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Multiplexed, label-free detection of biomarkers using aptamers and Tunable Resistive Pulse Sensing (AptaTRPS)

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ABSTRACT

Diagnostics that are capable of detecting multiple biomarkers are improving the accuracy and efficiency of bioassays. In previous work we have demonstrated the potential of an aptamer-based sensor (aptasensor) utilising Tunable Resistive Pulse Sensing (TRPS). Here, we have advanced the technique identifying key experimental designs for potential POC assays. The assay utilised superparamagnetic beads, and using TRPS monitored their translocations through a pore. If the surfaces of the beads are modified with an aptamer, the frequency of beads (translocations/min) through the pore can be related to the concentration of specific proteins in the solution. Herein, we have demonstrated the successful use of TRPS to observe the binding of two proteins, to their specific aptamers simultaneously. We describe a series of experiments illustrating key factors which we believe are integral to bead-based assays and demonstrate a general method for a multiplexed assay. In summary, we have explored the effects of beads size, concentration, potential bias, pH and aptamer affinity to enhance the sensitivity and practically of a TRPS aptasensor. The method utilises the fact the binding of the aptamer to the protein results in a change in charge density on the bead surface, the isoelectric point of the protein then dominates the mobility of the beads, creating a multiplexed assay termed AptaTRPS. By alteration of the applied potential to the instrument it is possible to produce a positive signal in a simple multiplexed assay.

1. Introduction

In recent decades there has been an increase in interest in rapid, affordable and user-friendly diagnostic techniques; driven by the rise in interest in personalised medicine, reduced assay times and the desire to provide home diagnostics (Chan et al., 2013). To make personalised medicine available to everyone, it is necessary to minimise the cost and labour intensity of assays so that they are affordable and available regardless of geographical location (Yager et al., 2008). Additionally, it is also desirable to minimise the time taken to yield results of an assay and to move towards point-of-care (POC) diagnostics and monitoring to improve decision-making. Key considerations in the choice of an appropriate technology are the ability to multiplex, assay time, cost, reliability and simplicity (Song et al., 2014).

Gold standard methods of analysis still heavily rely on PCR or antibody based technologies. A number of concerns remain with antibodies, as they: add cost and complexity to any assay; require the use of animals or cell culture for their generation; are susceptible to batch-to-batch variations; cause difficulties in handling and storage; and can have less than ideal sensitivity (Ledur et al., 1995; Tombelli et al., 2005). Their integration in POC technologies often gives the device a limited shelf life, and test substrates require refrigeration. A viable and accessible alternative to antibodies, addressing these concerns, would be a transformative technology. Aptamers are rapidly gaining interest as one alternative to antibodies (Fang and Tan, 2010; Levy-Nissenbaum et al., 2008; Mayer, 2009; Rowe et al., 2009). They offer an increased stability and ease of synthesis, they are far more cost-effective and reliable than their antibody counterparts and can easily be incorporated into diagnostic platforms. Full reviews on aptasensors can be found (Chiu and Huang, 2009; Cho et al., 2009; de-los-Santos-Álvarez et al., 2008; Famulok and Mayer, 2011) and whilst they have been criticised for repetition of choice in targets (Cho et al., 2009), this is likely due to authors keeping with tried and tested high affinity ligands to enable greater comparative power between new platforms.

One of the more recent technologies to implement aptamers is Resistive Pulse Sensing (RPS) (Rotem et al., 2012). A more recent form of RPS uses a polyurethane elastomeric membrane and is known as Tunable Resistive Pulse Sensing (TRPS) (Platt et al., 2012) in which the pore is able to be mechanically manipulated in real time to alter pore geometry (Roberts et al., 2010; Vogel et al., 2011). In brief, the set up and theory for RPS technologies is as

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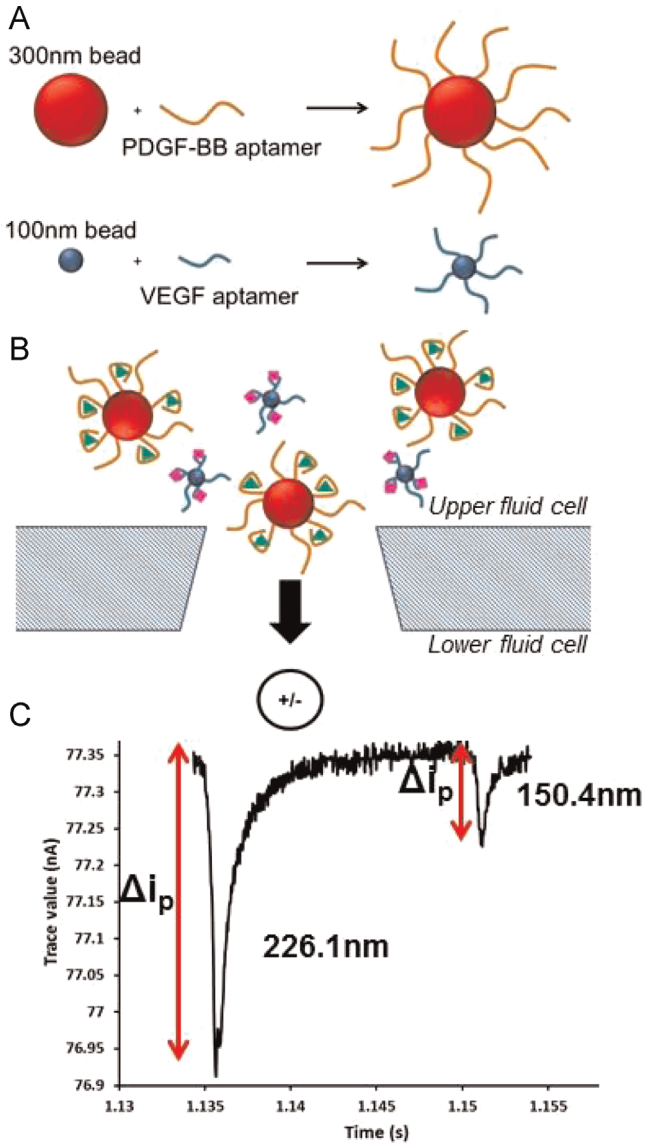

