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Rapid *Salmonella* detection using an acoustic wave device combined with the RCA isothermal DNA amplification method

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ABSTRACT

Salmonella enterica serovar Typhimurium is a major foodborne pathogen that causes Salmonellosis, posing a serious threat for public health and economy; thus, the development of fast and sensitive methods is of paramount importance for food quality control and safety management. In the current work, we are presenting a new approach where an isothermal amplification method is combined with an acoustic wave device for the development of a label free assay for bacteria detection. Specifically, our method utilizes a Love wave biosensor based on a Surface Acoustic Wave (SAW) device combined with the isothermal Rolling Circle Amplification (RCA) method; various protocols were tested regarding the DNA amplification and detection, including off-chip amplification at two different temperatures (30 °C and room temperature) followed by acoustic detection and on-chip amplification and detection at room temperature, with the current detection limit being as little as 100 Bacteria Cell Equivalents (BCE)/sample. Our acoustic results showed that the acoustic ratio, i.e., the amplitude over phase change observed during DNA binding, provided the only sensitive means for product detection while the measurement of amplitude or phase alone could not discriminate positive from negative samples. The method's fast analysis time together with other inherent advantages i.e., portability, potential for multi-analysis, lower sample volumes and reduced power consumption, hold great promise for employing the developed assay in a Lab on Chip (LoC) platform for the integrated analysis of *Salmonella* in food samples.

1. Introduction

Salmonella species are one of the primary candidates for foodborne disease (Salmonellosis) and pose a major threat for public health. Salmonellosis, which can be spread via food sources such as raw meat, eggs, dairy products, vegetables and even water [1,2], affects millions of people globally in an annual basis with severe symptoms that can potentially lead to death [3,4]. Apart from the above health issues, food-related outbreaks have a significant impact on the consumers' trust towards food-producers damaging the related food industry. For these reasons the development of strategies for food quality control and bacteria detection in food sources is of paramount importance.

The conventional culture method used nowadays to achieve the required sensitivity is time consuming and labor intensive as the detection and confirmation may take up to several days [5]. For this reason, alternative rapid methods have been developed, including two major types of methodologies: 1. Nucleic acid-based amplification techniques, employing various types of Polymerase Chain Reaction (PCR) [6], such as real time [7] and multiplexed [1], or isothermal methods, such as the NASBA [8] and LAMP [9]; and, 2. Immunology-based methods employing enzyme–based immunoassays, such as ELISA, EIA, immune-precipitation, immunomagnetic separation, flow cytometry etc. [10,11]. Nucleic acid methods are normally coupled with gel elrectrophoresis or fluorescence measurement for amplicons detection, while immunology-based approaches use optical or magnetic methodologies for the detection of the captured bacteria.

Recently, biosensors have also emerged as an attractive alternative in the pathogen detection field [12]. Biosensing devices employing optical, electrochemical or piezoelectric transducers combined with nucleic acid or whole bacteria detection, the latter through the use of antibodies, have been establishing as a very promising technology for the rapid, sensitive and selective detection of pathogens [13]. In the case of nucleic acid-based biosensors, the bacterial target-DNAs are normally coupled with DNA amplification prior to detection. Several examples can be found in the literature where *Salmonella* detection was demonstrated using Surface Plasmon Resonance (SPR) [14], electrochemical [15] or even simple lateral flow [16] biosensors, combined with

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antigen-antibody interactions [17]. A clear trend in the field of nucleic acid based biosensors for molecular diagnostics is the use of isothermal amplification methods as already shown in some cases (SDA, LAMP) [16,18] due to their potential for integration into portable, automated and multi-analysis systems.

