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Rapid and sensitive detection of multiple microRNAs in cell lysate by low-fouling surface plasmon resonance biosensor

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ABSTRACT

We report an ultra-low fouling surface plasmon resonance imaging (SPRi) biosensor for the rapid simultaneous detection of multiple miRNAs in erythrocyte lysate (EL) at subpicomolar levels without need of RNA extraction. The SPRi chips were coated with ultra-low fouling functionalizable poly(carboxybetaine acrylamide) (pCBAA) brushes having optimized thicknesses and directly functionalized with amino-modified oligonucleotide probes. We have characterized the effect of the brush thickness on the probe loading capacity: a loading capacity of $\sim 9.8 \times 10^{12}$ probes/cm^2 was achieved for pCBAA having a thickness of ~40 nm. The probe-functionalized sensor also exhibited a high resistance to fouling from ~90% EL samples (< 2 ng/cm^2). A two-step detection assay was employed for multiplexed miRNA detection in EL. Specifically, the assay consisted of (i) a sandwich-type hybridization of the probe-functionalized pCBAA with target miRNA in EL (bound to biotinylated oligonucleotides) and (ii) the capture of streptavidin-functionalized gold nanoparticles to the aforementioned biotinylated probes. We have demonstrated that this approach enables the detection of miRNAs in EL at concentrations as low as 0.5 pM. Finally, we have confirmed the detection of four endogenous miRNAs representing a set of potential miRNA biomarkers of myelodysplastic syndrome (MDS) in clinical EL samples (miR-16, miR-181, miR-34a, and miR-125b). The results revealed significantly higher levels of miR-16 in all the clinical EL samples compared to the other measured miRNAs.

1. Introduction

Mature microribonucleic acids (miRNAs) are ~19–24 nucleotide long noncoding RNAs that regulate gene expression in living cells by mediating targeted hydrolysis and translation inhibition of messenger RNAs (He and Hannon, 2004). The miRNAs present in erythrocyte lysate (EL) play an important role in erythropoiesis and final mRNA degradation (Farh et al., 2005; Felli et al., 2005; Georgantas et al., 2007). Although the complex mechanism of miRNA-induced regulation of gene expression is still not fully understood, numerous intracellular and circulating miRNAs have been linked to disease outcomes and prognosis (Iorio et al., 2005; Lu et al., 2005). Moreover, there has been increasing evidence over the last five years suggesting that changes in miRNA levels in bodily fluids and tissues are closely associated with the pathogenesis of most human malignancies, including myelodysplastic syndrome (MDS) or cardiovascular disease (Erdogan et al., 2011; Ikeda et al., 2007; Iorio et al., 2005; Lu et al., 2005; Wang et al., 2009).

Accordingly, miRNAs represent an attractive target as potential disease biomarkers. Although methods for the detection of nucleic acids are well established, there remain great challenges for the accurate and reliable quantification of miRNAs in bodily fluids. These challenges include miRNAs having short sequences, low concentrations of target miRNAs in analyzed samples, and interferences from complex sample matrices (Engels and Hutvagner, 2006; Gao et al., 2014).

Conventional methods for the detection of miRNA include quantitative reverse-transcription real-time polymerase chain reaction (RT-qPCR), microarrays, and northern blotting (Benes and Castoldi, 2010; Lagos-Quintana et al., 2002; Thomson et al., 2004; Wark et al., 2008). Unlike semi-quantitative northern blotting-based approaches (Kuchar et al., 2014; Valoczi et al., 2004), RT-qPCR can cover a broad dynamic range of miRNA concentrations with a relatively high sensitivity. Unfortunately, this method is rather time consuming and requires expensive and complex

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equipment. Furthermore, because highly purified samples are required, the extraction of miRNA from complex biological samples is necessary. Due to the lack of standardized protocols for miRNA extraction, this extraction step may lead to increases in variability and decreases in accuracy (McAlexander et al., 2013; Turchinovich et al., 2012). Microarrays enable parallelized detection of multiple miRNAs (Thomson et al., 2004); however, the complexity of miRNA labeling, insufficient sensitivity, long hybridization times, or challenges for standardization limit the routine use of microarrays in centralized laboratories (Liu et al., 2004).

Several biosensing methods have been proposed as alternative methods to detect miRNAs. These biosensors include those based on (photo)electrochemistry (Dong et al., 2012; Gao et al., 2013; Ge et al., 2014; Kilic et al., 2012; Li et al., 2014; Yin et al., 2014, 2012; Zhu et al., 2014), electrochemiluminiscence (Cheng et al., 2014; Liu et al., 2014), fluorescence quenching (Dong et al., 2014; Guo et al. ,2014; Ryoo et al., 2013), and surface plasmon resonance (SPR) (Sipova et al., 2010; Zhang et al., 2013). The majority of these studies involve the detection of miRNA either in buffer, diluted complex bodily fluids, or in aqueous solutions of reconstituted RNA extracts (Dong et al., 2014; Wen et al., 2013; Yin et al., 2014). Only a few of these studies involve the detection of miRNA directly in a complex biological sample (without the sample pre-treatment miRNA extraction step). For instance, Yin et al. reported the ultrasensitive detection of miR-21 in diluted human serum (LOD of 100aM for miR-21 detection in a buffer) using an electrochemical biosensor; however, this technique had rather long detection time (>4 h) (Yin et al., 2012). In addition, Ryoo et al. recently reported a fluorescence label-based optical sensor that utilized peptide nucleic acid (PNA) and nano-sized graphene oxide. The sensor was capable of multiplexed detection of miRNAs at picomolar levels in living cells (Ryoo et al., 2013).