Fig. 1. (A) Schematic of the multiplexed experimental process; two beads of different sizes are conjugated to either VEGF or PDGF. (B) Beads are incubated with a combination of concentrations of VEGF and PDGF for 30 min; as they bind to the target their velocity and frequency through the pore is monitored. (C) Recorded "resistive peaks" for two different sized beads; the blockade magnitude (Δi_p) of each peak is proportional to the volume of each bead and can therefore be calibrated to provide the diameter of each individual bead.

follows: a stable ionic current is established by two electrodes, separated by a pore; as beads/analytes translocate the pore they temporarily occlude ions, leading to a transient decrease in potential known as a "blockade event", examples of which can be seen in Fig. 1C. In the current arrangement, the pore is mounted laterally so that particles typically move from the upper fluid cell into the lower fluid cell, aided by an inherent pressure head due to 40 µl of liquid in the upper fluid cell of approximately 50 Pa (Willmott et al., 2012), and a positive or negative bias is applied via an electrode under the pore (see Fig. 1B). By monitoring changes in full width half maximum (FWHM), peak magnitude (Δi_p) and peak frequency (events/min) it is possible to elucidate the zeta potential (Vogel et al., 2012), size (Vogel et al., 2011), and concentration (Willmott et al., 2010) of colloidal dispersions in situ, as described in more detail elsewhere (Vogel et al., 2011). TRPS is well suited to detect DNA and DNA-protein interactions; previous work has provided proof-of-concept studies on the technology (Billinge et al., 2014) in which the velocity and frequency of aptamer-tagged beads changes upon the addition of the target analyte as the aptamer folds to a specific conformation whereby the target binds, shielding the surface charge and altering double layer structure, as has been demonstrated with the target thrombin (Billinge et al., 2014). A significant development to this method would be the ability to detect multiple proteins at once.

In the present study we develop an aptasensor to haemostatic proteins Vascular Endothelial Growth Factor (VEGF) and Platelet Derived Growth Factor (PDGF) which are both involved in various disease processes such as cancer (Wei et al., 2003), atherosclerosis (Matsumoto and Mugishima, 2006), and Alzheimer's (Tarkowski et al., 2002). Although circulating levels of VEGF have been found to be variable between individuals, between 9–150 ng/L (Jelkmann, 2001), VEGF levels have been demonstrated to be elevated in several aggressive types of cancer, such as ovarian (Li et al., 2004), haematological (Belgore et al., 2001) and gastric carcinoma (Karayiannakis et al., 2002) and VEGF measurement has been highlighted as both a potential prognostic or diagnostic marker (Jelkmann, 2001) and for comparison between pre- and post-operative cancer states (Karayiannakis et al., 2002; Li et al., 2004). PDGF levels have also been found to be elevated in several different forms of cancer and has recently been added to the Glasgow Prognostic Index, which measures inflammatory mediators, as an important indication of tumour and significant predictor of survival (Hamilton et al., 2014). Previous studies have measured both VEGF and PDGF levels in cancer patients undergoing anti-VEGF treatment (Madsen et al., 2012), however this was performed over two different systems, increasing the cost and time considerations of the assay.

To monitor two analytes simultaneously in one-step analysis we use the ability of TRPS to provide accurate particle-by-particle analysis. Its ability to accurately calculate the beads size as it translocates the pore, allows the data from each particle set to be separated according to their size. By using two differently sized beads and separately functionalising them with anti-VEGF or anti-PDGF aptamers, as displayed in Fig. 1A, it is possible to separate the individual signals based on Δi_p, Fig. 1C, and monitor changes to their frequency and speed, relating them back to the target analyte concentration.

2. Materials and methods

2.1. Chemicals and reagents

The following chemicals were sourced from Sigma-Aldrich, UK, without any further purification unless otherwise stated: Phosphate Buffered Saline (PBS – P4417), Tween 20 (P1379), Bovine Serum Albumin (BSA – A2153), and thrombin (T7513). DNA sequences were obtained from Sigma-Aldrich's custom oligonucleotides service as lyophilised powders and made up to a stock concentration of 100 pmol/µL: 5′ CAG GCT ACG GCA CGT AGA GCA TCA CCA TGA TCC TG-Biotin-3′ (anti-PDGF-BB aptamer) (Deng et al., 2013), 5′ ATA CCA GTC TAT TCA ATT GGG CCC GTC CGT ATG GTG GGT GTG CTG GCC AG-Biotin-3′ (VEa5 anti-VEGF aptamer) (Hasegawa et al., 2008). 5′ TGT GGG GGT GGA CGG GCC GGG TAG ATT TTT-Biotin-3′ (V7t1 anti-VEGF aptamer) (Nonaka et al., 2010). Human recombinant PDGF-BB and human $VEGF_{165}$ were purchased from Life Technologies, UK (PHG0044 and PHC9394, respectively). Water purified to a resistivity of 18.2 MΩ cm (Maxima) was used to make all solutions unless otherwise specified and 1 × PBST (0.05% Tween) was used as the buffer.