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miRNA purification with an optimized PDMS microdevice: Toward the direct purification of low abundant circulating biomarkers

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ABSTRACT

A reliable clinical assay based on circulating microRNAs (miRNAs) as biomarkers is highly required. Microdevices offer an attractive solution as a fast and inexpensive way of concentrating these biomarkers from a low sample volume. A previously developed polydimethylsiloxane (PDMS) microdevice able to purify and detect circulating miRNAs was here optimized. The optimization of the morphological and chemical surface properties by nanopatterning and functionalization is presented. Surfaces were firstly characterized by atomic force microscopy (AFM), X-ray photoelectron spectroscopy (XPS), fluorescamine assay and s-SDTB (sulphosuccinimidyl-4-o-(4,4-dimethoxytrityl) butyrate) assay and subsequently tested for their capacity to adsorb a fluorescent miRNA. From our analysis, modification of surface charge with 0.1% APTMS ((3-Aminopropyl) trimethoxysilane) and 0.9% PEG-s (2-[Methoxy-(polyethyleneoxy)propyl]trimethoxysilane) performed at 60 °C for 10 min was identified as the best purification condition. Our optimized microdevice integrated with real-time PCR detection, was demonstrated to selectively purify both synthetic and natural circulating miRNAs with a sensitivity of 0.01 pM.

1. Introduction

Circulating miRNAs are a class of endogenous small single-stranded RNAs (18–22 nt long) which emerged as key players in the post-transcriptional regulation of gene expression [1]. Synthesized in the cell nucleus, and active in the cytoplasm in the sequence-specific silencing of target messenger RNAs (mRNA), microRNAs can be secreted in circulation as cell-free entities. Circulating miRNAs, in fact, have been detected in virtually every body fluid including blood plasma [2,3]. According to current knowledge, circulating miRNAs are protected in the extracellular environment by vesicles (exosomes) [4] or protein complexes (e.g. AGO2) [5]. The negative regulation exerted by miRNA affects a variety of fundamental cellular processes including cell development and proliferation [6,7] and cell death [8]. These molecules have gained clinical relevance as their aberrant expression has been shown to correlate with the pathogenesis and progression of several diseases including cancer [9,10]. Among all, miR-21 is one of most studied 'oncomiR', whose overexpression has been detected in a variety of solid [11–14] and hematological tumors [15,16]. Due to their relevance combined to their easy accessibility, circulating miRNAs hold great potential as non-invasive biomarkers for the prognosis, diagnosis and assessment of response to treatment; for example, in cancer patients. Despite their potentialities, the development of a reliable assay based on circulating miRNAs is however mainly hindered by their low abundance in circulation, which is estimated to be around 0.017 pM in the case of the most abundant miRNA species [3,17,18]. In addition, the expression analysis of circulating miRNAs still presents many challenges, which are ascribable to a high degree of variability derived from individual factors and methodological approaches [19]. As a result, at present circulating miRNA expression levels are assessed by a plethora of complex and time-consuming laboratory protocols.

In this regard, a Lab-on-a-chip (LOC) approach could offer several advantages to the introduction of circulating miRNAs analysis in the clinical practice. Microfluidic microdevices, in fact, would allow to integrate in a cost-effective way a whole analytical process reducing its complexity, time of analysis, sample and reagents volumes. In this contest, a PDMS microdevice was previously developed in our lab for the purification and direct detection of cell-free miRNAs [20]. PDMS

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undoubtedly represents an attractive option for the realization of microdevices for biological applications. Its biocompatibility, its adaptability to a wide range of temperatures, chemical inertia, its propensity to be easily manufactured makes it an eligible cost-effective material for the making of disposable LOCs for clinical use [21–23]. Moreover, PDMS was demonstrated to have an intrinsic capacity of spontaneously and strongly adsorb nucleic acids [20,24]. This device is composed of a microchamber, which is fabricated by the assembly of a mold-cast droplet-shaped cavity and a PDMS support layer obtained by spin coating. Due to the facility of this material to undergo functionalization [25], Potrich et al. demonstrated that silanization with a positively-charged aminosilane significantly enhanced the adsorption of miRNAs to the internal surfaces of the microdevice. Based on this observation, the role of surface charge in the adsorption of miRNAs was indeed underlined. Moreover, it was shown that this peculiar interaction between miRNAs and PDMS was highly specific and could successfully be exploited for the extraction of circulating miRNAs from complex biological fluids [20]. Importantly, PDMS surfaces were found to simultaneously allow the purification and the detection of miRNA through a gold standard quantitative technique based on reverse transcription (RT) and real-time quantitative PCR (RT-qPCR). However, during deep characterization of internal surfaces of the microchamber, miRNAs adsorption was found to occur mainly on the lower surface obtained by spin coating, while the interaction of miRNA with the upper surface obtained by replica molding was found to be negligible. According to morphological investigations by atomic force microscopy (AFM), substantial differences in the nanometric roughness, with a significantly rougher upper surface, were detected. This striking difference in functionality between the two surfaces suggested that, beside surface charge, morphology can play a discriminative role in the interaction of miRNAs and that modulation of this parameter might positively affect its effectiveness of adsorption.