In this work, we present a label-free optical biosensor based on surface plasmon resonance imaging (SPRi) that allows for rapid (<1 h) and multiplexed detection of miRNAs in EL samples without the need for miRNA extraction or pre-amplification. The sensor employs carboxy-functional ultra-low fouling polymer brushes with amino-modified probes for a two-step assay, which utilizes sandwich-type hybridization in EL in addition to signal amplification by streptavidin-functionalized gold nanoparticles (S-AuNPs). We explore the effects of the brush thickness on the surface loading capacity and the resistance to fouling from EL. We targeted four miRNAs (miR-16, miR-181, miR-34a, and miR-125b) that represent potential myelodysplastic syndrome (MDS) biomarkers (McAlexander et al., 2013; Peng and Gao, 2011; Rhyasen and Starczynowski, 2012). After establishing a set of calibration curves for these miRNAs spiked in EL, we screened three clinical EL samples for the levels of native endogenous miRNAs.

2. Materials and methods

2.1. Reagents and erythrocyte lysate samples

The list of reagents used in this work and procedure of preparation of erythrocyte lysate samples is provided in the Supplementary material. Spherical gold nanoparticles (AuNPs) with a diameter of 35 nm were prepared by the reduction of $HAuCl_4$ with sodium citrate (Bastus et al., 2011). Bare AuNPs were modified with carboxyl-terminated alkanethiols and then streptavidin was covalently bound to the surface of AuNPs by amine coupling chemistry (see Supplementary material).

2.2. SPR sensors

Two SPR systems developed at the Institute of Photonics and Electronics (Prague, Czech Republic) were used in this study. A high-resolution SPR imaging (SPRi) system with polarization contrast and internal referencing (Piliarik et al., 2010) combined with dispersionless microfluidics (Springer et al., 2010) was used in the detection experiments. This system enables the simultaneous analysis of biomolecular interactions in 30 individual flow-through sensing spots by using two separate flow-cells with perpendicularly oriented flow chambers. The first (six-channel) flow-cell was used for surface functionalization, while the second (with six perpendicularly oriented channels) was used for the detection (see also Supplementary material). A four-channel spectroscopic SPR sensor (Homola et al., 2002; Vaisocherova et al., 2014) was used for the optimization of surface functionalization, fouling studies, and assay control experiments. Both SPR sensors were equipped with a temperature controller, enabling SPR measurements in a temperature interval of 5–40 °C with a baseline stability of 0.01 °C. In order to compensate for the effect of decreasing SPR sensitivity with increasing layer thickness (Homola, 2008), the SPR sensor response obtained from both sensors was calibrated using previously described procedures (Piliarik et al., 2010; Vaisocherova et al., 2014). A typical value of the correction factor for bare polymer brush thickness was 1.29 for a 30 nm thick pCBAA (RI = 1.401 RIU at 750 nm for wet surface (Vaisocherova et al., 2014)). For the SPR sensors used in this study and a resonant wavelength of about 750 nm, a 1 mRIU SPRi sensor response corresponds to a $\sim$5.9 nm response of the spectroscopic SPR sensor (Piliarik et al., 2010). In terms of surface coverage, a SPRi sensor response of 1 mRIU corresponds to a change in the surface coverage of $\sim$100 ng/cm^2 (Homola, 2006; Vaisocherova et al., 2007).

2.3. SPR sensor measurements

The poly(carboxybetaine acrylamide) (pCBAA) coatings were prepared by atom transfer radical polymerization (ATRP) according to a previously described procedure (Vaisocherova et al., 2008). These procedures were carried out under optimized conditions in order to obtain coatings with a wet thickness in the range of 13–45 nm (Supplementary material). The thickness of wet brushes was measured by means of the spectroscopic SPR sensor via a previously described procedure (Vaisocherova et al., 2014).

The pCBAA surface functionalization was performed using a flow-through regime without extra spotting. The procedure consisted of three steps: (1) activation of the functional carboxyl groups by the NHS/EDC method adjusted to the physical–chemical properties of pCBAA (Vaisocherova et al., 2014), (2) covalent attachment of amino-modified oligonucleotide probes ($probe_1$ in Fig. 2), and (3) deactivation of any residual reactive groups. The optimized procedure for surface functionalization is provided in Supplementary material.

In the spiked miRNA detection experiments, the sample with miRNA was mixed with biotin-terminated probes ($probe_2$) having a complementary base sequence to the target miRNAs and flowed along the $probe_1$-functionalized pCBAA surface at 15 °C for 10 min (step I. in Fig. 2). This step resulted in the formation of stable miRNA*$probe_1$–$probe_2$ complexes on the pCBAA-coated sensor surface. In the second step of the assay, the sensor response to miRNA was enhanced with S-AuNPs. The S-AuNPs were flowed along the surface for 25 min at 15 °C and bound to $probe_2$ via the streptavidin–biotin interaction (step II. in Fig. 2).

In order to obtain the calibration curves, we carried out the detection of four miRNA spiked samples (miR-16, miR-34a, miR-181, and miR-125b) in EL or PBS at target concentrations of 0.1 pM, 0.5 pM, 1 pM, 100 pM, and 500 pM. Specifically, the undiluted EL sample taken from a healthy individual was spiked with aliquots of EDTA (5 mM), NaCl (150 mM), the biotin-terminated probes to