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Dual detection system for cancer-associated point mutations assisted by a multiplexed LNA-based amperometric bioplatform coupled with rolling circle amplification

Ravery Sebuyoya [a,b,1], Alejandro Valverde [c,1], Ludmila Moranova [a], Johana Strmiskova [a,b], Roman Hrstka [a], Víctor Ruiz-Valdepeñas Montiel [c], José M. Pingarrón [c], Rodrigo Barderas [d], Susana Campuzano [c,*], Martin Bartosik [a,*]

[a] Research Centre for Applied Molecular Oncology, Masaryk Memorial Cancer Institute, Zluty Kopec 7, 656 53 Brno, Czech Republic
[b] National Centre for Biomolecular Research, Faculty of Science, Masaryk University, Kamenice 5, 625 00 Brno, Czech Republic
[c] Department of Analytical Chemistry, Faculty of Chemistry, Complutense University of Madrid, 28040 Madrid, Spain
[d] Chronic Disease Programme, UFIEC, Institute of Health Carlos III, Majadahonda, 28220 Madrid, Spain

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ABSTRACT

DNA point mutation in a *BRAF* proto-oncogene, V600E, is considered an important prognostic and predictive biomarker in various types of cancer, such as melanoma or colorectal cancer. We report here a novel electrochemical (EC) bioplatform for the analysis of *BRAF* V600E mutation coupled with rolling circle amplification (RCA) and locked nucleic acid (LNA) capture probes. A dual detection system was implemented, whereby two padlock probes complementary to either wild-type (wt) *BRAF* gene or DNA with V600E mutation (mut) led to amplification of wt or mut variant, respectively. Hybridization with specific LNA capture probes then increased the assay specificity, while EC detection provided rapid measurement times. The bioplatform was applied to analyze *BRAF* V600E mutation of cancer cells and tumor tissues from patients with melanoma or colorectal cancer. This is the first RCA-based EC bioplatform for *BRAF* analysis in a dual format without using PCR or sophisticated instrumentation.

1. Introduction

DNA point mutation in the genome refers to an addition, deletion, or substitution of a single nucleotide for another, arising from spontaneous DNA replication errors or from exogenous sources. The discrimination of DNA single point mutations is important for a broad spectrum of research studies, including fundamental research on gene structure and function, the study of genetic diseases and disorders, and species identification. Moreover, DNA point mutations in driver genes are closely linked to the onset and progress of humans' diseases, especially cancer. One important driver mutation is located in the *BRAF* proto-oncogene, where a single nucleotide substitution at the second position of codon 600 in exon 15 (GTG → GAG, i.e. T1799A) results in an amino acid exchange from valine (V) to glutamic acid (E) (termed V600E mutation). This mutation causes continuous activation of the B-Raf oncoprotein regardless of an external stimulus. The V600E mutation has been linked to various types of cancer, including melanoma, colorectal cancer, lung cancer, thyroid papillary cancer, multiple myeloma or hairy cell leukemia [1–3]. Although patients with mutated *BRAF* have usually worse prognosis, the *BRAF*-targeted therapy nowadays shows remarkable efficacy in *BRAF*-mutated melanoma, where the presence of a *BRAF* V600 mutation serves as predictive biomarker of therapy response [4,5].

Despite potentially serious effects of point mutations, they represent relatively subtle changes in the DNA structure, making their analysis challenging. Current method of choice is next-generation sequencing (NGS), enabling high-throughput screening of a large panel of point mutations across a whole genome [6]. However, NGS is still expensive, laborious and time-consuming, and it generates vast amount of data that are difficult to manage in clinical practice. Another option is to use low-throughput PCR-based techniques usually targeting a single point mutation (or a small panel of mutations), which are faster and less expensive than NGS, but also require large instrumentation and skilled

* Corresponding authors.
E-mail addresses: susanacr@quim.ucm.es (S. Campuzano), martin.bartosik@mou.cz (M. Bartosik).
[1] The first two authors have equal contribution to the work.

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personnel [7].

New biomedical technologies addressing the above-mentioned challenges are thus needed, ideally meeting the demands of precision medicine at the point-of-care. Bioassays and biosensors based on electrochemical (EC) analysis can be an interesting option, since they utilize inexpensive, simple and miniaturized instrumentation with the possibility of parallel measurements at electrode chips and arrays, while they allow rapid and highly sensitive determinations [7–11]. Numerous EC-based assays have been developed for DNA point mutation analysis, mostly focusing on *TP53* or *KRAS* genes that play crucial roles in cancer development or prediction of therapy outcome, respectively [12–16]. Less frequently, *BRAF* V600E mutation has also been a target of several EC assays [17–21]. However, the most competitive methods that were tested in cancer cell lines [18] or in plasma samples from oncological patients [21] required ARMS-PCR amplification, making them less suitable for point-of-care applications.

Compared to PCR, isothermal amplification techniques provide shorter amplification times, moderate constant temperatures without the need for thermal cycling, and better resistance to PCR inhibitors [22]. Techniques such as loop-mediated amplification (LAMP), strand displacement amplification (SDA), recombinase polymerase amplification (RPA), or rolling circle amplification (RCA) are being increasingly used in biosensor research, including analysis of DNA point mutations [15,20,23–25]. RCA is especially suitable for this purpose. During the RCA reaction, DNA of interest (target DNA) binds to both ends of a specially designed padlock probe (PP) that bears a phosphate moiety at its 5'-end. Upon successful hybridization, both ends of the PP come to a close vicinity for subsequent ligation reaction. The ligation occurs only when the sequences are fully complementary, creating covalently closed circular DNA that serves as a template for RCA. Even a single mismatch can be detrimental for the ligation and thus no RCA product is generated [26]. Principles, design and application of RCA in biosensing platforms have been recently reviewed [27]. There are several DNA point mutation assays combining RCA with EC measurement, such as a strategy based on nicking endonuclease-assisted target recycling to analyze *TP53* mutation [28], or an assay combining RCA with MutS protein and atomic transfer radical polymerization reaction to check for *KRAS* mutation [16]. However, these methods, not applied to the detection of point mutations in *BRAF*, only detected synthetic targets in human serum, with no application to cancer cell lines or patient samples.