Piezoelectric sensors, such as the Love wave geometry based on a Surface Acoustic Wave (SAW) device and the Quartz Crystal Microbalance (QCM) have also been applied extensively to pathogen detection and molecular diagnostics. The main advantages of acoustic sensors, i.e., the simple instrumentation required for signal measurement and ability to integrate with microfluidics [19] to a Lab-on-Chip platform for automated analysis, make them a significant alternative to optical and electrochemical sensors. Successful applications of the QCM acoustic devices include their use in combination with PCR or multiplex PCR for the detection of *Plasmodium* species [20,21], *Listeria* [22] and *Salmonella* [23], as well as isothermal methods such as the Rolling Circle Amplification (RCA) for the detection of Hepatitis B Virus (HBV) [24] and LAMP for the detection of type 58 human papillomavirus [25]. Love wave devices have also been combined with either isothermal amplification (LAMP) for the diagnosis of sexually transmitted diseases [26] or antibodies for bacteria detection [27].

In the last few years, a novel and simple method has been proposed for the detection of DNA using both the Love wave and QCM sensors. The method is based on the measurement of the acoustic ratio of single- or double-stranded DNA molecules loaded directly on the sensor surface [28,29,30]. Specifically, the acoustic ratio, expressed as $\Delta A/\Delta Ph$ or $\Delta D/\Delta F$ in the case of the SAW or QCM sensors, respectively (where A, Ph, D and F represent amplitude, phase, dissipation and frequency, respectively) was shown to be a measure of the size and shape of the DNA molecule attached to the device surface. The practical significance of the concept was demonstrated in previous works during the detection of specific mutations in the case of cancer biomarkers including single point ones [29]; the presence of pesticide-resistance genes in the malaria-transmitting anopheles insect [28]; the quantification of the expression of the ABCA1 gene in mice treated with a particular ligand [28]; and, the co-existence of various pathogens in the tomato plants [31]. In all the above cases, the detection of the target DNA took place upon the design and production through PCR of amplicons of a specific length, or two different lengths in the case of multiplexing [28,31]. In addition, the methodology was shown to be sensitive and inherently selective to the conformation of the target(s), while it was also free from the need for DNA denaturation and surface-hybridization. In the present study, the concept is applied and demonstrated to work for *Salmonella* DNA detection; in addition, for the first time the acoustic ratio detection approach is combined with an isothermal amplification step and specifically, the RCA method [32,33]. Moreover, a strategy based on on-chip DNA amplification was also tested and shown to work equally well, with the additional advantages of providing more integrated and faster analysis. This is the first time that such a detection platform is applied to *Salmonella* pathogen detection with a reported sensitivity going down to 100 Bacteria Cell Equivalent (BCE) as the starting template.

2. Materials and methods

2.1. Acoustic devices

Surface Acoustic Wave devices (SAW) operating at 155 MHz were prepared by photolithography. These devices were used to support a Love wave in a configuration employing a photoresist S1805 (Rohm and Haas, USA) waveguide layer of 1 μm thickness. A Network analyzer (E5061A, Agilent Technologies, USA) and a LabVIEW software (National Instruments, Austin, TX) were used for signal generation/detection and real-time monitoring of the acoustic signal. Prior to use, the polymer coated device surface was cleaned by air plasma etching (PDC-002, Harrick) for 150 s.

2.2. Preparation of circular DNA probes

10 μM of a linear padlock probe (5′-GCC TCT ACT CCA TCG TGC AGA TTC TAA *GCC AAA CAC AAC CCA TCA GGA* TCG ATC GCG CTA AGC GGC GTG GAT A-3′, Metabion International AG) were phosphorylated at the 5′ terminus using 5 Units of T4 Polynucleotide Kinase (New England Biolabs), 1 mM ATP and 5 μg BSA added up to a final reaction volume of 50 μL. The reaction was incubated at 37 °C for 30 min, followed by enzyme inactivation at 65 °C for 20 min. 5 μL of the phosphorylated padlock were then mixed with 5 units of AmpLigase (Epicentre), 2.5 μg BSA and *Salmonella* DNA template (genomic or chemically synthesized target sequence) into a final reaction volume of 25 μL. The mixture was incubated at 94 °C for 5 min followed by 20–40 cycles of 92 °C (1 min) and 62.5 °C (20–120 s) for circularization of the padlock probes. The DNA target sequence corresponded to a region of the *Salmonella PurE* gene with the following sequence: 5′-CCA CCG GAA TGC CGC GCG GCA TCT GCA CGA TGG AGT AGA GGC TAT CCA CGC CGC TTA GCG CAG CGC TTT GTA CCG GCA CG-3′.

