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Smart detection of microRNAs through fluorescence enhancement on a photonic crystal

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ABSTRACT

The detection of low abundant biomarkers, such as circulating microRNAs, demands innovative detection methods with increased resolution, sensitivity and specificity. Here, a biofunctional surface was implemented for the selective capture of microRNAs, which were detected through fluorescence enhancement directly on a photonic crystal. To set up the optimal biofunctional surface, epoxy-coated commercially available microscope slides were spotted with specific anti-microRNA probes. The optimal concentration of probe as well as of passivating agent were selected and employed for titrating the microRNA hybridization. Cross-hybridization of different microRNAs was also tested, resulting negligible. Once optimized, the protocol was adapted to the photonic crystal surface, where fluorescent synthetic miR-16 was hybridized and imaged with a dedicated equipment. The photonic crystal consists of a dielectric multilayer patterned with a grating structure. In this way, it is possible to take advantage from both a resonant excitation of fluorophores and an angularly redirection of the emitted radiation. As a result, a significant fluorescence enhancement due to the resonant structure is collected from the patterned photonic crystal with respect to the outer non-structured surface. The dedicated read-out system is compact and based on a wide-field imaging detection, with little or no optical alignment issues, which makes this approach particularly interesting for further development such as for example in microarray-type bioassays.

1. Introduction

MicroRNAs are short (18–24 nucleotides), single-stranded non-coding RNAs which act as post-transcriptional regulators of gene expression [1,2]. The alteration of microRNAs expression has been associated to a variety of pathological conditions such as cancer [3–5], neurodegeneration [6], cardiovascular disease [7], Hepatitis B virus infection [8], allergic inflammation and asthma [9], among others. Since microRNAs can be extracted from serum, plasma and other body fluids and their stability is not dramatically influenced by the storage conditions, they are considered as privileged biomarkers of pathologies [10–12]. However, due to their low abundance, detecting microRNAs is one of the new challenges of analytical technologies. A method that could enhance the microRNAs detection signal would therefore be of great interest.

Standard detection techniques of microRNAs rely on quantitative real-time reverse transcription polymerase chain reaction (qPCR), which could introduce bias due to the lack of an established normalization strategy [13] as well as to several other bias [14,15]. On the other hand, microRNAs analysis through microarray technology is attractive for high throughput and multiplex analysis [16], but the sensitivity and the normalization of hybridization efficiency are still demanding. In this context, the development of surface structures and detection instruments that can enhance the detection sensitivity of fluorescent assays would have two important outcomes: (1) increasing the signal/noise ratio of detection resulting in a greater statistical significance and (2) reducing the detection limits [17–20]. Although several optical methods have been proposed in the past demonstrating detection capabilities down to the single molecule level, the use of chip-

Abbreviation: PC, Photonic crystal; NA, Numerical aperture; miR, MicroRNA; GPTMS, 3-Glycidoxypropyltrimethoxysilane; Saline-sodium citrate (SSC) buffer 1 ×, 150 mM sodium chloride, 15 mM trisodium citrate (adjusted to pH 7.0 with HCl)

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based devices has attracted much interest because of their generally advantageous simplicity of use. For example, dielectric or metallic photonic surfaces are known to enhance or improve the fluorescence emission from biological assays [21–23].

The fluorescence enhancement mediated by a patterned one-dimensional photonic crystal (1DPC) with a suitable surface functionalization could increase both the sensitivity and the specificity of biomarker analysis [24]. The optimization of the surface functionalization is indeed crucial in order to achieve a highly reliable detection, since the probe immobilization as well as the passivation step and all the experimental conditions of the assay influence the sensitivity, specificity and detection limit of a biosensor. For this reason, this paper focuses on the preparation of an optimal functional surface and on the refinement of the hybridization protocols in order to set up the best experimental conditions for photonic crystal testing. Every step was carefully set up on commercially available epoxy-slides before moving to the photonic crystal. A proof-of-concept of the huge potentialities of this type of detection is reported. The photonic system here described is compact and could therefore be easily included in a reliable point-of-care device with well-defined diagnostic applications.

2. Materials and methods

2.1. Materials

3-Glycidoxypropyltrimethoxysilane (GPTMS), toluene and toluene anhydrous (99.8%), powders for buffered solutions and salmon sperm DNA were purchased from Sigma-Aldrich (USA) and used without any further purification. Anti-miR16 probes, i.e. DNA complementary to miR-16 with a reactive amino group at the 5′ end (anti-miR-16, Table 1), the same sequence labeled at the 3′ end (anti-miR-16 TAM), and the synthetic miR-16 functionalized at the 5′ end with a fluorescent dye (Alexa-546) were also purchased from Sigma-Aldrich (USA). Synthetic miR-21, miR-1246 and miR-1290 were produced by IDT Integrated DNA Technologies (Leuven, Belgium). NEXTERION® Slide E (Epoxysilane coating) and Nexterion hyb buffer were purchased from SCHOTT (Germany). Sequences of probe and target miR are reported in Table 1.