To improve discrimination ability of assays, DNA capture probes (CP) are often replaced with structural analogs, most notably locked nucleic acids (LNA). With their altered backbone structure but same base pairing abilities, LNAs show highly improved binding affinities towards complementary DNA sequences and also improved mismatch recognition abilities, making them especially useful in mutational analysis [29]. Few EC-based assays utilized LNA probes for an improved mismatch recognition [30,31], but they did not target *BRAF* V600E mutation and they relied on PCR amplification.

Most EC assays for analysis of DNA point mutations do not show feasibility in real samples, including cancer cell lines and patient samples. To address this challenge, we report here an EC bioassay for quick analysis of *BRAF* mutation status, i.e., for discrimination between wild-type and mutated V600E sequences. This was achieved by combining RCA that selectively amplifies either wild-type (wt) DNA sequence or the mutated form of *BRAF* DNA (V600E mutation, mut), hybridization with LNA capture probes on magnetic particles (MPs) that further increased assay specificity, and electrochemical detection on screen-printed electrode chips that provided desired sensitivity and rapid measurement times. The assay involved a dual detection system with two RCA padlock probes simultaneously targeting wt and mut DNA, thus reducing the number of false negatives. It was applied to check the mutation status of selected panel of cancer cell lines and tumor tissues from patients with melanoma or colorectal cancer with known *BRAF* status by innovatively applying a quick pre-amplification step using RPA reaction that generated short DNA fragments as an input material for RCA.

2. Material and methods

Chemicals and apparatus, RCA protocol, Protocol with magnetic particles, Amperometric measurements, Cell lines and patient samples, RPA protocol and High resolution melting analysis are described in detail in the Supporting Information.

3. Results

3.1. Assay principles

The current assay comprises several steps depicted in the assay workflow displayed in Fig. 1. In the first step (Fig. 1, step 1), either a wt double-stranded DNA or double-stranded DNA bearing V600E mutation in *BRAF* gene was mixed with the universal primer and specific padlock probe (PP). The PP was designed for both wt and mut sequences, i.e., the wt padlock probe (wtPP) contained adenine at its 3'-end for successful pairing with thymine at the position 1799 in the wt target. Conversely, the mutated padlock probe (mutPP) contained thymine at its 3'-end to pair with adenine in target V600E sequence (Table S1 in the Supporting Information). To open target DNA duplexes and facilitate binding with the primer and PPs, this step involved quick 5 min denaturation and cooling on ice.

In the second step (Fig. 1, step 2), the ligation reaction took place. PP was designed in such a way that two end regions of its sequence were complementary to a single stretch of the target DNA. Upon successful hybridization, both ends of the PP were covalently joined by the action of the T4 ligase enzyme. It should be noted that the ligation efficiently proceeded only if the 3'-end of the PP was complementary to its target, i.e., when wt target hybridized with the wtPP, and mutated target with the mutPP. Ligation efficiency was greatly reduced in the presence of mismatched bases at the mutation site (for wt target/mutPP pair and mut target/wtPP pair, respectively); this was the first level of a mismatch recognition. A product of the ligation reaction, which was a covalently closed circular padlock probe (PP), then served as a template in the following RCA reaction.

In a third step (Fig. 1, step 3) the RCA reaction was set up to amplify the covalently closed circular PP sequence from the previous ligation reaction. The phi29 polymerase in the reaction mixture extended the 3'-end of the primer that was bound to circular PP to generate huge RCA product containing thousands repeats that are complementary to the PP sequence. During the RCA reaction, phi29 polymerase also incorporated biotin-modified dUTP nucleotide into the growing RCA product for subsequent EC measurement. In absence of circular PP, no amplification occurred, and no RCA product was generated; this can be considered as a second level of a mismatch recognition.

The RCA product is very large, which may lead to less effective hybridization with LNA CPs at MPs in the next step. Indeed, some studies have attempted to measure the size of the RCA product [32–34] demonstrating that it ranges between 40 and 175 thousand nucleotides. Therefore, we implemented a fourth step (Fig. 1, step 4) involving an enzymatic fragmentation of the RCA product using MspI restriction endonuclease. This endonuclease specifically digests double-stranded DNA at the restriction site CCGG, which was intentionally inserted into both wtPP and mutPP during their design (see Table S1 in the Supporting Information). Digestion of otherwise single-stranded RCA product most probably occurs on the double-stranded portions of the amplicon (Fig. 1B, small blue and red boxes), as shown recently by Minero et al. who reported formation of secondary double-stranded structures within a huge single-stranded RCA product [35]. Moreover, digestion of RCA products with MspI enzyme was already described [36]. Gel electrophoresis analysis in Fig. S1 (in the Supporting Information) showed a successful digestion.