2.3. RCA reactions

RCA was performed using 5 μL of the ligation reaction (circular probes) supplied with 5 units of phi29 DNA Polymerase, 1× phi29 DNA Polymerase Reaction Buffer, 2.5 μg BSA, 200 μM dNTP mix, 400 nM biotinylated d-UTP (Metabion) and 400 nM of a 5′-biotinylated DNA primer (Metabion) in a 25 μL final reaction volume. All reagents were purchased from New England Biolabs, unless otherwise stated. RCA reactions were conducted for 30–60 min using a thermocycler at 30 °C or for 60 min at room temperature, followed by enzyme inactivation at 65 °C for 10 min. The DNA primer (5′-Biotin-TCCTGATGGGTTGTGTTTGG-3′) was selected to ensure that hybridization would occur only between the primer and the padlock. After the RCA reaction was over, 5 μL were analyzed in 1% agarose gel stained with GelRed (Biotium) along with 100 ng of λ DNA-BstEII DNA marker (New England Biolabs). Gels were visualized after 2 h running at 120 V–150 V using a UV station. The non-specific primer used as control for in situ RCA measurements was: 5′-Biotin-GTCACGGTGATCGATCCGGT-3′.

2.4. Acoustic measurements

All acoustic experiments were carried out under flow using a peristaltic pump with a flow rate of 20 μL/min. Each sample used for an acoustic measurement was diluted to the running buffer (PBS pH 7.4, 10 mM $MgCl_2$), with the exception of on-surface RCA, where no dilution was used. It should be noted that the undiluted viscous RCA reactions were causing big phase changes when loaded on the device surface. The first step of each experiment was saturation of the sensor surface with 200 μL of neutravidin (200 ng/μL, Invitrogen) followed by buffer rinsing. RCA reactions performed off-chip were directly pumped over the neutravidin coated sensor surface without purification followed by buffer rinsing. Performing on-chip RCA required the immobilization of the biotinylated DNA primer (200 nM) on neutravidin prior to the addition of 50 μL of the RCA reaction mix (this volume was required to fill the flow cell). The RCA mix lacking the DNA primer and the biotinylated-UTPs was pumped over the surface until filling the entire area and the flow was stopped for 60 min. Flow was then restored and the surface was washed with buffer.

In all measurements, changes in the amplitude (ΔA) and the phase (ΔPh) of the acoustic wave were monitored in real-time and used to calculate the acoustic ratio ($\Delta A/\Delta Ph$) for each sample separately.

2.5. Atomic Force Microscopy (AFM) imaging

RCA amplicon production at 30 °C was also confirmed by AFM imaging, either directly after amplification or after DNA purification using phenol-chloroform extraction and ethanol precipitation. AFM images

of positive and negative samples were compared before and after DNA purification. The four samples were deposited on freshly cleaved mica (RS Company) and were left there for 30 min at RT. Mica was rinsed with 1 mL of dd-H_2O twice and dried with nitrogen gas. Image visualization was carried out in air using the NanoScope IIIa system. The instrument operated in tapping mode and in ambient temperatures using Veeco tips (RTESP model, spring constant 20 N/m–80 N/m; resonance frequency 267 kHz–298 kHz; tip radius 10 nm), and a scan rate of 1 Hz–1.5 Hz. The scan sizes obtained ranged from 1 μm–5 μm.

3. Results

3.1. Rolling Circle Amplification method

RCA utilizes linear padlock probes that can be ligated into circular DNA molecules provided that the target sequence is perfectly matched. After ligation (circularization) a specific primer hybridizes to the circular probe and phi29 polymerase elongates the product (Fig. 1). When the circle is complete, the enzyme displaces the already existing product and proceeds with the reaction, rolling around of the target multiple times. To facilitate immobilization of the RCA products on the sensor surface, biotinylated d-UTPs were supplemented in a minimal amount in the reaction so that multiple biotins would be randomly inserted in the final product body. The isothermal amplification was conducted either off the device or directly on it at predefined positions. In both cases, the acoustic signal upon immobilization of the RCA product was monitored and evaluated. For this series of experiments, two types of templates were used a chemically synthesized oligo 80 nt long and isolated *Salmonella* genomic DNA.

