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3D-printed Point-of-Care Platform for Genetic Testing of Infectious Diseases Directly in Human Samples Using Acoustic Sensors and a Smartphone

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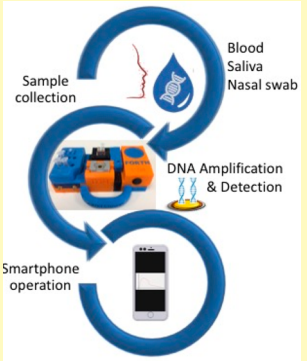
Supporting Information

ABSTRACT: The objective of this work is to develop a methodology and associated platform for nucleic acid detection at the point-of-care (POC) that is sensitive, user-friendly, affordable, rapid, and robust. The heart of this system is an acoustic wave sensor, based on a Surface Acoustic Wave (SAW) or Quartz Crystal Microbalance (QCM) device, which is employed for the label-free detection of isothermally amplified target DNA. Nucleic acids amplification and detection is demonstrated inside three crude human samples, i.e., whole blood, saliva, and nasal swab, spiked in with 10−100 *Salmonella* cells. To qualify for POC applications, a portable platform was developed based on 3D printing, integrating inside a single box: (i) simple fluidics based on plastic tubing and a mini peristaltic pump, (ii) a heating plate combined with disposable reaction tubes for isothermal amplification; (iii) a mini antenna analyzer operated through a tablet; and (iv) an acoustic wave device housing unit. The simplicity of the method combined with smartphone operation and detection, rapid sample-to-answer analysis time (30 min), and high performance (detection limit 4×10^3 CFU/ml) in three of the most important human samples in diagnostics suggest that the methodology could become a tool of choice for nucleic acid detection at the POC. In addition, the low cost of the platform and assay holds promise for its adoption in resource limited areas. The acoustic detection method is shown to give similar results with a standard colorimetric assay carried out in saliva and nasal swab but can also be used to detect nucleic acids inside whole blood, where a colorimetric assay failed to perform.



KEYWORDS: *acoustic biosensor, LAMP, quantitative nucleic acid detection, crude human samples, point-of-care, Salmonella testing, smartphone molecular diagnostics, resource limited areas*

The dominant model of infectious diseases testing throughout the world remains the centralized laboratory. However, healthcare is changing, partly as a result of economic pressures and also because of the general recognition that care needs to be more patient-centered.[1] Recently, advancements in *in vitro* molecular diagnostics have enabled the development of efficient, rapid, and sensitive systems, which potentially can help shift testing from the laboratory to the point-of-care (POC).[2] A major driver in the development of POC tests is also the ability to diagnose diseases at sites with limited infrastructure.[3] In principle, a POC device should be easy to operate and utilize reagents and consumables that are robust in storage and usage, require minimum sample preparation, minimal equipment, and offer sample-to-result times in minutes.[2]

POC testing can be based on the detection of either antibodies or nucleic acids. Antibody-based tests such as the lateral flow immunoassay (LFIA) for infectious diseases have revolutionized POC rapid testing due to their simplicity, instrumentation-free nature, and cost-effectiveness.[3] As a result, several LFIA tests have received Clinical Laboratory Improvement Amendments (CLIA) waivers, which is a prerequisite for POC use. One potential problem encountered with this technology is the need for high concentrations of the analyte which, in turn, raises concerns over a low clinical sensitivity and false-negative results.[3,4] Molecular technologies enabling nucleic acids testing are currently emerging as an attractive alternative to immunological tests due to their higher sensitivity and reliability.[3,5] However, for developing POC genetic testing systems, there are several technical challenges that need to be addressed. These are related to achieving appropriate integration of multiple crucial components while minimizing complexity, turnaround test-time, and cost, the

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Table 1. Acoustic Ratio (10^{-6}/Hz) Comparison

cells	water	blood	saliva	swab
0	0.0305 ± 0.010	0.0120 ± 0.001	0.0241 ± 0.003	0.0232 ± 0.001
10	0.0475 ± 0.003	0.0412 ± 0.002	0.0455 ± 0.001	0.0486 ± 0.001
50	0.0470 ± 0.002	0.0451 ± 0.002	0.0448 ± 0.002	0.0489 ± 0.001
100	0.0450 ± 0.002	0.0411 ± 0.002	0.0471 ± 0.003	0.0491 ± 0.001

latter especially in the developing countries. Combination of sample pretreatment with nucleic acids amplification and detection in an integrated platform is the greatest challenge. Until now, nucleic acids testing is almost exclusively performed in centralized laboratories using high-end instrumentation and skilled personnel. In contrast to LFIA, only few commercially available platforms exist as point of care CLIA-waived systems for DNA testing.[4,6] These are all based on fluorescent detection; reverse transcriptase polymerase chain reaction (rtPCR) or isothermal nucleic acid amplification; use of small to medium size benchtop, mostly expensive, equipment; and applications targeting mainly influenza A and B or flu viruses in nasal or nasopharyngeal swabs.[3] Moreover, of the three commercially available systems (Alere i, GenXpert Omni, and Cobas Liat), only two report ability for automatic connectivity. During the last years, isothermal amplification methods are becoming extremely popular for POC applications with the loop-mediated amplification (LAMP) method being an emerging isothermal method due to its low cost and ease of use by nontrained personnel. So far, the method has been shown to be fully compatible with microfluidics and microdevice technologies and has been combined with a whole range of detection methodologies, namely, optical, colorimetric, and fluorescent.[4,7,8]