In the next step (Fig. 1, step 5), digested RCA products were hybridized to either wt- or mut-specific LNA CPs, designed to significantly improve mismatch discrimination in comparison with unmodified DNA

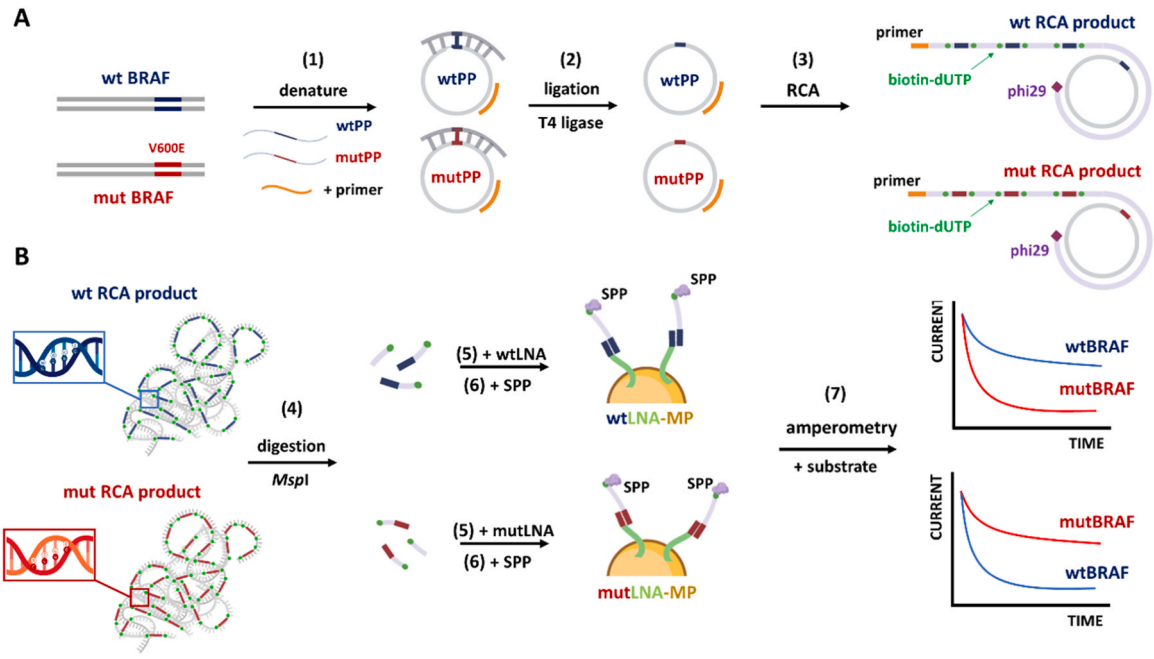

Fig. 1. Simplified workflow of the assay implemented for the analysis of *BRAF* V600E mutation: (A) isothermal amplification of wt or mut DNA targets using RCA reaction; (B) digestion of RCA products by MspI (occurring at secondary double-stranded structures, see zoomed area in the box), hybridization of the digested RCA products at LNA-modified magnetic particles (MPs) and amperometric monitoring of peroxidase reaction at electrode chips. PP – padlock probe; RCA – rolling circle amplification; SPP – streptavidin-peroxidase polymer.

probes [37,38], and covalently attached to carboxyl MPs via 5'-terminal amino group. LNA probe is a chimeric DNA/LNA with few LNA nucleotides inserted into the DNA sequence. In our case, the 15-nt long LNA probes were composed of 12 deoxyribonucleotides and 3 adjacently inserted LNA nucleotides (triplets) located in the middle of the probe around the mutation site (with the point mutation in the middle of the triplet, see Table S1 in the Supporting Information). Compared to DNA probes, LNA probes exhibit much larger differences in melting temperatures (ΔT_m) between fully complementary and single mismatched duplexes. Indeed, we performed a high-resolution melting experiment (Table S2 in the Supporting Information) that showed larger ΔT_m between LNA/DNA duplexes (i.e., between wtLNA CP/wt DNA target and wtLNA/mut DNA target, $\Delta T_m = 12.7$ °C) than between their DNA/DNA counterparts (between wtDNA CP/wt DNA target and wtDNA/mut DNA target, $\Delta T_m = 8.4$ °C). Binding of RCA products to their fully complementary LNA probes can thus be considered as a third level of mismatch recognition.

Thereafter, the MPs modified with the RCA product were incubated with streptavidin-peroxidase polymer (SPP) that interacted with biotin moieties incorporated within the RCA product (Fig. 1, step 6). SPP, which we used in our previous work [39], is a polymeric variant where the streptavidin protein is covalently conjugated to a polymerized form of horseradish peroxidase enzyme. SPP generated enhanced EC signals allowing better sensitivities than when using its monomeric counterpart, i.e., one HRP molecule per one streptavidin molecule (wt/mut signal ratio of 91 for SPP and 63 for the monomer, Fig. S2 in the Supporting Information). The recorded readout was obtained by monitoring activity of the peroxidase enzyme in the presence of hydroquinone (HQ) and hydrogen peroxide (Fig. 1, step 7). SPP enzymatically oxidized HQ to benzoquinone (BQ), which was reduced back to HQ at the carbon electrode surface. The resulting cathodic current was monitored by chronoamperometry in a multielectrode format on the chip where eight measurements can be performed simultaneously to speed up the whole readout (i.e., all eight samples were measured in 90 s), as we have shown previously [40–42]. The optimization experiments that searched for the best conditions in terms of reaction times, temperatures and concentrations of components can be found in Figs. S3-S8 (in the Supporting Information).

3.2. Control experiments

To evaluate the reliability of the methodology and the role played by each component, control experiments were carried out where one individual component per sample was excluded (Fig. 2). Without PP, negligible EC signals were obtained (sample 1) since no RCA product was formed. As expected, RCA was also unsuccessful without T4 ligase or phi29 polymerase (samples 2 and 3). When the MspI digestion was absent in the protocol, the recorded current dropped by ~20 %, indicating that the digestion step improved the hybridization efficiency with LNA probes (sample 4). The assay, however, can be used without the MspI digestion and thus it is possible to obtain satisfactory results at shorter time.

