Sample Preparation for Point of Care Molecular Diagnostics of STIs

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Abstract Brunel DoCLab is part of the *e*sti^2 consortium developing electronic self-testing instruments for sexually transmitted infections using nucleic acid amplification testing (NAAT). A proprietary sample collection device has been designed to integrate directly with a microfluidic cartridge. Cell lysis was conducted using a chemical method and nucleic acid purification was done on an activated cellulose membrane. The microfluidic device incorporates passive mixing of the lysis-binding buffers and sample. Preliminary results have shown extraction efficiencies for this new membrane of 69% and 57% compared to the commercial Qiagen extraction method of 85% and 59.4% for 0.1ng/µL and 100ng/µL salmon sperm DNA spiked in phosphate buffered solution. Preliminary extraction experiments in the passive mixer cartridges with lysis and nucleic acid purification showed extraction efficiency around 80% of the commercial Qiagen kit. Isothermal amplification was conducted using thermophillic helicase dependant amplification. A low cost benchtop real-time isothermal amplification platform has been developed capable of running six amplifications simultaneously. Work to integrate sample collection, nucleic acid extraction and isothermal amplification is currently underway.

Keywords: Microfluidics, Microengineering, Point-of-Care, Molecular Diagnostics

1. Introduction

The aim of this project is to develop rapid electronic self-testing instruments for sexually transmitted infections (*e*sti^2). Nucleic acid amplification testing (NAAT) is becoming increasingly popular within point of care tests (POCT) due to the rapid, sensitive and specific results obtained. An integrated microengineered platform (Figure 1) is in development for automated DNA extraction, isothermal amplification [1] and optical detection directly from raw samples such as urine, blood, swabs and saliva. The handheld device will automate sample processing and send results directly to clinicians for rapid diagnosis to expedite time to treatment. Sample collection and integration with preparation methods including nucleic acid extraction has inhibited the uptake of commercial POCT devices.

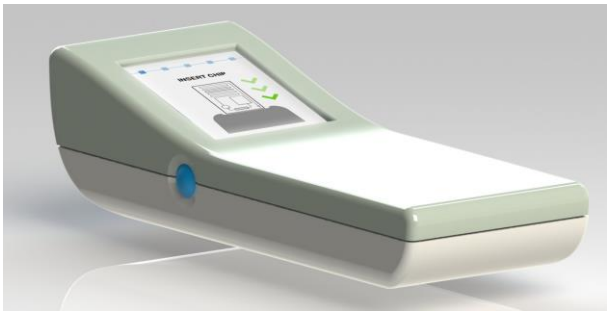


Fig. 1. Handheld device in development for rapid diagnostics of STIs

The group has developed, simple, easy to use, sample collection devices incorporating both urine and swab samples (Figure 2). The ease of use is imperative in self-tests as the user will take a self-collected sample which will integrate with the microfluidic cartridge which is plugged into the handheld device for automated processing.

Nucleic acid extraction for POCT devices

is dominated by solid phase extraction with chaotropic salts using silica membrane, columns [2, 3] and magnetic beads [4]; membranes require centrifugation whilst magnetic beads require an external magnet for active mixing. The other drawback to this method is the use of toxic guanidinium thiocyanate, which can inhibit downstream polymerase chain reaction (PCR) [5]. This paper reports a new method of DNA isolation using chitosan impregnated on an organic membrane inserted into a Polydimethylsiloxane (PDMS): glass prototyped microfluidic devices.

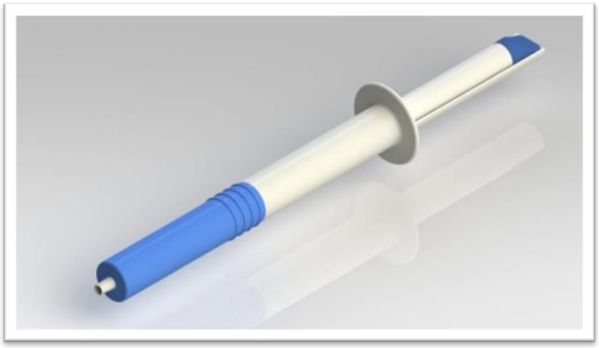


Fig. 2. User friendly sample collection device in development

Chitosan exists as a cationic polymer when the pH is less than 6.2 as the pKa value of protonated amino group at 6.2 confers to its polycationic behavior (Figure 3), whereas it is in its neutral form at pH values greater than this. At higher pH, the amine groups in chitosan are deprotonated (Figure 3). This chemical aspect of chitosan makes it an ideal candidate to capture and release DNA by a simple pH change [6,7]. The presence of phosphodiester bond in DNA makes it a strong acid with pKa less than 1.0 and facilitates its binding to chitosan by electrostatic interaction [8].