RCA is capable of producing single-stranded molecules of high molecular weight that collapse into a sphere [34]. To verify the outcome of the RCA protocol, we amplified at 30 °C 10^{16} chemically synthesized DNA copies corresponding to the *Salmonella PurE* gene and used AFM imaging to observe the morphology of the products after their adsorption on a mica surface (Fig. 2a, b). For comparison we also used a negative control reaction, lacking the target template (Fig. 2c, d). Positive samples can be clearly distinguished from the negative ones both before and after DNA purification within an area of 25μm^2 from the AFM images. The negative samples gave a clear surface as expected, whereas the positive ones showed light-colored dots which correspond to the RCA products, as they appear both before and after DNA purification. These products appear to form compact aggregates, as expected due to internal base-pairing of the single-stranded products of a diameter varying from 100 to 250 nm.

The formation of DNA aggregates of a high molecular weight was further verified by gel electrophoresis, where a smear that migrates very slowly was clearly visible, as expected by large DNA molecules. By using gel electrophoresis, it was also possible to verify the formation of RCA amplicons produced after 60 min at room temperature (data not shown).

3.2. Acoustic detection of RCA products

In a first set of experiments, off-chip prepared RCA products using chemically synthesized *Salmonella* DNA as target were loaded directly on the device surface without any further purification. Binding to the device was achieved through the biotin molecules incorporated in the amplified DNA and the presence of neutravidin protein, pre-adsorbed on the polymer surface. The real time addition of neutravidin and, subsequently, *Salmonella* DNA are shown in Fig. 3 as these steps were monitored by following the amplitude and phase of the acoustic wave. Amplitude and phase changes (ΔA and ΔPh, respectively) measured at equilibrium and after the addition of varying initial amounts of DNA (i.e., from 100 to 10^{16} copies) are presented in Fig. 4. The use of excess target (10^{16} copies) aimed towards the creation of enough products to saturate the surface.

Results show that regardless of the initial DNA used, the measured acoustic ratios did not vary significantly (average value 0.099 ± 0.016 dB/deg). In addition, in all cases the acoustic ratio of the RCA amplified DNA was at least 3.7 higher than that observed with the negative control (average value 0.028 ± 0.010 dB/deg), i.e., same reaction lacking the target DNA, leading to a clear distinction between the two samples.

RCA was also conducted at room temperature (RT) using the chemically synthesized targets, taking advantage of the fact that phi29 DNA polymerase can operate under such conditions, although at slower rates [35]. Indeed, the only difference between the RCA reactions was the operating temperature which in this case was ~25 °C instead of 30 °C, while all other parameters, including the ligation step were the same. Negative and positive (1000 copies of chemically synthesized target) samples, tested at least in triplicates gave an average acoustic ratio value of 0.061 ± 0.031 (dB/deg) and 0.121 ± 0.017 (dB/deg), respectively. Although negative and positive signals can be differentiated from each other, it was observed that RCA results have elevated acoustic values for RT experiments compared to the corresponding at 30 °C regarding the negative controls. This difference could be due to some degree of non specific amplification favored at room temperature. Nevertheless, these findings are in good agreement with the data from RCA reactions utilizing a thermocycler, showing both that RCA can operate equally well at RT and that our system is capable of detecting the bacterial DNA efficiently in all cases.