Furthermore, even when the RCA product was formed (as supported by the gel electrophoresis, see Fig. S1 in the Supporting Information), the amperometric signal was negligible in the absence of LNA CPs (i.e., MPs were used without attached LNA probes, sample 5). This confirmed that the binding of RCA products to MPs takes place solely via hybridization with the CPs. In addition, very low signal was obtained also when excluding SPP from the assay, suggesting that the enzymatic reaction is crucial for the signal generation (sample 6).

3.3. Analytical characteristics

After careful optimization of all key parameters, we constructed a calibration plot using wt target DNA (in combination with wtPP and wtLNA-MPs) up to 500 nM value (0, 0.1, 0.5, 1, 5, 10, 100, and 500 nM, Fig. 3A). Raw amperograms for these eight samples are displayed in Fig. 3C. A linear range was obtained between 0.5 nM and 10 nM with R^2 of 0.9994 and slope value of 1.82 ± 0.02 μA nM^{-1} (Fig. 3B). A limit of detection (LOD) of 55 pM was calculated as three times the standard deviation (obtained from ten blank measurements) divided by the slope value. A limit of quantification of 184 pM was calculated as ten times of standard deviation divided by the slope value.

In addition, we also ran the same samples on gel electrophoresis

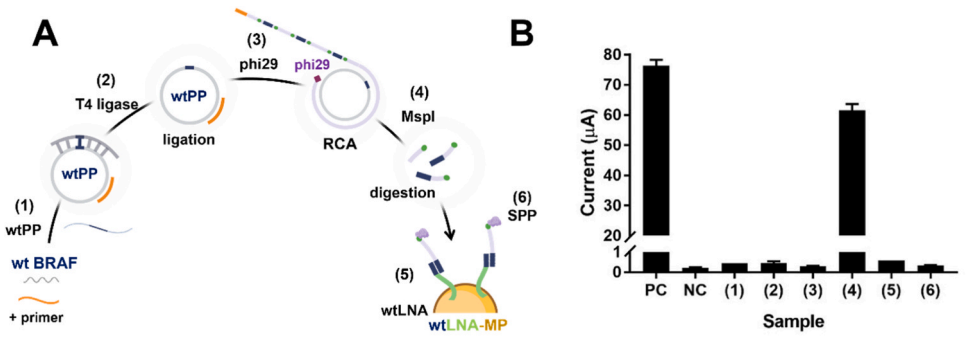

Fig. 2. Role of individual components and steps during the assay implemented for the analysis of *BRAF* V600E mutation. (A) Schematic overview of individual steps. (B) Amperometric responses. PC: positive control with all the components involved, using 25 nM wt target with wtPP and wtLNA MPs; NC: mut target; (1) no wtPP; (2) no T4 ligase; (3) no phi29; (4) no MspI; (5) no wtLNA probe; (6) no SPP.

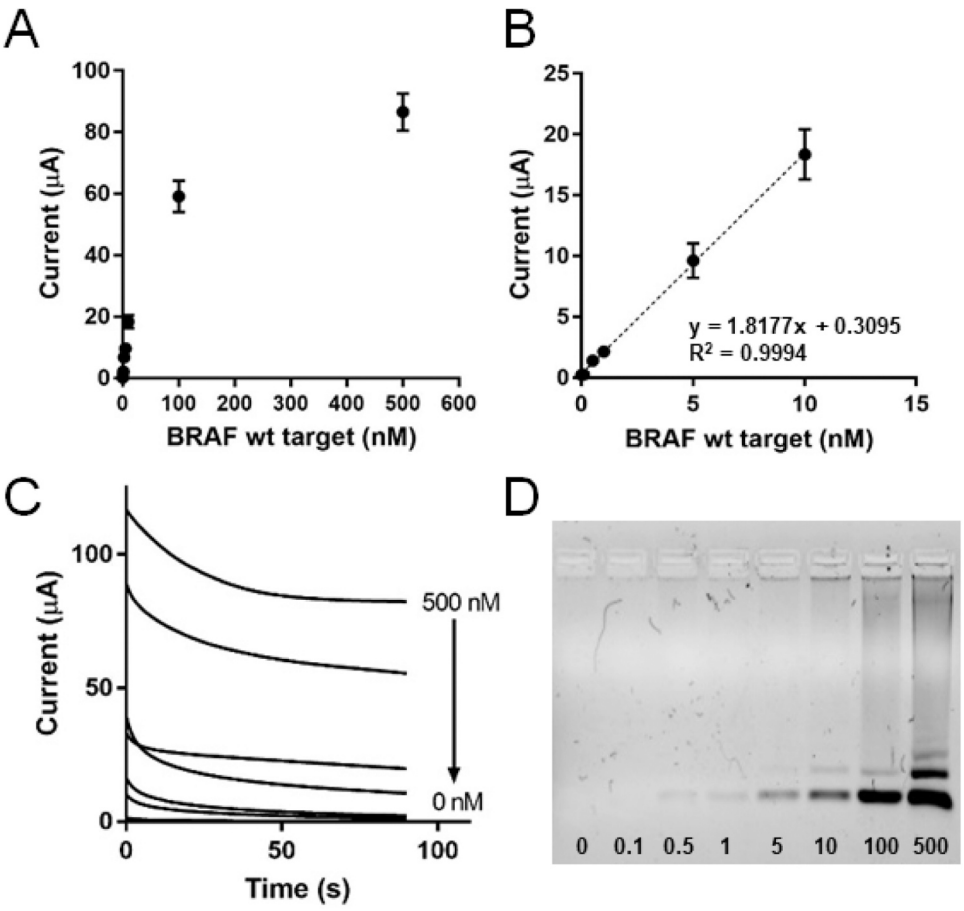

Fig. 3. Calibration plot for a wt target DNA using wtPP and wtLNA-MPs. The amperometric responses are plotted (A) as mean values with error bars from duplicate measurements. (B) Narrower range shows the linear range of the calibration plot, along with the linear equation and R^2 value showing a goodness-of-fit measure for linear regression models. (C) Raw amperograms for a concentration range from 0 to 500 nM. (D) Gel electrophoresis of corresponding digested RCA products. Numbers denote nanomolar concentrations of input DNA.

