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ARTICLE

A Microfluidic Device for Rapid Quantification of Cell-free DNA in Patients with Severe Sepsis

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A rapid and accurate method to identify severe sepsis patients at high risk of death is critically needed for clinical practice. In a recent study, the concentration of cell-free DNA (cfDNA) in blood was found to be a prognostic indicator for ICU mortality in patients with severe sepsis. But current DNA quantification techniques are time-consuming and involve extensive sample preparation. In this paper, we demonstrate a low-cost microfluidic device capable of rapid quantification of cfDNA ina small droplet (<10μl) of blood plasma and whole blood in 5 min using only electrical power. The cfDNA in samples is selectively labeled by PicoGreen, and is extracted and concentrated by electrophoresis into a gel by application of a DC potential of 9V. This device has potential as a prognostic tool for early and rapid assessment of septic patients.

Introduction

Sepsis is the systemic response to infection. It is a leading cause of morbidity and mortality in the western world. If infection is not recognized early and inflammation is not controlled with appropriate therapeutic interventions, progression of the septic condition may lead to organ dysfunction, such as renal failure. [1]. Many patients with severe sepsis require prolonged ICU treatment, which accounts for the highest resource consumption within any hospital. Current prognostication with severe sepsis is mainly focused on the use of clinical scoring systems, including the Multiple Organ Dysfunction Score (MODS) and Acute Physiology and Chronic Health Evaluation II (APACHE II) [2]. However, these scoring systems are not specific for sepsis, thus their predictive power is limited [3]. In addition, there currently exists no routine biomarker to aid in either the diagnosis or prognosis of patients with severe sepsis, even though over 170 biomarkers had been investigated by 2010 [4]. This has been a significant factor limiting the ability to provide timely care in a condition that is resource-intensive and carries a very high mortality. Recently, circulating cell-free DNA (cfDNA) in blood has been found to be a reliable indicator for predicting mortality in ICU patients [3,5].The study concludes that cfDNA concentration in blood is much higher in patients who died in ICU (non-survivors) compared with those who survived (survivors) based on data collected from 80 severely septic patients. The mean cfDNA levels in survivors (1.16±0.13μg/ml) was found to be similar to that of healthy volunteers (0.93±0.76μg/ml) ($p=0.426$), while that of non-survivors (4.65±0.48μg/ml) was notably higher ($p<0.001$) [3]. In combination with current scoring systems (e.g. Multiple Organ Dysfunction Score, MODS) and other sepsis biomarkers (e.g. Protein C and procalcitonin), cfDNA levels can potentially have stronger predictive power [3]. Therefore, rapid quantification of cfDNA in patient blood could enable physicians to identify patients who have a lower likelihood of survival and thus, require a more aggressive care plan. In addition, cfDNA quantification can assist ICU physicians, to utilize medical resources efficiently, thus lower the overall costs for patients and hospitals [6].

Although cfDNA quantification is a promising approach for clinical practice on severe sepsis patients in hospitals, current DNA quantification techniques (spectrophotometers and qPCR) cannot meet the requirements of the clinical application as they are time-consuming, and require labour-intensive sample preparation steps such as centrifugation and DNA-extraction [7, 8]. They significantly delay the prognostic results and limit the ability to provide timely care to the patients who are at high risk of death. Therefore, a point-of-care (POC) device which enables medical staff to quantify the cfDNA levels in severe sepsis patients in a real-time manner is needed. In this paper, we develop a microfluidic device for rapid DNA quantification. It concentrates fluorescently labelled DNA with a Direct Current (DC) electric field, and measures the fluorescent intensity which is directly proportional to the DNA concentration in the sample.

Working Principle and Device Design

The basic goal of the device is to: 1) quickly differentiate between survivors (~1μg/ml) and non-survivors (over ~5μg/ml) in severe sepsis patients based on cfDNA concentration in blood, and 2) measure the various cfDNA levels in those non-survivors. To realize this objective, several operations should be performed: 1) cfDNA in blood should be separated from the constituents that may significantly influence quantification results, such as red blood cells; 2) cfDNA should be concentrated for better quantification due to its low concentration in blood; 3) the concentration value needs to be transduced into an optical or an electrical signal which can be easily recorded and quantified. Based on these requirements, electrokinetic (EK)approach [9-15] was selected to extract and concentrate cfDNA in blood as it is a reagent-free process and relatively fast compared with other DNA extraction methods

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such as silica-based absorptive extraction [16-18], pH-induced DNA capture[19, 20], aluminium oxide membrane filtration [21], and DNA binding with functionalized microparticles [22, 23]. In the EK process, a DC voltage is applied to continuously transport the DNA present in the sample onto a gel, where its mobility is lower, in order to accumulate it over a certain period of time. A fluorescent intercalating tag attached to the DNA is used to quantify the amount of accumulated cfDNA and transduce the signal into the electrical domain. The combination of selective fluorescent staining of all the cfDNA in blood and its electrophoretic accumulation on the gel make it suitable to quantify cfDNA in a short period of time.

