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Purification of DNA/RNA in a Microfluidic Device

Andy Fan,

Department of Biomedical Engineering, Boston University, Boston, MA, USA

Samantha Byrnes, and

Department of Biomedical Engineering, Boston University, Boston, MA, USA

Catherine Klapperich

Department of Biomedical Engineering, Boston University, Boston, MA, USA, catherin@bu.edu

Abstract

Often, modern diagnostic techniques require the isolation and purification of nucleic acids directly from patient samples such as blood or stool. Many diagnostic tests are being miniaturized onto micro-sized platforms and integrated into microfluidic devices due to the economies resulting from smaller sample and reagent volumes. Often, these devices perform sample preparation in series with the diagnostic tests. The sample preparation steps are vital in order to purify the desired genetic material from potential inhibitors that can interfere with the outcome of the test. There are various techniques used to selectively capture the nucleic acids while washing away potential contamination (proteins, enzymes, lipids, etc.). Two of the most common forms of selective capture are based on nucleic acid binding to silica surface or on the precipitation of nucleic acids with or without the presence of a carrier species. Each of these methods can be performed in liquid phase or in a solid support such as an extraction column. Here we discuss both methods and address microfluidic applications.

Keywords

Sample preparation; Microfluidic device; Diagnostic tests; Micro-solid phase extraction; Purification of DNA/RNA; Blood lysis

1. Introduction

In a traditional medical lab setting, extraction and purification of nucleic acids (NA) from biologically complex samples, such as blood or organ tissues, often involves a common set of unit processes: (a) isolation of target cells from a heterogeneous matrix by fractionation, (b) lysis of the target cells, (c) isolation of NA from protein-laden lysate using additional fractionation steps, and (d) concentration of harvested NA. Before the advent of NA purification by solid-phase extraction with silica matrices (1, 2), the above tasks usually required cellular debris separations with centrifugation, handling of toxic substances such as phenol–chloroform, and time-consuming ice-cold precipitation of NA with alcohols. Even

with modern advances in bench top solid-phase extraction (SPE) methods offered by commercial entities such as Qiagen, Invitrogen, and others (3), a single limitation persists—the need for an accessible centrifuge, which implies access to a dedicated lab space with reliable power sources.

Recently, there has been a move toward miniaturization of diagnostics onto micro-sized platforms that include sample preparation steps (4–6). Besides the footprint advantage that microfluidic devices hold over dedicated lab equipment, the microscale platform allows the possibility of using a smaller patient sample size, automation of extraction (7) and most importantly, device portability that could potentially be the driving force toward future point-of-care diagnostics. To take advantage of the form factor while keeping the lysis chemistry simple and nontoxic, the majority of microfluidic NA extraction devices rely on the combination of a SPE column that captures and precipitates NAs and guanidine hydrochloride (GuHCl) or guanidine thiocyanate (GuSCN) as the lysis reagent (8, 9). In the presence of the guanidine cation, the most notable NA capturing technique is to utilize silica particles, which can be embedded within the column (10, 11) or kept as a separate layer from the column. An additional method of NA capture relies on a co-precipitant molecule, such as glycogen, which captures the NA and selectively precipitates it in the presence of a precipitating solvent, such as ethanol or isopropanol (12, 13) and small cation cofactor supplied by sodium chloride (NaCl), sodium acetate (NaOAc), ammonium acetate (NH₄OAc), or lithium chloride (LiCl) (8). Direct NA precipitation using this method, however, requires careful tailoring of the chemistry in order to avoid excess co-precipitation of cellular proteins and will not be considered in this chapter.

All of these sample preparation methods can take place on a fully integrated microfluidic chip or in small disposable systems. Additionally, these sample preparation methods do not differentiate between the type or source of NAs, resulting in broad-spectrum use of these techniques with various downstream applications such as PCR, ELISA, and ESI-MS (3, 14).

As will be detailed in Subheading 3.1, a simple, low-cost microfluidic chip can be constructed by thermo-compression bonding of two sheets of Zeonor 750R plastic. Briefly, channels are formed by patterning one sheet of Zeonor by hot-embossing with a noncompliant master mold made from SU8. The choice of Zeonor as the chip material was driven by two factors: the material is UV transparent and relatively chemically inert—both of which are advantageous attributes in most biological assays. This polymer is a cyclic polyolefin; similar materials with similar temperature and optical characteristics can be obtained from other suppliers. The bonded chips are filled with a pre-polymer solution and then exposed to UV light. After polymerization, the porogens and initiators are removed through washes with methanol and ethanol, and the resulting SPE matrix is equilibrated with lysis buffer prior to NA capture.