In our lab, we have pioneered the use of acoustic wave chips as an alternative means to detect DNA in various samples. We have demonstrated that acoustic wave sensors can provide real-time and quantitative information on bacterial DNA detection when combined with the PCR method[9−12] even within complex matrices such as milk mixed with nutrient broth.[12] In addition, we have combined acoustic detection with other DNA amplification methods such as the Rolling Circle Amplification,[13] Recombinase Polymerase Amplification (RPA),[14] and LAMP, the latter in a Lab-on-Chip (LOC) platform.[15] The current work describes the development of a simple, rapid, and sensitive method based on acoustic wave sensors, which is used to perform genetic material detection directly in three unprocessed human samples, i.e., whole blood, saliva, and nasal swabs. To achieve the above, label-free acoustic wave sensing was combined with the rapid and sensitive DNA isothermal LAMP amplification technique. However, in contrast to the use of a fully integrated LOC platform,[15] here we deliberately employed simple fluidics based on plastic tubing and microreaction wells for performing the amplification step. This alternative setup alleviates the need for expensive microfluidic chips and high precision instrumentation for accurate flow control. Moreover, to further demonstrate the potential of the technology for POC testing, the traditional expensive and bulky lab equipment normally used for DNA amplification and acoustic monitoring was replaced by miniaturized, portable, and inexpensive components; i.e., a mini-Peltier heating element for temperature controlling and an antenna analyzer for acoustic signal generation. Both parts were proven suitable for generating similar results to a conventional thermocycler and a network analyzer, respectively. Finally, by employing the three-dimensional (3D) additive manufacturing technology, all the above components were integrated, together with a mini peristaltic pump, into an inexpensive and lightweight prototype system, which was wirelessly connected to a tablet. This work represents a very attractive system for testing of human samples at the POC, with a technology readiness level (TRL 5) suitable for adoption by the end user.

EXPERIMENTAL SECTION

Acoustic Wave Sensors and Acoustic Measurements. Two different acoustic wave sensors, a surface acoustic wave (SAW), and a bulk wave quartz crystal microbalance (QCM) were employed. SAW devices, operating at 155 MHz, were prepared by photolithography and 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 LabVIEWsoftware (National Instruments, Austin, TX) were used for signal generation/detection and real-time monitoring of the acoustic signal. A quartz crystal microbalance (QCM, Q-Sense E4, Sweden) setup was also used with a crystal of 5 MHz fundamental frequency (thickness shear mode, TSM) and a working range of 5−65 MHz.

Real time acoustic experiments were carried out under flow using a peristaltic pump with a flow rate of 50 μL/min. Prior to use, both devices were cleaned by air plasma etching (PDC-002, Harrick) for 150 s. Each sample used for an acoustic measurement was diluted to the running buffer (PBS pH 7.4, Sigma). 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. For SAW measurements, changes in the amplitude (ΔA) and phase (ΔPh) of the acoustic wave were monitored and used to calculate the acoustic ratio ($\Delta A/\Delta$Ph) for each sample. For QCM measurements, the recorded signals are energy dissipation change (ΔD) and frequency shift (ΔF) for the seventh harmonic (i.e., 35 MHz), with frequency data not being divided by the harmonic number. The acoustic ratio ($\Delta D/\Delta F$) is also calculated from the recorded data. All measurements were repeated at least 3 times and were used to calculate the average and the standard deviation values reported in Figures 2, 3, and 7 as well as in Table 1; the acoustic ratio is represented by R.

3D-printed Acoustic Prototype. A 3D-printer (Taz6 LulzBot, USA) was used to build a unit for housing the necessary components for the analysis. The miniVNA PRO antenna analyzer (WiMo Antennen and Elektronik GmbH, Germany) and a wireless hand-held Vector Network Analyzer were used to perform acoustic measurements with both acoustic chips. This analyzer is capable of scanning and transmitting the data using an integrated Bluetooth module to a remote Tablet/Smartphone up to 100 m from the miniVNA PRO's location, using the Blue VNA Android application (Google Play store). A Peltier unit (SLVPU-20S) with an integrated fan and a temperature controller (VPE-35-5V), purchased from Takasago Electric, Inc. (Japan) was used for the LAMP amplification in combination with a stainless steel rack for holding eppendorfs. A QCM cell used for liquid flow measurements with a standard 5 MHz QCM device was purchased from AWSensors (Valencia, Spain). A SAW sensor setup described previously[16] was used in connection with either the miniVNA PRO or an Agilent network analyzer. A Raspberry Pi 3 Model B+ was purchased by Raspberry Pi foundation

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(UK). A mini peristaltic pump was purchased from Jobst Technologies (Germany).

