DP. HRP enzymes, glutaraldehyde and 0.6 μM of DP were mixed at the ratios of 1:1:3 and incubated at 37 °C for 20 minutes (mixture A). The covalent functionalization of single-walled carbon nanotubes (SWNTs) with pre-labelled DP and HRP were carried out based on a procedure disclosed in our earlier study.[18]

2.3 Cell cultures and mRNA extraction

The cell lines SUP-B15 and HL-60 were used as positive and negative cell lines, respectively for the isolation of p185 *BCR-ABL* fusion transcript (491 bp target). The cell lines were cultured according to the ATCC's recommendation. Briefly, both cell lines were cultured in IMDM medium supplemented with 20 % FBS in an atmosphere of 5 % CO_2 at 37 °C. Cultures were maintained by replacement of fresh medium for 2 times per week and the cell density was maintained between 5×10^5 and 2×10^6 cells mL^{-1}. The cells were pelleted by centrifuge at 125×g for 10 minutes. The mRNAs in the pelleted cells were extracted with an Oligotex Direct mRNA extraction kit. The extracted mRNA was stored at -80 °C. The concentration of mRNA was determined by measuring the absorbance at 260 nm at pH 7.0 and the fluorescence using Quant-iT RNA assay kit. The purity was estimated by the ratio of the absorbance at 260 nm and 280 nm at pH 7.5.

2.4 Synthesis of full-length p185 BCR-ABL single-strand DNA (p185-ssDNA)

2.4.1 Synthesis of the biotinylated double-strand DNA (dsDNA)

The total mRNA population was first reversely transcribed to cDNA using the Superscript First-strand Synthesis kit following the recommended protocol. Briefly, a 10 μL-mRNA/primer mixture (1 mM of dNTP mix, 0.5 μg of Oligo(dT), 100 ng of mRNA template) was heated at 65 °C for 5 minutes and cooled on ice for 10 minutes. The RT reaction mixture, which contains the denatured RNA/primer mix, 1×RT buffer, 5 mM $MgCl_2$, 0.01 M DTT, 40 units of RNaseOUT™ Recombinant RNase Inhibitor and 50 units of SuperScript™ II RT at a final volume of 20 μL, was incubated at 42 °C for 50 minutes. The reaction was terminated by heating at 70 °C for 15 minutes and then immediately chilled on ice for at least 10 minutes. One microliter (4 units μL^{-1}) of the RNase H was added into the final RT reaction mixture and incubated for 30 minutes at 37 °C to remove the mRNA before proceeding to the amplification of target cDNA. The primer set of FP and RP was used for the amplification of full-length fragment of p185 *BCR-ABL*. The 5'-biotinylated primer in reverse direction (i.e. RP) was used to introduce the biotin at the 5'-end of sense-strand DNA fragment for the separation of antisense-strand DNA from the sense-strand target. The PCR was performed on a Genius Techne PCR thermocycler (Techne Inc, Burlington, NJ) with a final volume of 50 μL consisting 2 μL of cDNA, 2.5 units of ExTaq DNA polymerase, 1× PCR buffer, 0.1 mM dNTP, and 1 μM of each RP and FP. The reaction was first incubated at 94 °C for 2 minutes. Then, it was carried out for 30 cycles at the following conditions: 94 °C for 30 seconds, 62 °C for 30 seconds, and 72 °C for 45 seconds and followed by a final extension step at 72 °C for 2.5 minutes. After the PCR amplification, the biotinylated PCR products were purified by agarose gel separation and the agarose digestion using β-agarase I. The β-agarase I was removed by phenol:chloroform extraction. The sample was also desalted and concentrated using Microcon YM-30 to improve the biotinylated purification of sense-strand DNA. The DNA sequencing analysis was carried to confirm the PCR product amplified from cDNA library was identical to the target gene.