From a point-of-care diagnostics stance, there are cases where a very small form-factor microfluidic device is not feasible in the field. For instance, in resource-poor locations where electricity is at a premium, an electrically actuated fluidic pump operating at lower pressures can be replaced by a manual bicycle pump operating at an excess of 80 psi. As a result of favoring portability and laboratory independent operation over automation and flow

accuracy, an existing chip design can be scaled up such that both the SPE column and the “microchannel” can be packaged together in a premade plastic vessel like a pipette tip. Preparation of such a non-chip-based extraction method is described in Subheading 3.2.

2. Materials

2.1. Microchip Components

1. Zeonor 750R, a thermoplastic polyolefin resin (Zeon Chemicals, Louisville, KY).
2. SU8 for making the master mold (Microchem, Newton, MA).
3. Metal sputtering system for titanium and aluminum coating of master mold.

2.2. Microchip Reagents for Bacterial DNA Recovery from Cell Culture or Blood

1. Test sample (e.g., infected blood or cell culture medium).
2. Resuspension buffer: 200 mM Tris–HCl, 2 mM EDTA, with sodium dodecyl sulfate (SDS) and Triton X-100.
For gram-negative bacteria: Resuspension buffer should contain 0.03% SDS/3.6% Triton X-100.
For gram-positive bacteria: Resuspension buffer should contain 0.3% SDS/7.2% Triton X-100.
3. Lysis reagent: 3 M GuSCN (from 6 M stock, Sigma Aldrich, St. Louis, MO).
4. Proteinase K (20 mg/mL stock in 50 mM Tris–HCl (pH 8.0), 10 mM CaCl₂); from Amresco (Solon, OH).
5. 70% Ethanol.
6. Nuclease-free water.

2.3. Reagents for Viral RNA Recovery from Plasma or Cell-Free Supernatants

1. Channel Buffer: 1.5 M GuSCN, 50% isopropanol, 1× RNASecure reagent. For 5 mL of channel buffer, mix 1.25 mL of 6 M GuSCN, 2.5 mL of 99% isopropanol, and 200 µL of 25× RNASecure reagent (stock is 25× from Applied Biosystems, Carlsbad, CA, Cat #AM7005) in a 15 mL tube. Use stock immediately.
2. Lysis buffer: 2 M GuSCN, 62.7% isopropanol, 1× RNASecure reagent. For 4.5 mL of lysis buffer, mix 1.5 mL of 6 M GuSCN, 3 mL of 99% isopropanol, and 180 µL of 25× RNA Secure reagent in a 15 mL tube. Use stock immediately.
3. 70% Ethanol.
4. Nuclease-free water.

2.4. Acid-Treated Silica Slurry

- 5 Silica particles (Sigma Aldrich, St. Louis, MO).
- 6 Ultra-pure water.

- 7 Hydrochloric Acid Solution: hydrochloric acid (32 wt%/vol, 10.2 M).

2.5. UV Activated Micro-Solid Phase Extraction Column

1. Grafting Solution: ethylene diacrylate (90% EDA), methyl methacrylate (99%, MMA), benzophenone (99%). Mix a 1:1 solution of EDA and MMA with 3% benzophenone.
2. SPE column pre-polymer solution: porogenic solvents 1-dodecanol and cyclohexanol, monomer solution butyl methacrylate (99% BuMA) and ethylene dimethacrylate (98% EDMA). Mix 24 wt% of BuMA, 16 wt% EDMA, 42 wt% 1-dodecanol, 18 wt% cyclohexanol. Add ~0.4 wt% of the UV-Sensitive Free-Radical Initiator: 2,2-dimethoxy-2-phenylacetophenone (99%, DMPAP). The DMPAP should be 1 wt% with respect to the monomers.
3. 0.7 μm Silica microspheres (Polysciences, Inc., Warrington, PA).
4. Methanol.
5. Absolute ethanol.

