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A surface plasmon enhanced fluorescence sensor platform†‡

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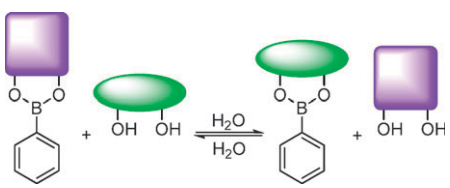
Surface-immobilised and fluorophore-labelled boronic acids were prepared and used for the specific detection of quencher-labelled diols.

The detection of small saccharides, such as glucose and fructose, has attracted considerable research interest in recent years, not only for the determination of blood glucose in subjects with diabetes, but also for use in industrial scenarios, such as fermentation processes and pharmaceutical manufacture.[1] Saccharides are of great importance in cellular processes, with the saccharides expressed on cell surfaces playing critical roles in intercellular signalling, antibody–cell recognition, virus–cell interactions and cancer pathogenesis.[2–4] It has been proposed that the study of oligosaccharides and the emerging field of *glycomics* may become as important as genomics and proteomics in the near future.[5]

Whilst highly selective saccharide sensor systems can be based on enzyme recognition events,[6] it has also been shown that saccharide selectivity can be built into synthetic boronic acid receptors that exploit the reversible boronic ester formation depicted in Scheme 1. Judiciously designed boronic acid motifs allow differentiation between fructose and glucose in solution by exploiting differences in receptor–saccharide binding constants.[7–10] Boron–hydroxy interactions have also been exploited in hydrogels for saccharide electrophoresis and colourimetric assays,[11–13] as well as finding applications as chiral NMR shift reagents and supramolecular building blocks.[14–16]

All sensor systems require the capability to both recognise an analyte and then to transduce the binding event into a "read-out" signal. Surface plasmon resonance (SPR) is a technique often used for the investigation and detection of biological analytes.[17] A related technique uses surface plasmon enhanced fluorescence in order to concomitantly obtain fluorescence spectra of the bound species.[18] A specific advantage of surface plasmon enhanced fluorescence spectroscopy (SPFS) is that the excitation of the fluorophore is through the surface plasmon and not from incident light, thus facilitating fluorescence spectroscopy without the interference of incident/refracted light. Additionally, SPFS is a sensitive technique, with limits of detection better than 10^{-12} mol dm^{-3}.[19] Streptavidin-appended gold surfaces have previously been shown to be ideally suited for the SPFS technique.[22] Our concept was to use a surface-appended fluorescent boronic acid, that, when exposed to an analyte diol appended with a quencher, would reduce the fluorescence output of the system, thus signalling its presence. Herein, self-assembled boronic acid hybrid systems for surface plasmon enhanced fluorescence detection of quencher-labelled diols is disclosed. When a quencher-tagged diol binds to a fluorescent boronic acid receptor, the fluorescence signal is quenched; therefore, read-out of diol binding is observed as a decrease in the total surface excited fluorescence. This generic sensing format is schematically illustrated in Fig. 1. The approach is similar to the 'molecular beacon' fluorophore—quencher pairs methodology used in quantitative PCR assays, such as Taqman®.

In order to assemble a sensor construct at a gold–streptavidin surface, the molecule we call FLAB (Fluorophore Linker boronic Acid Biotin) was prepared, as shown in Scheme 2. FLAB was prepared by coupling biotin to a mono-boc-protected diamine (linker), which was then subjected to reductive amination conditions in the presence of 4-formyl phenylboronic acid. Following this, an activated ester derivative of fluorophore Alexa Fluor® 647 (AF-647, Invitrogen) was tagged onto the molecule. The design incorporates a terminal biotin for attachment to the surface bound streptavidin, a boronic acid receptor and a fluorophore (Alexa Fluor® 647, ex_{max} = 647 nm). A quencher–diol conjugate was prepared from the commercial



Scheme 1 Equilibria depicting boronic ester–diol exchange. The relative equilibrium determines whether one diol displaces another in an aqueous medium.