3.2.1. On-chip RCA amplification and detection

Following our observation that RCA can take place at room temperature, a second set of experiments was conducted where, this time,

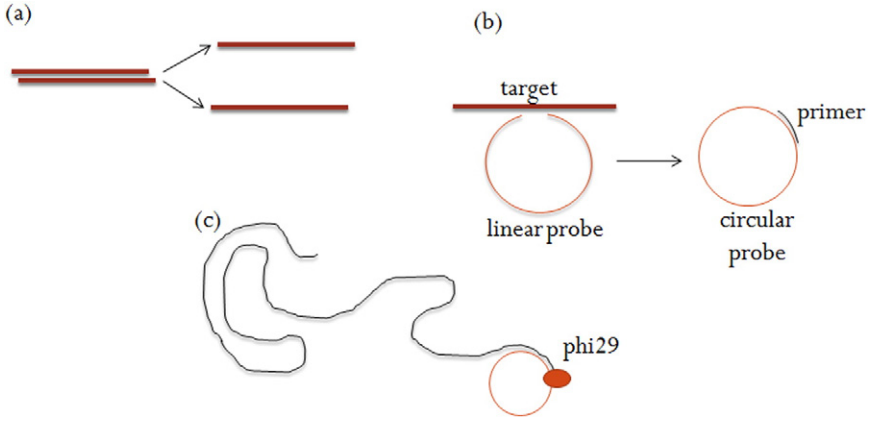

Fig. 1. Schematic depiction of the RCA method: (a) DNA target denaturation, (b) padlock hybridization to DNA target and circularization; primer hybridization and (c) primer elongation and DNA amplification with phi29 polymerase.

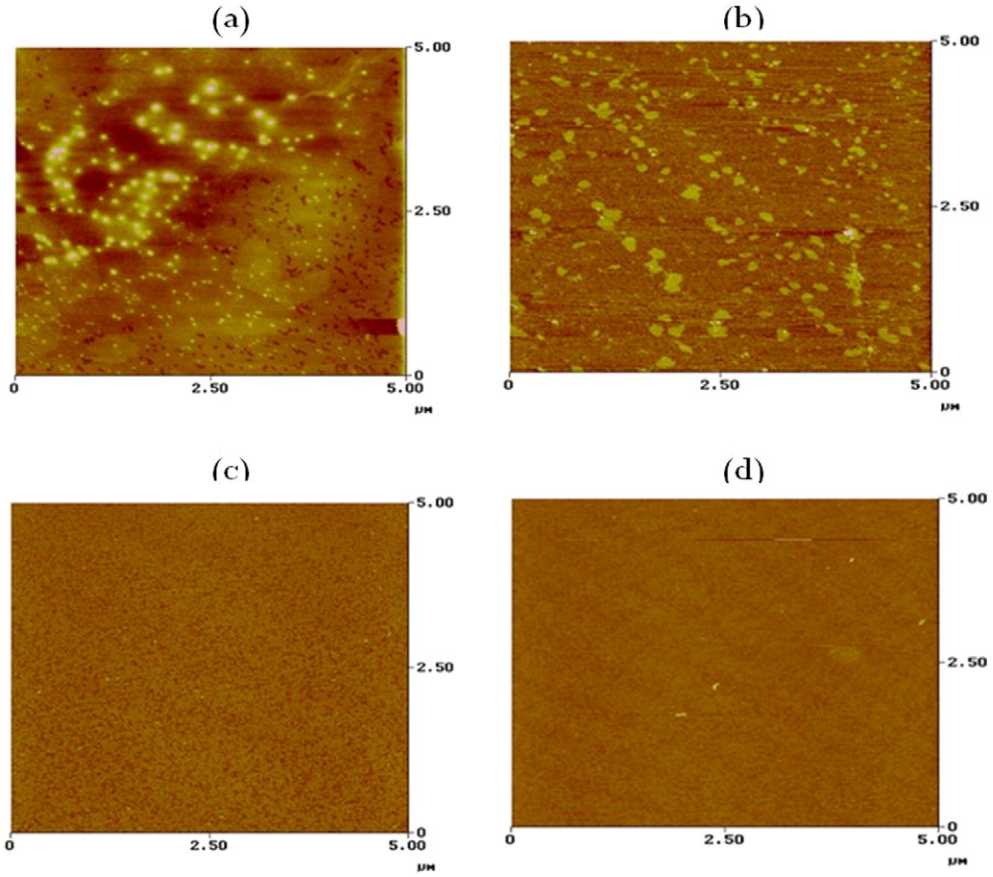

Fig. 2. AFM images of RCA products upon amplification of an initial template of 10^{16} DNA targets before (a) and after (b) purification; and the negative sample (without DNA targets) before (c) and after (d) purification.

