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DEVELOPMENT OF A DIFFUSION LIMITED MICROFLUIDIC MODULE FOR DNA PURIFICATION VIA PHENOL EXTRACTION

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ABSTRACT

Purification of Deoxyribonucleic acid (DNA) by organic-aqueous liquid extraction, also called phenol extraction, is a standard technique commonly utilized in biology laboratories. In order to minimize interaction energies, membrane components and proteins naturally partition to the organic (phenol) phase while the DNA stays in the aqueous phase, where it can be easily removed. In recent years, microfluidics has become a driving force toward more efficient and autonomous platforms for fluid based diagnostics, chemical reaction chambers, separation and preparation of biological materials.

In this work, fabrication, and performance of a long microfluidic device for DNA extraction are presented. The devices were fabricated using soft lithography to transfer lithographically defined features into a PDMS structure via replica molding. Stratified-flow experiments using a rhodamine dye conjugated bovine serum albumin protein (BSA) in an aqueous phase were conducted to demonstrate the ability to remove proteins from the aqueous phase into the phenol phase. Additionally, the study of BSA partitioning and DNA isolation in a two-phase system under stratified flow condition were presented, separately and conjunctly. Finally, protein partitioning and DNA recovery obtained with this device could be compared with other types of mixing and extraction such as mixing by droplet formation and electrohydrodynamic (EHD) instability.

INTRODUCTION

Phenol extraction is an organic-aqueous liquid extraction technique that separates DNA and RNA from cellular components and proteins based on their affinity to the different phases [1]. Briefly, the procedure consists of digestion of cells or tissue in a basic lysis buffer and the addition of equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v) mixture to the aqueous solution. The two phases are vortexed vigorously for 5 minutes to mix them. During this process, the cell components distribute into either the aqueous phase or the organic phase in order to minimize interaction energies. The cell membrane lipid components and proteins partition to the organic phase and DNA stays in the aqueous phase due to its polar nature having greater affinity to the aqueous phase. The separation then occurs at the organic-aqueous interface. Following partitioning, centrifugation is used to separate the two phases by density differences and the aqueous phase is removed with a micropipette tool. DNA is concentrated by precipitation in ethanol and resuspended in an aqueous buffer.

Microfluidic approaches have been the cutting-edge for automation and optimization of traditional analytical processes and point-of-care medical systems. Integrating cell lysis, purification, amplification and analysis of nucleic acids into a micrototal analytical system (μTAS) represents the driving goal of many research groups. Advances in microfluidics as well as in material sciences have made possible to rethink standard DNA purification procedures, using the attractive characteristics of microfluidic chips to provide simplicity, automation, and reduction in time, reagents and user handling.

To date, the research towards integrating multiple DNA handling processes in a simple microchip has been mainly focused to scale down the solid phase extraction (SPE) technique rather than the standard phenol extraction [2-11]. SPE is based on the absorption of DNA onto a silica solid phase under high salt concentration condition to separate DNA from the cell components. SPE microfluidic devices using silicon pillars [7, 8], silica beads [3, 9, 10] and porous silicon [11] have been presented and integrated with cell lysis, polymerase chain reaction (PCR) and capillary electrophoresis (CE) processes [4, 5, 9]. However, the first steps towards miniaturizing the phenol

extraction technique have been taken examining the organic-aqueous interface stabilization [12], the experiment and numerical simulation of droplet-based flows [13] and the mixing between phases using electrohydrodynamic (EHD) instabilities [14].

In two-phase systems, the flow profile is affected by the interfacial tension and the shear force. The viscous forces act tangentially to the interface elongating it and the interfacial forces act normally to the interface minimizing the interfacial area by inducing droplet formation [12]. The competition between these forces is described by the capillary number (Ca):

$$Ca = \frac{\mu U}{\sigma}$$

where μ is the dynamic viscosity, U is the mean flow velocity and σ is the interfacial tension. If the viscous forces are more significant than the interfacial forces, the multiphase flow behavior is parallel or stratified (Ca>>1). Segmented or droplet-based flow occurs when interfacial forces are predominant (Ca<<1). Other regimes appear in the transition between stratified flow and droplet-based flow such as pearl-necklace and pears regimes [15, 16].

Multiphase flow pattern are influenced by several factors. The fluid dynamics depends on interfacial forces between the fluids and channel walls [12, 17-19], individual fluid viscosities [15, 20], fluid velocities [15, 20-22], and the geometric characteristics of the channel [21].

