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Differential Gene Expression Using mRNA Isolated on Plastic Microfluidic Chips

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***Abstract*—Here we demonstrate the ability to perform differential gene expression experiments using messenger RNA (mRNA) isolated from crude cell lysates using a plastic microfluidic solid phase extraction column. The microfluidic columns (100µm by 100µm by 1.5 cm) were fabricated in a cyclic polyolefin by hot-embossing with an electroformed master-mold. The solid-phase consisted of a photopolymerized microporous monolith embedded with functional microparticles and covalently attached to the channel walls via photoinitiated grafting. For mRNA isolation from total RNA and direct mRNA isolation from cell lysates, oligo(dT) beads were embedded in the monolith. The extraction efficiency of the system is approximately 80% and the nucleic acid binding capacity of the silica solid-phase in this configuration is approximately 3.5 ng. The micro solid-phase was applied for the extraction and purification of mRNA from human liver total RNA and the isolation of mRNA from neonatal human dermal fibroblast cells (NHDF) and MCF7 breast cancer cell lysates. Differential gene expression between the two cell lines is demonstrated.**

I. Introduction

THE purification of nucleic acids from crude biological samples is a prerequisite to most genetic analysis methods including PCR amplification/detection, microarray analysis, DNA sequencing, etc. Recently, effort has been directed at automating the first step in genetic analysis, i.e. nucleic acid isolation and purification[1, 2]. Success in this area could streamline conventional bench-top procedures, which are technically demanding and labor intensive, and susceptible to human error and sample degradation.

For non-infectious disease detection (e.g. cancer, metabolic diseases, Alzheimer's disease, etc.), it is important to be able to evaluate both stable (DNA) as well as unstable (mRNA) markers to understand the molecular underpinnings of disease pathogenesis, progression and response to therapeutics. Extraction of mRNA is an important component of gene expression studies, and the success of large-scale gene expression experiments will require effective strategies for high-throughput purification of mRNA from small amounts of starting sample. The quantification of gene transcripts can be performed by use of total specimen RNA or only mRNA [3, 4]. However, if mRNA is used, both sensitivity and accuracy of transcript detection improves, particularly for the quantification of rare genes [5]. Most of the mRNA extraction techniques take advantage of the presence of a poly(A) tail in all eukaryotic mRNA. The extraction procedure involves binding of the poly(A) tail to immobilized oligo(dT) matrices followed by washing and elution steps.

Nucleic acid (DNA and mRNA) extraction procedures have been successfully miniaturized and incorporated in microfluidic chips using many different techniques. DNA purification in microfluidic systems has been previously demonstrated in glass microchips with microchannels packed with silica beads, silica based sol-gel, or combination of both [6-8]. The sol-gel/silica bead mixtures have been shown to have very good extraction efficiencies and reproducibility in microfluidic systems. However, the sol-gel process involves high temperatures and is not always suitable for use in polymeric devices. Packing silica beads in a polymer chip is a viable alternative design to the one presented here, and would require the addition of a weir structure in the channel. Recently, Landers and his co-workers demonstrated a device that incorporates sample preparation, amplification and detection in a single chip [14]. The DNA isolation in the device was performed with a silica bead packed column held in place with an etched weir. Others have demonstrated integrated systems using different sample preparation techniques [2, 9, 10]. Quake demonstrated mRNA isolation using paramagnetic oligo-dT beads in a PDMS (polydimethylsiloxane) chip [11].

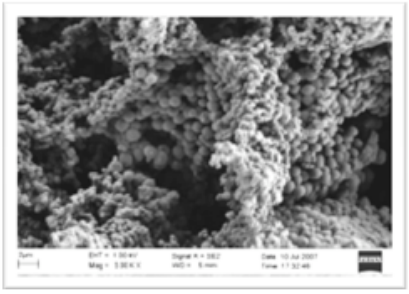

Figure 1: SEM micrograph of the porous polymer monolith embedded with 1 µm oligo(dT) beads taken at 3000X.

We are interested in fabricating devices composed entirely of polymer parts. The main driver for this materials selection is the ability to compression or injection mold polymer parts from a single microfabricated master, thus we are developing methods for sample preparation specifically

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tailored to full plastic assemblies [12, 13].

In this paper, we describe the fabrication of a micro solid-phase extraction (μSPE) method for isolation of total RNA and mRNA only in plastic microchips. The micro solid-phase was applied for the extraction and purification of mRNA from human liver total RNA and the isolation of mRNA from neonatal human dermal fibroblast cells (NHDF) and MCF7 breast cancer cell lysates. Differential gene expression between the two cell lines is demonstrated.