circular DNA amplification was attempted directly on the acoustic device surface. The experimental setup involved again surface activation through adsorption of neutravidin, followed by subsequent binding of the biotinylated primer for initializing the Rolling Circle Amplification. Circular padlocks were mixed with phi29 enzyme and the resulting mix was loaded on the surface under flow. When the surface was completely covered with the amplification mix, the flow was stopped for 1 h during which the circular padlocks hybridized on the primers and amplification was performed (Fig. 5). After 1 h, flow was restored and buffer was used to wash any unbound molecules before measuring the acoustic signal. Negative samples were also tested, i.e., samples identical to the positive ones except for the target which was not included during ligation (negative control 1). As a result, circular padlocks could not be formed which led to no RCA product. Moreover, an additional negative control for specificity was also tested, where, in this case, the solution contained circular padlocks but the primer on the

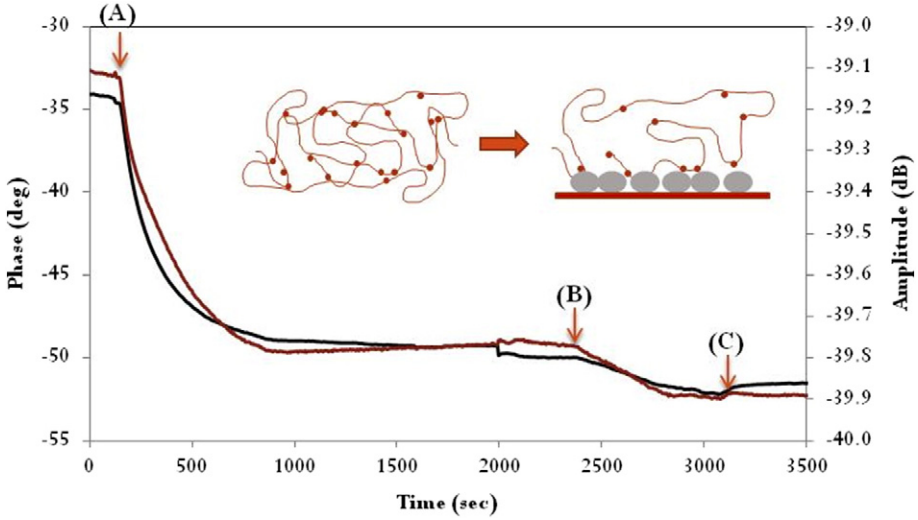

Fig. 3. Real time acoustic detection of RCA products created off-chip at 30 °C (red line: amplitude; black line: phase). (A) Adsorption of neutravidin on sensor surface, (B) immobilization of RCA products through biotin-neutravidin interaction, (C) buffer rinsing step. Inset: schematic depiction of biotinylated RCA product binding to the neutravidin-coated sensor surface via multiple biotin molecules.

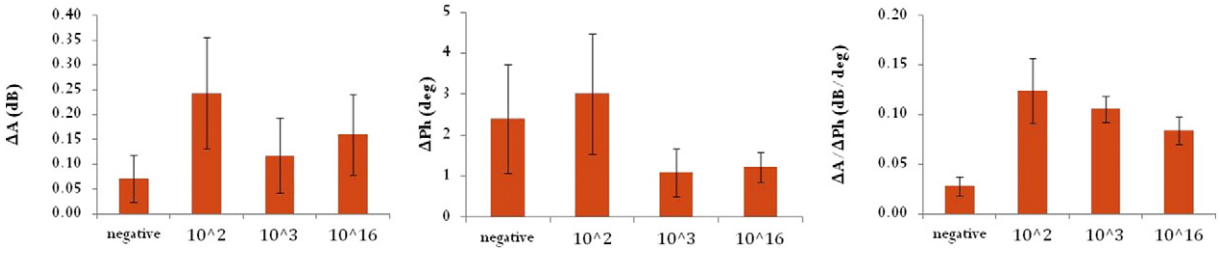

Fig. 4. Acoustic detection results for RCA products prepared off-chip at 30 °C using from 100 up to 10^{16} DNA target copies. The three charts display the measured values related to changes in the amplitude (dB), phase (deg) and calculated acoustic ratio (dB/deg).

surface was not complementary to them, prohibiting RCA (negative control 2). Experiments were repeated at least 3 times for each sample and results, regarding the measured acoustic ratios from real time graphs, are summarized in Fig. 6.

