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Technical Note

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Simultaneous Purification and Fractionation of Nucleic Acids and Proteins from Complex Samples Using Bidirectional Isotachophoresis

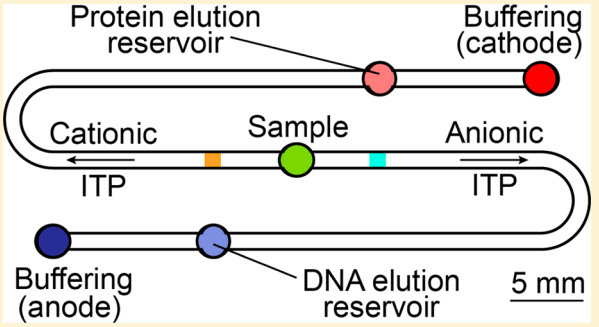
Yatian Qu,[†] Lewis A. Marshall,[‡] and Juan G. Santiago*[,†]

[†]Department of Mechanical Engineering, Stanford University, Stanford, California 94305, United States

[‡]Department of Chemical Engineering, Stanford University, Stanford, California 94305, United States

Supporting Information

ABSTRACT: We report on our efforts to create an on-chip system to simultaneously purify and fractionate nucleic acids and proteins from complex samples using isotachophoresis (ITP). We have developed this technique to simultaneously extract extracellular DNA and proteins from human blood serum samples and deliver these to two separate output reservoirs on a chip. The purified DNA is compatible with quantitative polymerase chain reaction (qPCR), and proteins can be extracted so as to exclude albumin, the most abundant protein in serum. We describe significant remaining challenges in making this bidirectional method a robust and efficient technique. These challenges include managing channel surface adsorption of proteins, identifying the cause of observed reductions in low molecular weight proteins, and dealing with nonspecific binding of proteins and DNA.



Accessing correlated information between nucleic acids and proteins is important for investigating a complex biological system. Consider the classic view of the central dogma of molecular biology,[1] which is that coded genetic information from DNA is transcribed into mRNA (mRNA), and then proteins can be synthesized using the information in mRNA as a template. This basic construct suggests a full understanding of biological processes including aging, gene regulation, and phenotypic expression of mutant genes requires correlated genomic and proteomic studies. These studies should therefore benefit from simultaneous purification and isolation of nucleic acids and proteins from the same sample, particularly when the sample is precious and limited in volume.

Current simultaneous extraction methods include phase separation and precipitation using guanidinium salts with organic solvents[2−6] and using column-based extraction kits.[7,8] One example is the longevity study of Riol et al., who performed simultaneous extraction of nucleic acids and proteins from human lymphocytes cells of individuals in the 92−101 year age range. They needed a simultaneous isolation method because the longevity study requires investigation at all levels, DNA, RNA, and protein, and they were limited by the samples they could obtain from individuals at advanced ages. They used chloroform to separate sample into three phases, then precipitated nucleic acids from aqueous solution by using isopropanol and dialyzed soluble proteins from an organic phase.[6] As an alternative to phase separation and precipitation methods, both Tolosa et al.[8] and Morse et. al[7] presented a method using commercially available column-based nucleic acids purification kits and followed by protein precipitation and centrifugation of flow-through eluent liquid to simultaneously extract nucleic acids and proteins from human tissues. All these reported methods require long processing time, are very labor intensive, and require the use of hazardous chemicals. They also all require sample volumes of hundreds microliters to several milliliters. Clearly, further improvement on recovery time, sample consumption, and automation is desired for simultaneous extraction methods.

Here, we present work toward a novel integrated sample preparation technique to simultaneously extract nucleic acids and proteins from complex biological samples using isotachophoresis (ITP). ITP is an electrophoretic technique that both separates and preconcentrates ions based on their electrophoretic mobility.[9] ITP is a robust sample preparation method and has been recently extensively applied to extraction and purification of both DNA[10−12] and RNA targets from a variety of samples including blood, urine, and cell culture.[12] ITP sample preparation of nucleic acids has also been shown to be compatible with downstream assays including qPCR and hybridization reactions.[12−15] We initially presented some of the current results in Qu et al.[16] We here include additional experimental observations and discussions around remaining challenges.

