Sensors and Actuators B 155 (2011) 437–445

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

Oligonucleotide immobilization using 10-(carbomethoxy)decyl-dimethylchlorosilane for mRNA isolation and cDNA synthesis on a microfluidic chip

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ARTICLE INFO

Article history:
Received 17 December 2009
Received in revised form
20 December 2010
Accepted 22 December 2010
Available online 30 December 2010

Keywords:
Microfluidic device
Oligonucleotide immobilization
Silane
mRNA detection
PCR

ABSTRACT

Fabrication of microfluidic systems capable of extracting and isolating nucleic acids from biological samples and preparing them for downstream applications within the same device is of interest as on-chip sample preparation reduces the time and effort expended on multi-step benchtop procedures. A microfluidic chip capable of cell lysis, nucleic acid extraction and immobilization has been developed in our laboratory. This report focuses on substrate development and chip integration of the nucleic acid immobilization platform. The immobilization region was initially developed using poly-adenylated mRNA released from normal human lymphoblastoid cells. After immobilization, the mRNA remains functional for amplification by the reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR (qPCR) gene expression analyses. Detailed surface functionalization, chemical modification, and covalent oligonucleotide immobilization methods are described. Vapor and liquid deposition techniques for Pyrex functionalization by 10-(carbomethoxy)decyl-dimethylchlorosilane (CMDCS) were developed and an optimal method was determined for microfluidic chip application. CMDCS substrate depositions and modification were characterized by atomic force microscopy and contact angle goniometry, respectively. Oligonucleotide immobilizations and mRNA bindings were determined by fluorescence microscopy. Functional, selective platform integration within the microfluidic chip was confirmed by standard RT-PCR and qPCR systems.

1. Introduction

Development and integration of functional immobilization platforms are essential for genomic detection systems [1–11], since it provides a milieu for DNA or RNA molecules to be selectively recognized and captured for system processing (i.e. hybridization). The integrated immobilization platform should have the ability to be applied in a consistent, repeatable manner, be able to isolate and detect only the nucleic acid of interest, ensure that immobilized oligonucleotides remain functional, and withstand heating and cooling temperatures needed for the required process. Numerous strategies describing covalent anchoring of 5′ or 3′ modified oligonucleotides via self-assembled monolayers (SAMs) to various solid supports have been evaluated [1–3,8,11–15]. Glass is a commonly selected substrate [1,2,8,12,15] because it is relatively inert but may become activated with the introduction of surface chemistries [16], it is relatively stable at the required hybridization temperatures [8], and lithography procedures are standardized. SAMs such as silanes are desirable because they are commercially available, are highly reactive with surface hydroxyl, oxide, and silicon dioxide groups, and can be modified to work in a variety of experiments [1,8,12–14,16–19].

Fabrication of microfluidic systems for upstream nucleic acid processing and/or downstream PCR processing has been reported [20–30]. Upstream processing is of paramount importance since nucleic acid integrity must be maintained throughout sample extraction, capture, isolation, purification, and release. Sample types and techniques applied to microfluidic chips for upstream processing are varied. Several groups of investigators have demonstrated upstream nucleic acid processing using mammalian cell

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doi:10.1016/j.snb.2010.12.043

lines [21,25–27,29], bacterial cells [22], viral cells [29], human whole blood [21,30], as well as DNA [28] and RNA [20,23] in a buffered solution for PCR-based microfluidic chip applications. Furdui and Harrison described a method in which a microfluidic device was used to capture and isolate T-cells from whole blood on-chip through the use of magnetic beads [21]. After T-cells were released, DNA extraction was achieved off-chip with the aid of magnetic beads, followed by off-chip PCR analysis. Another microfluidic system capable of separating white blood cells (WBCs) from whole blood, DNA extraction, isolation, and amplification was described by Han et al. where DNA capture occurred by chemically modified silica beads [30]. On-chip upstream processing of RNA has been presented on surfaces involving oligonucleotide immobilization through solid-phase extraction (SPE) methods [23,28,29] and magnetic beads [20,25–27]. Although DNA is more stable, isolation of DNA from samples is not always advantageous since it is RNA (more specifically mRNA) that provides information regarding gene expression within cells [31]. Moreover, since RNA is less stable and more prone to enzymatic digestion when whole blood is used for upstream nucleic acid processing, WBCs are separated from all other cells prior to nucleic acid extraction [21,30].

As part of our efforts to develop a field-portable qPCR device, microfluidic chips were fabricated in our laboratory. One chip is dedicated completely to upstream nucleic acid processing and the other chip is for DNA amplification. This report focuses on the development and preparation of the nucleic acid processing chip. Within this microfluidic chip the following steps occur: (1) the chip surface is chemically modified for oligonucleotide immobilization, (2) mRNA is extracted from the WBCs in a whole blood sample (without the need for a pre-WBC separation/isolation step), (3) mRNA is captured and enriched, and (4) cDNA is generated through reverse transcription. Finally we describe a solid-phase extraction (SPE) process for mRNA capture that to our knowledge has not been previously presented for use in a microfluidic application.