A microfluidic device designed for this purpose consists of two PDMS layers that intersect each other as shown in Fig. 1. The top layer contains a sample channel with a dimension of 4mm (L) × 100μm (W) × 60μm (D). The bottom layer contains an accumulation channel with a dimension of 4mm (L) × 500μm (W) × 160μm (D). The two channels are positioned perpendicular to each other, and meet at an intersection where the two channels are fluidically connected. The accumulation channel is pre-filled with 1% agarose gel, while the sample liquid is loaded in the sample channel. Two electrodes are placed in the gel inlet (anode), and at the sample outlet (cathode) as depicted in Fig. 1. When a potential is applied, the DNA from the sample in the sample channel gets selectively transported to the intersection of that channel and the gel in the accumulation channel and gets concentrated at that location.

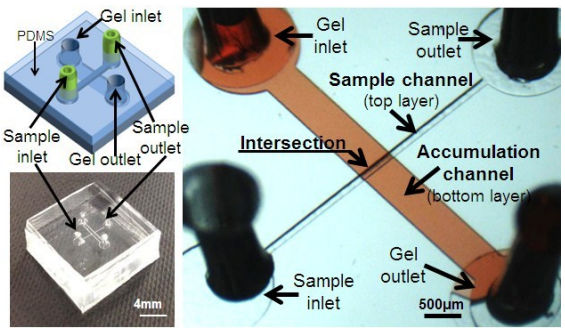

Fig. 1 Schematic of the device and experimental configuration: the sample channel (top) intersects with the accumulation channel (bottom). A DC power supply is connected between the sample outlet and the gel inlet. The accumulation channel is filled with 1% agarose gel (red), and sample fluid is loaded in the sample channel.

The contents of the blood sample are influenced by 3 main electrokinetic forces in a spatially non-uniform DC electric field such as the one in this geometry, namely: the electrophoretic (EP) force, the dielectrophoretic (DEP) force and the electroosmotic (EO) force.

EP force is the net Coulomb force a charged particle and its electric double layer (EDL) ions experienced in the electric field. A negatively charged particle in the sample channel, typically a DNA molecule, will move towards the anode under the EP force. In this device, DNA moves into the gel at the intersection.

Contents in blood also experience DEP forces due to a polarization process in the non-uniform electric field. Most of them, including DNA and blood cells, are subjected to a negative DEP force, which is directed to a local electric field minimum. In the sample channel, particles will move away from the intersection due to the higher electric field there. The DEP force is proportional to the particle volume and the gradient of the electric field squared. The magnitude of force reduces dramatically with the scaling down of the particle size as compared to other forces that are dependent on size. Therefore, the DEP force is usually ignored on small biomolecules such as DNA, while it can be a dominating force for blood cells.

In the sample channel, an EO flow of around 200μm/s is estimated to be generated due to the EDL formed at the surface of the PDMS [24]. The EO force refers to the drag force acting on the particles because of the EO flow. However, in the plasma or blood sample, the EO flow can be significantly reduced by the absorption and interaction between the proteins or cells and the channel surface [25]. As a result, the actual electroosmotic flow will be very small and is ignored for the DNA motion analysis.

According to the analysis above, the transport of cfDNA in the blood sample should be dominated by EP force which drives it into the gel at the intersection. However, the DEP force may have a significant influence on the blood cells, and move them away from the gel at the intersection. As a result, an electric field established at this intersection between a channel and gel can potentially be used to separate cfDNA from other constituents of the blood sample.

Experimental Materials and Methods

Experimental Setup

The device was placed on a fluorescent microscope (Model 500 Lumascope) with a 10x lens. A droplet of heated 1% agarose gel (BioShop) was dropped at the gel inlet of the accumulation channel. Then an empty syringe was used to aspirate the gel solution from the gel outlet. During the aspiration, liquid gel solution fills the accumulation channel completely, while the sample channel stays clear due to aspiration of air from it. Once the gel solution cooled down and gelled (~20seconds), the sample solution was injected into the sample channel using a clean 1 ml syringe (BD Luer-Lok^{TM}Tip) connecting with a 20G needle (BD PrecisionGlide^{TM}). Silver wires (0.2mm diameter, Warner Instruments) were inserted into the gel inlet and sample outlet as electrodes, and were connected with a DC power supply (Keithley 2410). The accumulation channel was connected with anode, while the sample channel was connected with cathode. During experiments, a positive DC voltage was applied, and the device was covered with a black cloth in order to reduce background light interference from outside. The fluorescent microscope was connected with a laptop, through which the DNA accumulation process can be observed and recorded. The brightness of the light source (LED) in the microscope was set as 29 using the software, which represented an illuminance of around 1.0×10^4 lux, measured with a digital lux meter (LX1332B, Vicimeter Technology CO., LTD.). Brightness was maintained the same throughout all the experiments.

Experimental Method

Quant-iT™ PicoGreen® dsDNA Reagent and Kits (Life Technologies) were used to prepare fluorescently labelled DNA samples. Studies have shown that PicoGreen is a very sensitive stain for DNA quantification, even with the presence of most contaminants [26], and this is crucial for our purpose. Clinical samples, including whole blood and blood plasma of healthy donors and patients, were received from a collaborative project, DNA as Prognostic Marker in ICU Patients Study. The study

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was approved by the Research Ethics Board of McMaster University and Hamilton Health Sciences, Hamilton (REB approval 10-532). In each experiment, the prepared sample liquid was stored in a testing tube which was wrapped with aluminium foil, and was left in room temperature for 10 minutes to make sure DNA and PicoGreen were thoroughly intercalated.

