A NUCLEIC ACID EXTRACTION METHOD FOR POINT OF CARE DEVICES
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ABSTRACT
A method has been developed using chitosan impregnated on membranes reducing the complexity of nucleic acid extraction. This can be automated in a microfluidic system prior to nucleic acid amplification and detection. The procedure is pH dependent and involves the binding of DNA at pH 5.0 with DNA release at pH 9.0. The results showed extraction efficiencies of 63% and 82% for input samples of 100ng/µL and 0.1ng/µL respectively in comparison to benchtop Qiagen extraction results of 59% and 85%.

KEYWORDS: Point of care diagnostics, nucleic acid extraction, chitosan

INTRODUCTION
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 (Fig. 1) is under 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 POC devices. This paper discusses a nucleic acid extraction method which will be integrated into a single use, closed loop microfluidic cartridge that will incorporate amplification and detection; this will be used with the handheld device for rapid detection of sexually transmitted infections (STIs).

THEORY
Nucleic acid extraction for POC 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 Polymethyl Methacrylate/Polydimethylsiloxane (PMMA)/(PDMS) microfluidic device. Chitosan, a deacetylated form of chitin, has been shown to adsorb DNA in microfluidic devices by anion exchange [6–8]. This method significantly reduces the complexity associated with nucleic acid extraction as DNA is bound to the membrane under acidic conditions and is eluted in alkaline conditions. This differs from work reported by Landers group [6, 7] by immobilizing the biopolymer on an organic membrane, utilizing a different cross-linking reagent and a higher percentage of chitosan.

EXPERIMENTAL
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°C for one hour. The membranes were inserted into a microfluidic chamber with a total volume of 100µL.
The microfluidic device incorporated 4 parts (Fig. 2): a PMMA chamber with weirs manufactured using hot embossing and a micromill, a PDMS disc to seal the chamber, a clamp to bond the PMMA and PDMS temporarily and the chitosan membrane. This setup allows the chambers to be reused with easy removal and exchange of membranes from the device.

The membranes were tested by spiking TE buffer with salmon sperm DNA (Sigma, UK) with concentrations of 200ng/µL and 0.2ng/µL. The buffers and sample were loaded into a length of 0.5mm tubing (Altec, UK) so that they could be flowed in sequence into the microfluidic chamber. MES buffer (10mM) was prepared at pH5.0, 1v/v% or 2v/v% Triton-X 100 was added to the solution to aid wetting of the membrane surface. Samples were flowed over the device using a piezoelectric pump (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, this was left for 20 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. Concurrent experiments were conducted using a Qiagen commercial DNA extraction kit for comparison.

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. 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. 3), these membranes were impregnated with 4w/v% chitosan and cross linked with GA.

![Figure 2: Experimental setup with the microfluidic device holder, clamp system, extraction membrane and micropump for nucleic acid extraction](image)

![Figure 3: Extraction results for the new membrane compared to a Qiagen spin column extraction with varying concentrations of DNA.](image)

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GPTMS cross linking gave more homogenous results, extraction efficiencies up to 58% have been shown for 0.1 ng/µL using a 1 w/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%.

CONCLUSION
These preliminary results show higher extraction efficiencies than previously published results of 75% from whole blood [6] and 69.1% in an artificial urine solution [8], however, spiked samples are yet to be tested. The method is extremely low cost and simple to setup and offers a simplified protocol for extraction of nucleic acids that can be placed into a fully integrated POC diagnostic system.

ACKNOWLEDGEMENTS
The authors would like to thank MRC for funding the esti2 project, grant number G0901608.

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