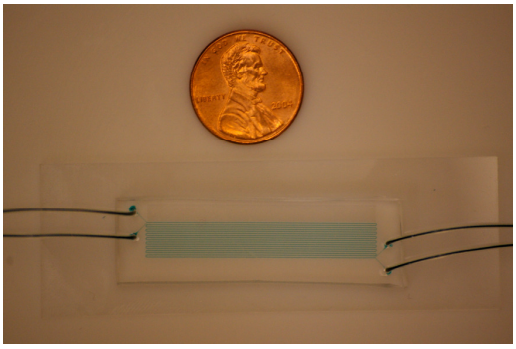
This work presents the development of a dual-inlet serpentine microchannel used as platform for DNA extraction by organic solvents. The main aims were to analyze protein partitioning between the aqueous and the organic phase and DNA purification within the device by passive diffusion through stratified flows.

EXPERIMENTAL

Device Fabrication:

The serpentine device consisted of a long microchannel where the two phases were co-infused into a converging channel geometry with channel width, height and length of 80 μm, 20 μm and 57 cm, respectively (Fig. 1.a). The microchannel was fabricated using soft lithography to transfer lithographically defined features into a polydimethylsiloxane (PDMS) structure via replica molding [23]. The master mold was lithographically patterned on a spin coated film of negative photoresist SU-8 (MicroChem, Newton, MA). The PDMS structure was made by mixing elastomer base and elastomer curing agent (Sylgard 184 Silicone Elastomer Kit, Dow Corning, Midland, MI) at a ratio of 10:1 and poured on top of the master mold. After getting rid of air bubbles by using a vacuum chamber, the PDMS cast was cured in an oven at 65 ˚C for 2 hours. The PDMS structure was peeled off the master mold containing the microchannel pattern and punched off at the reservoirs to allow the entrance and exit of the fluids. The PDMS structure was then irreversibly bonded to a glass slide by using a corona discharge generator. The device was connected with Tygon tubes (Small Parts Inc., Miami Lakes, FL) and sealed with glue (J-B KWIK, Sulphur Springs, TX), specially selected due to their compatibility with the organic phase. In Fig. 1.b, a photograph of the microfluidic device is shown.

a)



b)

Fig. 1. a) Geometry of the device. The aqueous phase and the organic phase were introduced into the main channel separately by the two inlets and collected at the end from two outlets. b) Photograph of the device.

Experimental procedure:

The infusion of the aqueous and organic phases, contained in glass syringes (Hamilton, Reno, NV), was made by using syringe pumps (Harvard apparatus, Holliston, MA) to control the flow rates. The aqueous phase consisted of a buffer solution, phosphate buffered saline (PBS) with the addition of sodium dodecyl sulfate (SDS), from J. T. Baker Chemical Co., Phillipsburg, N J, at a concentration of 0.5% (w/v), to reduce the surface tension and achieve interfacial stabilization with the organic phase [12].

The analytes introduced in the aqueous phase were bovine serum albumin protein (BSA) (Omnipur, fraction V; EMD Biosciences) conjugated with rhodamine fluorescent dye and 2-log DNA ladder (0.1 to 10.0 kb; New England Biolabs, Beverly, MA) labeled with YOYO-1 fluorescent dye (Molecular Probes, Invitrogen).

5-6-carboxy-tetramethylrhodamine (TAMRA) succinimidyl ester dye (Fluka, Switzerland) was dissolved in Dimethyl Sulfoxide (DMSO) (Amresco, Solon, OH), added to the BSA solution and mixed using a rotary shaker for 30 minutes. Then, the labeled solution was filtered through a PD-10 desalting column (Sephadex G-25M; GE Healthcare) to remove the free dye from the solution. The output flow collected with the

labeled proteins was stored at -20˚C and diluted with PBS before use. The protein concentration was 0.3 µg/µl, which had enough fluorescence signal to be captured by the camera while preventing precipitation of proteins or clogging in the microchannel due to high concentrations.

YOYO-1 was intercalated within the DNA backbone at a ratio of one molecule of dye per five base pairs of DNA (1:5), similarly as described in the literature [24]. The average molecular weigh of the base pair used was 650 grams/bp-mole. The DNA was diluted in TE buffer (10 mM tris-HCl, 1 mM EDTA, from Rockland Inc., Gilbertsville, PA). The dye solution was added conjunctly with B-mercaptoethanol, an oxygen scavenger that protect DNA-dye complex from oxygen radicals, at a final concentration of 4% (v/v). The incubation of DNA and the dye occurred at room temperature in the dark and for a minimum of two hours. The final DNA concentration was 0.075 µg/µl.