2.4.2 Purification of sense p185-ssDNA

The sense strand DNA was separated from the magnetic bead-bound biotinylated-antisense strand of PCR product. The recommended procedure of the BioMag® Plus Streptavidin was modified to capture the biotinylated PCR products to the streptavidin-coated magnetic beads. The amount of the biotinylated dsDNA PCR product was first determined by Quant-iT dsDNA HS assay kit measured by a Qubit fluorometer (Invitrogen Corp.). The biotinylated PCR product was coupled on the magnetic beads at a ratio of 35 pmol dsDNA per mg beads in the coupling/wash buffer (0.01 M PBS, pH 7.4, 1 % BSA, 0.1 % NaN_3 and 1 mM sodium EDTA). The coupling reaction was carried out at 4 °C for overnight with gentle mixing and subsequently washed three times with ice-cold coupling/wash buffer. The non-biotinylated sense strand DNA was separated by suspending the dsDNA-bound beads in 10 mM Tris-HCl buffer (pH 8.0) and heating at 95 °C for 5 minutes. The beads carrying the antisense DNA were magnetically separated immediately. The supernatant containing the sense strand DNA was collected and concentrated using Microcon YM-30 by centrifugation at 15000×g. The concentrated sense strand DNA sample was analyzed by agarose gel electrophoresis and quantified by RQ-PCR with a 7900HT Fast Real-time PCR System (Applied Biosystems).

2.5 Sandwich hybridization

The hybridization procedures outlined below were adapted from our earlier work.[18] Each hybridization reaction involved was carried out in a 1.5 mL microcentrifuge tube. The separation of beads or bead-bound conjugates from solution was carried out by placing the microcentrifuge tube on the magnetic rack (Pierce Biotechnology, Inc.) for 3 to 5 minutes. The supernatant was removed while the beads were magnetically held at the vertical sidewall of tube.

2.5.1 Targets hybridization

Immobilization of CP to carboxylic acids functionalized beads (COOH-beads) were carried out based on a procedure disclosed in our earlier study.[18] An amount of 2.5 μL CP-beads was mixed with 5 μL of target samples and 50 μL of hybridization buffer (Gold Hybridization Buffer, 8 % blocking agent, 0.5 M sodium chloride) for each reaction tube. The mixture was incubated at 29 ^{o}C for 30 minutes. The hybridized beads were washed once with 100 μL with secondary wash buffer (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0).

2.5.2 Hybridization with CNT-based labels

Five μL of CNT labelling mixture were topped up with hybridization buffer to a total volume of 50 μL for each reaction. The mixture was added to the hybridized beads that had been washed and incubated at 29 ^{o}C for 30 minutes. After the second hybridization step, the resulting beads were washed twice each with 100 μL of primary wash buffer (75 mM sodium chloride, 7.5 mM sodium citrate and 0.4 % sodium dodecyl sulfate, pH 7.0) and 100 μL of secondary wash buffer. Before the electrochemical measurement, the hybridized beads in each tube were stored in 1×PBS buffer (pH 7.4) at 4 ^{o}C.

2.6. Samples assay

2.6.1 HRP enzymatic reaction

The HRP activity coupled to the successful hybridization events was quantified by end-point assay. The PBS solution used to store the hybridized beads was discarded. Twenty-five μL of *o*AP-H_2O_2 substrate solution were added to each reaction tube and incubated for 5 minutes at room temperature. Immediately after the 5 minutes of enzymatic reaction, the resulting HRP catalyzed enzymatic product solution was separated from the magnetic beads in the microcentrifuge tube and loaded onto the SPCE for an electrochemical measurement. This enzymatic reaction was carried identically for all control samples.

2.6.2 Electrochemical detection

The electrochemical measurement of HRP-catalyzed enzymatic product was carried out by SWV. This analysis was performed using an electrochemical analyzer CHI 620C (CH Instruments, Austin, TX) connected to a personal computer. A three-electrode configuration was employed for all electrochemical measurements. The SPCE (AndCare, Durham, NC) consisted of three built-in electrodes on a 1.3 cm × 3.0 cm × 0.05 cm plastic substrate with screen-printed carbon serving as a working electrode (0.4 cm diameter) and counter electrodes, and silver as the reference electrode. The SWV scanning was performed from 0.00 to -0.60 V with a step potential of 0.004 V, an amplitude of 0.025 V, and a frequency of 25 Hz.