LAMP Amplification in Crude Samples. Single donor human whole blood (Catalog No.: IPLA-WB1) was purchased from Innovative Research (USA). Saliva from healthy donors and a nasal cavity swab from a single healthy donor were purchased from Lee Biosolutions, USA. A volume of 2.5 μL of whole blood or saliva was mixed with 1 μL of bacterial cells followed by cell lysis at 95 °C for 5 min or with the addition of Triton-X 100 at a final concentration of 0.01% and 10 min incubation at room temperature. The swab stick was first placed in a 1.5 mL tube with 500 μL of chromatography grade water (Li Chrosolv, Germany), followed by the ejection of the end part. The tube was incubated at 95 °C for 5 min in a heated mixer with shaking at 1200 rpm. Then 2.5 μL of the solution was mixed with 1 μL of bacterial cells followed by thermal lysis at 95 °C. All experiments were performed with an attenuated strain of *Salmonella enterica* serovar Typhimirium. *Salmonella* was grown overnight in Luria−Bertani (LB) medium; cultures were subsequently measured spectrophotometrically (OD600) and adjusted at OD600:1 corresponding to a cell concentration of 1−1.5 × 10^9 CFU/mL. Serial dilution of the above cells suspension by LB was carried out to reach the required concentration. The *Salmonella* invasion gene invA was targeted by a set of six primers (Metabion, Germany),[15] two outer (F3 and B3), two inner (FIP and BIP) and two loop (Loop-F and Loop-B). The FIP primer was biotinylated at the 5′-end. The LAMP reagent mix in a total volume of 25 μL contained 12.5 μL of WarmStart or Colorimetric WarmStart 2 × Master Mix (New England BioLabs), 1.8 μM FIP and BIP, 0.1 μM F3 and B3, 0.4 μM Loop-F and Loop-B, and 2.5 μL of sterile water or any crude sample mixed with 1 μL of cells. LAMP was performed at 63 °C, and the amplification products were analyzed using electrophoresis on a 2% agarose gel containing GelRed (Biotium) and visualized under UV light.

RESULTS AND DISCUSSION

Stability and Performance of LAMP Reactions. LAMP amplification of the invA gene of *Salmonella* was achieved in a thermocycler with a maximum volume of 2.5 μL (10% crude sample dilution) of whole blood, saliva, or swab. No enzymatic inhibition in any of the above crude samples was observed, as verified by gel electrophoresis. The LAMP mix was found to be stable at room temperature for 3 days without affecting the efficiency of the amplification reaction (Figure S1). This result is of high significance since the stability of the reagents is an important prerequisite for POC applications. LAMP amplification of 10 cells (8 ± 3 cells verified by the bacteria plating method) in 2.5 μL of sample was achieved with all three types of samples within 30 min of reaction time. Higher numbers, 50 or 100 cells, produced detectable signals during gel electrophoresis within 20 min (Figure S2). The same results were reproduced using the colorimetric LAMP kit apart from the case of whole blood, which showed complete inhibition most likely due to the low buffering capacity of the mix.

LAMP Amplification Combined with Acoustic Measurements. Initially, we employed the Quartz Crystal Microbalance device with Dissipation monitoring (QCM-D) for optimizing the acoustic detection assay. Acoustic results included monitoring changes in both the frequency (ΔF) and dissipation (ΔD) of the wave as well as their ratio, i.e., $\Delta D/\Delta F$. The acoustic ratio, i.e., the normalized acoustic energy dissipation per unit mass, has been shown to be a qualitative and/or quantitative measure of the conformation or way of attachment of biomolecules on the device surface.[17−19] This measurement has been proven in several cases to be more sensitive than frequency measurement alone and capable to discriminate specific from nonspecific binding.[12] In all cases, the acoustic experiments involved the monitoring of the binding of biotinylated LAMP products to a neutravidin modified device surface. Figure 1 shows the real-time binding

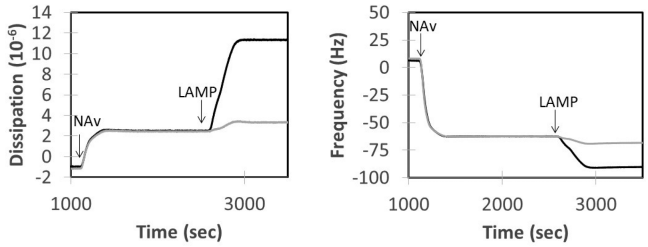

Figure 1. Real-time frequency and dissipation curves during the addition of neutravidin (NAv) and a LAMP reaction containing 10 bacteria (black line) or 0 bacteria (gray line).

curves during the adsorption of neutravidin followed by the addition of a LAMP reaction containing bacteria cells diluted in PBS buffer by a factor of 10. The negative control is also depicted, i.e., application to the neutravidin-coated surface of a LAMP reaction free of any bacteria.