The organic phase was made of a mixture of three components: phenol, the main solvent, chloroform, and isoamyl alcohol, at 50, 48 and 2% volume concentration, respectively. The mixture was purchased from Pierce (Rockford, IL) and used as received. The organic phase viscosity was 3.52 x 10^{-3} Pa-s and the interfacial tension with the aqueous phase was 0.1 mN/m.

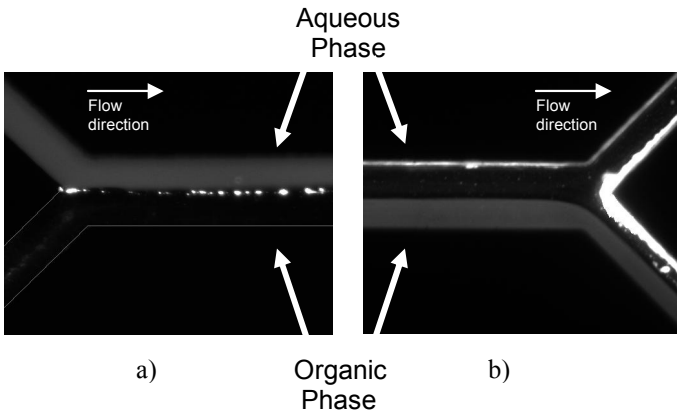


Fig. 2. Images of the two phases at the entrance (a) and at the outlet (b) of the device. Rhodamine fluorescent dye labeled BSA is infused at the entrance and removed into the phenol phase at the outlet

RESULTS AND DISCUSSION

A flow rate of 2 µl/min for the aqueous phase and 0.6 µl/min for the phenol phase were utilized to produce a stable stratified flow profile. At the inlet, the labeled BSA was introduced in the aqueous and diffused into the organic phase through the device and collected at the outlet (Fig. 2). Using a charge-coupled device camera (PowerView 1.4 MP, TSI), images at different positions in the serpentine device were taken to track the protein partitioning from one phase to the other. The image processing was prepared using ImageJ software (NIH Image). The images were then analyzed, obtaining the average intensity of the aqueous phase at different downstream position and normalized with the average intensity at the inlet. The fluorescence profiles demonstrate the ability of the module to extract proteins from the aqueous phase (Fig. 3).

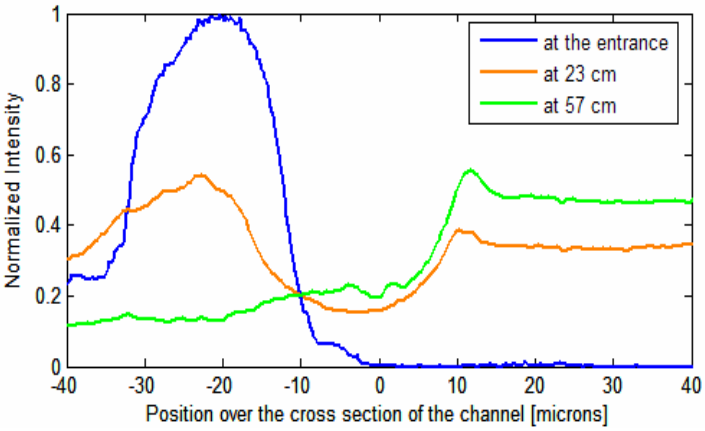


Fig 3. Cross section fluorescence profiles at different downstream positions using Rhodamine fluorescent dye conjugated BSA in the aqueous phase. The aqueous phase is located at the negative values and phenol phase at the positive values. These intensity profiles show the protein extraction across the width of the channel. The variations in maximum intensity at the aqueous and the organic phases are due to the difference in absorbance properties of these two liquids.

During these experiments with rhodamine fluorescent dye labeled BSA, the aqueous phase outputs were collected and analyzed by spectrophotometry to evaluate the total partitioning of rhodamine conjugated BSA from the aqueous phase to the organic phase at the outlet. According to the image analysis and the spectra, the protein concentration in the aqueous phase through the device was depleted by approximately 75% of the initial protein concentration.