3. Results and discussion

3.1 Electrochemical detection and signal amplification principles

Fig. 1 illustrates the principle of electrochemical hybridization assay amplified by CNT-based labels for the detection of nucleic acid targets. The targets include the short oligonucleotides (i.e. TG, 46 bp) and full-length sequence (i.e. p185-ssDNA or mRNA, 491bp) bearing the human leukemia p185 *BCR-ABL* oncogene. The recognition of targets was based on a sandwich hybridization assay performed on magnetic beads (Fig. 1A). The CPs were immobilized on the beads (CP-beads) via diimide-activated covalent interaction. Upon addition of samples to the CP-beads, the target was captured on the bead surfaces through the hybridization of CPs with a specific region (373 to 390 bp) of the p185 *BCR-ABL*. Following the hybridization of the target, the target-bound hybrids were tagged with the CNT-based labels via specific hybridization of DPs with another region (401-418 bp) of the target. The use of multiple-HRP functionalized labels increases the amount of signal per unit of target, and hence, is capable of providing a much better detection than that of conventional HRP labels. The activity of captured HRP in association with the amount of targets was quantified by SWV analysis measuring the electroactive enzymatic product in the presence of *o*-AP-H_2O_2 substrates solution (Fig. 1B). Reaction product of HRP-catalyzed oxidation of *o*-AP in acidic medium by H_2O_2 is expected to be 3-aminophenoxazone.[29] We have previously studied the voltammetric characteristics of HRP-*o*-AP-H_2O_2 enzyme-substrate system.[30] This electroactive enzymatic product was reduced at a disposable SPCE and yielded a well-defined reduction peak at ca. - 0.34 V (Fig. 1C). The resulting reduction currents output is proportional to the amount of targets.

3.2 SWV detection of leukemic oligonucleotide targets amplified with CNT-based labels

The analytical performances of CNT-based labels for the electrochemical detection of leukemia targets at concentrations of 8.3×10^{-14} – 1.7×10^{-9} M and negative control were examined under optimized conditions. The responses to TG samples were concentration dependent as the measured SWV signals increased proportionally with target concentrations. An exceptionally wide dynamic range over four orders of target concentration was achieved. It constitutes the high-range (dotted linear curve in Fig. 2A) and low-range (enlarged as in Fig. 2B, $< 1 \times 10^{-10}$ M) linear

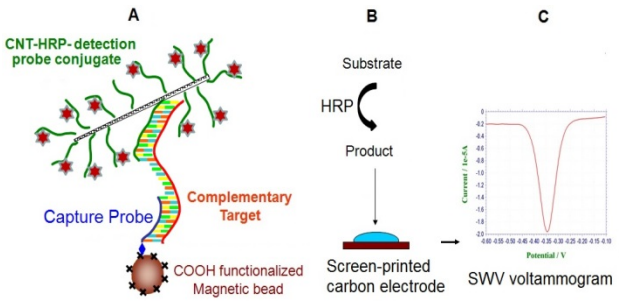


Fig. 1. Schematic representation of signal-amplified electrochemical detection of nucleic acid targets based on CNT-based labels. (A). Sandwich hybridization assay performed on magnetic beads; (B). The targets were quantified by measuring the electroactive enzymatic product, formed by the HRP catalyzed reaction with *o*-AP-H_2O_2 substrate solution using a screen-printed electrode; (C). The resulting SWV voltammogram indicates a well-defined reduction peak for TG/p185-ssDNA/mRNA targets.

plots. Fig. 2A and 2B (right) display typical electrochemical response of the assay with increasing concentrations of target (2A: 8.3×10^{-11} to 1.7×10^{-9} M, 2B: 0 to 8.3×10^{-11}; top to down.

Well-defined voltammetric peaks are observed and the peak intensities increased with the increase of target concentrations. A negligible signal was observed in the control samples, i.e. in the absence of target (Curve a in Fig. 2B, right). The high degree of labelling of a single hybridization event by the novel CNT-based labels amplifies the hybridization signal dramatically, in a way that was not achievable by employing conventional single HRP labeled probes. The detection limit of 8.3×10^{-14} M (1 pg mL^{-1}) TG was achieved. Such a detection limit corresponds to 5×10^{-18} mol or ~ 3×10^{6} molecules in a 60 µL-hybridization solution, which is comparable to those of an electrogenerated chemiluminescent (ECL) assay amplified by the ECL labels-loaded polystyrene beads (1 fM)[10] and an impedimetric sensing amplified by ALP-catalyzed precipitation of insoluble products (50 fM).[31] Our previous work found that the colorimetric hybridization assay with conventional HRP labels resulted in higher detection limit[18] at 1×10^{-9} M. The optimized electrochemical hybridization assay with CNT-based labels as signal amplifier demonstrated a remarkable 12000-fold enhancement in assay sensitivity. This measurement is more sensitive than those amplified by CdS-loaded CNT labels (6.6 pM)[16] and 2-generation dendritic-like liposome assemblies (0.1 pM).[32] The high sensitivity exhibited by our proposed CNT-based labels was with good reproducibility. A series of eight repetitive measurements of 5.8×10^{-11} M TG yielded reproducible signals with relative standard deviations (RSDs) of 7.7 %.