During each experiment, fluorescent images were collected at specific time points (0, 30s, 1min, 2min, 3min, 4min, and 5min) to monitor the change of the fluorescence at the intersection, while minimizing the errors introduced by photobleaching of the dye. The collected fluorescent pictures were processed using ImageJ (1.48v) to characterize the fluorescent intensity. The original RGB pictures were transferred into 8-digit grey scale format which has a value range of 0~255. The brighter the fluorescence was, the higher the grey scale value was for each pixel. A mean grey scale value was calculated at a pre-selected area enclosing the intersection edge. After subtracting the fluorescent intensity value of the sample channel background, the new value was used as a parameter representing the fluorescent intensity of the accumulated DNA.

Experimental Results and Discussions

Demonstration of DNA Accumulation

In order to demonstrate that DNA can be efficiently accumulated on the gel at the intersection region, a proof of concept experiment was performed. A 1μg/ml DNA spiked plasma sample was used in this experiment. It was prepared as follows: 1) the original λ-DNA solution in the kit was dilutedto20μg/ml using TE buffer. This concentration was confirmed using a spectrophotometer (NanoDrop 2000); 2) it was added to the blood plasma from a healthy donor to achieve a final concentration of 4μg/ml; 3) this sample was mixed with 200-fold diluted PicoGreen reagent in a 1:3 volume ratio to obtain a DNA spiked plasma sample with a final concentration of 1μg/ml. During the experiment, no voltage was applied for the first 5 minutes after sample loading, while 9V was applied for the next 5 minutes. The experiment was repeated 3 times with a new device used in each repeat.

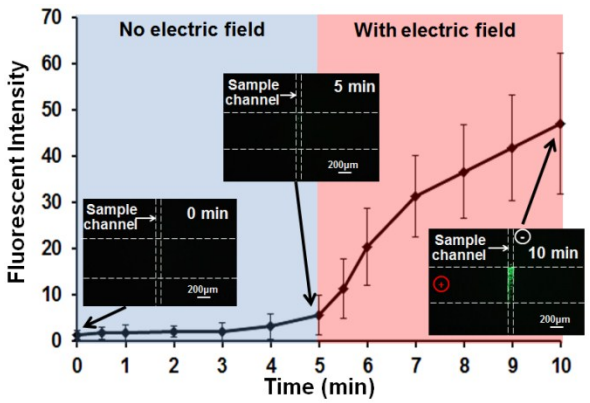

Fig. 2 Experiment demonstrating DNA accumulation. The curve shows the timely change of fluorescent intensity at the intersection with no voltage applied (blue) and with 9V applied (red). The fluorescent images at time points of 0, 5, and 10min were shown. Dash lines represent the outlines of the sample channel and the accumulation channel; error bars represent standard deviation (SD), n=3.

Fig. 2 shows the change of fluorescent intensity at the intersection obtained over the duration of the experiment. When no voltage was applied (blue area), no increase in fluorescence intensity can be observed ($p=0.2281$, $\alpha=0.01$ *between 0min and 5min*). But after applying9V (red area), a rapid increase of the fluorescent intensity can be observed from 5min to 7min, followed by a slower and more linear increase from 7min to 10min.

DNA at the intersection was immediately transported to the gel by EP force upon the application of the electric field, which led to a rapid increase during the first minute. After the initial accumulation, the region close to the gel is depleted of the DNA and rate of accumulation will depend on the rate of electrophoretic transport of DNA from the bulk solution leading to establishment of a steady flux. This experiment demonstrates that DNA could be effectively concentrated at the intersection in the presence of other biological contents (such as proteins) in 5 min by applying 9V on the device.

The electric field distribution at the intersection was simulated using commercial software (COMSOL Multiphysics v4.3) in order to understand the influence of various EK forces on the sample. The electrical boundary conditions set were as follows: The electrode in the sample channel was set at ground potential, and the other electrode in the accumulation channel was set as 9V. All the other channel surfaces were set to insulating boundary condition using zero charge node option in COMSOL. The material parameters, including relative permittivity (ε) and electrical conductivity (σ), were set as blood plasma ($\varepsilon=70$, $\sigma=0.01S/m$) for the sample channel [27], and water ($\varepsilon=78$, $\sigma=5.5\times10\text{-}6S/m$) for the accumulation channel [28]. Properties for silver (Ag) electrodes were chosen from the material library in the software. The electric field model was solved based on 2 assumptions: charge conservation and uniform temperature. The EP force and DEP force were calculated at the high field intersection region using the simulated electric field intensity and distribution. The simulation was also compared with the experimental results to better understand the concentration mechanism.