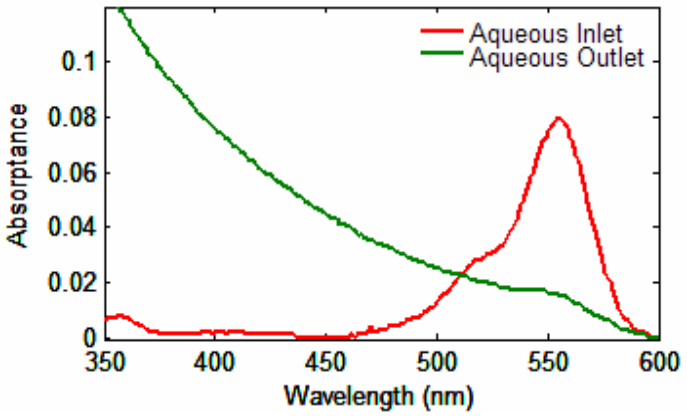


Fig 4. Absorption spectra of the aqueous phase inlet and aqueous phase outlet. Only rhodamine conjugated BSA is present in the aqueous inlet.

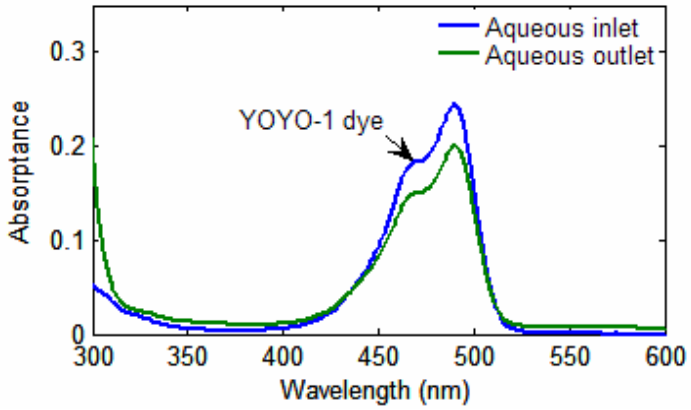
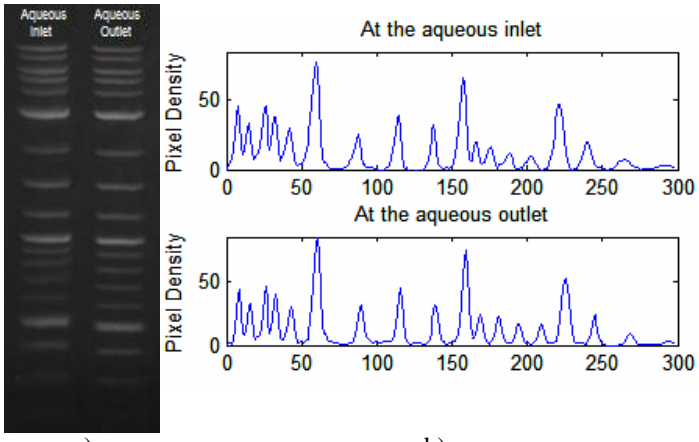


Fig 5. Absorption spectra of aqueous phase inlet and outlet using YOYO-1 labeled DNA ladder in aqueous inlet.

A second experiment was conducted using only DNA in aqueous phase to analyze its interaction in the two-phase system. The results obtained by spectrophotometry show a high recovery of DNA at the aqueous outlet, as was expected due to its low affinity with organic solvents (Fig. 5). To analyze the concentration of DNA at the inlet and output samples, gel electrophoresis at 1% agarose concentration was performed. From the image shown in Fig. 6a, the inlet and outlet samples seem to have approximately the same DNA concentrations. ImageJ was used to plot the pixel density in each lane (Fig. 6b).



a) b)

Fig. 6. Incorporation of only DNA in the aqueous phase: a) Agarose gel electrophoresis analysis of inlet and outlet samples, b) Pixel intensity analysis of the gel lanes.

The next step was to mix BSA and DNA together in the aqueous phase. Absorption spectra analysis and gel electrophoresis analysis were conducted as in the previous experiment (Fig 7 and Fig. 8, respectively).