(Fig. 3D), showing good correlation between the two methods, but as expected, the gel electrophoresis exhibited worse sensitivity (bands not visible for concentrations lower than 0.5 nM) compared to the EC bioassay (with LOD of 55 pM). The reproducibility of the assay was tested from the parallel measurements of eight 5 nM wt DNA target replicates. A relative standard deviation (RSD) value of 4.9 % was obtained.

3.4. Dual detection using wt and mut padlocks

As mentioned above, optimization studies were carried out using a wt padlock targeting wt sequence of the *BRAF* gene, and a clear discrimination between wt (positive control, higher signal) and mut (negative control, lower signal) samples was observed (Fig. 2, PC and NC columns). However, it is useful to devise a dual detection system with two different padlock probes, one targeting the wt sequence and the other targeting mut sequence of the *BRAF* gene. We hypothesized that a DNA sample containing wt sequence when interrogated with both padlocks, would produce a larger signal for wtPP and much lower signal for mutPP, and vice versa, the sample containing mut sequence would produce a larger signal when using mutPP and lower signal for wtPP. A clear benefit of this approach would be a lower number of false negatives, i.e., the sample should not be negative for both padlocks, but positive for either wtPP or mutPP. If the sample was negative for both padlocks, the assay did not work as expected and needs to be repeated (for instance, amplification with the PP could have failed, pipetting error could occur, etc.). This is in contrast with the single padlock approach, where it would be difficult to distinguish whether negative result was

due to a mismatch presence (real negative sample) or due to failure of the amplification (false negative sample).

Therefore, we designed these two padlocks, differing in only one nucleotide at the 3'-end of their sequence. The data shown in Fig. 4 A indicate that the dual detection approach was indeed feasible. Optimized conditions were used with minor modifications. We increased the temperature of RCA product hybridization at MPs from RT to 50 °C. This modification ensured that the two positive controls, samples a (wt target + wtPP + wtLNA) and h (mut target + mutPP + mutLNA) yielded larger amperometric signals, while all other combinations (b-g, negative controls) were suppressed as much as possible. Even at 50 °C, we observed appreciable signals for two negative controls where the RCA product was generated, i.e., the sample d (mut target + mutPP + wtLNA) which was suppressed by ~66 %, and the sample e, which was suppressed by ~71 %. It seems that when the RCA products are successfully formed (samples a, d, e, h), they bind not only to the matching LNA CPs (a, h), but a fraction of the product may hybridize to non-matching CPs as well (d, e). Eventually, it was a fine-tuning of the temperature and ionic strength during the hybridization that enabled the significant reduction of the amperometric signals to only 5 % (sample b), 1 % (sample c) and 33 % (sample d) compared to matching wt sample a (set to 100 %), and to 29 % (sample e), 2 % (sample f) and 3 % (sample g), compared to matching mut sample h (100 %). Analytical signals of the two positive controls (columns a and h), although clearly larger than the negative controls, did not show similar intensities when compared to each other. This difference (wt sample was ~25 % larger than mut sample) was attributed to different efficiencies in some of the steps, most likely in DNA ligation, coupling of LNA probes to MPs, or hybridization of RCA products to LNA probes.

Clinical samples are often heterogeneous and may contain not only DNA from tumor cells harboring *BRAF* mutation, but also from nearby cells with unmutated wt *BRAF* status. This is even more pronounced in a liquid biopsy when circulating tumor DNA (ctDNA), which represents only a small fraction of total circulating free DNA (cfDNA), is analyzed [43]. Hence, we tested how sensitive is our assay towards mutated DNA in the presence of abundant wt DNA by adding small increments of mut DNA (from 1 % to 50 %) to a constant amount of wt DNA (100 %), as shown in Fig. 4 B. The obtained results indicate that it was possible to distinguish mut *BRAF* target in 100-fold excess wt (i.e., 1 % mut DNA in wt DNA) and the signal from mut DNA was steadily rising with further additions. The very low signal obtained for sample denominated as "0 % mut", which was the pure wt DNA, confirmed excellent recognition abilities of mutPP and mutLNA towards mutated DNA. In fact, as shown in the inset of Fig. 4B, the results demonstrate close to linear semi-logarithmic dependence on the current on %mut in wt target ($R^2 = 0.9812$) that could be exploited in future studies for quantification purposes.