The simulation results are presented in the supplementary material. It shows that when a potential is applied (9V), the electric field established in the top sample channel is nearly 10 times that in the accumulation channel. Since the electrophoretic force transporting the DNA is proportional to the electric field, this geometry promotes transport of DNA in the sample channel towards the intersection region and a relatively weak transport of the DNA in the accumulation channel. Also, due to a much lower DNA mobility in the gel compared with that in the sample fluid, DNA can be well trapped and accumulated at the interface of the sample channel and the accumulation channel. The simulation also shows that the electric field gradient reaches its maximum at the intersection edge, and drops significantly in other areas. In the supplementary material, the directions of the EP force and the DEP force acting on the constituents in blood are demonstrated. It shows that the EP force drives DNA into the gel at the intersection, while the DEP force drags DNA away from the gel. The magnitude of the forces is determined by the profile of the electric field, which further affects the accumulation of DNA.

The experimental results obtained also show a similar accumulation profile as expected based on simulation (Fig. 3). The EP force reaches its maximum at the intersection edge, where the fluorescent intensity is the highest. It indicates the DNA is accumulated mostly at the intersection edge due to the stronger EP force.

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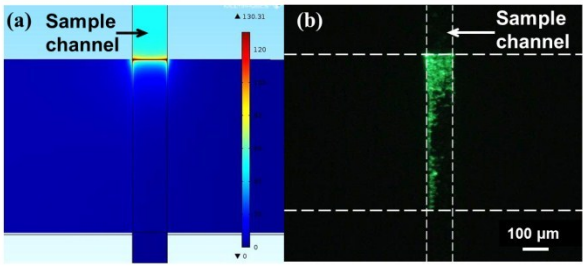

Fig. 3 Comparison of the simulation and the experimental results: (a) the simulated electric field distribution on the bottom plane of the sample channel. The colour band demonstrates the intensity of electric field, which reaches the maximum at the intersection edge; (b) the DNA accumulation result using the 1μg/ml DNA spiked plasma sample. The dash lines are the edge of the sample channel and the accumulation channel.

Effect of Applied Voltage on DNA Accumulation

The voltages applied between the sample channel and accumulation channel determines the electric field strength and its distribution in the device, which directly affects the motion of DNA molecules. As analysed above, the dominant force for DNA molecules at the intersection is EP force. The electric field strength within the channel can be increased by increasing the voltage, which leads to larger EP forces on DNA. Therefore DNA molecules can be accumulated faster at the intersection at higher voltages reducing the time required for DNA quantification.

In total 3 different voltages (3V, 9V, and 15V) were applied for 5 min respectively using a ~1.6μg/ml DNA spiked plasma sample. The sample was prepared using the same method as in the last section. The results (Fig. 4) show that a small increase in the fluorescent intensity can be observed between 3V and 9V ($p=0.0172$, $\alpha=0.01$) while the variation in the measurements decreased. Further increase in the potential to 15V caused the intensity to decrease ($p=0.7355$, $\alpha=0.01$ *between 3V and 15V;* $p=0.354$, $\alpha=0.01$ *between 9V and 15V*) along with a much larger variation in accumulation. Similar results were observed using 5μg/ml DNA spiked plasma sample (data not shown).

According to the results above, the fluorescent intensity is the highest when a potential of 9Vwas applied throughout the accumulation process. However, based on simulation and the force calculation, the EP force should have been the strongest at 15 V.

This unexpected result is likely due to the excessive heat generated in the channels when a relatively high voltage (15V) is applied for 5 minutes. According to the numerical simulation results, the electric field strength at the intersection reaches around 20kV/m when 9V was applied and over 30kV/m when using 15V. The high electric field strength formed is comparable to that normally used in capillary electrophoresis (CE), in which Joule heating can yield radial temperature gradients [29]. However, no heat management strategies were applied in our device as those used in CE, thus temperature variations and ineffective heat dissipation in the channels can cause the mobility changes of the contents in the sample liquid [30]. This could potentially be the cause for reduction in accumulation at 15V.

Based on the results in this experiment, an optimal potential of 9V was used in subsequent experiments.

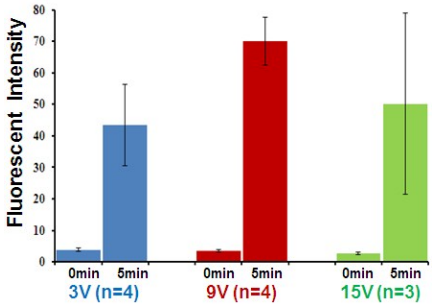

Fig. 4 Fluorescent intensity values at the beginning (0 min) and the end (5 min) of DNA accumulation with 3 different voltages applied. Error bars represent SD.

Quantification of DNA Accumulation

The rate of accumulation of DNA and the net amount accumulated over a certain period of time should be proportional to the initial concentration of the DNA present in the channel. To characterize it, and to demonstrate the feasibility of distinguishing between1μg/ml and 5μg/ml DNA, a series of experiments with different concentrations of DNA in buffer sample was performed. Here λ-DNA was diluted in 1x TE buffer and mixed with PicoGreen dye to obtain two sample solutions with DNA concentrations of 5μg/ml and 1μg/ml, which were used to mimic the cfDNA level in survivors and non-survivors of severe sepsis patients respectively. This sample contains no proteins, cells or other contaminants. The electric potential was applied for 4 minutes, and the results are shown in Fig. 5.