In this study, an in-depth analysis of the role of individual players, surface roughness and charge, involved in the adsorption of miRNAs was first of all analyzed on PDMS planar surfaces. The refinement of these surface properties is, in fact, necessary to improve the purification performances of the microdevice and lower its sensitivity in order to approach the detection of scarcely concentrated but informative circulating miRNAs. To tackle this issue, modifications of the PDMS surface morphology were performed by replica molding and AFM was employed for the characterization of the resulting nanopatterns. In a parallel analysis, the modulation of the positive surface charge was achieved by functionalization with increasing concentrations of APTMS ((3-Aminopropyl)trimethoxysilane) mix with a neutral PEG silane (2-[Methoxy(polyethyleneoxy)propyl]trimethoxysilane). To establish the entity of chemical modifications, X-ray photo electron spectroscopy (XPS), fluorescamine assay and s-SDTB (sulfosuccinimidyl-4-[2-(4,4-dimethoxytrityl)]butyrate) assay were applied to the panel of modified surfaces. Upon morphological and chemical modifications, their impact on the adsorption of miRNAs was established through a functional assay which makes use of a synthetic and fluorescent miRNA. Based on these outcomes, the most efficient modifications were applied to the microdevice and tested for their effectiveness in the purification of both synthetic and circulating miR-21 from real human plasma samples. Quantification of the purified miRNA was performed on-chip by RT-qPCR.

2. Material and methods

2.1. Materials

(3-Aminopropyl)trimethoxysilane (APTMS), ethanol 99.8%, fluorescamine powder ≥ 98% (TLC), and *N*,*N*-Dimethylformamide (DMF) were acquired from Sigma-Aldrich. 2-[Methoxy-(polyethyleneoxy)propyl]trimethoxysilane, tech-90, with 6–9 C_2H_4O units, called PEG-s, was acquired from Fluorochem (UK). Sulphosuccinimidyl-4-o-(4,4-dimethoxytrityl) butyrate (s-SDTB) powder was provided by Apollo Scientific (UK), while polydimethylsiloxane (PDMS) (SYLGARD® 184 elastomer) was obtained from Dow Corning Corporation (USA). Thermo Fisher Scientific supplied the TaqMan® MicroRNA Reverse Transcription Kit, the TaqMan® MicroRNA Assay-hsa-21, the TaqMan® Universal PCR Master Mix. Synthetic fluorescently-labelled hsa-miR-21 (5′-Alexa488-UAGCUUAUCAGACUGAUGUUGA-3′) was purchased from IDT Integrated DNA Technologies (Belgium). Distilled DNAse/RNase free water was acquired from Gibco™ (USA). Finally, the APL 1 buffer derived from the QIAamp UCP Pure Pathogen Blood kit, was provided by Qiagen (Germany).

2.2. PDMS planar surfaces fabrication

PDMS planar surfaces were produced in house either by spin coating or replica molding. In both procedures, 10 parts of PDMS base were mixed with 1 of its curing agent and the mixture was hand stirred for 5 min and subsequently degassed for 30 min under vacuum until bubble-free.

For the PDMS substrates obtained by spinning, silicon substrates ($1 \times 1\ cm^2$) were employed as support. Silicon plates underwent an argon plasma treatment of 2 min at 2 mbar with 6.8 W of power applied to the RF coil to remove organic contaminants and hydroxylate the surface, thus allowing PDMS to better adhere. Silicon substrates were subsequently coated with PDMS by means of a spin coater (Model WS 400B-6NPP/LITE, Laurell Technologies Corporations, USA) using the following protocol: 5 s at 2000 rpm and 1 min at 8000 rpm.

For the surfaces produced by replica molding, PDMS was mold-cast alternatively on three different silicon-based templates coated with a 3 nm film of titanium and an anti-adhesion aluminum layer (20 nm): native silicon oxide (here referred as N), Reactive Ion Etching (RIE)-treated silicon (called R) and Plasma-enhanced chemical vapor deposition (PECVD) silicon oxide (P). These masters ($1 \times 1\ cm^2$) were positioned on an aluminum holder, covered with PDMS mix, and degassed for 30 min under vacuum to remove any trapped air.

Both after spinning and mold casting, surfaces were cured in a convection oven (BINDER Inc., USA) to allow PDMS polymerization. In this study the curing step was performed at 110° or 130 °C for an hour.

2.3. PDMS planar surfaces functionalization and characterization

PDMS planar surfaces were functionalized with a range of silane mixtures containing APTMS and PEG-s silane through a wet functionalization procedure (Table 1). Prior to silanization, in order to favor the exposure of their hydroxyl groups, PDMS substrates were treated with a H2O:HCl:H2O2 = 5:1:1 (v/v/v) solution for 5 min at room temperature, washed intensively three times in ultrapure water and dried with a nitrogen flux. Shortly after hydroxylation, PDMS surfaces were incubated with the organosilane mixture of choice dissolved in ethanol absolute in a hermetic glass kettle for 10 min at 60 °C. For the mixtures with lower amounts of APTMS (< 1%), PEG-s was added to ensure a

Table 1
Nomenclature of the surfaces employed in this study. For each abbreviation the percentage content of APTMS (A) and PEG-s (P) used during the wet functionalization procedure is reported. A bare non-functionalized (NF) surface is intended as negative control.

Surface acronyms	% APTMS (v/v)	% PEG-s (v/v)
NF	0	0
1P	0	1
A-4	0.0001	0.9999
A-3	0.001	0.999
A-2	0.01	0.99
A-1	0.1	0.9
1A	1	0
10A	10	0