When both DNA and BSA are present, the absorption spectra (Fig. 8) show partitioning of BSA to the organic phase, similar to the experiments without DNA (Fig. 5). However, in Fig. 7, high absorption at the YOYO-1 dye wavelength is present in the organic outlet instead of the aqueous phase where DNA is expected to be, based in the experiment without BSA (Fig. 5). Nevertheless, gel electrophoresis results in Fig. 8 determined that DNA is present in the aqueous outlet, and not in the organic outlet, leading to the hypothesis that YOYO-1 dye intercalation could be affected by the presence of organic solvents and could be partitioning to the organic phase. Future works will involve the study of different DNA tags to monitor DNA presence within the microdevice as well as the extraction efficiency.

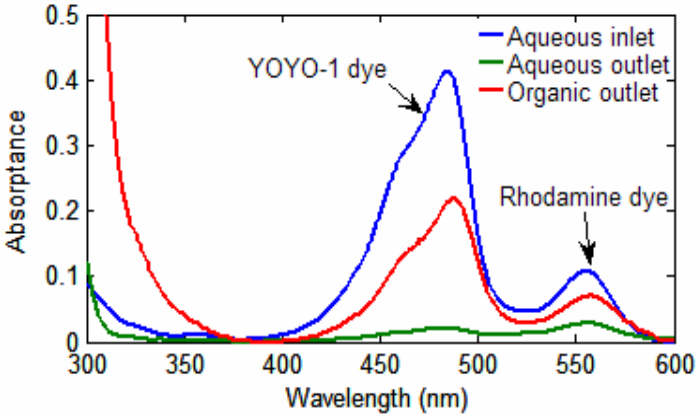
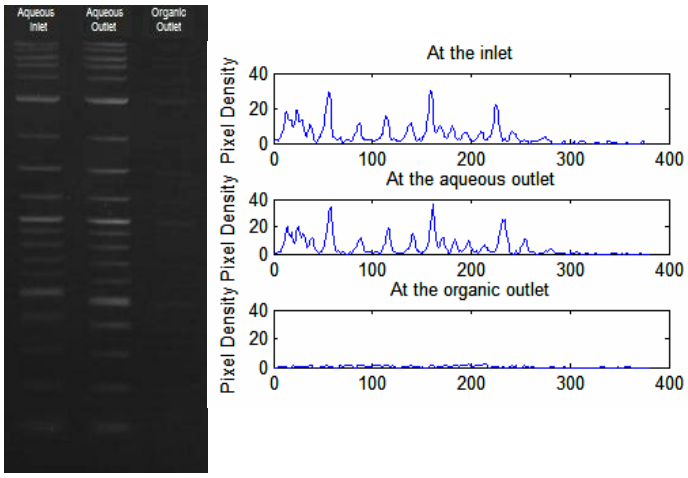


Fig. 7. Absorption spectra of the aqueous phase inlet and outlet. Both rhodamine conjugated BSA and YOYO-1 labeled DNA are present in the aqueous phase inlet.



a) b)

Fig. 8. Incorporation of DNA and BSA in the aqueous phase: a) Agarose gel electrophoresis analysis of inlet and outlet samples, b) Pixel intensity analysis of the gel lanes.

Gel electrophoresis analysis results show that, in proportion, the aqueous outlet intensity is larger that the aqueous inlet intensity with outlet-to-inlet ratio of 1.07 (Fig. 8b). The higher DNA concentration at the aqueous outlet could be either due to evaporation during the collection of the sample, uneven loading of the gel, or to a concentrating effect due to the presence of the organic phase. Nevertheless, only a very small amount of DNA was found in the organic outlet (9%); consequently, the purification efficiency using stratified flows was approximately 90%.

The diffusion-limited microfluidic device shown in this work has demonstrated its ability to separate proteins from an aqueous solution and isolate DNA. Further improvements to this study could involve convective enhancements such as by using droplet formation and EHD instability. Those enhancements could also reduce the extraction time and the channel length.

CONCLUSIONS

In this study, partitioning of proteins and DNA isolation were evaluated in a dual-inlet serpentine microfluidic device under stratified flow conditions. Protein partitioning and DNA extraction were analyzed, separately and conjunctly. The two-phase microfluidic device has demonstrated the extraction of proteins from the aqueous phase into the organic phase. The protein partitioning efficiency was approximately 75% with minimal loss of DNA, determined by gel band intensity. According to the spectrophotometry results, YOYO-1 dye seems to partition to the phenol phase which will require a further study of other DNA dyes. Droplet formation and EHD instability could be used to enhance the convective transport of proteins to the interface.

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