3. Methods

3.1. Preparation of Microfluidic Chip with Embedded Micro-Solid Phase Extraction Column Applied to Bacterial DNA Extraction from Blood

A schematic of the chip-making process is shown in Fig. 1.

1. For preparation of the master mold: pre-clean 100 mm diameter p-type (100) Si wafers with piranha solution (1:3 vol ratio of 30 wt% hydrogen peroxide and 98 wt % sulfuric acid) and spincoat 100 μm of SU8 onto the wafer surface. After a 30 min prebake at 95°C, the appropriate channel features are formed using contact mask lithography. In general, the SPE columns are fabricated inside of straight microfluidic channels. The channels do not need to have a specific type of sidewall (i.e., both isotropic and anisotropic techniques can be used). After pattern development, post-bake the wafers for 1.5 h at 175°C (see Note 1).
2. To aid the subsequent removal of the hot-embossed plastics from the SU8 mold after embossing (see Note 2), a bilayer of 500 Å titanium/1,000 Å of aluminum should be sputtered onto the SU8 surface. The completed SU8 mold can be reused 2–5 times before structural degradation occurs from repeated hot-embossing.
3. To construct Zeonor microchannels, the Zeonor sheets to be used for subsequent hot-embossing can be made by melting raw Zeonor pellets in a heat press at 90°C (20°C above the glass transition temperature of Zeonor 750R) against two flat surfaces. Shims (spacers) can be used to create sheets or plaques of the desired thickness; otherwise, extruded sheet material can also be purchased directly through vendors.

¹Following the post-bake period, the masters had a negative pattern of the channels and had glass-like mechanical properties (1).

²The sputter coating for the master is an optional step but the aluminum coating seems to help with the removal of the master after embossing. Of course, electroplating is a much more robust, but also more expensive method of achieving the same effect.

4. Starting from a sheet of Zeonor, microfluidic channels with 100 μm depth and either 100 or 150 μm in width can be created by hot-embossing the Zeonor against the SU8 master using a heat press at 90°C and 250 psi for 5 min. The Zeonor-SU8 duplex is then cooled for 2 min at room temperature prior to manual separation (see Note 3).
5. Following separation, drill two, 1.5-mm diameter wells at each end of the microchannel to form the input and output ports.
6. To encapsulate the microchannel, an identically sized piece of Zeonor was thermally bonded to the hot-embossed Zeonor sheet on a hot-press at 70°C, 250 psi for 2 min.
7. In order to make the intra-channel solid-phase matrix, the channel walls should be treated with grafting agent to increase sidewall adhesion to the polymer monolith, due to the surface chemistry of the Zeonor plastic (15). This can be accomplished by filling the channels with the grafting agent, followed by UV-irradiation in an oven for 10 min at a wavelength of 254 nm and at 200 mJ/cm^2 .
8. After UV-irradiation, remove the excess solution from the channels by washing with 100 μL of methanol; between 10 and 33 column volume-worth (CV), where 1 CV equates to the SPE monolith volume.
9. After grafting and washing, the SPE-proper can be made by filling the channels with the SPE column pre-polymer solution and 15 wt% of 0.7 μm silica microspheres (wet volume, see Note 4) and then UV-irradiated for 2 min at 200 mJ/cm^2 . Typically, 3–10 μL of the SPE column pre-polymer solution is used to make a SPE.
10. After the second UV-irradiation step, wash out any excess porogen and SPE column pre-polymer solution with 100 μL (10–33 CV) of methanol followed by 200 μL (20–66 CV) of absolute ethanol.
11. The first step of using the on-chip SPE column for the application of bacterial DNA extraction from blood is sample collection and off-chip lysis. To simulate bacteremia, where bacteria are found in human whole blood, one must first take an overnight culture (or take log-phase cultures at $\text{OD}_{600} \sim 0.5$) and make serial dilutions of 10^1 – 10^5 CFU/mL. Then, spin down the 1 mL—worth of cells at $6,800 \times g$ for 10 min, decant the supernatant, and resuspend each bacteria pellet in 100 μL of human whole blood.
12. By adding 150 μL of resuspension buffer to the blood–bacteria matrix, indiscriminant cellular lysis commences with both SDS and Triton-X denaturing the plasma membranes of cells and the bacteria cell walls.

³The master/substrate were cooled on an aluminum plate and separated manually when the plastic was no longer soft.