3.3 Discrimination of non-complementary and mismatched sequences

The specificity of the assay for the detection of target gene was further evaluated by replacing or mixing the fully complementary target (TG) with non-complementary (NTG), one-base (M1) or three-base (M3) mismatched sequences. Under optimal conditions, the proposed assay resulted in excellent assay specificity. The assay showed null responses to the blank control (TGs were replaced by ultrapure water) and negative control (8×10^{-7} M NTG), compared to the favourable response yielded by a 1000-fold less concentrated TG sample (Fig. 3A). A similar response of TG was obtained even in the presence of NTG at a 1000-fold higher concentration.

Fig. 3B shows the ability of assay in discriminating the mismatched sequences. Samples containing equal amount (8×10^{-11} M) of single-component TG, M1 or M3 and the proportionally mixed TG and M1 (TG+M1) and TG and M3 (TG+M3) were tested. The detection yielded a negligible response to M3, similar to that of blank control. Significant increase of response to TG+M3 mixture was not observed compared to that of single-component TG sample, which indicates the successful discrimination between the perfectly matched (i.e. TG) and mismatched sequences (i.e. M3).

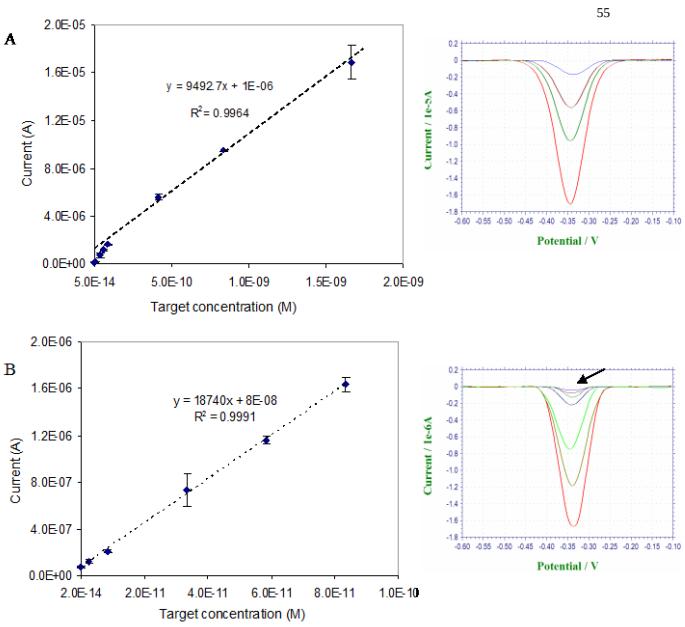


Fig. 2. Calibration curve for the electrochemical quantitation of leukemic DNA target amplified by the CNT-based labels. The dynamic detection range constitutes (A). the high-range linear plot (left) and the corresponded square-wave voltammograms (right), and (B).the enlarged low-range linear (left) and the corresponded voltammograms (right). Curve a shows the response for the control sample without the targets. The error bars are based on standard deviation with n = 3.

However, we observed a similar magnitude of response between single-component TG and M1 as well as the doubled response of TG+M1 mixture. Such similarity may occur as the thermo-stability and stringency of one-base mismatched hybrids resembled the perfectly match hybrids, in such that M1 could also hybridize to the CP-beads and result in ineffective discrimination between TG and M1. Additional efforts are generally required to achieve robust detection of single nucleotide polymorphisms (SNPs). As examples, Di Giusto *et al.* used redox-labelled nucleotide terminators,[33] while Patolsky used enzyme labels[34] in connection to the single-base extension (SBE) technique for detecting known point mutations. When the mutation site corresponded to the labelled chain terminator, the captured extension product led to an electrical signal.