Furthermore, we checked whether the assay was able to selectively discriminate the *BRAF* gene from other genes. Fig. 4 C shows the expected large signals from the positive controls (wt *BRAF* DNA using wtPP, black bar; mut *BRAF* DNA using mutPP, grey bar), while negligible currents were obtained from all mismatched DNA samples, including single-mismatched samples (mutPP + wt target, wtPP + mut target) and fully noncomplementary DNA samples (short sequences from *KRAS* gene and viral HPV16 DNA). In fact, this almost complete selectivity towards fully complementary sequences (slightly better for wt probes) is remarkable and can be attributed to the different levels of mismatch recognition imparted by RCA, the use of LNA probes, and their particular design (see also Table S2, HRM analysis). Moreover, we analyzed both wt and mut *BRAF* targets in complex mixtures made of

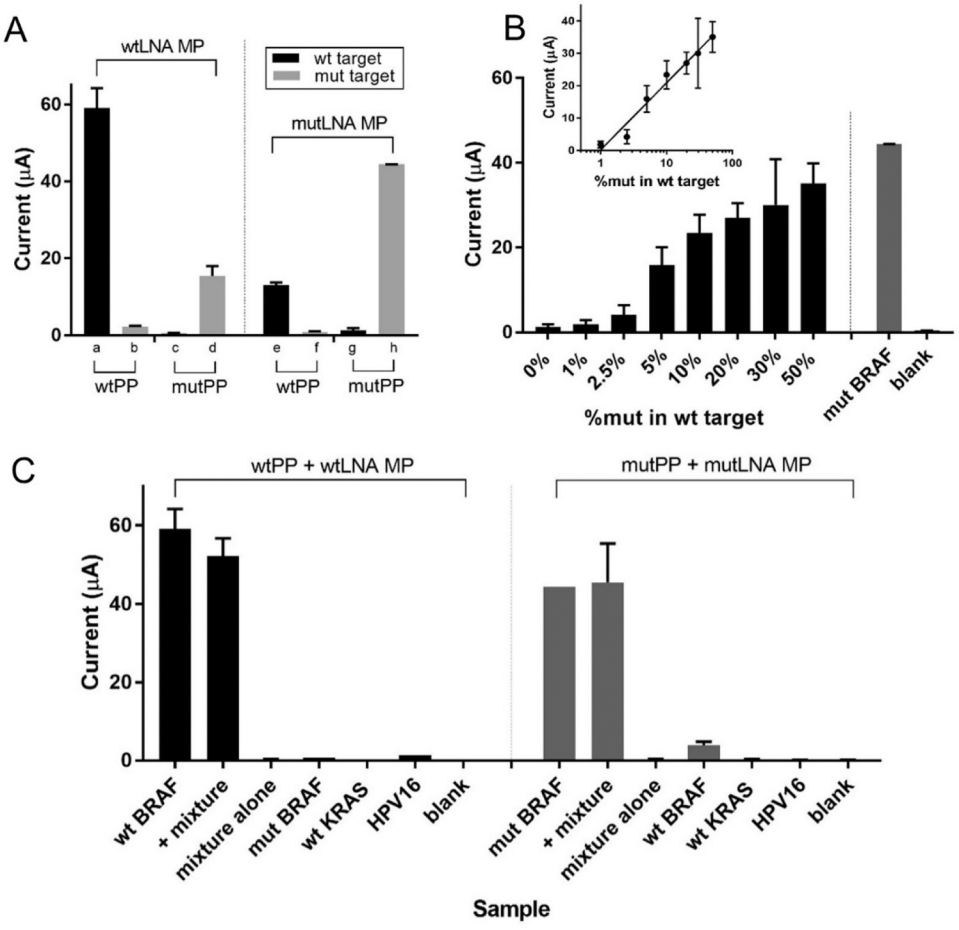

Fig. 4. (A) Dual detection approach using wtPP or mutPP to interrogate *BRAF* mutation status of the wt and mut target DNA. Columns (a) and (h) are positive controls with the highest currents, columns (b-g) should be suppressed as much as possible. (B) Addition of mut target to an excess of wt target (black bars). Analysis was performed using mutPP and mutLNA MPs. Grey bars represent positive control (100 % mut target) and negative control without target (blank). Inset: A semi-logarithmic dependence showing a linear trend. (C) Selectivity experiment showing non-complementary DNAs or their mixtures, interrogated with both wtPP (left, black bars) and mutPP (right, grey bars). Sample "+mixture" is a coctail of *BRAF* targets (wt and mut) and non-complementary *KRAS* DNA and HPV16 DNA; sample "mixture alone" is the same coctail without corresponding *BRAF* target.

these non-complementary sequences. For wt *BRAF* target, the mixture contained mut *BRAF*, *KRAS* DNA and HPV16 DNA; vice versa, for mut *BRAF* target, the mixture contained wt *BRAF*, *KRAS* DNA and HPV16 DNA. Fig. 4 C shows that a successful recovery of complementary targets in the mixtures was achieved (88 % recovery for wt DNA and 102 % for mut DNA), while mixtures alone provided only negligible signals.

3.5. Real samples analysis

To evaluate applicability of the bioassay in clinical settings, we analyzed *BRAF* mutation status in DNA extracted from various cancer cell lines, harboring either wt sequence or V600E mutation. Three cell lines were interrogated using the dual detection approach with both wtPP and mutPP (Fig. 5, left). The A549 lung cancer cell line harbors a wild-type *BRAF* in both alleles (wt/wt), HT-29 cell line is a *BRAF* V600E heterozygote (wt/V600E) and A375 melanoma cell line is a mutated homozygote (V600E/V600E), having the mutation in both alleles. The obtained results fully reflected this; the A549 DNA (wt/wt) interrogation exhibited a large amperometric signal using wtPP and a negligible signal from mutPP. Conversely, A375 DNA (V600E/V600E) gave rise to a much larger signal using mutPP and a low signal from wtPP. Moreover, HT-29 DNA (wt/V600E) yielded comparable currents for both wtPP and mutPP. These results suggested a good discrimination ability of the dual assay.