To our knowledge, the concept of simultaneous purification of nucleic acids and proteins using ITP was first discussed by

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Young et al. in a patent application in 2010.[17,18] The Young et al. application included a few sentences around the idea of simultaneous cationic and anionic ITP to extract and separate nucleic acids and proteins. However, they show no experimental or theoretical data showing counter-migrating ITP processes into channels leading from a single reservoir as we do here. We note also the Young et al. patent mostly emphasizes the coelution of nucleic acids and proteins into a so-called "single volume". Here, we demonstrated the first application of ITP in a bidirectional process to simultaneously extract and isolate DNA and proteins into two respective output volumes. We demonstrate this in the extraction and fractionation of gDNA and plasma proteins from human blood serum. We show that the DNA is sufficiently purified to be immediately compatible with polymerase chain reaction (PCR). Further, the protein extraction can be configured to exclude albumin, a highly abundant species with little analytical value. We implement this in a bidirecitonal ITP chip with a single input reservoir that accommodates an 8 μL sample, two output reservoirs, and a 25 min run time. We conclude by describing remaining challenges in creating a robust simultaneous extraction system and recommendations for future work.

MATERIALS AND METHODS

Chip Design and Preparation. We designed and fabricated the novel microfluidic device shown in Figure 1a to demonstrate the technique. The device includes two "C" shaped channels leading from a single sample input reservoir at the center. We fabricated separation devices from polydimethylsiloxane (PDMS) and glass slides by using soft lithography.[19] The mold of the device was fabricated by the Stanford Microfluidics Foundry (Stanford, CA). The channels are 1 mm wide and 100 μm deep. Each of two branches leading from the center is about 40 mm long, with a volume of 3.8 μL for each branch. A cationic ITP channel branch collects positively charged protein species. An anionic ITP channel branch collects nucleic acids, which are strongly negatively charged. Each branch has its own elution reservoir, where the respective biomolecules can be pipetted off the chip. Each elution reservoir is 2.5 mm in diameter and 1.8 mm in depth, with a volume of approximately 8 μL. Connected downstream and in series with each elution reservoir are separate buffering reservoirs. These each contains high concentration buffers to provide additional pH-buffering capacity, without requiring high ionic strength in the extracted sample mixture. Figure 1b summarizes our extraction process with human blood serum samples.

Prior to first use of each microfluidic system, we rinsed the channel with the following successively: methanol (5 min), deionized water (DI) (2 min), 1 M HCl (5 min), DI (2 min), 1 M NaOH (10 min), and DI (2 min) to initially condition the channel surface. Between experiments, we rinsed the channel with 10% household bleach to remove residual nucleic acid contaminations, followed by washes of DI (2 min), NaOH (2 min), DI (2 min), HCl (2 min), and DI (2 min) to remove adsorbed proteins. Before loading sample and buffers, we rinsed the channel with UltraTrol "Low Norm" dynamic precoatings (Target Discovery, Inc., Palo Alto, CA) for 2 min, followed by air drying for 2 min to suppress electroosmotic flow and protein adsorption onto PDMS channel walls.

Human Blood Serum Samples. We prepared serum samples from whole blood samples collected from healthy donors in nonanticoagulated tubes at the Stanford Blood Center (Stanford, CA). We let fresh human blood samples clot under room temperature for 30−60 min and then removed the clot by centrifuging at 1500*g* for 15 min. We collected the serum, which was the resulting supernatant, apportioned it into 200 μL aliquots, and stored at −80 °C.