2.2. Photonic structure and optical setup

The photonic structure is a dielectric multilayer that is surface-patterned with an array of linear gratings. The use of such a photonic substrate allows an enhancement and a beaming of the emitted radiation toward the observer, in such a way that the fluorescence collection is largely improved. Twenty alternating layers of Ta_2O_5 and SiO_2, with ($n=2.1$) and low ($n=1.45$) refractive index respectively are deposited on a thin glass substrate by plasma ion assisted process under high vacuum conditions. The Ta_2O_5 and SiO_2 layers are 95 and 137 nm thick respectively, while the top SiO_2 layer is 127 nm thick. On top of the stack a pattern of linear gratings with a period of $\lambda=530$ nm is fabricated by electron beam lithography, followed by a sputter deposition of SiO_2 (K.J. Lesker PVD 75 DC Magnetron Sputtering). Gratings are 250 μm × 250 μm in size and 100 nm high. The detection is performed by means of a simplified fluorescence microscope, wherein fluorescence is excited with a doubled frequency Nd:YAG laser beam (wavelength $\lambda=532$ nm) that is collimated onto the sample through a NA=0.1 objective. The laser homogeneously illuminates a single grating. Illumination is incident from the glass substrate and can be polarized either parallel or perpendicular to the grating groves. This arrangement allows for the on/off switching of the resonant excitation of fluorescence through the coupling to the photonic crystal optical modes. On the air-side, the fluorescence emitted in the free-space is collected by a NA=0.2 objective. After laser filtering, a fluorescent image of the photonic crystal surface is obtained. An analyzer is also employed for polarization-filtering of the collected fluorescence.

Table 1
Sequences of probe and target miRNAs.

Name	**Sequence** (5′–3′)
Anti-miR-16	[AmC6F]CGCCAATATTTACGTGCTGCTA
Anti-miR-16 TAM	[AmC6F]CGCCAATATTTACGTGCTGCTA[TAM]
miR-16	[A546]UAGCAGCACGUAAAUAUUGGCG
miR-21	UAGCUUAUCAGACUGAUGUUGA
miR-1246	AAUGGAUUUUUGGAGCAGG
miR-1290	UGGAUUUUUGGAUCAGGGA

2.3. Set up of probe-immobilization and hybridization conditions

2.3.1. On epoxy-coated microscope slides

The anti-miR probe was directly immobilized on slides with a microarray spotter (BioOdyssey™ Calligrapher™ Miniarray Spotter, Biorad) in custom arrays with a capillary pin having a diameter of 360 μm. The probe concentration spanned a range from 3 to 200 μM and was immobilized in 0.5 M sodium phosphate pH 7.4. Before immobilization, the probes were thermally treated (95 °C for 1 min) in order to unfold the sequences and promote the covalent bonding to the epoxy groups on the surface. After spotting, the slides were left overnight in the spotter chamber with 65% controlled humidity. Then, the slides were washed in the same immobilization buffer and passivated in ethanolamine (1–100 mM concentration) for 30 min. After washing in 0.5 M sodium phosphate pH 7.4 and ultrapure water, different concentrations of microRNAs (0.5–1000 nM) were diluted in Nexterion hyb buffer and thermally treated at 60 °C for 1 min. Next, 0.1 μg/μl of Salmon Sperm DNA was added to the solution and incubated at 48 °C for 1 h in a hybridization chamber (Corning) in dark. The excess of microRNA was removed by washing the slides in 2 × SSC+0.1% SDS, 2 × SSC, 1 × SSC, and finally in ultrapure water. The fluorescence of slides was measured with the fluorescence microscope Leica DMLA (Leica Microsystems, Germany), equipped with a mercury lamp and fluorescence filter N2.1 (Leica Microsystems, Germany). A cooled CCD camera (DFC 420C, Leica Microsystems, Germany) was used to acquire the images, which were analyzed with Fiji software [25].

For the quantification of microRNA hybridized on slides, 100 μM anti-miR probe was spotted on epoxy-slides which were passivated with 1 mM ethanolamine and used for the hybridization of 0.5 μM miR-16_AF546 in the conditions described above. After washes, the slides were imaged with the fluorescence microscope. A dehybridization solution (10 mM Tris/HCl+0.1M NaOH) was added for 5 min at room temperature in order to remove the hybridized miR-16, which was collected and measured with a spectrofluorimeter (FluoroMax-4, Horiba Jobin Yvon), using an excitation wavelength of 550 nm and recording the emission spectrum from 560 to 800 nm. This step was repeated two times. The area between 570 and 590 nm was integrated and the obtained value was compared to a calibration curve, previously worked out.

2.3.2. On the photonic crystal

Immediately before silanization, the photonic crystal was cleaned with a pirana solution (3:1 v/v of H_2SO_4:H_2O_2) to remove organic contaminants. Then, the photonic crystal was treated with 0.01% (v/v) GPTMS in toluene anhydrous at 60 °C for 10 min, washed several times in toluene and dried in a stream of nitrogen,