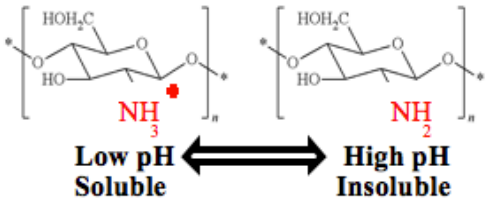


Figure 3: Conformational changes and solubility of chitosan molecules corresponding to pH variation

Polymerase chain reaction (PCR) was the first method employed for nucleic acid amplification testing (NAAT). More recently isothermal amplification methods have been developed that utilize enzymes for DNA strand separation [1]. An isothermal method was chosen for this project as it removes the requirement for rapid heating and cooling steps required in PCR, therefore, less power is consumed within the handheld device. Optical detection of amplified DNA was chosen as this could be implemented in a low cost manner in a handheld device and allows real time visualization of the NAAT, the benefit of this over other methods is that the original sample load can be quantified.

2. Platform Development

A low cost isothermal amplification and optical detection platform has been developed incorporating a resistive heating element, low cost photodiodes, LEDs and optical filters (Figure 4). The control and data acquisition was conducted using two Arduino Mega2560 microcontroller boards (Arduino, Italy).

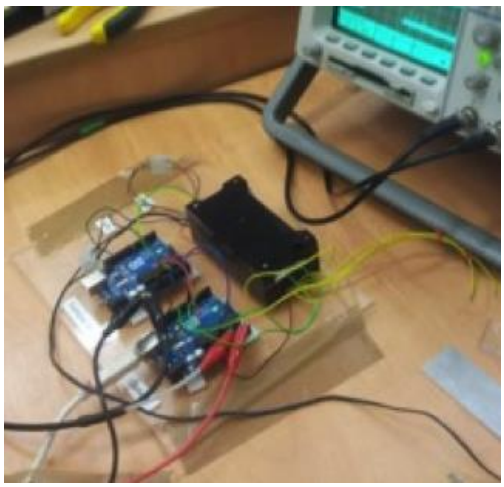


Fig. 4. Small footprint, low cost, isothermal amplification platform.

A resistive heating element was designed and developed from a two-layer printed circuit board (Fotoboard 2, PrecisionMicro, Birmingham, UK) using standard PCB etching methods. A surface mount thermistor was integrated centrally on the heating element to monitor and control temperature changes in

the heater. An aluminium plate was attached to this heating element using a heat transfer adhesive (TermoGlue, AG Termopasty Grzegorz Gasowski, Poland) as shown in Figure 5, to function as an isothermal plate to dissipate the heat evenly to the chip and thermistor. The heating element produced sufficient energy to reach 65^{o}C required to perform the helicase dependent isothermal NAAT in ≈ 90 seconds.

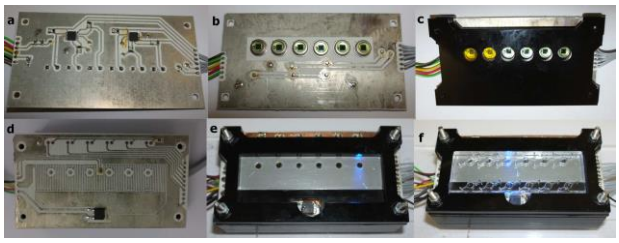


Fig. 5. Isothermal amplification platform.