LAMP Amplification Time. In a first series of experiments, we performed the LAMP in the thermocycler for 20, 25, and 30 min for three different concentrations, i.e., 10, 50, and 100 cells per 25 μL of LAMP reaction. Figure 2 shows the effect of

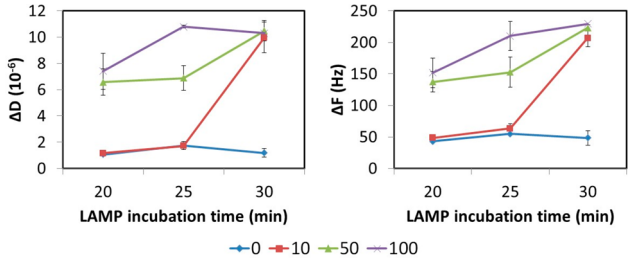

Figure 2. Acoustic response during the loading of LAMP reactions containing 0, 10, 50, and 100 *Salmonella* cells incubated for 20, 25, and 30 min at 63 °C. The blue line corresponds to the background signal when no cells are present.

amplification time to the acoustic sensor response, where both acoustic signals represent end-point measurements obtained at the specific amplification time. For the cell-free LAMP reactions (blue line), which do not contain biotinylated LAMP amplicons, the changes in dissipation and frequency are constant for all three incubation times. This background signal was also observed previously and attributed to the biotinylated primers contained in the amplification cocktail as well as other ingredients of the reaction mix.[10] When 10 cells (red line) were used as the template, a positive signal (above the background) was monitored after 30 min of LAMP-incubation time. When 50 (green line) or 100 (purple line) cells were included, a positive signal was monitored from as early as 20 min of incubation time. It can also be observed that, within 30 min, all three concentrations seem to have reached the maximum signal, while for the 100 cells the saturation point has already been reached from 25 min. It should be noted that for higher starting bacteria concentrations (10^3−10^4 per reaction) the amplification time can be reduced to only 10 min (data not shown).

LAMP Employing Human Samples Combined with Acoustic Measurements. In a second set of measurements,

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we investigated the effect of the human sample matrix on the acoustic detection method. Whole blood, saliva, or nasal swabs spiked with 10, 50, or 100 bacteria were injected directly in the LAMP cocktail and incubated for 30 min in a thermocycler; this time was previously shown (see Figure 2) to be sufficient for both LAMP amplification and acoustic signal saturation. One point of concern was the presence of enzyme inhibitors in the crude samples, which could potentially affect the efficiency of amplification. A second point was the potential interaction of the sample matrices with the acoustic sensor surface. It was unknown whether such interactions could cause nonspecific acoustic signal changes that could mask the signal of the immobilized biotinylated amplicons. For this reason, three different surfaces were tested for their biocompatibility toward the direct application of crude human samples. These were a PLL-*g*-PEG surface, i.e., a protein passivation coating previously employed for the detection of *Salmonella* DNA from a crude sample (milk)[15] and two biotin binding surfaces using either a streptavidin or a neutravidin layer. The last two have been used extensively during the binding of biotinylated molecules from a buffer solution;[20−22] however, to our knowledge they have not been tested before for their ability to selectively bind biotinylated biomolecules from a rich crude medium. For blood-containing samples (1−2.5 μL of whole blood), the neutravidin-coated device gives the best discrimination capability among all three tested surfaces (Figure S3). However, the PLL-*g*-PEG appears to be the worst, providing no capability to discriminate between a positive and a negative sample due to the excess amount of human genomic DNA interacting directly with the poly-L-lysine.

Based on the above, the neutravidin modified surface was selected for all subsequent experiments in the three human samples. Figure 3 summarizes the frequency and dissipation

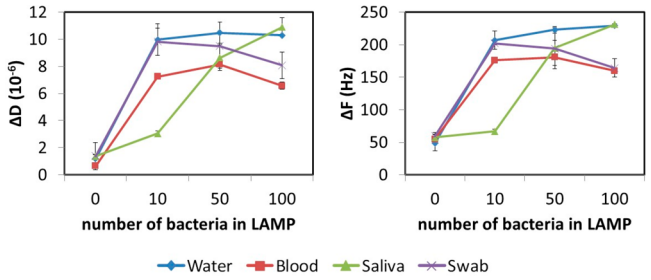

Figure 3. Acoustic response during the loading of LAMP reactions containing 2.5 μL of water, whole blood, saliva, or nasal swab spiked with 0, 10, 50, and 100 *Salmonella* cells and incubated for 30 min at 63 °C.

shifts that were monitored during this series of measurements. It is apparent that both acoustic signals (ΔD or ΔF) have reached a saturation level for all three numbers of tested bacteria in blood and swab samples; saliva samples have also reached a saturation level with the 50 and 100 cells but not with the 10 cells. This outcome shows that the efficiency of the amplification reactions is not affected by the presence of inhibitors in any of the crude samples. In addition, nonspecific binding on the optimized neutravidin surface is minimum, causing no interference with the amplicons detection.