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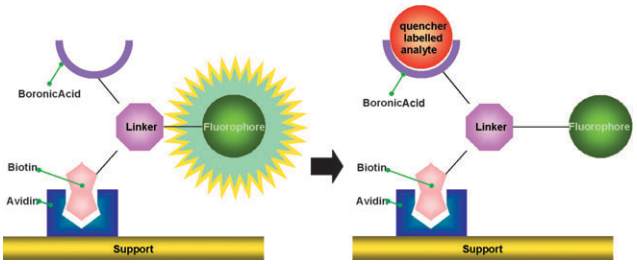


Fig. 1 Schematic of quencher-appended FLAB attached to a support *via* biotin–avidin binding. Quencher-appended analyte reduces fluorescence signalling its presence.

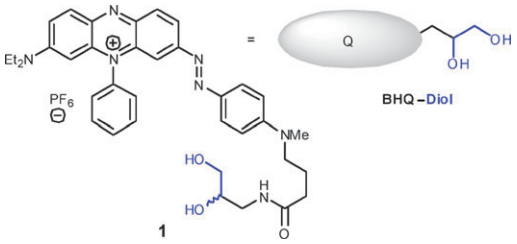


Fig. 2 The BHQ-3 quencher–diol conjugate.

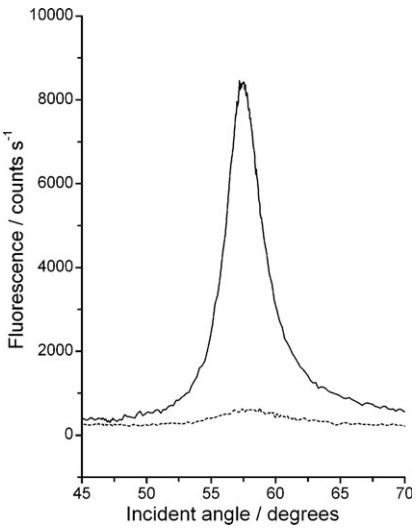


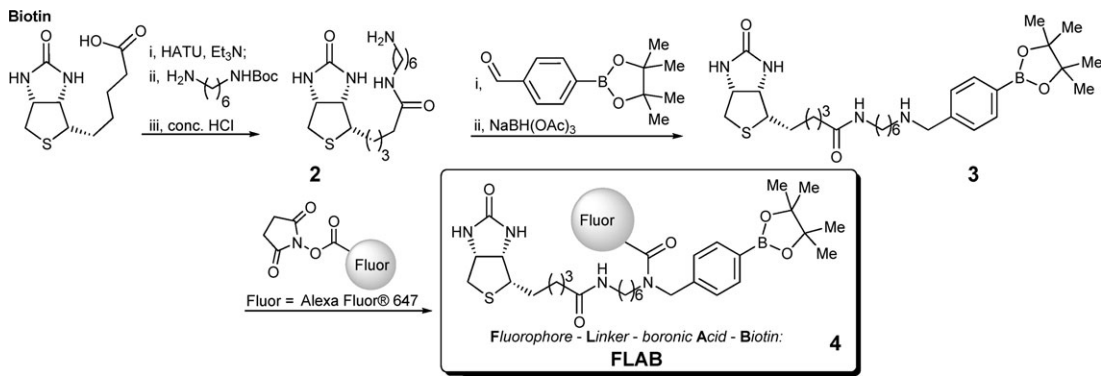
Fig. 3 The surface plasmon enhanced fluorescence signal before (dashed line) and after (solid line) the binding of 5 μmol dm^{-3} FLAB to the biotin–streptavadin matrix.

quencher for Alexa Fluor® 647, BHQ-3 (Biosearch Technologies, Inc.), which was purchased as its *N*-hydroxy succinimidyl ester, which allowed quantitative conversion to a diol-appended variant by reaction with 3-aminopropane-1,2-diol (Fig. 2). The quencher diol conjugate thus prepared can be considered as a model for diol-containing substrates that may be quencher-labelled, such as saccharides and oligosaccharides, which may be important markers in biological scenarios.