3.4 Detection of p185-ssDNA targets

The proposed method was also examined for the detection of full-length target gene in biological samples. In this study, mRNA containing the target gene, i.e. p185 *BCR-ABL* was extracted from SUP-B15 cell line. Sense-strand DNAs synthesized from the mRNA templates (i.e. p185-ssDNA) were used as standards. This single-stranded p185-ssDNA was purified from the biotinylated double-stranded PCR products (detailed at Section 2.5).

Fig. 4 displays the typical electrochemical response with increasing concentrations of p185-ssDNA. Well defined voltammetric peaks are observed with low concentration of p185-ssDNA. The peak currents increased with the increase of concentrations. A negligible signal was observed in the control sample (in the absence of p185-ssDNA). The resulting calibration plot of the currents versus p185-ssDNA concentrations (inset) is linear over the range of 4.8×10^{-16} to 1.2×10^{-14} mol of p185-ssDNA and is suitable for quantitative analysis. The sample loading beyond the upper limit was saturated (data not shown). The detection limit of 1.0×10^{-16} mol (in 60 μL) of p185-ssDNA was achieved (estimated based on signal-to-noise ratio of 3). This detection limit corresponds to approximately 33 pg of target gene, which is comparable to that reported earlier for the detection of a 388-bp-long DNA identifying *Salmonella spp* (50 pM).[35] It was noted that by incorporating the TG as internal reference, the detection limit of full-length target (491 bp) was 3 to 5-fold higher than that of short TG target (46 bp). This implies that the selection and preparation of single-strand DNA standard of similar or identical length and sequence is crucial for accurate quantitative nucleic acids assay.

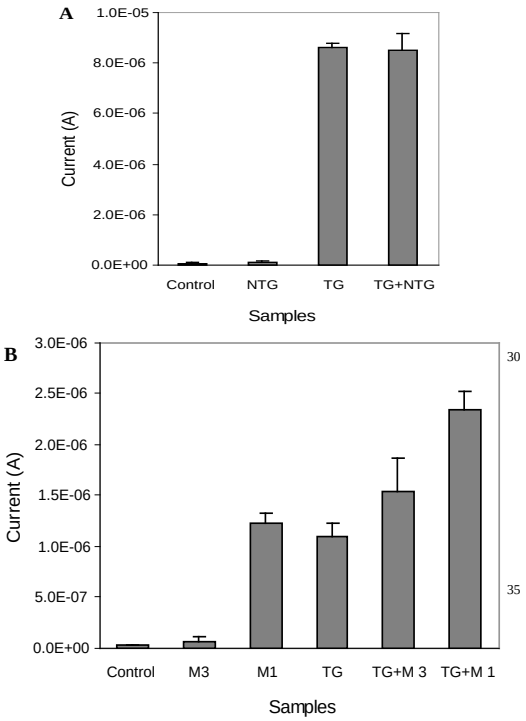


Fig. 3. Specificity of the electrochemical detection of leukemic DNA targets amplified by CNT-based labels. (A). Discrimination between complementary target (TG) and non-complementary target (NTG). The concentrations used: 8×10^{-10} M of TG, 8×10^{-7} M of NTG, a mixture containing 8×10^{-10} M TG and 8×10^{-7} M NTG (TG+NTG). Error bars: standard deviation with n = 4. The control samples contain only ultrapure water. (B). Discrimination between TG and mismatched sequences. M1 and M3 denote one-base and three-base mismatched samples respectively Single-component samples contain 8×10^{-11} M of TG, M1 or M3. The mixtures of TG+M1 and TG+M3 contain 8×10^{-11} M each of TG and M1 (or M3). Error bars: standard deviation with n = 3.

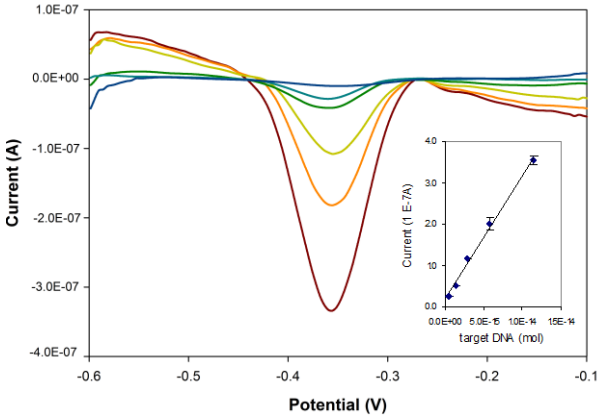


Fig. 4. Typical SWV responses with increasing concentrations of p185-ssDNA From top to bottom, the concentrations of target is 0, 4.8×10^{-16}, 1.4×10^{-15}, 2.9×10^{-15}, 5.7×10^{-15} and 1.2×10^{-14} mol, respectively. Inset shows the resulting calibration plot. Other conditions of SWV analysis and hybridization assay were detailed in the Experimental Section. All curves and value of data points were obtained after the substrate background current subtraction and baseline correction. Error bars are based on standard deviation with n = 3.