By calculating the acoustic ratio, we noticed that all positive samples (even with the 10 cells in saliva) have an average value of 0.0457 (10^{-6}/Hz). This suggests that amplicons produced inside all four samples have the same shape and size and are bound to the device surface in the same way (through biotin−neutravidin interactions). Interestingly, the values obtained with the negative controls, although lower than corresponding positive ones, depend on the testing sample; in water it is 0.0305 (10^{-6}/Hz), in saliva and swab 0.0236 (10^{-6}/Hz) and in whole blood 0.012 (10^{-6}/Hz) (Table 1). The lowest ratio in the latter case indicates that blood ingredients such as platelets, albumins, globulins, etc., result in a much tighter and less dissipative surface-bound layer than in saliva and swab. Overall, acoustic results (Figure 3 and Table 1) point out that any of the two acoustic parameters (frequency or dissipation) or their combination (acoustic ratio) can be used for discriminating infected from noninfected samples when using the QCM-D device.

Comparison of Acoustic Results with a Colorimetric Assay. For comparison of the acoustic assays with another technique, a pH-dependent LAMP colorimetric kit based on the Phenol Red dye was also employed. This dye allows the shift of the initial pink color of a LAMP reaction to yellow due to the release of protons during DNA amplification.[23] The assay allows observation of three different colors, i.e., pink, orange, and yellow, which can be correlated to arbitrary color change units of 0, 1, and 2, respectively (Figure 4, left).

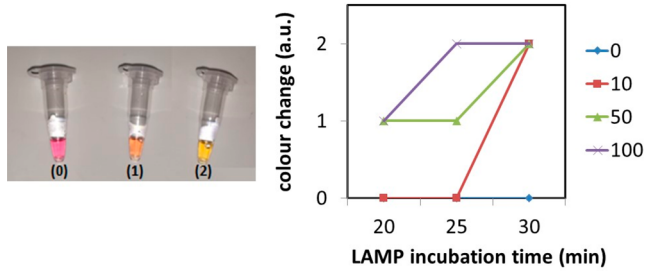

Figure 4. (left) Three different colors that could be visualized during end-point colorimetric LAMP. (right) Color change for various cell amounts and incubation times.

Performing the LAMP reactions with the three different cell numbers (10, 50, and 100) and for the three different incubation times gave the same pattern (Figure 4, right) as the one observed during the acoustic measurements (Figure 2). Saliva and swab samples also gave similar colorimetric results to the acoustic ones but not whole blood. Whole blood did not allow any color observation due to the high background of hemoglobin, which dominates. This observation is a clear advantage of the acoustic method for point-of-care applications since whole blood is a valuable source of genetic material associated with infectious agents.

Building a 3D-printed POC Prototype for Smartphone Operation. While the previously shown acoustic method is simple, rapid, and sensitive, it is based on relatively expensive benchtop equipment such as a thermocycler and a network analyzer. For POC applications, portability and low instrumentation costs are of great importance. For this reason, an alternative solution for performing the LAMP amplification and the acoustic detection was investigated and evaluated. A Peltier unit was employed to replace the thermocycler and a hand-held vector network analyzer, the miniVNA PRO antenna, for recording acoustic measurements. The miniVNA system can be controlled with a smartphone or tablet via Bluetooth connection. In addition, an acoustic sensor docking station was used for housing either a Surface Acoustic Wave

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(SAW) or a QCM device together with a mini peristaltic pump for driving the fluid sample from the LAMP reaction tube over the device surface. Data was collected in both a transmission and reflection mode for the SAW and QCM device, respectively.

The different components mentioned above (miniVNA, acoustic cell, Peltier unit, and mini peristaltic pump) were placed into a portable modular system that would be suitable for infectious diseases' POC testing based on the developed acoustic detection methodology. The portable plastic housing unit was manufactured by using 3D-printing, a fast and inexpensive technology allowing the creation of a lightweight but also durable at elevated temperatures prototype (Figure 5).

