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Quartz crystal resonant sensor (QCRS) model for label-free, small molecules—receptor studies

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Studying the binding mechanisms of potential drug candidates to receptors of interest is of huge importance to the pharmaceutical industry and laboratory workers worldwide. Reliable arrangements for following interactions between low molecular weight samples and their potential receptors without the requirement for labelling are uncommon. Quartz crystal resonant sensors (QCRS) are becoming established as label-free tools for observing the interactions between species in liquid media. Here we describe a new model based upon QCRS technology and novel thiol surface chemistry, capable of detecting the interactions between low molecular weight sugars and their host receptors in real-time.

Introduction

The mechanisms by which potential drug candidates bind to specific receptor targets are fundamental to the development of new therapeutics within the pharmaceutical industry. Likewise the attachment processes of small molecules to host proteins or guest receptors are at the heart of many academic and industrial research programs. Historically many methods have been employed to follow the attachment of drug to receptor, however, currently almost without exception this type of work is carried out in high throughput assays using either radio-isotopic labelling or fluorescent tagging. Radionuclear methods require that an isotope of a commonly occurring element within the compound structure, such as nitrogen, is introduced whilst the synthesis is taking place.[1] Whilst this introduces no additional molecular mass to the compound, the techniques required are expensive, time consuming and potentially harmful, dealing with radioactive materials, albeit with short half-lives, and the cleanup of contaminated waste.[2]

Fluorescence based methods however, require that an additional 'tag' be attached to the favoured compound prior to interaction with substrate.[3] The tag has fluorescent properties which may be monitored directly by fluorimetry. Recently, suspicions have been raised regarding the limitations of such tags with regard to their liability toward blocking or competition for desired binding sites, possibly leading to the discovery of false results within assays.[4]

Questions broached over the safety, efficiency and cost of these two methods have led to the requirement for new techniques capable of following potential interactions between species, without the need for prior labelling or tagging.

Many solutions have been proposed, however to date, only two distinct methods have shown promise and although relatively young technologies, both are now in everyday use within the pharmaceutical industry.

Isothemal titration calorimetry (ITC) measures the energy transition as heat which takes place when a binding event occurs between two species.[5] Both receptor and ligand remain label-free, with time and concentration courses being plotted allowing both qualitative and quantitative measurements to be recorded. Although many instruments have fully automated sample handling systems, sample requirements can be greater than rival technologies at present.

Surface plasmon resonance (SPR) in its simplest incarnation measures the change in refractive index of a liquid system in real-time.[6] Receptors are attached to the working dielectric surface, most commonly silver or gold, by a host of surface chemistries,[7] with potential ligands of interest being passed over the surface, most commonly *via* an aqueous or semi-aqueous buffer flow. Hutchinson presents the principles of SPR in an excellent review.[8] Changes in the angle of the reflected plasmon beam are a function of the refractive index at the liquid–surface interface with binding events being recorded as distinct, residual shifts from the baseline index measurement.[9] Both strength and rate of interaction can be determined with appropriate controls, often run automatically within the multi-channel flow cell assemblies of the most modern instrument.[10]

Sensitivity with SPR is claimed to be as good as low nanomolar with species below M_r 400,[10] although this is dependent upon the refractive index of the individual species. Limitations on the depth to which the evanescent wave can penetrate the surface bound sample and hence the effective sensitivity, can also limit the usefulness to species bound close to the surface (< 150 nm), as the wave decays exponentially from the working dielectric surface.[11] SPR is now becoming accepted as a useful medium-to-high throughput tool within drug discovery programs.

Amongst other more esoteric new techniques, quartz crystal resonant sensors (QCRS) are emerging as label-free sensors for a wide range of applications. QCRS sensors can trace their origins back to the pioneering work in piezoelectricity of the Curies.[12] Modern devices employ the inverse piezoelectric effect, that is, the application of a potential difference *via* transition metal electrodes on either face, to elicit a change in the dipole moment of the crystal lattice within a piezoelectric material and hence a resonance around a specific frequency by the addition of a low noise sine wave.[13] The majority of current devices are based upon bulk acoustic wave, AT-cut, 1–10 MHz quartz wafers, specifically designed to have low temperature coefficients at room temperature for timing applications within the electronics industry.[14]

Sensor applications were first developed in the 1960s when Sauerbrey found a linear relationship between deposited film mass and frequency change in high vacuum metal deposition apparatus[15] using quartz crystals as thickness monitors.