A low-cost photodiode (BPW21, Centronic, Surrey, UK) with a high gain operational amplifier (OPA4750, Texas Instruments, Dallas, USA) with 1GΩ feedback resistor was utilised to perform the optical detection. The photodiodes were coupled to the device via a 15mm long section of hand polished, unjacketed 3000µm plastic optical fiber (Edmund Optics, Barrington NJ, USA). A long pass filter cut from orange glass (OG515, Schott AG, Mainz, Germany) was placed between the photodiode and the optical fiber. The optical fiber was aligned through a hole on the heating element and on the isothermal plate to rest flush to the upper surface of the isothermal plate where the underside of the microfluidic chambers will rest. 3mm LED (L-7104QBC-D, Kingbright, New Taipei City, Taiwan) which illuminated the chip orthogonal to the optical fiber was used to perform the excitation at 470nm.

The final device was constructed using the elements described and 6 layers of black PMMA (Figure 5) with a recess for the placement of the microfluidic cartridge to create a low cost platform for isothermal amplification and detection.

3. Methodology

Chitosan was dissolved in 2v/v% acetic acid with varying weight percentages of chitosan (1-5w/v%). Various grades of Whatman chromatography paper were used as membranes, thus a hybrid plastic paper microfluidic device was created. Membranes were added to the solution and cross-linked using either 1v/v% glutaraldehyde (GA) or 0.1v/v% (3-Glycidoxypropyl) methyldiethoxysilane (GPTMS). The membranes were left in solution for eight hours, removed and thoroughly rinsed in 10mM acetic acid. Membranes were dried in an oven at 60^{O}C for one hour. The membranes were inserted into a microfluidic chamber with a total volume of 100µL.

Microfluidic molds were designed using Solidworks CAD software and saved as STL files. The molds were created using 3D printing techniques with an Objet 30 Pro (Statasys, US) using jetted photopolymer deposition. QSil 218 PDMS (ACC Silicones, UK) was mixed at a ratio of 1:10 for 2 minutes and then degassed using a centrifuge for about 4 minutes. The molds were cleaned by rinsing with iso-propanol alcohol and then DI H_2O to remove contaminants; they were then dried using nitrogen. The mold was placed into a custom made stainless steel frame which was cleaned in the same manner. After degassing the PDMS it is poured gently into the mold and left in the oven at a set temperature of 45° C for 4 hours. The cured PDMS was removed from the mold; a handheld corona treater, BD20-AC (Electro-Technic Products Inc., US), was used to bond the PDMS to a 75x50mm glass slide (Sigma Aldrich, UK). The nucleic acid extraction membrane was placed in the chamber and the treated surfaces are then pressed together and placed in an oven at 45° C for 8 hours.

The microfluidic device incorporates sample and buffer inlets, a pre-mixing chamber, passive serpentine mixer and nucleic acid extraction membrane (Figure 6).

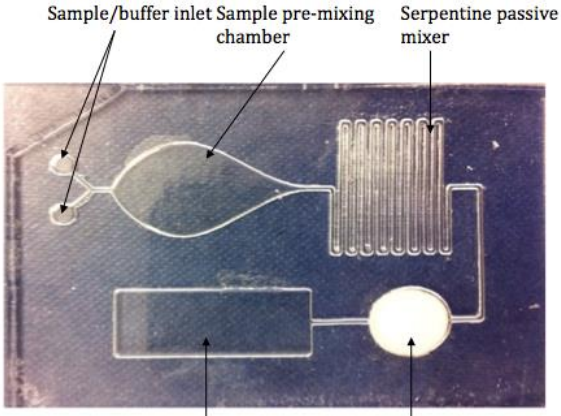


Fig. 6. Credit card sized microfluidic extraction cartridge.

Membranes were tested by spiking TE buffer with salmon sperm DNA (Sigma, UK) with concentrations of 100ng/µL and 0.1ng/µL. Further tests were conducted incorporating lysis by purifying DNA from *Escherichia coli* (*E.coli*) spiked phosphate buffered solution using four designs of passive mixing-lysis-extraction cartridges (Fig. 6). The buffers and sample were loaded into a length of 0.5mm tubing (Altec, UK) and were flowed in sequence into the microfluidic chamber. MES buffer (10mM) was prepared at pH5.0 (pH3.0 if alkaline lysis was required). Samples were flowed over the device using a piezoelectric pump/s (Bartels Mikrotechnik GmBH, Germany) controlled using an Arduino; samples were eluted using 10mM TRIS buffer at pH 9 and pH 9.5. A volume of 25µL MES buffer was passed over the membrane at a flow rate of 1µL/s. This was left in the chamber for 5 minutes to ensure protonated amino groups were formed on the chitosan surface. The sample (25µL) was flowed directly into the chamber with MES buffer solution and was left for 5 minutes. The solution was removed from the chamber by flowing air into the device at 3µL/s. The elution buffer (50µL) was flowed into the device at 1µL/s and left for 10 minutes, this was removed and the extraction efficiency was calculated by measuring the concentration of DNA that was eluted from the membrane. This was compared to the original sample. Measurements were made using a NanoDrop Spectrophotometer and Qubit HS dsDNA assay with a Qubit Fluorometer (LifeTechnologies, US). Concurrent experiments were conducted using a Qiagen mini prep (Qiagen, UK) commercial DNA extraction kit for comparison.