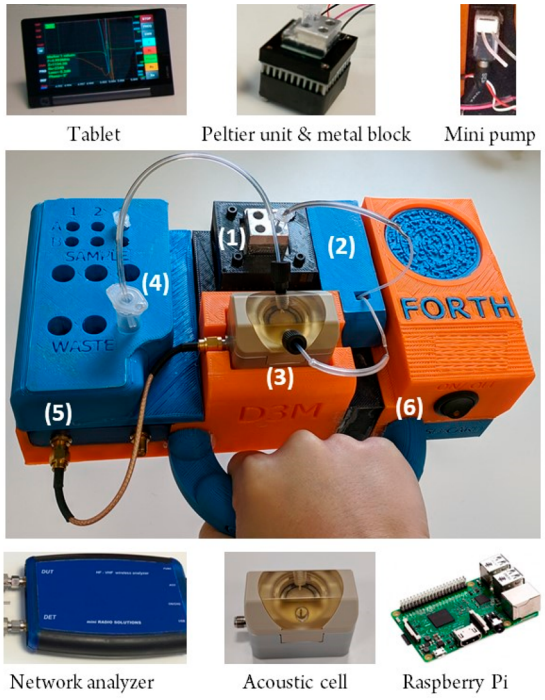

Figure 5. Three-dimensionally-printed prototype system and its components. The amplification tube is placed inside the metal block on top of the Peltier unit (1). When heating is completed, the peristaltic pump (placed inside 2) drives the sample to the acoustic cell (3) and finally to the waste tube (4). The mini network analyzer is placed inside position (5). The Raspberry Pi (6) controls the system with the help of a tablet.

Moreover, a Raspberry Pi was employed to control the analyzer, the temperature of the Peltier unit, and the pump through a developed web user interface (see SI). The modular configuration of the prototype allows customization of the measuring sequence; i.e., the addition of sample preparation steps, use of an alternative to LAMP isothermal DNA amplification method, and use of an acoustic docking station for housing other chips, for example, a High Fundamental Frequency (HFF) QCM or Lamb wave device. Last but not least, changes in the prototype design can be readily and inexpensively implemented within one working day compared to systems produced by standard injection molding, CNC machining, or lithography techniques.

Evaluation of the 3D-prototype Parts during Nucleic Acid Amplification and Detection. For the evaluation of the new 3D-printed prototype, a metal stand for holding 0.2 mL eppendorf tubes attached on the metal surface of a Peltier heating element was employed for performing thermal lysis and LAMP amplification. The efficiency of DNA amplification within the above module was compared with that from a thermocycler using gel electrophoresis (Figure 6A) and

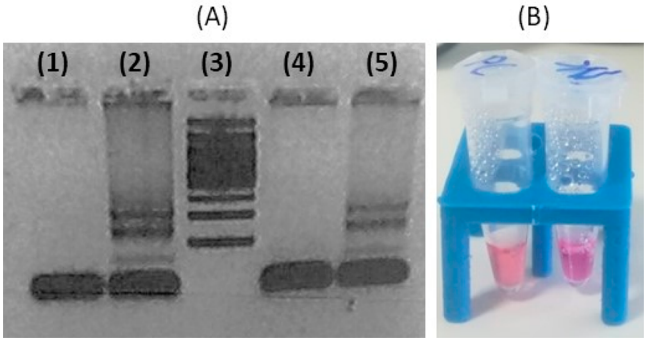

Figure 6. Comparison of LAMP amplification efficiency in the thermocycler and Peltier module. (A) Image of a 2% agarose gel. Lanes 1 and 2: LAMP negative and positive reactions in a thermocycler, respectively; lane 3: DNA ladder; lanes 4 and 5: LAMP negative and positive reactions in the Peltier module, respectively. (B) Colorimetric results of a positive (left tube, 100 bacteria in saliva) and a negative (right tube) LAMP reaction performed in the Peltier module for 20 min.

colorimetric analysis (Figure 6B). Results verified that the currently developed Peltier unit with the metal mini-eppendorf holder has the same efficiency as a thermocycler unit, making it an attractive alternative for POC settings.

Following amplification, the 3D-printed prototype was tested toward the direct acoustic detection after LAMP amplification of blood samples spiked with 0 (negative) or 10 (positive) *Salmonella* cells, according to the method described previously and a SAW device. Based on the data shown in Figure 7/left, the acoustic ratio could discriminate

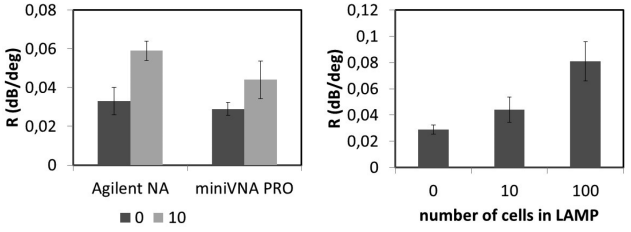

Figure 7. (left) Comparison of acoustic ratio results retrieved with the Agilent network analyzer and the miniVNA PRO (p = 0.029 from triplicate measurements). (right) Acoustic ratio results obtained with the miniVNA pro during measurements of LAMP reactions spiked with different number of bacteria cells.

the positive from the negative sample by using the miniVNA PRO in transmission mode. Comparing these results with acoustic data obtained with a standard network analyzer, we found that the latter gives slightly better results; this is attributed to the fact that, for the miniVNA PRO, the sensitivity in the phase and amplitude is 1 deg and 0.1 dB, respectively, while for the network analyzer these numbers are 0.01 deg and 0.001 dB. Furthermore, the measurements with the miniVNA are end-point ones and not real time, which would allow signal correction of the often-observed drift. It is noted that phase change was not a sensitive measurement when using both analyzers, i.e., there was no difference

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between positive and negative samples (Figure S4). This is in agreement with our previous observations where phase change was not able to distinguish between positive and negative signals.[13] However, with the miniVNA and through acoustic ratio measurements we have managed to discriminate the two and even retrieve quantitative information regarding the presence of 10 or 100 bacteria in the reaction mix (Figure 7/right).