More recently, groups have been working with quartz crystals within the chemical[16] and biological[17] analytical fields. Initial work was hampered by early low amplitude oscillator designs, limiting the use of the technology commonly called quartz crystal microbalances (QCM), to the gas phase. However, in the last few years developments within the electronics field have allowed for downstream improvements in oscillator technology, which have permitted the use of quartz devices in liquid environments without significant attenuation of the resonant frequency.[18] Applications including agglutination reactions,[19] antibody-peptide binding[20] and RNA interactions[21] have been published.

Initial attempts to model liquid phase frequency behaviour to early models derived from the work of Sauerbrey[15] have been plagued with inconsistencies due to interference from solution density and other viscoelastic phenomena. No universal model is currently available to explain frequency behavior; however a number of differing approaches are discussed in an excellent review by Janshoff *et al*.[22] Despite the lack of a universal model, QCRS based sensor technology, although in its relative infancy has the potential to provide high quality, label-free binding data in an aqueous environment for a wide range of applications.

In an effort to further develop the electronic and instrumental apparatus behind the QCRS technology, a binding model was required upon which to test hypotheses and base development decisions, which closely mimic the conditions under which a fully developed instrument would be expected to perform. Intrinsically, to be of use, this would require the binding of low molecular weight (<500) compounds or drug fragments to target receptors previously attached to the sensor surface by one of a range of attachment mechanisms. Ideally a degree of regeneration would also be premeditated in order to make the most use of the bound receptor.

The work presented here uses well characterised thiol based technology allowing for a relatively straightforward attachment mechanism to the gold electrode placed on the crystal surface.[23] Thiols are well known to spontaneously form stable monolayer systems when brought into contact with several transition metals.[24] Organic solutions of thiols (1–10 mM) form well ordered, compact, homogeneous monolayers within a period of twenty hours contact time.[25] For this work thiol compounds with appropriate headgroup chemistry were unavailable. Therefore, a medium chain length thiol with a boronic acid head group, *N*-(3-dihydroxyborylphenyl)-5-mercaptopentanamide was synthesised.[26]

Boronic acid moieties are known to form pH dependent reversible cyclic esters with vicinal diol groups.[27] This gives the opportunity to study simple sugar adhesion close to the sensor surface, but also by linking nicotinamide adenine dinucleotide (NAD) to the boronic moiety followed by an oxoreductase such as glucose dehydrogenase (GDH), for which NAD is a co-factor, to follow the interaction of glucose with GDH at a considerable distance from the surface (and mimic a receptor–drug interaction). The β-NAD has two vicinal diol moieties on one of its faces which potentially allows interaction on its opposite face whilst anchored to the surface *via* the boronic acid.[28] The dehydrogenases may be regarded as good drug-receptor mimics being specific for low molecular weight, stable readily available species such as glucose, maltose and lactose.[29]

The work presented involves the application of a novel thiol, β-NAD and GDH sandwich to produce a multilayer arrangement upon the crystal surface, followed by the investigation of low molecular weight sugar interaction with the GDH, and the possibility of re-generation *via* breakage of the pH dependent acid–diol ester linkage.

Experiments were carried out using both QCRS and SPR technologies.

Experimental

Apparatus

QCRS experiments were carried out using a custom built instrument (GlaxoSmithKline Instrument Design Technology Group, Welwyn, UK). Novel oscillator circuitry,[30] was based on auto-gain controller (AGC) principles, in which crystals are driven within a self-sensing circuit which automatically controls the gain levels applied to the driving voltage.

AT-cut quartz crystals (10 Mz) in HC49U holders were obtained, pre-coated with a 5 nm nickel adhesion flash and 300 nm gold, from SES Piezo Ltd (Porstmouth, UK).

A custom laboratory built liquid flow cell was used for all experiments. Crystals were sandwiched between two 0.5 mm, medical grade silicone sheets (Dow Corning), with appropriate apertures to allow the formation of a void above both faces. The silicone sheets were gently clamped between two Perspex blocks to form the flow cell designed to allow liquid to flow over one face of the crystal, the other face referenced to air. PEEK polymer inlet and outlet tubes were recessed into the upper perspex block to allow the introduction of liquids. Flow cell volume was calculated at 20 μl.