Prior to incorporating the methods into the microfluidic chip for testing, it was necessary to examine and characterize hydroxylated substrates modified with 10-(carbomethoxy)decyldimethylchlorosilane (CMDCS), oligonucleotides (dT_{25}), and polyadenylated RNA (poly-A RNA). Functionalization with CMDCS is promising since it provides an immobilization milieu, whereby, the amine-modified oligonucleotide may be covalently anchored to the surface in a few steps. This method also provides an alternative to the use of epoxy-based silanes for the covalent attachment of amine-modified oligonucleotides, since binding with the epoxy ring is a moisture sensitive process [8,32]. Functionalized CMDCS substrates for the immobilization of polymers have been demonstrated [18,33]. Previously, Bras et al. [18] described a process of directly binding poly(ethylene glycol)$_{20}$ to a CMDCS functionalized surface for indirect substrate–oligonucleotide immobilization. The modification of substrates by CMDCS for chemical grafting and direct oligonucleotide immobilization have also been presented [34,35]. The CMDCS deposition method used in these studies could not be readily transferred to our microfluidic chip for oligonucleotide immobilization [18,28,29]. Thus, we investigated alternative CMDCS deposition methods that would be applicable for microfluidic chip integration. CMDCS functionalization was confirmed by AFM to examine surface topography and contact angle measurements to determine the extent of surface hydrophobicity. Immobilized dT_{25} oligonucleotides and poly-A RNAs were examined by fluorescence microscopy with the aid of SYBR Green II (SGII) and Rhodamine-dUTP labeled mRNA, transcribed *in vitro* from an expression vector, respectively. Lymphocyte lysis, mRNA isolation, and cDNA synthesis within the microfluidic chip were confirmed with conventional RT-PCR and qPCR systems.

2. Experimental

2.1. Surface functionalization and characterizations

2.1.1. Substrate preparation

Pyrex 7740 wafers were diced into 100 mm^2 pieces. Diced substrates were cleaned by immersion in piranha solution [H_2SO_4/H_2O_2, 3:1 (v/v)] at ambient temperature, then rinsed with copious deionized water, immersed in ethanol, and finally, individually dried with a stream of nitrogen. Substrates were then stored in a nitrogen-purged desiccator until CMDCS depositions, which were performed within 24 h of substrate cleaning.

2.1.2. Chemical vapor deposition (CVD) of CMDCS

Substrates were placed in a dry-seal vacuum desiccator with 10 ml of CMDCS (Gelest; CMDCS and substrates were never in direct contact). A vacuum was obtained by pumping at 14.2 psi for 15–30 min and then the desiccator was transferred to a 60 °C incubator for 17–18 h. After vacuum release, CMDCS-coated substrates were thoroughly rinsed in methanol to remove unbound material and dried with a stream of nitrogen. Samples were re-stored in the nitrogen-purged desiccator until CMDCS activation.

2.1.3. Liquid deposition of CMDCS

Substrates were prepared in 10 ml CMDCS solutions, in anhydrous toluene (Sigma–Aldrich), and examined at various concentrations, times, and temperatures. After deposition, substrates were thoroughly rinsed in anhydrous toluene, water, and methanol, and then each was dried with a stream of nitrogen. Samples were re-stored as described in Section 2.1.2.

2.1.4. CMDCS activation

Subsequent to CMDCS deposition, substrates were immersed in 1 N HCl for 1 h at ambient temperature, rinsed with copious water, and dried with a stream of nitrogen. This was performed within 72 h of CMDCS deposition and immediately prior to oligonucleotide immobilization.

2.1.5. Oligonucleotide immobilization on activated CMDCS substrates

Oligonucleotides were obtained from Integrated DNA Technologies (IDT) with the following sequence: 5′-/5AmMC6-T_{25}-3′, where the 5′-end amino modifier, 5AmMC6, represents a phosphate bound to the terminal thymine that is linked to a terminal amine end-group by five methyl groups. Lyophilized samples were reconstituted and diluted with DNase/RNase-free water (Invitrogen). CoverWell perfusion chamber gaskets (9 mm diameter and 2 mm depth, Invitrogen) were cut to fit each 100 mm^2 sample to localize a region for oligonucleotide immobilizations. In the localized region, 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer with 0.9% NaCl (pH 4.7, Pierce), 100 mM 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), 100 mM *N*-hydroxysulfosuccinimide (sulfo-NHS), and oligonucleotides were applied to the activated CMDCS surface for 2 h at ambient temperature on an orbital shaker, and then thoroughly rinsed with MES buffer. Samples were stained in 1× SYBR Green II (SGII, Invitrogen) in 1× PBS for 15 min in the dark on an orbital shaking platform and then rinsed with copious MES buffer. Finally, samples were fixed on microscope slides with ProLong Gold antifade reagent (Invitrogen), stored in the dark to cure for at least 24 h, and analyzed within 72 h of slide preparation.

2.1.6. Isolation of GM15036 total RNA

The GM15036 normal human lymphoblastoid cell line (Coriell Institute for Medical Research) was cultured in RPMI 1640 (Gibco) supplemented with 15% FBS (Atlanta Biologicals), 1%