II. Experimental Methods

A. Materials

Zeonex690R was obtained as gift from Zeon Chemicals L.P., Louisville, KY. Butyl methacrylate (99%, BuMA), ethylene dimethacrylate (98%, EDMA), methyl methacrylate (99%, MMA), 1-dodecanol (98%), cyclohexanol (99%), benzophenone (99%, BP), and 2, 2-dimethoxy-2-phenylacetophenone (99%, DMPAP) were purchased from Sigma-Aldrich (St. Louis, MO). Guanidinium thiocyanate (GuSCN) containing lysis buffer (Buffer RLT) was purchased from Qiagen Inc. (Valencia, CA). The oligo(dT) beads were obtained from GenElute™ mRNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO). SDS (sodium dodecyl sulphate) and proteinase K were purchased from Sigma-Aldrich (St. Louis, MO). PEEK (Polyetheretherketone) capillaries of 360 μm o.d. and NanoPort™ assemblies for chip-based fluidic connections were purchased from Upchurch Scientific (Oak Harbor, WA). The primers and probes were purchased from Applied Biosystems, Foster City, CA, and are listed in Tables 1 and 2.

Gene ID	Gene Name	Part Numbers
GAPDH	Human GAPD Endogenous Control	4333764F
HGPRT	Human HPRT1 Endogenous Control	4333768F
18S rRNA	Eukaryotic 18S rRNA Endogenous Control	4319413E

Table 1: TaqMan® Assays used to analyze mRNA isolated from total RNA

Gene ID	Gene Name	Assay ID
COL1A1	Collagen, type I, alpha1	Hs00164004_ml
COL3A1	Collagen, type III, alpha1	Hs00164103_ml
CDH1	Cadherin 1, type 1, E-cadherin	Hs00170423_ml
ESR1	Estrogen receptor 1	Hs00174860_ml

Table 2: Gene Expression Assays.

B. Formation of Micro Solid Phase Extraction Columns

The chips were fabricated in Zeonex® (Zeonex690R) by hot-embossing with an electroformed master mold (NiCoForm, Inc., Rochester, NY). The microchip fabrication process has been described elsewhere [13]. The solid-phase was prepared within the microchannels by means of surface modification via photografting followed by in situ photopolymerization [12, 13]. Briefly, the microchannels were filled with MMA and 3% benzophenone. The chip was then UV-irradiated for 12 min at 254 nm (CL-1000 UV Crosslinker, UPV Inc., Upland, CA). The grafting step was followed by the preparation of a porous poly(butyl methacrylate-co-ethylene dimethacrylate) monolith within the grafted channels consisting of BuMA (15% wt), EDMA (10% wt), 1-dodecanol (52.5 % wt), cyclohexanol (22.5 % wt), DMPAP (1% wt with respect to monomers) and the functional microparticles (15% with respect to the total volume of the pre-polymer mixture). After the microchannels were filled with monolith precursor mixture, the chip was UV irradiated at 200 mJ/cm^2 for 1.1 min to initiate polymerization, and then washed with methanol. The porous polymer monolith was embedded with 1 μm oligo dT30 coated polystyrene beads. Figure 1 shows and SEM micrograph of a monolith embedded with oligo(dT)-polystyrene beads.

C. Cell Culture

For primary NHDF culture, human neonatal foreskins from circumcisions performed at Brigham and Women's Hospital (Boston, MA, USA) were obtained. Cells were extracted from the foreskins using standard methods. The cells were trypsinized and split several times, after which they were frozen down with 1 % DMSO and stored for later use.

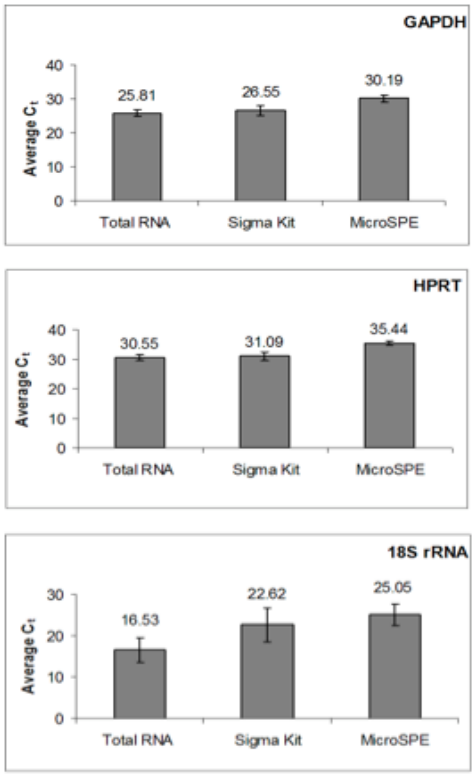

Figure 2: Average threshold cycle (C_t) values obtained with total RNA versus the Sigma kit and our μSPE columns for GAPDH mRNA, HPRT mRNA and 18S rRNA (n=4). A higher threshold cycle corresponds to less substrate RNA.