Frequency was recorded at one second intervals using a Fluka 6685 frequency counter (Fluka, Sweden), capable of sub Hz resolution and connected *via* a National instruments PCIIa GPIB card to a personal computer operating Fluka Timeview data acquisition and control software. Temperature was controlled at 30 ± 0.5 °C by encompassing the instrument in a thermostatically controlled oven (Denley, UK). Liquid handling was achieved using a Shimadzu 9A dual piston HPLC (Shimadzu, Japan), with a Rheodyne 7025 (Rheodyne, Cotati, CA USA), six way injection valve with 20 μl loop, in-line between pump and flow cell. Flow rates of 5 μl min^{-1} were used in all experiments. PEEK polymer tubing, fittings and injection loops were used throughout in order to reduce non-specific binding of samples.

SPR measurements were performed using a prototype three channel Kretchmann configuration instrument,[31] excited *via* a 632.8 nm He–Ne laser. Liquid flow within a rectangular flow cell, (calculated volume = 320 μl), was achieved using an inbuilt syringe driver working at 4 μl s^{-1}, an equivalent rate to that employed with the QCRS instrument.

SPR substrates were made in-house on 'piranha' solution cleaned glass slides (20 °C, 10 min, 4:1, H_2SO_4–H_2O_2 (care required, potentially explosively incompatible with organic materials). Glass surfaces were treated by evaporative coating of a 3 nm nickel adhesive layer and 38 nm gold dielectric layer.[32] All experiments were carried out at 30 ± 0.1 °C affected by an in-built temperature controller.

Sessile drop contact angle measurements were performed using a laboratory built, single element projection system. Droplets (2 μl) of HPLC grade reverse osmosis (RO) water were used throughout.

Reagents

N-(3-dihydroxyborylphenyl)-5-mercaptopentanamide was synthesised according to a route described previously.[26]

β-Nicotinamide adenine dinucleotide (NAD, 99% crystalline, M_r = 663.4), glucose dehydrogenase (Type 12 from Torula yeast, 330 units mg^{-1}, M_r = 120 kDa), HPLC grade

ethanol, potassium hydrogen phthalate and hydrochloric acid were all obtained from Sigma-Aldrich (Poole, Dorset, UK).

D-Glucose, dihydrogen potassium phosphate and disodium hydrogen phosphate, all Analar grade, were obtained from Merck BDH Laboratory Chemicals Ltd (Poole, Dorset, UK).

Sorenson's phosphate buffered saline (0.2 M, pH 7.2) and potassium hydrogen phthalate–HCL buffer (0.1 M, pH 2.4), were prepared, filtered (0.2 μm, cellulose acatate) and degassed by nitrogen sparge (30 min) followed by ultrasonication (15 min).

All buffers were made up with and washing steps performed using HPLC grade reverse osmosis water (Purite Stillplus HP, 18+ MΩ).

Methods

Formation of *N*-(3-dihydroxyborylphenyl)-5-mercaptopentanamide SAMs (self-assembled monolayers) on gold coated QCRS sensors and SPR slides. Gold surfaces on QCRS sensors and SPR slides were cleaned using freshly prepared aliquots of 'piranha' solution (10 min at 20 °C), followed by copious water washing, a final rinse with ethanol and dried in a stream of nitrogen. Cleaned substrates were then submerged in 1 mM ethanolic solutions of *N*-(3-dihydroxyborylphenyl)-5-mercaptopentanamide for periods of 24 h at 22 °C. Upon removal samples were given a gentle rinse with ethanol and dried in a stream of nitrogen. All prepared samples were used immediately.

In order to observe dynamically the chemisorption of the SAM agent to a fresh gold surface, experiments were performed in which the flow cell volume of the SPR instrument was first filled with ethanol as a background. The ethanol was replaced with the 1 mM solution of the boronate derivative in ethanol and the SPR angle changed with time monitored for the maximum available instrumental time of nine hours.

Coupling of β-NAD followed by GDH to the SAM boronated surfaces. QCRS substrates and SPR slides, both previously coated with *N*-(3-dihydroxyborylphenyl)-5-mercaptopentanamide as described and uncoated controls were mounted in their respective instrumental flow cells and pH 7.2 Sorenson's buffer streams established in both cases to yield baseline conditions ($<\pm 0.2$ Hz, 15 min QCRS, <0.5 mda (millidegree angle) units, 10 min SPR). Solutions (10 mg ml^{-1}) of β-NAD prepared in buffer were introduced to the running buffer and allowed to pass through the respective flow cells. Frequency and SPR angle were recorded within each instrument. High NAD concentrations were employed as subsequent experiments required that the surface be saturated.