When applied a QCM device in the corresponding docking station, we used the mini-network analyzer in a reflection mode for measuring changes in the resistance at a fixed frequency. Since resistance and dissipation are essentially equivalent parameters,[24] we assumed that it would be possible to discriminate infected from noninfected samples based solely on the resistance change, as demonstrated in Figure 3. For this purpose, we prepared LAMP reactions with saliva spiked with 0 or 100 bacteria as before. The lysis and the 20 min LAMP amplification steps were performed in the Peltier module followed by acoustic detection with the portable QCM cell and the miniVNA analyzer operated via a tablet. For the noninfected samples, the resistance change was equal to 7 ± 1 Ohms, while for the infected ones, it was measured to be 27 ± 8 Ohms (data not shown), which can be considered as more than a clear difference.

Comparison of Acoustic Method with Other Sensors and Methodologies Used for DNA Detection at the Point-of-Care. The merits of the reported acoustic method are compared with other methods used for the detection of LAMP amplified nucleic acids. Currently, electrochemical sensors have emerged as a powerful methodology for nucleic acid detection at the point-of-care, promising to replace lab-based optical detection and cumbersome real time PCR. These systems, often combined with microfluidics for sample pretreatment and LAMP amplification, have been shown to be able to achieve impressive analytical performance.[25,26] Examples include the integrated microfluidic electrochemical (IMED) DNA chip, demonstrated to be able to detect $<10^3$ CFU/mL of *Salmonella* in unprocessed blood (8.5%) in <2 h;[27] the microfluidic electrochemical quantitative-LAMP (MEQ-LAMP), shown to be able to detect in real time and in a single step 16 (or calculated equivalent of 640 CFU/mL) LAMP amplified amplicons of *Salmonella* in <1 h;[28] or the real time microfluidic multiple electrochemical LAMP (μME-LAMP) for the detection of 16 cell copies/μL of *Klebsiella pneumonia* and *Mycobacterium tuberculosis* bacteria in 45 min.[29] In the current work, acoustic wave sensors based on the SAW or QCM device are demonstrated to have a similar limit of detection (LoD), i.e., 4×10^3 CFU/mL of *Salmonella* in all three cases of unprocessed human samples and a very rapid analysis time of <30 min. This detection limit is slightly elevated to the LoD of other acoustic methods combined with LAMP (2×10^3),[30,31] probably due to the fact that our work uses nonpurified crude samples. Our reported limit of detection, even if it changes in infected patients' samples as opposed to spiked-in human specimens, falls well within the clinically relevant concentrations (10^3–10^4 CFUs/mL or the fM range); this range is relevant for the detection of blood or upper respiratory tract infections caused by pathogens such as Dengue or Influenza viruses.[32] By increasing the LAMP reaction volume, we could use 5 to 10 μL of whole blood and thus improve the LOD by a factor of 2 to 4. A similar approach has been suggested for the IMED electrochemical microfluidic platform, which is also limited by the volume of the microfluidic chamber.[27]

In terms of simplicity, to the best of our knowledge, two are the simplest nonoptical methods reported so far including LAMP amplification and nucleic acid detection. Both are integrated and concern, the first one the MEQ-LAMP, a real time electrochemical technique, which comprises a single microfluidic chamber for LAMP and electrochemical detection;[28] the second an acoustic LOC[15] where *Salmonella* amplicons are detected upon LAMP amplification in a separate microfluidic channel. Both methods comprise a single pipetting step for the end-user to load the sample with the rest of the analysis performed automatically. The method presented here employs two steps; 1. loading the sample in the reaction vessel and, upon completion of the LAMP reaction, 2. immersing the tubing inside the vessel to transfer the sample via a mini-peristaltic pump on the device surface. In our opinion, the extra step employed in the reported acoustic method is not adding any significant extra time and labor; moreover, it is compensated by the simplicity, flexibility, and cost effectiveness of the fluidics employed in the latter case. Indeed, several concerns have appeared in literature regarding the ability of soft-lithography-based technologies and microfluidic chips to be effectively mass-produced at a low cost.[33]

One important aspect of the current work is the demonstration that acoustic detection is not affected by the presence of complex matrices such as whole blood, which is known to contain PCR inhibitors and fluorophore quenchers. This allowed the direct use of crude samples with only a quick thermal (or chemical) lysis step without the need for a more complex and time-consuming nucleic acid extraction procedure. The used 10% sample dilution is among the highest reported in literature. With real time PCR, a 5% dilution of whole blood has been previously reported.[34] However, in this work, an unusual concentration of 40× SYBR Green dye was required leading to excessive background fluorescent signal. Patterson et al, reported a 8.5% dilution of whole blood with a microfluidic system (3 μL in 35 μL of LAMP total).[27] In another recent work, the ability to detect RNA directly from 40% whole blood[35] was demonstrated. This involved a first round of PCR amplification with 40% whole blood followed by a second round of nested real time PCR for detection; the nested PCR was performed with 1 μL of the first reaction mix transferred in 20 μL final volume, corresponding to 2% whole blood dilution for real time detection.