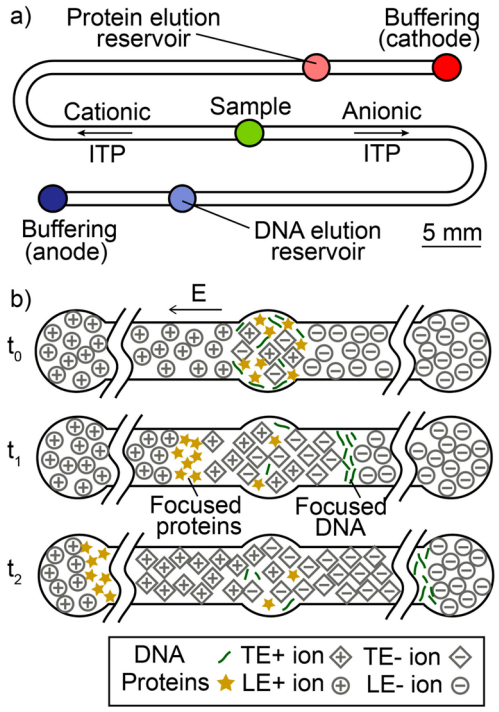

Figure 1. (a) Device design for simultaneous purification of nucleic acids and proteins from serum using simultaneous cationic and anionic ITP processes. The channels were 1 mm wide and 100 μm deep, with a volume of 3.8 μL for each branch. Each reservoir held approximately 8 μL of liquid. (b) Simultaneous extraction process in schematic of system (buffering reservoirs not shown). Serum sample was mixed with sample buffer and pipetted directly into the sample reservoir. An electric field was applied, and the DNA and proteins were extracted into each separation channel and focused at anionic and cationic ITP interfaces simultaneously, respectively. Purified DNA and proteins eventually reached each elution reservoir and were collected for off-chip PCR and SDS-PAGE.

Buffer Preparation. We prepared two leading electrolyte/trailing electrolyte (LE/TE) extraction buffers prior to each experiment. The LE+/TE− buffer contained 0.01% Tween 20 in 40 mM MES and 20 mM sodium hydroxide at pH 6.0. The LE−/TE+ buffer contained 0.01% Tween 20 and 500 nM SYTO 64 in 36 mM 6-aminocaproic acid and 18 mM hydrochloric acid at pH 4.4. We added Tween 20 to both buffers to facilitate protein solubilization. The buffer chemistry was optimized to minimize the difference of elution times of DNA and proteins by performing numerical simulation using Stanford Public Release Electrophoretic Separation Solver (SPRESSO).[20] To prepare the sample, we added 5 μL of serum into 45 μL of sample buffer, which contained 0.01% Tween 20, 500 nM SYTO 64, 2500 pg/μL sonicated salmon sperm DNA (300−2000 bp), and 1.9 μM Yellow Fluorescent Protein (YFP) in 10 mM MES and 10 mM 6-aminocaproic acid at pH 5.2. The SPRESSO tool was used to design for a pH of 5.0 for the adjusted TE zone in the protein channel. At this low pH value in this zone, proteins with p*I*s of roughly 5.0 and below will have a mobility value lower than the TE cation.

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Therefore, these proteins are excluded from ITP focusing zone. This includes albumin with a p*I* of 4.7−5.0.[21−23]

To provide additional buffering capacity, we prepared two higher concentration LE/TE buffers (LE+/TE−, 500 mM MES, 250 mM sodium hydroxide; LE−/TE+, 500 mM 6-aminocaproic acid, 250 mM hydrochloric acid) with 25% Pluronic F-127. Pluronic F-127 is a temperature-sensitive gel which acts like a liquid at 0 °C and a solid at room-temperature. We use it to suppress pressure-driven flow.[12]

Tween 20, NaOH, MES, 6-aminocaproic acid, and HCl were obtained from Sigma-Aldrich (St. Louis, MO); SYTO64 was obtained from Life Technologies (Grand Island, NY); YFP was purchased from BioVision, Inc. (Milpitas, CA); sonicated salmon sperm DNA was obtained from Agilent Technologies, Inc. (Santa Clara, CA). All solutions were prepared in UltraPure DNase-/RNase-free DI water (GIBCO Invitrogen, Carlsbad, CA).