Periods of 15 min were allowed for unbound material to be flushed from the flow systems before injections of GDH (1 mg ml^{-1}) prepared in buffer were introduced in both instruments, this concentration having been found capable of surface saturation. Another 15 min were allowed before both QCRS and SPR sensors were challenged with a series of glucose injections (0.5×10^{-7}–0.6×10^{-6} M), into the buffer stream.

In separate experiments, injections of GDH were made under identical conditions to the above over QCRS and SPR sensor surfaces treated with *N*-(3-dihydroxyborylphenyl)-5-mercaptopentanamide but with no NAD present.

Contact angle measurements were obtained at each stage of the monolayer, β-NAD, GDH architecture using the sessile drop procedure.

Regeneration of surfaces. Surfaces treated with SAM, β-NAD, GDH and glucose were exposed to a change in buffer conditions by dropping from pH 7.2 Sorenson's PBS to pH 2.4 potassium hydrogen phthalate–HCl buffer for a period of 15 min, then reverting back to Sorenson's PBS buffer.

Results and discussion

Real time SPR studies following the chemisorption of *N*-(3-dihydroxyborylphenyl)-5-mercaptopentanamide to gold substrates have previously been shown to produce a distinct two-stage adsorption process evident within the initial 4 h period of the experiments.[26] An overall two-stage SPR angle change of 0.4 degrees for the binding of *N*-(3-dihydroxyborylphenyl)-5-mercaptopentanamide agrees well with that reported for dodecanethiol of 0.45 degrees,[33] with differences in the degree of surface packing, possibly due to stearic hinderance involving the relatively bulky phenyl boronic acid head groups affecting the overall refractive index shift.

Fig. 3 shows the change in frequency for the attachment of β-NAD (10 mg ml^{-1}), to gold QCRS electrodes coated and uncoated with *N*-(3-dihydroxyborylphenyl)-5-mercaptopentanamide. It can be seen clearly that β-NAD attaches strongly to the SAM coated gold eliciting frequency decreases of 180 ± 15 Hz ($n = 3$). This is in contrast to the response of an equal concentration of material allowed to come into contact with an uncoated plain gold surface, whereupon no change in frequency was observed, strongly suggesting that no NAD had bound to the Au surface.

This would indicate that the boronic SAM were oriented with borate groups in contact with the aqueous phase. Further supporting this, NAD adsorption was observed to boronic acid treated gold SPR slides.[26]

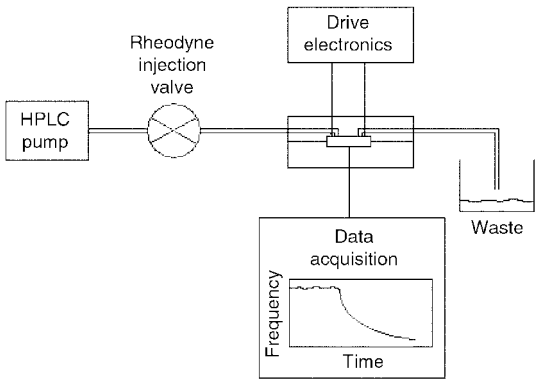

Fig. 1 Schematic configuration of quartz crystal resonant sensor (QCRS) apparatus.

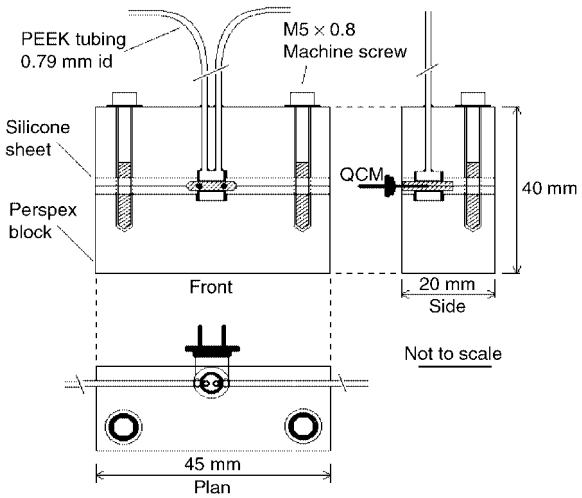

Fig. 2 QCRS flow cell apparatus.

Multiple injections of β-NAD for both QCRS and SPR experiments gave no further residual shifts in frequency or SPR angle, respectively, suggesting strongly that a single injection at high concentration was capable of binding to all borate sites exposed by the SAM.