⁴The silica microspheres come suspended in water so the volume is measured and spun down in a centrifuge at $2,500 \times g$ for 10 min. The supernatant was removed and the resulting pelleted silica was dried overnight at 115°C. The pellet was then broken up into a powder to be mixed with the porogen/monomer solution.

13. Add 200 μL of the lysis reagent (3 M GuSCN), vortex to mix, and add enough Proteinase K to a final concentration of 0.8 mg/mL. At this stage, the final concentration of GuSCN is 1.33 M, which is around the upper limit where both Proteinase K and GuSCN can function synergistically as a protein denaturant and, at the same time, protein digestion can take place without proteinase K itself being denatured by the chaotropic agent. Furthermore, the guanidinium cations in the mixture also act as cofactors in NA-silica binding.
14. For optimal lysis and digestion, the mixture can be incubated at 50–60°C for 30–90 min.
15. Condition the on-chip SPE column with loading buffer for 5 min (around 30 μL total, or 10 CV) prior to introducing the sample for extraction. For the description of sample loading, we will assume the volume of the SPE column to be 3 μL .
16. Pipette the blood–bacteria lysate into a 1 cm^3 syringe. Then, using a syringe pump, load the lysate into the microchannel at 10–15 $\mu\text{L}/\text{min}$. The flow-throughs can either be collected for analysis or discarded.
17. Wash the microchannel with approximately 300 μL of 70% ethanol (~10 CV) to remove excess proteins that may have adsorbed onto the column.
18. Finally, elute the DNA from the column using nuclease-free water. Typical elution volumes range from 25 to 100 μL (8–30 CVs).

3.2. Preparation of UV Activated SPE Column in Pipette Tips Applied to Viral RNA Extraction from Cell-Free Supernatants

1. To the solid-phase extraction (SPE) column pre-polymer solution, add 15 wt% of 0.7 μm silica microspheres (wet volume, see Note 4).
2. Aliquot the pre-polymer mixture in 50 μL portions into 250 μL non-filter pipette tips (see Note 5). To polymerize the solution, the pipette tips are placed in a 254 nm UV oven and irradiated for 2 min at 200 mJ/cm^2 . During this time, the solution will turn from a clear liquid to a solid white monolith.
3. After the polymerization is complete, wash the monoliths with 100 μL methanol (2 CV) followed by 200 μL (4 CV) of absolute ethanol (see Note 6). For the sake of discussion we are now assuming that 1 CV equates to 50 μL worth of SPE matrix.
4. For performing the viral RNA extraction from cell-free supernatants using the SPE column the pipette tip is first equilibrated with the channel buffer; fluid flow can be driven by compressed nitrogen or air at a range between 40 and 80 psi depending on the desired flow rate. After the channel buffer wash, the pressure source is disconnected, the pipette tip opening exposed, and then loading of the viral sample can proceed (see Note 7).

⁵The SPE pre-polymer solution can be applied to various column geometries and volumes. Certain geometries require treatment to help prepare the surface so that the SPE column does not get pushed out of the channel under pressure. Surface preparation can be achieved through the use of a grafting solution or by mechanically scoring the inside surface of the channel (12).

⁶The best way to store the SPE columns is in a dry, sealed bag with a desiccant packet.

5. Mix the virus sample at 1:1 with the lysis buffer, hand-invert the tube at least 20 times to mix, and commence lysis by incubating for 15 min at room temperature. Next, manually pipette the lysate into the SPE column, refit the compressed air source onto the SPE-tip complex and push the entire lysate through the SPE column. The flow-through fractions can either be collected for further analysis or discarded.
6. After the sample mixture passes completely over the column, wash the column with 500 μ L (10 CV) of 70% ethanol solution to remove excess salts and proteins that may have precipitated onto the column.
7. After the wash, the column is dried with air for 2 min to remove excess solvent.
8. Finally, elute the RNA from the SPE column by washing with several CV-worth of nuclease-free water (see Note 8).

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⁷The input sample can be virions in a protein solution or in some other biological matrix such as blood. This protocol can also be performed using a chip-scale SPE column.

⁸The rate of elution should be kept slow and consistent (approximately one drop every 5 s) to ensure the captured RNA is exposed to water long enough to return into solution from its precipitated form on the SPE column.

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Fig. 1.
Fabrication process of the microfluidic chip with embedded SPE column.