Extraction. At the start of each experiment, we filled the cationic ITP channel with LE+/TE− buffer and anionic ITP channel with LE−/TE+ buffer and then emptied the reservoirs with vacuum. We filled the each buffering reservoir with 8 μL of high concentration buffers. We then pipetted 8 μL of LE+/TE− buffer into the protein elution reservoir and 8 μL of LE−/TE+ to DNA elution reservoir. We loaded 8 μL of serum sample into sample reservoir. We placed 0.5 mm diameter platinum wire electrodes into each buffering reservoir (and connected to high voltage leads). We applied +10 μA to the system and recorded applied voltage over time using the Keithley 2410 source meter (Keithley Instruments, Inc., Cleveland, OH) interfaced with a computer running custom MATLAB (Mathworks, Inc., Natick, MA) code. After each experiment, we pipetted out extracted samples and stored these at −80 °C for further analysis.

Imaging Systems. We monitored the ITP zone using epifluorescent microscopy (see below). We imaged focused nucleic acids zone using SYTO 64 fluorescent dye (Ex./Em. 599/619 nm). The focused protein zone was monitored by imaging spiked YFP (Ex./Em. 525/538 nm).

The visualization process was performed on an inverted epifluorescence microscope (Nikon Eclipse TE300) (Nikon, Tokyo, Japan) equipped with a 4× objective (UPlanApo, NA 0.16; Nikon, Tokyo, Japan). A mercury lamp (model C-SHG; Nikon, Tokyo, Japan) was used for excitation. We used filter cubes optimized for detection of YFP (XF105-2, Omega Optical, Inc., Brattleboro, VT) and SYTO 64, and a 0.63× demagnification lens (Diagnostic Instruments, Sterling Heights, MI). We captured images using a 1300 × 1030, 12-bit, interline CCD camera (MicroMAX-1300Y, Princeton Instruments, Trenton, NJ). We controlled the camera using WinSpec (Princeton Instruments, Trenton, NJ) and processed the images with custom scripts in MATLAB (R2012a, Mathworks, Natick, MA).

qPCR. We performed off-chip quantitative polymerase chain reactions (qPCR) to verify the purification of our ITP extracted nucleic acids. For this, we added 3 μL of DNA extracted from ITP to a PCR tube containing 10 μL of 2× Fast SYBR Green PCR master mix (Applied Biosystems, Carlsbad, CA), 7 μL of DNase free water, and 150 nM primers targeting the human gene BRAC2. The forward (5′- CACCTTGTGATGTTAGTT-TGGA-3′) and reverse (5′- TGG AAAAGACTTGCTTGGT-ACT-3′) primer sequence reagents were purchased from Integrated DNA Technologies (Coraville, IA). We performed qPCR using MiniOpticon Real-Time PCR Systems (BioRad, Hercules, CA) with the following thermal profile: 20 s initial hold at 95 °C and 40 cycles composed of 3 s denaturation at 95 °C and 30 s annealing and extension at 60 °C. We obtained post-PCR dissociation curves using the same instrument.

SDS-PAGE. We used sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to analyze the extracted proteins. SDS-PAGE was performed with 4%−12% Bis-Tris gel (Life Technologies, Grand Island, NY) and MES SDS running buffers. Prior to electrophoresis, we reduced samples by mixing 1 μL of protein samples with 5 μL of 4× lithium dodecyl sulfate (LDS) buffer, 1 μL of sample reducing reagent, and 13 μL of DI and incubated at 70 °C for 10 min. Then 20 μL of final solution for each sample was loaded onto the gel. Bis-Tris gel, running buffers, sample buffer, and reducing reagent are all from Life Technologies (Grand Island, NY). Electrophoresis was carried out in XCell SureLock Mini-Cell system (Life Technologies, Grand Island, NY) at 200 V for 35 min. We performed silver staining (SilverXpress staining kit, Life Technologies) to stain the gel after electrophoresis according to manufacturer's protocol. The gel was imaged on a light box by using stereoscope (Olympus D-HSM 2077) (Olympus, Tokyo, Japan) equipped with a CCD camera and a Barlow lens.