Sessile drop contact angle shifts of 64 ± 3° to 37 ± 2° (n = 6 ± s), were recorded over a period of two hours for the adsorption of *N*-(3-dihydroxyborylphenyl)-5-mercaptopentanamide from ethanolic solution to the gold substrates[26] and are similar to those reported by Barnes[34] for gold and also the binding of long-chain methyl terminated thiols to gold. These were followed by a further drop to 26 ± 1° (n = 6 ± s) for the binding of β-NAD. These results indicate modification to the surface as a result of addition of these compounds in a layered structure. No shifts in contact angle were observed for β-NAD if the SAM borate layer was not present suggesting that this surface modification was boronic acid–diol dependent.

Fig. 3 also shows that GDH (1 mg ml^{-1}) binds rapidly and tightly to pre-bound β-NAD on the QCRS sensor surface. In control experiments (Fig. 3), no binding of GDH was observed for surfaces coated with *N*-(3-dihydroxyborylphenyl)-5-mercaptopentanamide only. Similar patterns were observed when using SPR for NAD attached to boronic acid treated surfaces, upon exposure to GDH.[26] Multiple injections of GDH gave no greater residual shifts in either QCRS frequency or SPR angle respectively.

Regeneration of the surface bound systems was attempted in order to allow consecutive multiple experiments to be carried out, without the requirement to generate a fresh boronic SAM layer on a new substrate for each run. Fig. 4 and 5 give details of typical experiments where β-NAD and GDH have been attached to a SAM coated QCRS and SPR substrates, respectively, with buffer systems having then been cycled as described previously.

During the switching between buffers, large shifts in frequency were observed prior to returning to the initial baseline with pH 7.2 Sorenson's buffer. Differences in the viscosity and density of the two buffers would be expected to alter the frequency, however the return to the initial baseline strongly suggests that hydrolysis of the boronic acid–vicinal diol cyclic ester has taken place, leading to a desorption of the β-NAD–GDH complex and hence a return to the original baseline. Similar changes were observed during SPR experiments due to changes in the refractive index between the two buffer systems. Between T = 500 s and T = 1500 s in Fig. 4, sequential injections of fructose, sucrose and maltose were made at 1800 s intervals to investigate the specificity of the GDH. No residual baseline frequency changes were observed. Further, subsequent injections of β-NAD and GDH showed strong binding resulting in a drop in frequency for QCRS and rise in angle for SPR (results not shown). This indicates that regeneration of the surface neither removes the boronic thiolate in its entirety, nor greatly affects its capability to re-form the cyclic ester with fresh material. For both QCRS and SPR, the shifts obtained for NAD binding after regeneration were around 10 ± 2%, n = 3 (SPR) and 15 ± 5%, n = 2 (QCRS); smaller than those observed prior to regeneration suggesting that more than a single regeneration step may be required in order to achieve full regeneration. Fig. 6 gives a typical frequency–time trace for the exposure of a SAM treated QCRS electrode with previously bound β-NAD and GDH to a 5 × 10^{-7} M concentration of glucose. This gave a large initial decrease in frequency recovering to a stable residual frequency decrease of around 7 Hz, which although small is easily distinguishable from the ≈ 0.2 Hz background noise on the baseline. Further, results for a series of glucose concentrations gave a linear relationship (r^2 = 0.986) over the range 0.5 × 10^{-7}–0.6 × 10^{-6} M. An association constant K_A = 7.0 × 10^{-4} M^{-1} and limit of detection = 1 × 10^{-8} M were calculated for the glucose–GDH binding.

Estimation of the Michaelis constant from a Lineweaver–Burk plot (glucose concentration 3 × 10^{-6}–100 × 10^{-6} M), for the glucose–GDH reaction yields. K_m = 0.13 × 10^{-3} M (r^2 =

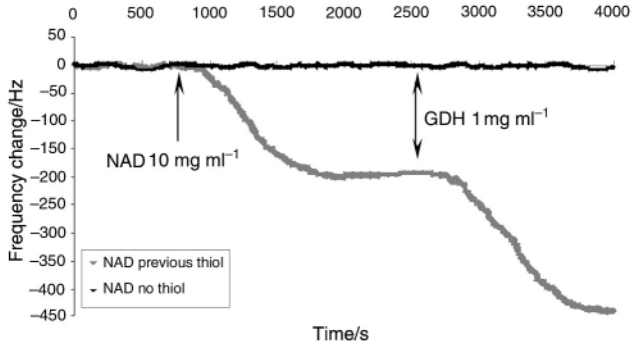

Fig. 3 Frequency change due to the addition of NAD and GDH to 10 MHz gold QCRS electrodes with and without prior exposure to *N*-(3-dihydroxyborylpheny)-5-mercaptopentanamide (pH 7.2 Sorenson's PBS; flow rate = 5 μl min^{-1}, temperature = 30 °C).

