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Novel surface plasmon resonance sensor for the detection of heme at biological levels via highly selective recognition by apo-hemoglobin ☆

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ABSTRACT

We have developed a novel surface plasmon resonance (SPR) biosensor for heme detection that utilizes the reconstitution of the heme cofactor with apohemoglobin (apoHb), hemoglobin from which the heme has been removed, as the sensing mechanism. The binding is highly specific, efficient and generated very strong SPR signals. This is the first report that uses immobilization of the apoprotein in a hydrophilic polymer matrix and senses the corresponding cofactor by SPR. This is also the first report of high sensitivity heme detection in real time by SPR and the sensing surface is re-generated many times without loss of sensitivity or selectivity. The sensing surface was fabricated by covalent immobilization of hemoglobin in a polyacrylic acid matrix in situ, which allowed for a high concentration of protein to be located in the plasmon detection range on the Au chip. Removal of the heme from the hemoglobin–polymer conjugate (Hb–PAA) resulted in a surface anchored apoHb–polymer conjugate. The limit of detection was approximately 2 μM or 1.30 μg/mL, which is relevant for biological heme levels (1–50 μM for hemolytic pathological conditions). This apoHb–polyacrylic acid system demonstrates a new concept in SPR detection with the use of protein cofactor binding pockets for analyte detection. The methodology that we developed here may be extended for the detection of a number of other cofactor molecules with high sensitivity, selectivity and low detection limits. In future, such sensors could be useful for the development of point-of-care devices to detect biologically important small molecules.

1. Introduction

The growing importance of early detection presents a need for fast, inexpensive, rapid, disposable and sensitive biosensors that reach ultralow detection limits while maintaining high stability, selectivity and suitability for point-of-care use. Development of such devices allows for a widespread and preventative disease detection, epidemic control and treatment, especially in developing countries. For example, malaria plagues certain parts of the world which is partly due to the difficulty of providing prompt diagnosis, access to laboratory support and high cost of diagnosis [1]. Malaria diagnostics would greatly benefit from an immediate point of care diagnosis of this debilitating tropical disease, as the current gold standard test for malaria detection is microscopy of blood stain films to confirm the presence of malaria parasites by a trained pathologist [2]. An alternative method for malaria diagnosis can be the detection of elevated heme levels in blood serum. Initial diagnosis of malaria could greatly benefit from a quick, reliable, easy detection method, at low cost. Another motivation is to develop rapid, reliable detection of heme to investigate its role in other pathological conditions such as ischemia reperfusion, sickle cell disease and porphyria [3–5]. Biological elevated levels of heme during hemolytic processes are in the range of 1–50 μM [6–9]. Heme detection is also relevant for the investigation of a range of cellular biological functions such as cellular signaling [4].

Presently, methods exist for heme detection but require the use of toxic reagents, strict storage conditions or complex instrumentation, all of which do not relate well to reliable, low cost, high sensitivity, multiplexed, real-time testing [1,10].

Abbreviations: SPR, Surface plasmon resonance; apoHb, Apohemoglobin; Hb–PAA, Hemoglobin–polyacrylic acid conjugate; TNT, Trinitrotoluene; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; NHS, N-hydroxysuccinimide; PAA, Polyacrylic acid; PEG, Polyethylene glycol; SAM, Self-assembled monolayer; μRIU, Micro refractive index unit; PBS, Phosphate buffered saline; HPLC, High performance liquid chromatography; MS, Mass spectrometry; ANS, 1-anilino-8-napthalene sulfonate

☆ *Novelty statement*: This is the first report that demonstrates a new concept in SPR detection with the use of protein cofactor binding pockets for heme detection. This is also the first report of high sensitivity heme detection in real time by SPR.

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For example, the most commonly used heme detection method, pyridine hemochrome, utilizes an excess of pyridine and heme in the reduced state to form a strongly absorbing hemochromogen complex [11]. The heme concentration is determined from the absorbance of the hemochromogen at 557 nm [9]. Spectrophotometric methods are limited by sample pureness and can require various separation requirements and also do not provide real-time, automated analysis.

SPR-based approach for heme sensing would provide a technique that offers rapid real time measurements and they could be translated to point-of-care detection [12,13]. SPR utilizes the surface plasmon wave generated when an intense beam of laser light passes through a prism and hits gold surface (Scheme 1A). The plasmon wave propagates ~300 nm above the gold surface. Therefore, when binding events occur at the Au surface, the refractive index changes and this in turn shifts the SPR resonance angle. SPR is a highly sensitive, real-time method for the detection of a variety of ligands and proteins. Use of SPR also voids the need for labeling of the analyte, such as fluorescent tags, which can increase the number of steps required to process samples. SPR is most commonly used in the biological field for studying protein–protein interactions, protein–antibody and ligand binding kinetics [14–18]. SPR is also used as an analytical sensing technique in food monitoring, medical diagnostics and environmental testing [17,19]. Most often, an antibody or aptamer is tethered to the Au surface and SPR signal is generated upon selective binding of an antigen or the pathogen, drug, hormone or a protein [20–22]. The SPR signal is dependent on the analyte molecular size, as well as concentration.