Separate microfluidic devices were designed and manufactured as previously to test the isothermal NAAT and real time optical detection. NAAT tests were conducted using a one step thermophillic helicase dependant amplification (tHDA) reaction (Biohelix, US). Preliminary results on the platform were done using the primers and positive control provided with the tHDAIII kit. The master mix was prepared in ratios as described in the test manual [9]. Primers, MGF3 (forward) and NGR3 (reverse) were used at a final concentration of 7.5nM each and 20pg control template pCNG1 was added. Reagents were mixed and pipetted manually into the 6 well microfluidic device (Figure 7) that contains reaction chambers of with a 25µL volume. The microfluidic cartridge was placed onto the pre-heated isothermal plate at 68°C and data collection was initiated. To ensure the specificity of the test, electrophoresis of the reaction mix at the endpoint was run on 2% agarose gels (Sigma Aldrich, UK) with an ethidium bromide stain (Sigma Aldrich, UK)

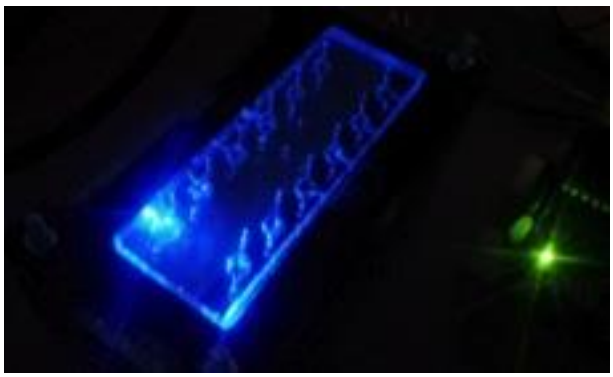
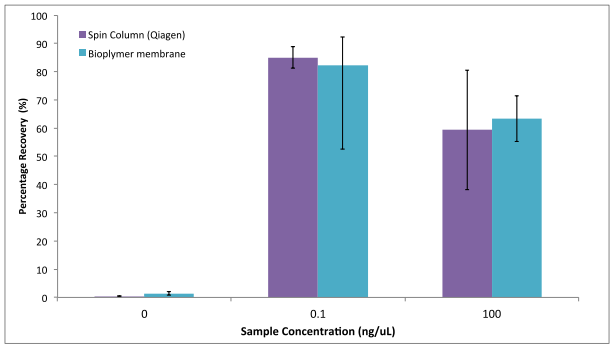


Fig. 7. PDMS glass microfluidic device for 6-plex amplification and detection

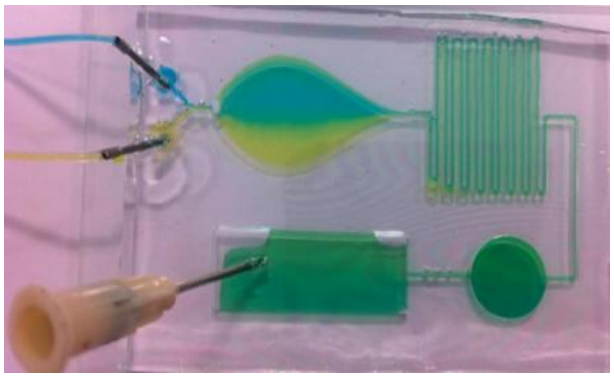
4. Results and Discussion

Cross-linking chitosan using GA gave the highest extraction results. However, it was noted that cross-linking was uneven across the