While several integrated platforms have been reported for performing nucleic acid detection at the POC, fewer works have demonstrated a suitable instrumentation, i.e., hand-held, reliable, and inexpensive. In our work, the use of plastic tubes (e.g., Eppendorfs) as the LAMP microreaction vessel and tubing for the fluidics is compatible with a mini peristaltic pump eliminating the need for more expensive syringe pumps and/or valves. Together with the miniVNA and a Peltier heating element, a fully portable system weighting at least 2 orders of magnitude less than a standard benchtop equipment was constructed. The fabrication cost of the portable unit is calculated to be <$2.5K based on commercially available parts, with the most expensive part being the acoustic housing unit ($2K); the above prices can be further reduced for large scale production. Consumables are calculated to be $2.5 per test based on commercial prices for LAMP reagents and an acoustic chip fully reusable for 100 times. A simpler alternative to recycling the sensors could be to use the High Fundamental

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Frequency QCM (HFF QCM) based on the much affordable MESA technology (current fabrication cost of $1 per chip).[36] Moreover, the application of 3D-printing for manufacturing the portable prototype is fully compatible with the need for a digital, rapid, and environmentally friendly prototyping technique of medium volume production.[37] In addition, the low capital investment for the instrumentation and very affordable price of the prototype are extra assets of additive manufacturing.

Finally, we consider the combination of the 3D-printed prototype with smartphone detection to be another asset of our methodology compared to the present state-of-art. So far, the vast majority of smartphone based bioanalytical systems are devoted to optical biosensors for microscopic bioimaging and fluorescence or colorimetric biosensors in blood, sweat, tears, urine, and saliva.[38−40] In addition, the few reported electrochemical smartphone-coupled sensors are targeting mainly electrolytes and metabolites.[33,38] The demonstrated ability of the 3D-printed acoustic platform to employ a smartphone for controlling the system operation (temperature, fluids, and acoustic) and displaying the results is fully compatible with the need of a hand-held nucleic acid detector for rapid analysis at the point-of-care. Potential communication via the network and incorporation of the Internet of Things concept presents an added value of the system for future development.

CONCLUSIONS AND PROSPECTS

In this work, a novel methodology for nucleic acid POC testing was developed that fulfils most of the ASSURED criteria of the World Health Organization,[41] i.e., is affordable, specific, user-friendly, rapid, robust, and deliverable to end-users. The methodology, although relying on the use of equipment, can partially bypass the equipment-free criteria, by offering inexpensive and lightweight hardware miniaturized parts, integrated through 3D printing into a real POC diagnostic system. Moreover, the prototype is customized to be capable of performing the whole sequence of the acoustic detection method while keeping the cost of the required consumables at minimum. The demonstrated capability of the method to detect the presence of 10 bacteria in 2.5 μL of crude human samples such as whole blood, saliva, or nasal swabs in less than 30 min is a clear advancement for molecular diagnostics at the POC and in low income settings. In addition to *Salmonella* employed in the current work for the proof-of-principle, the same concept can be applied to DNA or RNA viruses since the LAMP mix displays reverse transcription as well as strand displacement and DNA polymerase activities. For example, the methodology could become applicable to sexually transmitted diseases (Chlamydia), lower respiratory tract infections (Streptococcus), or other applications such as single nucleotide polymorphisms (SNP) detection for human pharmacogenetics and screening for cancer related mutations and miRNAs.

Overall, to the best of our knowledge, this is the first demonstration of a fully autonomous 3D-printed platform able to perform DNA LAMP amplification and acoustic detection at the POC directly in crude samples and with the use of a smartphone. Following the demonstrated prototype evaluation in the lab and under ideal conditions, we anticipate to proceed to target DNA analysis under real-life conditions by end-users in the near future.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssensors.9b00264.

LAMP amplification products with LAMP mix stored at RT. LAMP products after amplification for 20 min. Comparison of PLL-*g*-PEG, NeutrAvidin, or Streptavidin surface coating. Comparison of phase measurements using an Agilent NA and the Mini VNA Pro (PDF)

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The manuscript was written through contributions of G.P. and E.G. The design of the experiments was performed by G.P., A.K.P., and E.G. The experiments were performed by A.P., M.N., K.P., G.I.T., and G.K. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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