Fig. 5(a) clearly shows that the fluorescent intensity of both samples (1μg/ml and 5μg/ml) increased with time. The shape of the DNA accumulation in the gel followed the distribution of the electric field as seen in the simulation. There was higher accumulation close to the top edge of the intersection region and some of the DNA migrated towards the anode with increasing time. The measurement of fluorescent intensity with time (Fig. 5(b)) also showed a clear difference between 1μg/ml and 5μg/ml samples. It indicates that the accumulation of DNA in a sample with concentration of 1μg/ml was relatively linear and slow throughout the 4 minutes, while that of 5μg/ml sample increased rapidly within the first 30 seconds followed by a slower increase and a plateau after 3min.The plateau was formed due to the saturation of the fluorescent signals at the intersection. Fig.6(c) shows that during the accumulation process, the fluorescent intensity of 1μg/ml sample increased from average 12.4 to 82.0, while that of 5μg/ml sample increased from average 51.3 to 201.4. Before applying an electric field, no significant difference between 1μg/ml and 5μg/ml sample could be observed ($p=0.0527$, $\alpha=0.01$). But after 4 minutes DNA accumulation, the average fluorescent intensities were significantly different ($p=3.7\times10\text{-}4$, $\alpha=0.01$). The dash line indicated a threshold value of 130 could be set to distinguish the two concentrations.

Interference of Proteins on DNA Quantification

It has been shown that1μg/ml and 5μg/ml DNA in buffer samples can be rapidly distinguished using the device. But other constituents in blood plasma (mostly proteins) may affect the accumulation and quantification process. Therefore, DNA spiked plasma sample was prepared to simulate the blood plasma collected from ICU patients in hospital, and was used to investigate the interference of proteins on DNA quantification.

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This sample contains other biomolecules like proteins in the DNA accumulation process. The sample preparation method is similar as preparing the DNA in buffer sample, except that the DNA solution was added to human blood plasma instead of TE buffer.

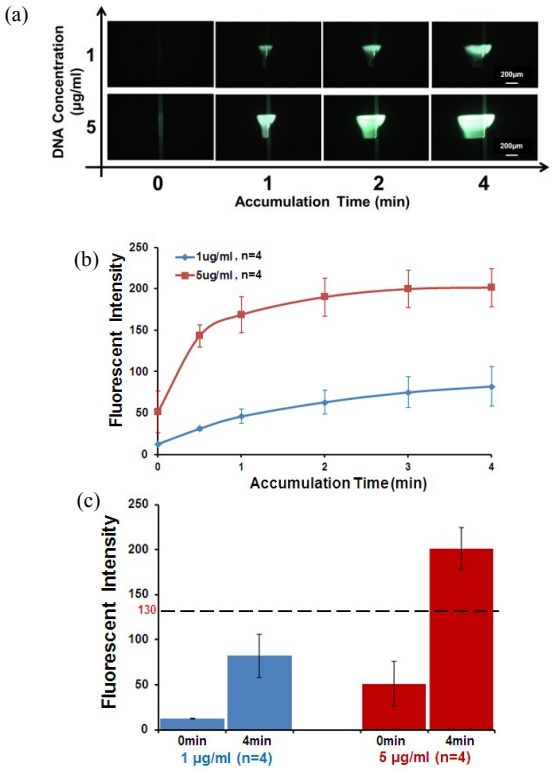

Fig. 5 Experimental results using DNA in buffer sample: error bars represent SD. 9V was applied; (a) fluorescent images taken at time intervals of 0, 1, 2, 3 and 4min demonstrating the DNA accumulation process at the intersection with 1μg/ml and 5μg/ml DNA concentration; (b) fluorescent intensity curves during DNA accumulation; (c) fluorescent intensity values at the beginning (0min) and the end (4min) of DNA accumulation.

As shown in Fig. 6, the fluorescent signals of 5μg/ml and 1μg/ml samples both increased at the intersection. After the DNA accumulation in 4 minutes, the fluorescence of 5μg/ml sample was much stronger than that of the 1μg/ml sample. The fluorescent intensity of 1μg/ml DNA showed a linear increase which was similar in trend as that of DNA in buffer sample but reduced in magnitude. The fluorescent signals of 5μg/ml sample increased rapidly within the first minute followed by a smaller slope in the rest accumulation period. This trend is comparable with the fluorescence curve of 5μg/ml DNA in buffer sample. The 1μg/ml sample reflects an increase in fluorescent intensity from average 1.7 to 11.5 during the 4 minutes electrokinetic accumulation, while the 5μg/ml sample demonstrates a faster increase from average 2.7 to 50.1. There was no significant difference ($p=0.0444$, $\alpha=0.01$) between 1μg/ml and 5μg/ml samples before applying the electric field (0min), but a highly significant difference ($p=4.5\times10^{-8}$, $\alpha=0.01$) could be seen after applying 9V for 4 minutes on the device. A threshold value of 30 could be set to distinguish the samples.

Comparing the data between DNA in buffer samples and the DNA spiked plasma samples, at the end of DNA accumulation, the fluorescent intensity values of DNA in buffer samples are 82.0±24.0 (1μg/ml) and 201.4±23.0 (5μg/ml), while values of the DNA spiked plasma samples are 11.5±7.2 (0.8μg/ml) and 50.1±10.4 (4.2μg/ml). The values are clearly different between the two groups of samples even though the experimental setups are identical.