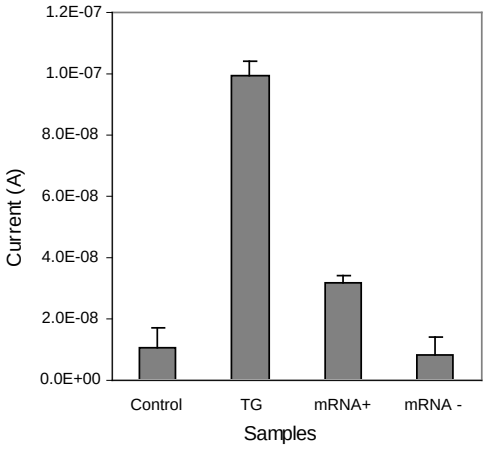


Fig. 5. Direct electrochemical measurement of p185 *BCR-ABL* mRNA fusion transcript. $mRNA^+$ and $mRNA^-$ refer to the mRNA samples extracted from the positive leukemia cell line SUP-B15 (ca. 65 ng) and negative cell line HL-60 (ca. 53 ng), respectively; TG refers to the complementary target DNA; only ultrapure water was added to the control sample. Conditions as for Fig. 4. Error bars are based on standard deviation with n = 3.

3.5 Detection of p185 *BCR-ABL* mRNA fusion transcript extracted from cell line

An mRNA pool may contain thousands of genes. A specific gene may comprise of only 0.01 to 3 % of the entire mRNA pool.[36] Without PCR amplification of target gene, our signal-amplified assay is capable of direct detection of p185 *BCR-ABL* fusion transcript in mRNA samples, which was also coupled to an excellent specificity. In this study, mRNAs containing the target gene ($mRNA^+$) were extracted from SUP-B15 cell line whereas those obtained from HL-60 cell line were used as a negative control mRNAs ($mRNA^-$). TG oligonucleotides sample was

incorporated as an internal positive control. As shown in Fig. 5, the $mRNA^+$ (ca. 65 ng) samples yielded a positive response (SNR = 2.9) in a similar way to that of TG samples. In general, repetitive detection of $mRNA^+$ with the amounts varied from 65 to 170 ng could result in the SNR of 2.9 to 11. In contrast, we observed a negligible response to the $mRNA^-$ samples (ca. 53 ng) similar to that of blank control (absence of TG and mRNA). The null responses were consistently observed in five repetitive measurements of $mRNA^-$ with the amounts of 53 to 150 ng. Our direct measurement unambiguously detect target gene from as little as ca. 65 ng of mRNA compared to the PCR assays that usually need 50 to 500 ng of mRNA sample (Invitrogen recommendation) and gel electrophoresis or northern blot that used micrograms of mRNA sample for a reliable analysis.

Conclusion

We have demonstrated a novel signal-amplified electrochemical nucleic acids assay using the numerous HRP-loaded CNT labels for the detection of human ALL-related oncogene, p185 *BCR-ABL*. The CNT-based labels bearing the 18 bp-detection probes specific to the target oncogene were applicable not only for the detection of short oligonucleotides but also the full-length sequence of p185 *BCR-ABL* ssDNA and mRNA fusion transcript. The use of such labels greatly amplifies hybridization signal and enables the detection of full-length p185 *BCR-ABL* transcripts at sub-femtomole levels, which corresponds to picograms of target gene. Without relying on RT and PCR amplification, the ultrasensitive assay demonstrated for the first time, a direct detection of p185 *BCR-ABL* mRNA fusion transcript from as little as nanograms of mRNA population extracted from SUP-B15 cell lines. The high specificity of assay successfully discriminated perfect match targets from a 3-base mismatch sequence and the noncomplementary sequences present as pure oligonucleotides or mRNA population extracted from negative ALL cell lines HL-60. The proposed approach that utilized HRP-tagged CNT-based labels can also be detected readily by fluorescent and luminescent methods. Such extension could pave the way for ultrasensitive *in situ* hybridization and imaging of specific sequences in the cells. In combination with the use of a well-quantified standard, this approach could provide a simple and sensitive quantitative tool alternative to the RQ-PCR. Gene expression analysis could also be realized by incorporating the house-keeping gene as an internal standard. The approach described herein has a great potential for ultrasensitive, simple and low cost nucleic acids detection in early disease and POC diagnostic application.