Fig. 6 indicates that RCA can, indeed, be performed on-chip and monitored acoustically with good specificity when compared to the control reaction, i.e. the same amplification mixture which does not include the non-complementary immobilized primer. The measured acoustic ratio after amplification (0.102 ± 0.022 (dB/deg)) was comparable to that obtained during the off-chip amplification reaction, indicating the formation of DNA aggregates of the same size and shape. In addition, both negative controls resulted in smaller acoustic ratios and of the same value, indicating that no products were produced in either case. However, of interest was the fact that both ratios obtained for the negative controls in Fig. 6 are much higher (almost double) than the corresponding one obtained during off-chip amplification. This may be due to some degree of non specific amplification favored by room temperature.

4. Discussion

In this work we demonstrate for the first time that the Love wave acoustic device can be used in combination with an isothermal amplification method to detect *Salmonella* amplified DNA on the device surface without the need for DNA purification after amplification or use of labels for signal transduction. It appears that even 100 copies as a starting material is sufficient for acoustic detection based on the measurement of the acoustic ratio. It should be noted that the reactions with this low copy number of targets could not be visualized with electrophoresis, although they were detected with the acoustic system, stressing the increased sensitivity of the acoustic methodology compared to the conventional gel electrophoresis detection or other reported methods [6].

Moreover, the acoustic ratios for the positive samples are constant (within experimental error), regardless of the amount of the initial template, in agreement with theory and previous studies [36]. Most importantly, it is clearly shown that the phase or amplitude alone cannot be used to discriminate negative from positive samples in the particular RCA complex medium; however, the ratio of ΔA/ΔPh appears to allow such discrimination (Fig. 4). The small ratio of the control suggests a much more tightly non-specifically bound layer of adsorbed RCA ingredients as opposed to the specifically bound more surface-protruding DNA molecules to the neutravidin surface via biotins. The same holds true for on-chip amplification and detection (Fig. 6).

Additionally, we have shown that chemically synthesized and genomic targets are both suitable for our analysis. This is in agreement with other works showing that RCA can be used with plasmids [37,38] as well as chemically synthesized padlocks that require ligation. We believe that our approach can be successfully applied to real samples containing whole bacterial cells by including a step of bacterial lysis (for example thermal lysis) in order to obtain padlocks for the RCA reaction, since post-amplification treatment is not a factor in the presented approach. These advantages highlight the robustness of our method and allow total experimental time to be further reduced. Of particular