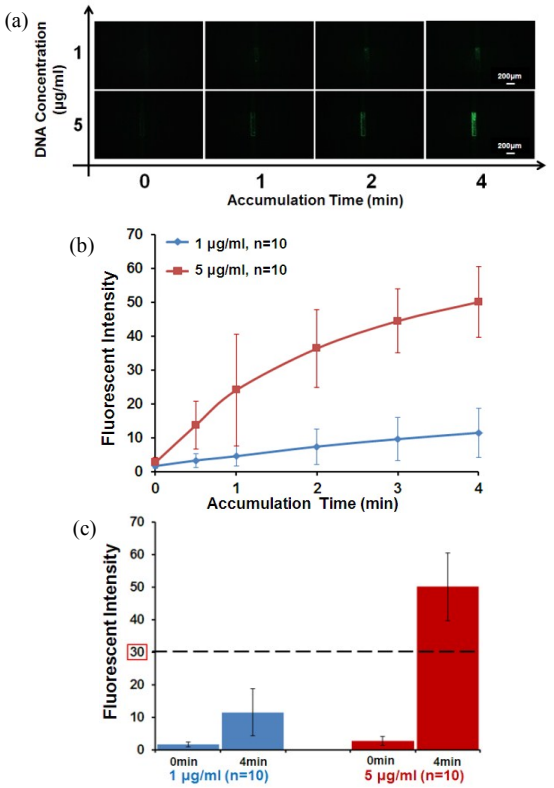

Fig. 6 Experimental results using DNA spiked plasma sample: error bars represent SD. 9V was applied; (a) fluorescent images taken at time intervals of 0, 1, 2, 3 and 4min demonstrating the DNA accumulation process at the intersection with 0.8μg/ml and 4.2μg/ml DNA concentration; (b) fluorescent intensity curves during DNA accumulation; (c) fluorescent intensity values at the beginning (0min) and the end (4min) of DNA accumulation.

Two main features are worth noting in the results. One is that the variation of the fluorescent intensity of the DNA spiked plasma samples is larger than that of the DNA in buffer samples: the relative uncertainties of the DNA spiked plasma samples are ~62.6% (1μg/ml) and ~20.8% (5μg/ml), while the relative uncertainties of DNA in buffer samples are ~29.3% (1μg/ml) and ~11.4% (5μg/ml). The other feature is that the average values of the fluorescent intensity of the DNA in buffer samples are significantly higher than that of the DNA spiked plasma samples.

There are 3 possible reasons for the results obtained: 1) DNA binds with plasma proteins. DNA in buffer can bind freely with PicoGreen molecules and can move freely between the solution and the gel under the influence of electric field. Thus the accumulation of DNA in the gel is repeatable and reflective of the concentration of the DNA in the sample. In the plasma sample, due to the presence of various plasma proteins, DNA-proteins complexes can be formed. For example, it has been shown that strong bindings can happen between human serum albumin (HSA) and DNA G-C bases and the backbone

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phosphate groups after mixing HSA and DNA solution [31]. These interactions between DNA and proteins can compete with DNA-PicoGreen binding, which potentially leads to a decreased fluorescence using the DNA spiked plasma sample. DNA-protein complexes may also have a different electrophoretic mobility, and could reduce the accumulation of DNA in the gel. 2) PicoGreen molecules interact with other constituents in plasma. PicoGreen molecules may have interactions with proteins or other contaminants as well. Therefore, less dye is available for PicoGreen-DNA binding. For example, a characterization study has shown that presence of proteins such as bovine serum albumin (BSA) can affect the intensity of the fluorescent signals of PicoGreen in a sample although the linearity of PicoGreen signal is not affected. It implies a similar change may probably occur in plasma samples. 3) The DNA-protein complex can be affected by an increased DEP force. The DNA-protein complex formed has a smaller charge-to-volume ratio than that of a single DNA molecule. Therefore, the DEP forces are more significant and could reduce the mobility of the DNA-protein complex towards the gel. Thus the net force driving DNA into the gel becomes smaller, which led to an impaired and less stable DNA accumulation process.

DNA Quantification using Clinical Blood Plasma

Clinical samples from severe septic patients were used to further validate the device performance and experimental setup. It was also used to investigate the interference of proteins in the samples. Here, plasma samples from 3 patients with different cfDNA levels (6μg/ml, 10μg/ml, and 20μg/ml) that have been quantified by conventional DNA concentration and UV absorbance measurement were selected. Plasma from 3 healthy donors were also used but not pre-measured, thus the original cfDNA concentrations remained unknown. The clinical plasma samples were processed by mixing with the 10-fold diluted PicoGreen reagent with a 10:1 volume ratio (plasma: PicoGreen). The samples were injected into the sample channel and 9V was applied for 5 minutes.