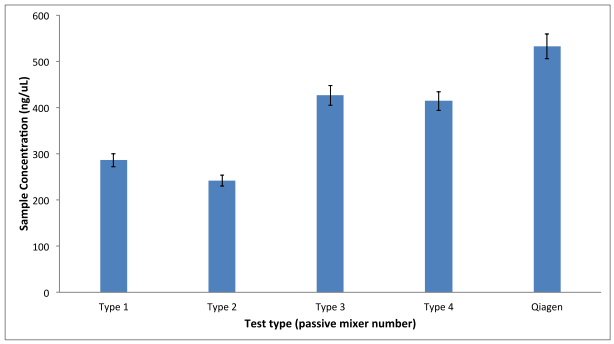
surface of the membrane and large variation in the extraction efficiency was seen. The extraction efficiency was lower when high DNA concentrations were used, at 100ng/µL both the Qiagen and chitosan membranes showed lower extraction efficiencies, the membrane may be saturated and unable to adsorb more DNA.



(a)



(b)



(c)

Fig. 8. (a) Extraction results for the new membrane compared to a Qiagen spin column extraction with varying concentrations of DNA; (b) Passive mixing device; (c) Passive mixing device results for DNA extracted from E.coli cells

The results showed extraction efficiencies of 63% and 82% for 100ng/µL and 0.1ng/µL respectively in comparison to benchtop Qiagen extraction results of 59% and 85% (Fig. 8a), these membranes were impregnated with 4w/v% chitosan and cross linked with GA. GPTMS cross linking gave more homogenous results, extraction efficiencies up to 58% were obtained for 0.1ng/µL, using a 1w/v% chitosan membrane. At higher w/v% chitosan when cross-linked with GPTMS, it was difficult to reverse the protonation of the amino groups and therefore, elute DNA from the membrane at pH 9.0; hence, the elution buffer pH was increased to pH 9.5, which did allow release of bound DNA. However, efficiency was lowered to <30%. Early experiments showed poor wetting of the chitosan membrane when the MES buffer was introduced, therefore, Triton-X 100 was added to MES protonation buffer, this significantly increased extraction efficiency from 35% to 44.6%. The passive mixing device showed good mixing prior to entering the extraction chamber (Fig. 8b). Four designs were tested and mixing analysis was conducted with ImageJ. Preliminary extraction experiments in the passive mixer cartridges showed results with lysis/extraction efficiency around 80% of the Qiagen kit (Fig. 8c).

The resistive heating element provided precise and stable heating of the microfluidic cartridge, a small thermal gradient across the device was seen but this was less than 0.8°C. The system was able to detect 20pg of the pCNG1 template and repeatability across the device was at a high level (Figure 9).

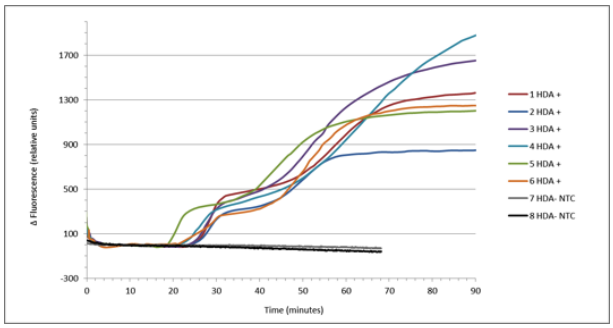


Fig. 9. HDA amplification curves from the isothermal amplification platform, positive and negative reactions for 20pg pCNG1

5. Conclusions

The work presented here shows a low cost, rapid nucleic acid extraction, isothermal amplification and detection. Nucleic acid extraction showed similar results to the commercial extraction kits. Work is ongoing to further optimise the results. The isothermal amplification platform and resistive heating element proved a robust method to amplify nucleic acid using isothermal assays and has shown repeatable results. The simple optics setup demonstrated high sensitivity detection of the tHDA reaction. The group is now developing a new microfluidic cartridge with integrated nucleic acid extraction, which fits into the current low cost isothermal amplification platform (£80); this will have the ability to prove the sample-in-to-answer-out capability of the platform. The platform will be further developed to create a robust handheld device with disposable microfluidic cartridges and simple easy to use sample collection at much lower cost than comparable systems creating a true self-test for STIs.

6. Acknowledgements

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7. References

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