As a proof of concept, eight clinical samples obtained from tumor tissue of patients with either colorectal cancer or melanoma, where *BRAF* mutation status was determined by sequencing (Table S4), were analyzed (Fig. 5, middle). Three patients with no V600E mutation (wt/wt) gave considerably larger signals when using wtPP than when using mutPP (patients 1–3); two patients with confirmed V600E mutation in both alleles (mut/mut, patients 7–8) exhibited much larger signals for mutPP than for wtPP. These results agreed perfectly with sequencing data. In addition, three patients with confirmed V600E mutation in ~50 % of the DNA sample and were thus considered heterozygous (wt/mut), gave mixed amperometric signals, i.e., the signals appeared for both wtPP and mutPP. This was also in agreement with sequencing, although heights of the signals were not always similar for both padlocks, as would be expected. This was probably due to the variable ratio of tumor/non-tumor tissue taken during the biopsy (details in Table S4) and the large heterogeneity of the clinical samples. Again, we cannot also exclude possible differences in efficiency of RCA reaction or during LNA coupling to MPs. This issue should be resolved by increasing the panel of clinical samples which we plan to address in following work.

When working with cell lines and patient samples, we included a DNA pre-amplification step using RPA technique to obtain short DNA fragments from *BRAF* gene that were used as a target for subsequent RCA reaction. When using long genomic DNA, as in our case, it is often useful to first obtain shorter fragments to facilitate binding with padlock probe [44], since RCA is more suitable for short targets [23,45]. Indeed, Fig. S9 shows that without any pretreatment, the RCA itself was not sufficient to amplify DNA from cell lines, and thus we tested nonspecific fragmentation by ultrasonication or enzymatic pre-amplification of the *BRAF* gene with PCR or RPA to obtain shorter templates. The option of ultrasonication was unsuccessful, but good results were obtained especially with RPA reaction that generated approx. 200 bp long RPA products from genomic DNA (see gel electrophoresis in Fig. S10). RCA reaction with PCR amplicons has also worked, but less efficiently than RPA pre-amplification (Fig. S9). These results confirmed that RPA pretreatment, innovatively proposed in this work, was essential to obtain good results in the analysis of clinical samples and probably explain why other reported RCA-based assays coupled with an EC detection for DNA point mutations (summarized in Table S5) did not prove results in these challenging applications. Although some works summarized in Table S5 yielded lower detection limits than that achieved in the developed method, none of them used actual biological samples, but instead relied on spiking of a synthetic gene into human serum. Moreover, these methods employed only single detection strategy (targeting either wt or mut, but not both), and required a longer assay time. To our knowledge, this is the first time that an RCA-based electrochemical bioplatform has demonstrated the ability to simultaneously detect point mutation status (wt/V600E) in cancer cells and tissues. The determination requires only 100 ng of genomic DNA and no sophisticated instrumentation or biohazardous materials, making it a fairly simple and affordable protocol that can be implemented in a low-resource settings.

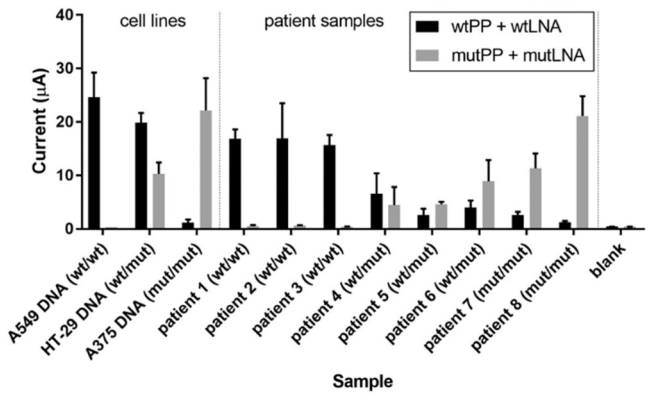

Fig. 5. Analysis of cancer cell lines (lung cancer cell line A549, colorectal cancer cell line HT-29, and melanoma cancer cell line A375) and tumor tissue samples from eight patients, using the dual detection approach. *BRAF* mutation status of cell lines and patients is shown in parenthesis. Negative control (blank) is shown as well.

4. Conclusions

A fast analysis of *BRAF* mutation status could be very helpful in deciding on the appropriate treatment modality, as well as when evaluating prognosis or prediction of therapy response in various types of cancers. Moreover, the possibility of detecting a small amount of mut *BRAF* DNA in an excess of wt *BRAF* DNA is considered of great relevance for tumor heterogeneity and for early diagnosis and/or detecting the presence of minimal residual disease [3,18]. EC-based bioassays are particularly suitable due to the simple and inexpensive instrumentation involved, fast analysis times, and option of miniaturization and multiplexing. Importantly, other available EC bioassays do not show feasibility in real samples, especially in tumor samples from patients, which is crucial when evaluating the potential clinical use.

We addressed this issue by developing a dual detection system for interrogation of *BRAF* V600E point mutation with no need for PCR amplification. Instead, we combined isothermal RCA, LNA capture probes and amperometric measurements that helped us to reach good sensitivity, selectivity as well as reproducibility of the assay. Detection range spanned four orders of magnitude, with limits of detection and quantification of 55 pM and 184 pM, respectively. A great selectivity towards fully complementary DNA as compared to single mismatched DNA, based on discriminatory abilities of ligation reaction, RCA reaction as well as LNA capture probes, has been demonstrated both with synthetic model and panel of cell lines. The developed assay was then applied to patient samples by using a quick RPA pre-amplification step. Our results obtained with patient samples perfectly corresponded to the sequencing data. In this study, we focused on DNA from tumor tissue samples. We are aware that the assay was not applied to liquid biopsy for analysis of ctDNA, where the target amount may represent only a small fraction of total cell-free DNA (usually 0.1–10 %). Therefore, further optimization of the protocol might be required to enhance sensitivity due to low levels of ctDNA in blood. However, the results shown in Fig. 4 B are of interest since they demonstrate that as little as 1 % of mut DNA in the sample could be detected.