Acknowledgements

The work was done at Pacific Northwest National Laboratory (PNNL) supported partially by a laboratory directed research and development program and partially by grant number U54 ES16015 from the National Institute of Environmental Health Sciences (NIEHS), NIH. The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of the Federal Government. The research described in this paper was performed at the Environmental Molecular Sciences Laboratory, a national scientific user facility sponsored by the U.S. Department of Energy's (DOE's) Office of Biological and Environmental Research and located at PNNL. PNNL is operated by Battelle for DOE under Contract DE-AC05-76RL01830. A.C. Lee gratefully acknowledges the awards of National University of Singapore (NUS) Postgraduate Research Scholarship and PNNL fellowship as well as the helpful information on leukaemia provided by Dr Allen Yeoh (NUS) and co-workers. The authors would like to acknowledge Dr. Swee-Ngin Tan and Dr. Guodong Liu for the helpful discussion during this work.

Notes and references

[a] *Pacific Northwest National Laboratory, Richland, Washington 99352, USA*
[b] *Department of Biological Sciences, National University of Singapore, 14 Science Drive 4, Singapore 117543*
[c] *School of Mechanical and Materials Engineering, Washington State University, Pullman WA 99164, USA*
[d] *Department of Paediatrics, National University of Singapore, 5 Lower Kent Ridge Road, Singapore*

[*] *Corresponding authors: Email: yuehe.lin@pnnl.gov, Tel: 01-509-371-6241, Fax: 01-509-371-6955; and Email: dbsltm@nus.edu.sg, Tel: 65-6516 2913, Fax: 65-6779 2486*

1 J.P. Radich, *Best Practice Res. Clin. Haematol.*, 2002, **15**, 91.
2 A.S. Advani and A.M. Pendergast, *Leukemia Res.*, 2002, **26**, 713.
3 F. Divsar and H. Ju, *Chem. Comm.*, 2011, **47**, 9879.
4 M. K. Borucki, J. Reynolds, D. R. Call, T.J. Ward, B. Page and J.J. Kadushin, *Clin. Microbiol.*, 2005, 43, 3255.
5 J. Detmer, R. Lagier, J. Flynn, C. Zayati, J. Kolberg, M. Collins, M. Urdea and R. Sánchez- Pescador, *J. Clin. Microbiol.*, 1996, 34, 901.
6 W. Zhang, A.M. Asiri, D. Liu, D. Du, Y. Lin, Tr. Anal. Chem., 2014, 54, 1
7 N. N. Kariuki, J. Luo, M.M. Maye, S.A. Hassan, T. Menard, H.R. Naslund, Y. Lin, C. Wang, M.H. Engelhard, C. Zhong, Langmuir, 2004, 20, 11240
8 a) H. Wang, J. Wang, C. Timchalk, Y. Lin, Anal. Chem., 2008, 80, 8477; b) G. Liu, J. Wang, R. Barry, C. Petersen, C. Timchalk, P.L. Gassman, Y. Lin, Chem. Euro. J., 2008, 14, 9951; c) Z. Li, Y. Wang, J. Wang, Z. Tang, J.G. Pounds, Y. Lin, Anal. Chem., 2010, 82,7008
9 J. Wang, R. Polsky, A. Merkoci and K.L.Turner, *Langmuir*, 2003, **19**, 989.
10 W. Miao and A.J. Bard, *Anal. Chem.*, 2004, **76**, 5379.
11 K.A. Edwards and A.J. Baeumner, *Anal. Chem.*, 2007, **79**, 1806.
12 a). G. Liu, H. Wu, A. Dohnalkova and Y. Lin, *Anal. Chem.*, 2007, **79**, 5614; b). G. Liu, Y. Lin, J. Am. Chem. Soc., 2007, 129, 10394
13 P. Bhattacharya, D. Du, Y. Lin, J. R. Soc. Interface, 2014, 11, 20131067.
14 M. Trojanowicz, *Trends Anal. Chem.*, 2006, **25**, 480.
15 Y. Lin, S. Taylor, H. Li, K.A.S. Fernando, W. Wang, L. Gu, B. Zhou and Y.P. Sun, *J. Mater. Chem.*, 2004, **14**, 527.
16 J. Wang, G. Liu, M. Rasul Jan and Q. Zhu, *Electrochem. Commun.*, 2003, **5**, 1000.
17 X. Yang, Y. Lu, Y. Ma, Z. Liu, F. Du and Y. Chen, *Biotechnol. Lett.*, 2007, **29**, 1775.
18 A.C. Lee, J.-S. Ye, S.N. Tan, D.P. Poenar, F.-S. Sheu, C.K. Heng and T.M. Lim, *Nanotechnology*, 2007, **18**, 455102.
19 J. Wang, G. Liu and M. Rasul Jan, *J. Am. Chem. Soc.*, 2004, **126**, 3010.
20 B. Munge, G. Liu, G. Collins and J. Wang, *Anal. Chem.*, 2005, **77**, 4662.