NHDF cells and MCF7 breast cancer cells (ATCC, Manassas, VA, USA) were grown to ~ 70% confluence in DMEM supplemented with 10% calf serum and 1% pen/strep in 25-cm^2 polystyrene tissue culture flasks (BD Falcon™, BD Biosciences, San Jose, CA, USA). Cells were washed in 1X PBS, trypsinized, and further diluted in culture medium to stop the trypsinization reaction. After counting the cells, the cells in the suspension were pelleted at 1500 rpm for 5 min and then suspended in the lysis buffer (0.5% SDS mixed with 10 mg/ml proteinase K in 50:1 ratio) to a concentration of 10^6 cells/mL.

D. Column Efficiency Analysis

The efficiency and reproducibility of the microfluidic mRNA purification procedure was first investigated using human liver total RNA (Ambion, Inc., Austin, TX, USA). RNA amounts were quantified using 260 nm absorbance reading (Eppendorf® Biophotometer, Eppendorf, Westbury, NY, USA). The microfluidic purification procedure was compared against a commercially available mRNA isolation kit (GenElute™ mRNA Miniprep Kit, Sigma-Aldrich, St. Louis, MO, USA). The Sigma kit uses oligo dT30

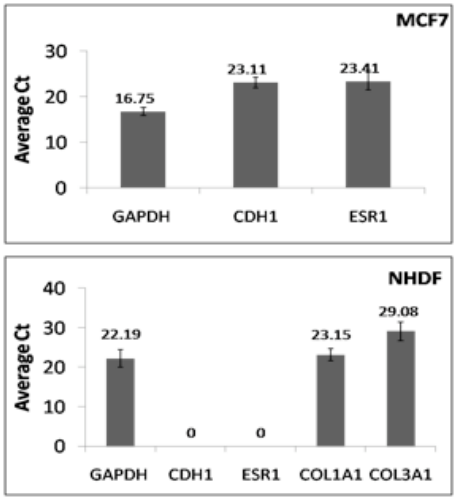

Figure 3: The average threshold cycle (C_t) values of PCR amplification assays from mRNA isolated directly from MCF7 cells (top) and NHDF cells (bottom) using the oligodT µSPE system (n=3 extractions for each gene).

covalently linked to 1 µm polystyrene beads to capture polyadenylated mRNA by hybridization. The kit utilizes a spin filter and centrifugal sedimentation to separate the beads from the load buffer, wash solution and the final eluent. The RNA sample volume required for the Sigma assay is 250 µL and the extracted mRNA is eluted in 100 µL of elution buffer. The procedure takes about 40 min and requires multiple wash and elution steps.

The microfluidic mRNA extraction procedure also follows a load-wash-elute type separation methodology. The sample, wash solutions and elution buffer were passed through the microchannels at a flow rate of 300 µL/hour with a KDS100 syringe pump. The plastic chip was placed on top of a hot plate (VWR® 375 Hotplate Stirrer, VWR, West Chester, PA, USA) for the steps that have to be performed at 70°C. Prior to starting the extraction procedure, the chip was placed on the hot plate at 70° C for several minutes to equilibrate the system. The total RNA sample was mixed with the binding solution from the Sigma kit to a concentration of 10 µg/mL. 15 µL of the sample (i.e. 150 ng of total RNA) was passed through the channel for 2 min at 70° C and incubated for 3 min at 70° C. The chip was then removed from the hot plate and placed at room temperature for 10 min. The wash buffer was then flowed through the channel for 5 min to remove contaminants. Finally, the elution buffer (10 mM Tris-HCL, pH 7.5.), pre-warmed to 70 °C, was flowed through the channel for 5 min and 25 µL of eluent was collected. For the elution step, the chip was again placed on the hot plate at 70 °C. The total time required for the entire purification process is 25 min. The concentration of total RNA used for the Sigma kit was 600 ng/mL, so that the amount of the total RNA used in both the systems were the same (i.e. 150 ng) in order to facilitate a direct comparison of the Sigma kit with the µSPE system.

The eluted mRNA was reverse transcribed off-chip using the SuperScript® III First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). PCR amplification was performed using an ABI 7500 Real Time PCR machine. The primers and probes for human GAPDH gene (Glyceraldehyde 3-phosphate dehydrogenase), human HPRT1 gene (Hypoxanthine phosphoribosyltransferase 1) and eukaryotic 18S rRNA was purchased from Applied Biosystems (TaqMan® Endogenous Controls, Applied Biosystems, Foster City, CA).