The results (Fig. 7) shows that after 5 minutes DNA accumulation with 9V applied across the channels, the average fluorescent intensity of the healthy samples did not increase. In the case of patient samples the intensity increased for all the concentrations (6μg/ml: from 6.5 to 13.4; 10μg/ml: from 10.6 to 25.1; and 20μg/ml: from 33.4 to 169.3). There was no significant difference between the 6μg/ml patient sample and the samples from healthy donors before applying the electric field ($p=0.0126$, $\alpha=0.01$). But at the end of DNA accumulation, a highly significant difference could be detected between them ($p=0.004$, $\alpha=0.01$). This result demonstrates that a more reliable distinction between the two samples can be obtained after applying the electric field. The patient samples with relatively high cfDNA concentrations (10μg/ml and 20μg/ml) were significantly different from the healthy donor samples, even before applying electric field ($p=7.9\times10^{-7}$ between healthy sample and 10μg/ml sample; $p=0.0019$ between healthy sample and 20μg/ml sample, α=0.01). After applying the electric field for 5 minutes, the distinctions remained highly significant ($p=5.0\times10^{-5}$ between healthy sample and 10μg/ml sample; $p=3.6\times10^{-4}$ between healthy sample and 20μg/ml sample, α=0.01). However, the fluorescent intensities between the 6μg/ml and 10μg/ml patient sample were not statistically different both in the beginning ($p=0.0110$, $\alpha=0.01$) and at the end ($p=0.0134$, $\alpha=0.01$) of DNA accumulation. These results have shown that healthy donor samples and the severe sepsis patient samples could be differentiated using our method.

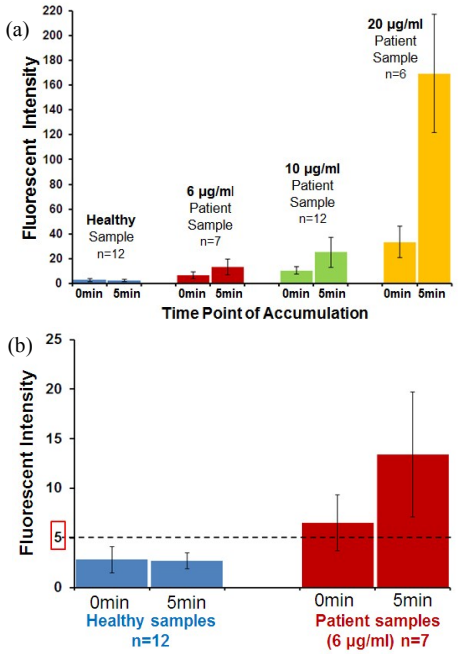

Fig. 7 Experimental results using the clinical plasma sample: error bars represent SD. 9V was applied; (a) fluorescent intensity at the beginning (0 min) and the end (5 min) of DNA accumulation; (b) fluorescent intensity of the healthy sample and 6μg/ml patient sample.

The fluorescent intensity of the 6μg/ml clinical plasma sample is much lower (13.4±6.3), compared with the 5μg/ml DNA in buffer sample (201.4±23.0) and the 5μg/ml DNA spiked plasma sample (50.1±10.4). The relative uncertainty of the 6μg/ml clinical plasma (≈47.0%) is larger as well. This further shows that the interference of proteins and other interferences on the cfDNA accumulation and quantification. There are 2 other factors that may contribute to this result besides the reasons mentioned in the section of DNA spiked plasma sample. The first one is the individual variations of the clinical plasma samples. The clinical plasma samples used in this experiment were collected from different patients and healthy donors. Therefore, the samples may not be homogeneous and will have individual variations. The amount of protein and other constituents in plasma could possibly change with patients which could indirectly affect the DNA accumulation process. The second possibility is the interference of histone in the patient plasma samples. Increased histone levels have been found in samples from severe septic patients, due to possible apoptotic or necrotic cells. Histone is able to keep cfDNA tightly coiled, thus the binding between DNA and PicoGreen molecules can be significantly affected. Meanwhile, there are other non-histone proteins involved in sepsis process, which can potentially bind with DNA molecules in plasma from septic patients. These mechanisms can lead to less DNA-PicoGreen binding, which further lead to a decreased fluorescent intensity.

DNA Quantification using DNA spiked Whole Blood

The ultimate goal of this device is to complete cfDNA quantification directly in whole blood sample, which can further simplify the sample preparation procedure and realize a true point-of-care testing. Before moving to the clinical whole

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blood sample, preliminary experiments were conducted using DNA spiked whole blood sample, which contains all the constituents in blood including blood cells and proteins. Here λ-DNA was mixed with whole blood from a healthy donor, followed by mixing it with 10-fold diluted PicoGreen reagent with a 1:1 volume ratio to obtain a final concentration of 5μg/ml and 1μg/ml DNA spiked in whole blood. A control sample was prepared by mixing 10-fold diluted PicoGreen and whole blood with no extraneous DNA added.

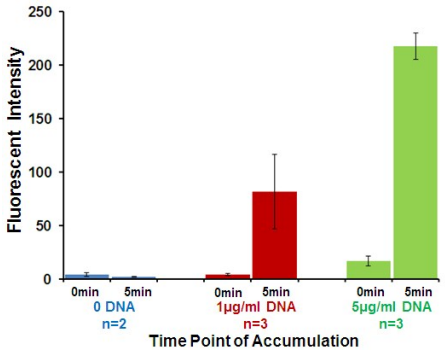

Fig. 8 Fluorescent intensity at the beginning (0min) and the end (5min) of DNA accumulation using DNA spiked whole blood sample: error bars represent SD. 9V was applied. The concentration of 0μg/ml represents whole blood with no extraneous DNA added.