Surface plasmon resonance has been used to reach low levels of detection (attomolar) for important biomarkers such as prostate specific antigen [23] and carcinoembryonic antigen [24], both of which are larger molecular weight analytes. However, detection of small molecules having a molecular weight less than a few thousands by SPR is difficult due to the small change in refractive index upon binding to the surface-bound biorecognition element. Often, indirect competitive immunoassays are used to detect small molecules such as trinitrotoluene (TNT) and 2-hydroxybiphenyl [13,25]. In the case of TNT detection, an antibody specific to TNT was used in conjunction with TNT–ovalbumin conjugate as a surrogate ligand. In the absence of TNT, the antibody and protein bind and give rise to a fairly large SPR signal. Although, when TNT is present the binding event is blocked and the SPR signal is decreased. [25]. Many SPR assays utilize a "sandwich method" or extra macromolecule to enhance the signal obtained from small molecule binding events. Molecularly imprinted hydrogels have also been used as a method for the development of a high sensitivity glucose SPR sensor [26]. With small molecule detection by SPR, a post-binding amplification strategy is commonly employed to enhance the SPR signal. Examples of these include nanoparticles [27–30], secondary antibodies and catalyzed precipitation reactions [13,31,32], which are coupled to the analyte binding on the SPR chip.

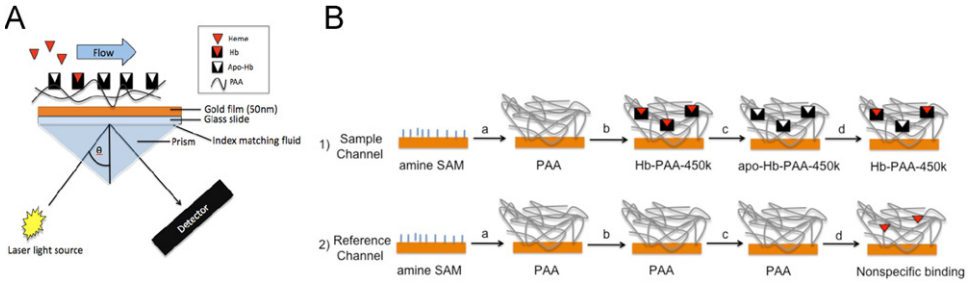
We recently demonstrated the stability and biological function of hemoglobin–polyacrylic acid (Hb–PAA) conjugates. The polymer and the protein are attached to each other randomly, which resulted in nanonetworks and these networks provided new opportunities for sensing applications. The use of PAA as an immobilization matrix for Hb is beneficial in providing multiple attachment points, improved mechanical stability and water retention within the protein–polymer film on the Au-chip surface. The use of PAA allows for a range of potential chemistries that can be used to alter PAA properties such as hydrophobic modification. We have previously shown that PAA covalently attached to Hb enhances thermal stability of Hb [33].

In this work, highly specific heme-binding pocket of apohemoglobin (apoHb) was chosen to develop the SPR sensor. The principle of detection is quite simple. It involves: (a) the removal of heme from Hb followed by, (b) exposure of the heme-depleted Hb (apoHb) to solutions of free heme, which resulted in the reconstitution of heme with apoHb, its natural host. This reconstitution event provides an excellent signal. Here, we show that the apoHb–PAA conjugate can be used effectively for the sensitive, selective and rapid detection of heme in aqueous solutions. This is the first report of SPR-detection of heme, where we take advantage of reversible heme binding to the apoHb–PAA conjugate.

2. Experimental

2.1. Materials and instrumentation

Poly(acrylic acid) (450 kDa), bovine hemoglobin, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), hemin chloride, ethanolamine and acetone were obtained from Sigma-Aldrich and used as received. β-mercaptoethylamine hydrochloride and hydrogen peroxide were obtained from Fisher Scientific. Surface plasmon resonance (SPR) traces were recorded on an SR7000DC dual channel flow SPR spectrometer from Reichert Analytical Instruments (NY, USA) with a semi-automatic injection setup with 500 μL PEEK injection loop and Harvard Apparatus flow pump. The flow cell was setup with a 'Y' connector as to facilitate parallel flow through the sample and reference channels. The SPR data were collected at 25 °C. Phosphate buffered saline pH 7.4 (PBS, 0.1 M phosphate, 0.1 M NaCl, 2.7 mM KCl) was thoroughly degassed and used for all manipulations. Bare gold chips were obtained from Reichert (catalog #13206060). The data were analyzed using the Scrubber 2.0 software (BioLogic Software, Australia) followed by Kaleidagraph for plotting.



Scheme 1. (A) Cartoon of SPR sensor, (B 1) Sample Channel—(a) attachment of PAA to amine monolayer with EDC/NHS, (b) immobilization of Hb in PAA matrix, (c) removal of heme from Hb (sample channel only) and (d) injection of heme solution resulting in heme reconstitution (B 2) Reference Channel—(a) attachment of PAA to amine SAM with EDC/NHS, (b) channel blocked off, (c) acid/acetone wash, and (d) injection of heme solution to determine nonspecific binding [34–36].