E. mRNA Isolation from Cells

The µSPE columns described above were used to directly isolate mRNA from NHDF and MCF7 lysates. The extracted mRNA was reverse transcribed to cDNA and used as template for PCR amplification of CDH1 (E-cadherin 1), ESR1 (estrogen receptor 1), COL1A1 (collagen type I, alpha 1), and COL3A1 (collagen type III, alpha 1) mRNAs. The overall efficiency of mRNA selection was evaluated using RT-PCR analysis of high-abundance GAPDH and low-abundance HPRT gene mRNAs, again using an 8µL (32%) aliquot of the eluate for each gene. Control total RNA was used as a RT-PCR control and control isolations from the cells were performed using a GenElute™ mRNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA).

The cells were disrupted with SDS/proteinase K digestion to release RNA and eliminate RNases prior to mRNA selection. The cells were incubated with the lysis buffer at 65 °C for 10 min and then mixed with 32 µL of 5M NaCl. The mixture was then loaded into the µSPE column for 2 min at 70 °C, incubated at 70 °C for 3 min, followed by incubation at room temperature for 10 min. The column was washed with low-salt wash buffer (0.5 mM NaCl, 100 mM Tris-HCL, pH 7.5, 0.1% SDS) for 5 min, and then the elution buffer (10 mM Tris-HCL, pH 7.5.), pre-warmed to 70 °C, was flowed through the channel for 5 min at 70 °C and 25 µL of eluent was collected.

The eluted mRNA was reverse transcribed off-chip using the SuperScript® III First Strand Synthesis System and PCR

amplification was performed using an ABI 7500 Real Time PCR machine. The primers and probes specific for human E-cadherin (CDH1), estrogen receptor 1 (ESR1), collagen, type 1, alpha I (COL1A1) and collagen, type III, alpha 3 (COL3A1) were designed by Applied Biosystems (TaqMan® Gene Expression Assays, Applied Biosystems, Foster City, CA). The Assay ID for the gene expression assays are listed in Table 1.

III. Results

The average Ct values for mRNA selected by the μSPE procedure and the Sigma kit are shown in Figure 2. There was an average Ct difference of 3.64 (GAPDH) and 4.35 (HPRT) between the two methods, which corresponds to an average 16-fold difference in substrate between the two extraction methods. For the dermal fibroblast cells, the PCR amplification of mRNAs of GAPDH, CDH1, ESR1, COL1A1, and COL3A1 genes were evaluated. For the MCF7 cells, the mRNAs of GAPDH, ESR1 and CDH1 genes were assessed. Figure 3 shows the mean Ct of amplification to evaluate the relative abundance of these mRNAs. It was found that both ESR1 and CDH1 were highly expressed by the MCF7 cells, which is typical of this human breast cancer cell line. In the dermal fibroblast cells, CDH1 was undetectable as expected, since these cells are contact inhibited. The gene-expression results also corroborated published observations suggesting that COL1A1 and COL3A1 mRNAs are expressed abundantly in the neonatal human dermal fibroblasts.

IV. Discussion

These results indicate that the mRNA yield from the μSPE method was lower than the yield from the Sigma kit, which is designed for larger sample volumes. The capture efficiency of the μSPE system is dependent on the residence volume of the porous monolith and the amount of oligo(dT) beads exposed to the loaded sample. The lower yield of the microfluidic system is believed to be due to the significantly smaller sample volume inside our μSPE column, and potentially saturation of the oligo(dT) beads. It is also possible that not all of the oligos on the oligo (dT) beads survived the light directed monolith polymerization process. It is expected that increasing the length and/or the cross-sectional area of the separation channel will increase the capture efficiency of the μSPE column and prevent loss of mRNA copies in future applications. It may also be possible to pack more beads into the channel to provide more capture surface area.

The micro solid-phase extraction columns presented here may eventually be coupled with on-chip PCR, microarray or DNA sequencing modules to form an integrated genetic analysis device for point-of-care clinical diagnostics. The low-cost of the microchip fabrication process and the simple procedure for generating the μSPE columns make this system practicable for one-time use, disposable applications. The in situ photografting process and the porous polymer monolith chemistry used here are very versatile and can be used to entrap any micro- or nanoparticles to form functionalized solid-phases within UV transparent thermoplastic microchips. The μSPE columns recovered mRNA of both abundant and rare genes, which validates that the original mRNA profile was conserved during the isolation. We demonstrated that the procedure can isolate PCR amplifiable mRNA templates directly from the whole cell lysates of two human cell lines without having to isolate total RNA as an intermediate step. The extraction procedure itself was simple and required minimal user handling. The entire purification process for isolation of mRNA required about 10 min and the isolation of mRNA took roughly 25 min. The procedure is flexible enough for use in a variety of different applications where the preconcentration of nucleic acids is necessary prior to downstream processing.

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