RESULTS AND DISCUSSION

We imaged both nucleic acids purification (Figure S-1a in the Supporting Information) and protein extraction (Figure S-1b in the Supporting Information) during the ITP process by the visualization method described above. Human serum samples contain inherently low concentration of extra-cellular nucleic acids.[24] Therefore, solely for the purpose of ITP zone visualizations (and not for the target template in Figure 2), we spiked serum sample with sonicated salmon sperm DNA to enhance the fluorescence signal. We also performed separate experiments with the same system buffers but using a smaller, alternate version of this device which provided the ability to

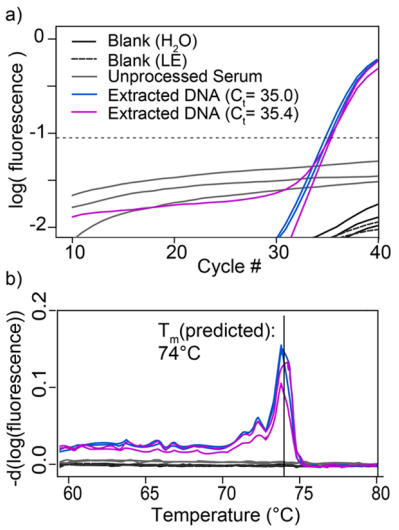

Figure 2. (a) qPCR results in which extracted nucleic acids were amplified using primers for a 201-bp section of the endogenous copies of the BRAC2 gene in the human genome. PCR successfully amplified the extracted DNA samples, while control samples of water and unprocessed serum did not amplify. (b) The resulting amplicons had a melting temperature of 74 °C, which matches the prediction from the Promega amplicon melting tool.

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capture both the cationic and anionic ITP zones in single images (see the Supporting Information).

Regarding the elution times in each experiment, typically the DNA ITP zones reached their elution reservoir prior to the arrival of proteins at their respective reservoir. However, the very low electric fields in the reservoir volume slows these zones dramatically. This allows proteins to reach their respective elution reservoir. The elution times for DNA and proteins were roughly 20 and 25 min, respectively.

Figure 2a shows qPCR results of both ITP extracted sample and unprocessed serum sample. We showed that ITP successfully recovered sufficient endogenous cell-free DNA to detect the BRAC2 gene via qPCR. Unprocessed serum showed no amplification in qPCR, as expected due to the effects of PCR inhibitors present in serum such as immunoglobulin G (IgG).[12] We did not observe amplification in negative control PCR reactions in which we analyzed nuclease-free water and LE−/TE+ buffer as templates.

The dissociation curves of PCR products showed melting temperatures very near 74 °C (Figure 2b). This melting temperature matches the prediction from the Promega amplicon melting tool (Promega Corporation, Madison, WI).[25] One phenomenon worth noting is that we have observed DNA−protein complex formation during sample preparation. We attribute this complex formation to unspecific bonding between nucleic acids and proteins.[26] This complex formation leads to possible DNA loss and results in low florescence signals of DNA zones in the ITP process (Figure S-4 in the Supporting Information). For example, to release DNA from bonding proteins, both Kondratova et al.[10] and Persat et al.[9] performed sample pretreatment using Proteinase K and SDS to digest serum proteins. Such an approach, however, is not applicable to our current purpose since we here wanted to preserve the integrity of (folded) proteins. Despite possible DNA loss due to DNA−protein complex formation, we nevertheless purified sufficient DNA targets for qPCR detection in a 6 μL extracted volume.