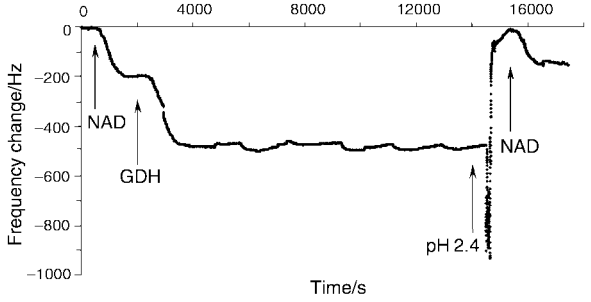

Fig. 4 Frequency change due to addition of NAD (10 mg ml^{-1}) to a 10 MHz gold QCRS electrode previously SAM conded followed by a pH 2.4 regeneration step and a repeat NAD injection (pH 7.2 Sorenson's PBS buffer, flow rate = 5 μl min^{-1}, temperature = 30 °C).

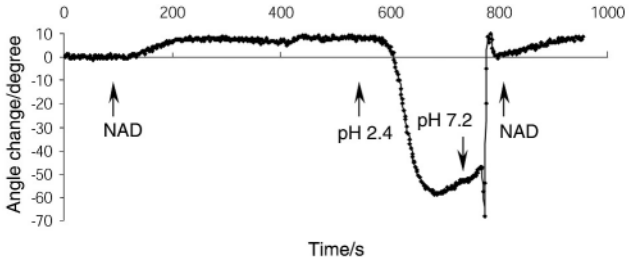

Fig. 5 5 SPR angle change for the binding of NAD (1 mg ml^{-1}) to a SAM coded gold slice followed by boronic codiol cleavage with a pH 2.4 phthdate–HCl buffer wash and subsequent NAD injection. (Flow rate = 5 μl min^{-1}, pH 7.2 Sorenson's PBS, temperature = 30 °C).

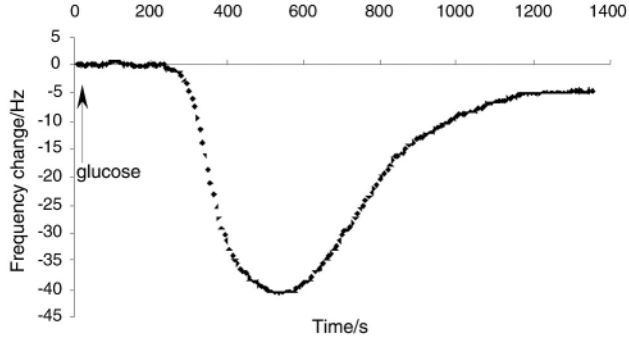

Fig. 6 Frequency change upon the addition of glucose (5 × 10^{-7} M) to a SAM coated gold QCRS substrate previously exposed to NAD and glucose dehydrogenase. (Flow rate = 5 μl min^{-1}, pH 7.2, Sorenson's buffer, temperature = 30 °C).

0.966), which is a little low when compared to the literature value of $5.0 \pm 0.3 \times 10^{-3}$ M,[35,36] but may be related to similar reductions in binding constants observed in SPR systems and attributed to mass transport effects due to flow cell design.[37,38] However, no response was discernible for glucose binding under identical conditions using the SPR instrument, possibly due to changes in refractive index of the GDH–glucose complex being below the limit of detection for our instrument, though clearly responses were obtained for the binding of NAD and GDH sequentially.

Conclusions

This study has shown, by use of a novel drug–receptor mimic, the capability of QCRS technology to monitor, in real-time, the interactions of very low molecular weight compounds, at relatively low concentrations, with very much larger host molecules and without the requirement for pre-emptive labelling of any kind. It has been seen that with correct chemical modification, a robust, re-usable surface can be formed, with simple regeneration protocols, onto which a multi-layer system can be built. This potentially avoids the pitfall of ligand attachment too close to the host substrate, which might hinder interaction and leads towards more natural conditions for biochemical interactions with *in vitro* models.

This study has shown QCRS technology to be a potentially robust screening system for the interactions of small molecules, which may be of great interest to the pharmaceutical and biomedical industries in order to investigate drug-receptor interactions within a label-free environment.

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