In conclusion, we believe that the proposed methodology could be a

compelling alternative to established molecular diagnostics techniques mainly in terms of simultaneous detection of presence of either wt or mut DNA. The combination of isothermal techniques and simple inexpensive electrochemical instrumentation makes the assay potentially useful in point-of-care settings by avoiding thermal cycling and bulky equipment. Moreover, the versatility of the strategy and the multiplexing capacity of both the sensor platforms and the electrochemical instrumentation make it easily transferable to the development of electrochemical ELISA plates (96 detections) capable of simultaneously interrogating other point mutations in clinical specimens with the repercussions that this would have in the diagnosis, prognosis and precision treatment.

CRediT authorship contribution statement

Ravery Sebuyoya: Methodology, Investigation, Formal analysis, Validation. **Alejandro Valverde:** Investigation, Formal analysis, Validation. **Ludmila Moranova:** Investigation, Writing – original draft. **Johana Strmiskova:** Investigation. **Roman Hrstka:** Funding acquisition, Writing – review & editing. **Víctor Ruiz-Valdepeñas Montiel:** Conceptualization, Formal analysis, Writing – review & editing. **José M. Pingarrón:** Conceptualization, Writing – original draft, Writing – review & editing. **Rodrigo Barderas:** Methodology, Resources, Writing – original draft, Writing – review & editing. **Susana Campuzano:** Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing. **Martin Bartosik:** Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.snb.2023.134375.

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Ravery Sebuyoya received his MSc. degree from Kaohsiung Medical University, Taiwan and is currently a Ph.D. student at the Masaryk University in Brno, Czech Republic. His topic of doctoral thesis is "Bioelectrochemistry in molecular oncology", which he carries out at the Masaryk Memorial Cancer Institute (Brno). He focuses on development of electrochemical biosensors for detection of DNA point mutations using various isothermal amplification techniques.

Alejandro Valverde obtained his Ph.D. in Advanced Chemistry from the Complutense University of Madrid (UCM) in 2023. Since 2017 he is working on the Analytical Chemistry Department at the Faculty of Science UCM as member of "Electroanalysis and Electrochemical Biosensors" research group. His research focuses on the development of electrochemical bioplatforms for the determination of cancer-related and emerging diseases biomarkers for clinical diagnosis.

Ludmila Moranova has worked at the Masaryk Memorial Cancer Institute in Research Centre for Applied Molecular Oncology (Brno, Czech Republic) since 2017. In 2022, she obtained her Ph.D. degree at Masaryk University with the Dean Award and the Vice-rector Award for excellent results. She is currently leading her own research project focused on development of new detection methods for human cytomegalovirus.

Johana Strmiskova is currently a Ph.D. student at the Masaryk University in Brno, Czech Republic. Her scientific interests include detection of oncoviral biomarkers and DNA point mutations by using isothermal amplification techniques and electrochemical detection. She conducts the research at the Masaryk Memorial Cancer Institute in Brno.

Roman Hrstka is an Associate Professor in the field of molecular and cellular biology and a Group leader at the Masaryk Memorial Cancer Institute, where he focuses on gynaecological malignancies, metastasis development and drug screening. He is also a national coordinator of BBMRI.cz large research infrastructure, which is a full member of the pan-European consortium of biobanks BBMRI-ERIC, internationally established as part of the European research infrastructure ESFRI Landmarks.

Víctor Ruiz-Valdepeñas Montiel works as Assistant Professor at the Analytical Department of the Chemistry Faculty of the Universidad Complutense de Madrid. He belongs to the "Electroanalysis and Electrochemical (Bio)sensors" Research Group. His current research lines include the design, set-up, and characterization of smart, low-cost, and user-friendly bioelectronic devices for n-plexed sensing of multi-omic biomarkers to address the most pressing analytical challenges in the clinical and foodomics fields.

José M. Pingarrón is Professor of Analytical Chemistry and member of the group "Electroanalysis and electrochemical(bio)sensors" at Complutense University of Madrid. His research lines include the development of nanostructured electrochemical platforms (enzyme, immuno- and genosensors) for single or multiplexed determination of relevant biomarkers. He is Senior Honorary Advisor of the journal Electroanalysis and since 2017 Fellow of the International Society of Electrochemistry. Currently he holds the position of Secretary General of Universities in the Spanish Government.

Rodrigo Barderas received his Ph.D. in Chemistry (Biochemistry and Molecular Biology) from the Complutense University of Madrid (Spain) in 2004. In 2017 got a Tenured Scientist position and in 2022 was promoted to "Investigador Científico" at the Instituto de Salud Carlos III. He is currently the Head of the Functional Proteomics Unit of the Chronic Disease Programme. His areas of interest include the identification of diagnostic, and prognostic markers and new targets of intervention in chronic diseases of high prevalence by using proteomics and phage display, with a special focus on colorectal cancer.

Susana Campuzano is Professor at the Department of Analytical Chemistry of the Chemistry Faculty of the Universidad Complutense de Madrid (Spain) and Head of "Electroanalysis and Electrochemical (Bio)Sensors" (GEBE-UCM) research group. Her areas of interest include the development of affinity-based electrochemical bioplatforms with potential for multiplexed and/or multi-omics determinations in precision medicine and nutrition.

Martin Bartosik obtained his Ph.D. at the Masaryk University in Brno in 2012 and is currently a Head of the Bioelectrochemical lab at the Masaryk Memorial Cancer Institute. His research interests include development of new methods in molecular biomedicine, especially electrochemical assays and biosensors for the detection of various tumor biomarkers and their validation in clinical samples.