To study the performance of protein extraction, we compared samples extracted via ITP with samples directly applied to the protein gel, as shown in Figure 3. We recovered at least 17 protein bands over a range of molecular weights through the ITP process from the original sample. As expected, unprocessed serum samples showed a strongly overloaded albumin band on SDS-PAGE gel. In contrast, ITP extracted proteins showed successful exclusion of albumin, confirming the p*I*-based exclusion of albumin described above was effective. We here note a significant challenge in this work: the concentrations of proteins with molecular weights less than about 22 kDa were significantly reduced after ITP extraction. These proteins are barely detectable in the gel analysis (cf. Figure 3). We hypothesize that the strong attenuation of low molecular weight proteins may be due to surface adsorption onto channel walls,[27] the attachment of these small proteins to albumin,[28] or both of these effects.

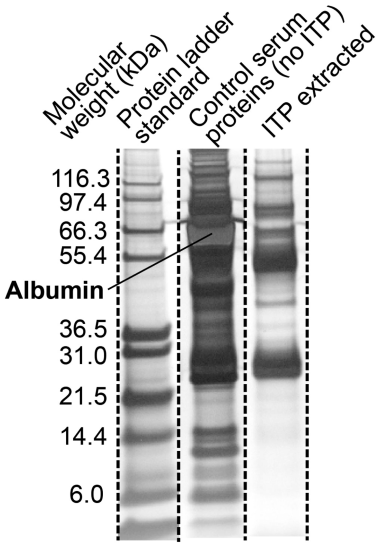

Figure 3. Typical silver-stained SDS-PAGE results. Images of gels for standard protein ladder, an unprocessed (original) serum sample, and a sample of proteins extracted using ITP. We recovered at least 17 visible protein bands over a range of molecular weights. Protein bands below roughly 22 kDa are very faint but detectable. Albumin is the most concentrated band in the original serum sample but is not visible in the selectively extracted sample.

SUMMARY

We report here on our efforts toward developing a novel bidirectional ITP technique to simultaneously extract nucleic acids and proteins from complex biological samples. We demonstrated the design and performance of a chip to implement this technique, which included a two separation channels "fed" by a central input sample reservoir, two output elution reservoirs, and two separate buffering reservoirs. The assay can be implemented with minimal manual steps, small sample volumes, and can automate the extraction and purification of nucleic acid and proteins in less than 25 min.

We demonstrated the technique to purification of DNA and proteins from human blood serum samples. We showed that the extracted endogenous copies of the BRAC2 gene DNA was sufficiently purified from the abundant PCR inhibitors present in serum. We also showed that our low pH separation conditions were efficacious in excluding proteins of p*I*s less than about 5.0, including abundant albumin.

To our knowledge, our technique is the first demonstration of simultaneous extraction of nucleic acids and proteins from a single biological sample into separate elution volumes using two counter-migrating ITP zones. Despite the current demonstration, we stress that application of our approach to serum still faces significant challenges. These include loss of DNA due to DNA−protein complexes. More importantly, we observed a strong reduction of proteins of molecular weights less than about 22 kDa, which may be caused by surface absorption, protein−protein interaction, or both. Important future work would include developing ITP compatible chemistry to reduce interactions between nucleic acids and proteins, reduce interactions within proteins themselves, and suppress protein absorption onto channel surfaces, all ideally while maintaining protein integrity. We hypothesize this will require extensive and labor-intensive variations of buffer chemistries and supporting experiments similar to what we present here. Hence, we present the current work as a possible guide for such explorations. Further, finite injection strategies (samples injected directly into finite lengths of channels and not just into reservoirs) can likely be used to optimize extraction efficiency.

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■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 650-723-5689. Fax: 650 723-7657. E-mail: juan.santiago@stanford.edu.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Y.Q. and L.A.M. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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