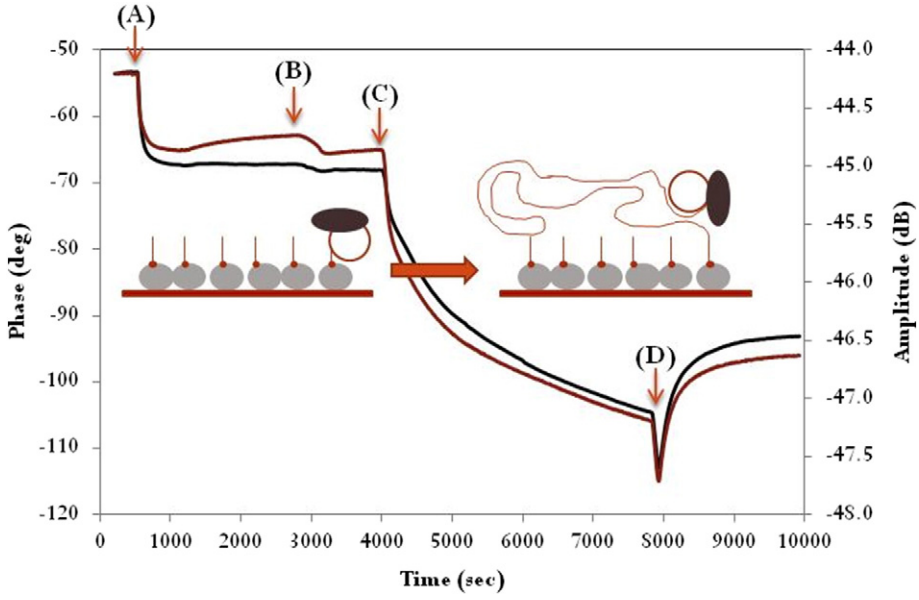

Fig. 5. Real-Time plot of RCA on-chip at RT (red line: amplitude; black line: phase). (A) Surface saturation with neutravidin. (B) Binding of a biotinylated primer to neutravidin. (C) Loading of the RCA mix (circular padlock, phi29 polymerase etc.) on the sensors surface – incubation for 60 min in static mode. (D) Buffer rinsing under flow. Inset: Schematic depiction of the device surface during on-chip RCA amplification and detection.

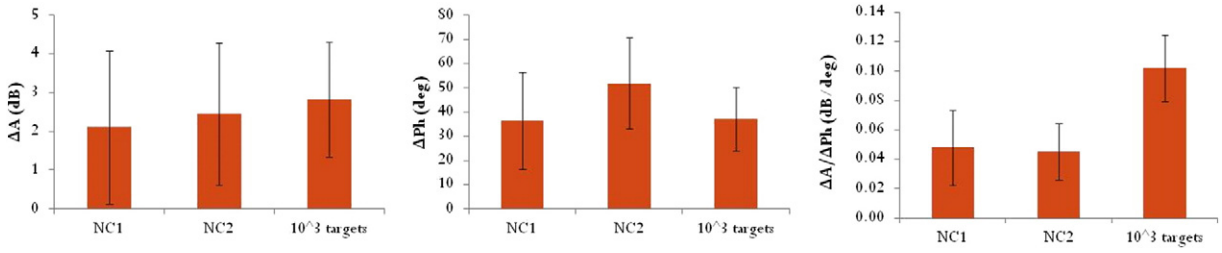

Fig. 6. Acoustic ratio average values and standard deviations for experiments where RCA was conducted on-chip. Negative control 1 (NC1) represents reactions with no circular probes, whereas negative control 2 (NC2) represents reactions where the probes were present but there was no complementarity with the immobilized primers for initializing the RCA. Positive samples represent RCA reactions with 1000 copies of chemically synthesized target as a starting template.

interest is also the relatively short analysis time; this is currently 0.5 and 1 h for the off and on chip amplification, respectively. Obviously, the successful parallel performance of DNA amplification and detection on the SAW surface is a significant improvement of the proposed assay holding potential for further integration to a portable and fast diagnostic platform.

Alternative isothermal amplification methods can also be proven very effective when combined with the SAW technology, as they may provide advantages over RCA such as the ability to be conducted without the need of circular templates and consequently reducing detection limits even further. However, one significant advantage of the RCA method is the fact that amplification can be performed rapidly enough even at room temperature which translates into reduced power consumption and no need for expensive equipment like thermocyclers. Temperature control is a challenge for integrated platforms, since temperature handling increases perplexity of the system. This fact highlights the advantage of RCA or any other isothermal amplification method over conventional tools like PCR, consequently simplifying temperature challenges.

5. Conclusions

In this work, *Salmonella* DNA detection was achieved using a Love wave acoustic biosensor combined with an RCA amplification step performed either off-chip or directly on the sensor surface. Detection limits were satisfactory, scaling down to 100 BCE as a starting template, with the potential to be reduced upon further optimization. Our results provide further evidence on the analytical value of recording the acoustic ratio of amplitude versus phase, as opposed to each measurement alone, since only the ratio could discriminate between positive and negative samples. Overall, the combined isothermal and acoustic detection system is very promising as it may allow further integration with microfluidics in order to design LoC platforms for food safety analysis.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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