21 X. Yu, B. Munge, V. Patel, G. Jensen, A. Bhirde, J.D. Gong, S.N. Kim, J. Gillespie, J.S. Gutkind, F. Papadimitrakopoulos and J.F. Rusling, *J. Am. Chem. Soc*., 2006, **128**, 11199.

22 J. Wang, G. Liu and Y. Lin, *Analyst*, 2006, **131**, 477.

23 Y. Wang, P.P. Joshi, K.L. Hobbs, M.B. Johnson and D.W. Schmidtke, *Langmuir*, 2006, **22**, 9776.

24 J.P. Tosar, G. Brañas and J. Laíz, *J, Biosens. Bioelectron*., 2010, **26**, 1205.

25 K.J. Odenthal and J.J. Gooding, *Analyst*, 2007, **132**, 603.

26 A.C. Lee, Z. Dai, B. Chen. H. Wu, J. Wang, A. Zhang, L. Zhang, T.M. Lim and Y. Lin, *Anal. Chem.*, 2008, **80**, 9402.

27 H. Xie, Y.H. Yu, F. Xie, Y.Z. Lao and Z. Gao, *Anal. Chem.*, 2004, **76**, 4023.

28 E.N. Kadnikova and N.M. Kostic, *J. Mol. Catal. B: Enzym.*, 2002, **18**, 39.

29 D. Goncalves, R.C. Faria, M. Yonashiro and L.O.S. Bulhoes, *J. Electroanal. Chem.*, 2000, **487,** 90.

30 A.C. Lee, G. Liu, G. C.K. Heng, S.N. Tan and T.M. Lim, *Electroanalysis*, 2008, **20**, 2040.

31 F. Patolsky, A. Lichtenstein and I. Willner, *Chem. Eur. J*., 2003, **9**, 1137.

32 F. Patolsky, A. Lichtenstein and I. Willner, *J. Am. Chem. Soc*., 2001, **123**, 5194.

33 D.A. DiGiusto, W.A. Wlassoff, S. Giesebrecht, J.J. Gooding and G.C. King, *J. Am. Chem. Soc*., 2004, **126**, 4120.

34 F. Patolsky, A. Lichtenstein and I. Willner, *Nat. Biotech.*, 2001, **19**, 253.

35 F. Lucarelli, G. Marrazza and M. Mascini, *Langmuir*, 2006, **22**, 4305.

36 In *Oligotex® Handbook*, 2nd edn.,Qiagen, 2002, pp. 94.

37 Y. Zhuo, R. Yuan, Y.Q. Chai and C.L. Hong, *Analyst*, 2010, **135**, 2036.

38 J. Zhang, Y.Q Chai, R. Yuan, Y.L. Yuan, L.J. Bai and S.B. Xie, *Analyst*, 2013, **138**, 6938.

39 R. Malhotra, V. Patel, J.P. Vaqué, J.S. Gutkind and J.F. Rusling, *Anal. Chem.*, 2010, **82**, 3118.

40 Y. Xiang, Y.Y. Zhang, B.Y. Jiang, Y.Q. Chai and R. Yuan, *Sens. Actuators B*, 2011, **155**, 317.