Accumulation of DNA from these samples in the device at a potential of 9V for 5 minutes showed that the 3 concentrations can be clearly separated after electrophoretic concentration (Fig. 8). The fluorescent intensity difference between the 0μg/ml sample and the 1μg/ml sample increases substantially after DNA accumulation ($p=0.058$, $\alpha=0.01$) compared with before applying the electric field ($p=0.9711$, $\alpha=0.01$); the difference between the 5μg/ml sample and the 0μg/ml sample is not significant before DNA accumulation ($p=0.0278$, $\alpha=0.01$). However, the difference becomes significant ($p=0.0010$, $\alpha=0.01$) after accumulation.

It can be seen that the p values are relatively large due to the small number of experiments conducted (n), especially for the 0μg/ml sample ($n=2$). However, the data provides a demonstration that the whole blood of healthy donors, which has a similar cfDNA concentration as survivors, can be directly distinguished from the whole blood of non-survivors in severe sepsis patients. It should be noted that the electric field in the sample channel at the applied potential of 9V is smaller than that required for haemolysis.

Immobilization of PicoGreen Reagents

Although the sample preparation process has been significantly simplified by using the device, current experiments still require pre-mixing of the fluorescent dye reagents and the sample liquid, which can complicate the point of care use of this device. To realize this goal, PicoGreen dye must be integrated with the device in advance.

PicoGreen reagent (50-fold diluted) from the kit was injected into the channels before gel filling and sample loading. The device was then wrapped with aluminium foil with a small opening to let the liquid evaporate. Upon leaving the device in a dark and ventilated environment for overnight, the solution in the channels was fully evaporated, leaving a residue of dried PicoGreen on the channel walls. These molecules could later be re-suspended upon loading of sample liquids, and intercalate with cfDNA molecules in the samples. After the gel filling, 5μg/ml DNA spiked plasma sample was loaded into the sample channel without pre-mixing with PicoGreen, followed by applying 9V for 5min. A control test was conducted by using a device that had no PicoGreen deposited, and the results were compared.

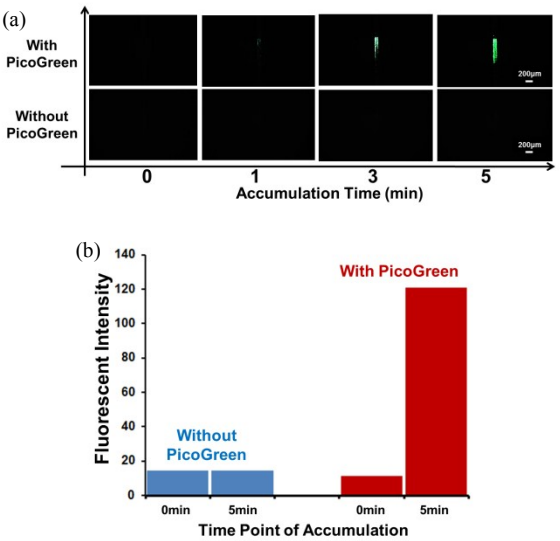

Fig. 9 (a) Fluorescent images taken at time intervals of 0, 1, 3, and 5min with PicoGreen integrated device (With PicoGreen) and regular device (Without PicoGreen); 9V was applied. The sample was 5μg/ml DNA spiked plasma with no PicoGreen pre-mixed; (b) fluorescent intensity at the beginning (0 min) and the end (5 min) of DNA accumulation.

Based on Fig. 9, it was clear that with PicoGreen molecules pre-deposited in the channels, fluorescence could be detected and the accumulation of cfDNA was observed. In comparison, the device without PicoGreen shows no observable fluorescence during the whole process since there was no binding of DNA and the dye.

This experiment demonstrated the possibility of dye immobilization in the device, which could potentially simplify the sample preparation steps. The re-suspension of PicoGreen and its effect on device performance has to be carefully studied. The concentration of PicoGreen used, the distribution of the dried dye inside the microchannels and the process of drying must be optimized to ensure that sufficient sensitivity is obtained while still minimizing the cost of the reagents.

Conclusions

This paper presents a novel device enabling a simplified rapid DNA quantification method directly in blood plasma, which is potential to be used in whole blood samples as well. The device is low-cost (PDMS based), simple structured, has low power consumption (9V), requires a small sample volume (<10μl), and can complete the quantification in 5min. These characteristics make the device a good candidate for Point-of-care prognosis of sepsis condition. The experimental results showed that clinical plasma from healthy donors (contains similar cfDNA levels as survivors of severe sepsis patients) are able to be distinguished from that of non-survivors of severe sepsis patients. Further studies are required to use this device for accurately quantifying samples with various cfDNA

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concentrations. The DNA accumulation process can be affected by proteins and cells in blood, but the repeatability of experiments was found to be suitable.
Future work will focus on the characterization using clinical blood samples from patients. The device will be modified to meet a better quantifying sensitivity and resolution.

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Notes and references

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