The sensor surface was prepared by the self-assembly of biotin-terminated tri(ethyleneglycol) hexadecanethiol (Asemblon™) onto a 50 nm-thick gold film for 2 h from a 500 nmol dm^{-3} solution in ethanol. Following self-assembly, the surface was rinsed with ethanol, dried in a stream of N_2 and incorporated into a Teflon® flow cuvette, with a prism below the gold and an uppermost quartz window. Details of the experimental approach are detailed elsewhere.[20] Streptavidin (500 nmol dm^{-3}, Sigma) was added and the binding followed in real time for 40 min by SPR. The surface was subsequently rinsed in PBS buffer. FLAB was added to the cuvette under continuous circulation for 16 h. Following FLAB binding, the surface was fully rinsed to remove unbound FLAB. Fig. 3 shows the surface enhanced fluorescence signal before and after the attachment of FLAB to the biotin–streptavadin matrix, indicating that the fluorophore had been appended to the surface as desired.

SPFS was employed to excite the surface-immobilised FLAB. The FLAB was bound *via* a streptavadin–biotin matrix ~6 nm away from the gold surface. Surface plasmons were excited by prism coupling in the Kretschmann configuration. Fluorescence was excited by the evanescent tail of the surface plasmon wave, which was in turn excited by laser light at 633 nm. SPFS has been shown to increase the fluorescent emission of fluorophores by up to 16-fold compared to classical light excitation, and has been used in a number of prototype sensor assays.[21] One key advantage of SPFS, in addition to sensitivity enhancement, is that only fluorophores situated within the plasmon evanescent wave (within ~150 nm of the gold substrate) are excited, helping to differentiate between surface and bulk response.

The addition of the diol quencher to the surface-immobilised FLAB at concentrations ≥ 1 μmol dm^{-3} gave a sharp reduction in fluorescence (Fig. 4). The SPR reflectance at a fixed angle also increased following quencher binding. The refractive



Scheme 2 Synthesis of FLAB **4**. Fluor refers to AF-647 (Invitrogen).

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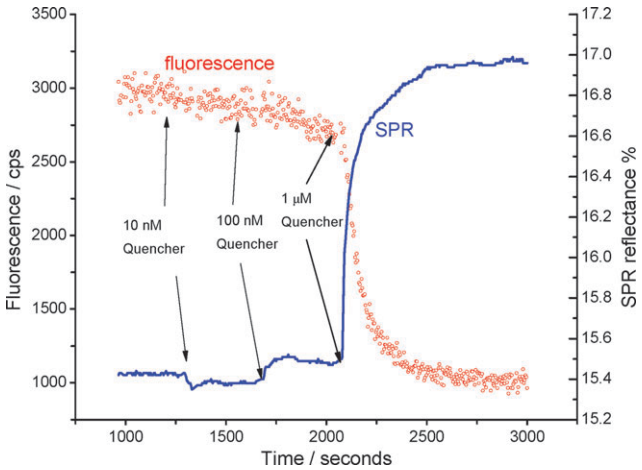

Fig. 4 A plot of the combined SPR and SPR enhanced fluorescence, showing quenching of FLAB following addition of 20 μmol dm^{-3} BHQ-3 diol quencher.

index of the surface film increases as **1** binds to the surface immobilised FLAB. This leads to a concurrent increase in reflectivity measured by SPR spectroscopy. Further aliquots of diol quencher reduced the fluorescence output of the system still further until a concentration of 5 μmol dm^{-3} quencher reduced the fluorescence signal to the background level, indicating that all of the binding sites on the sensor surface were occupied (Fig. 5).

The binding curves were modelled and fitted using the expression for simple bimolecular association at a surface (eqn (1)). R_t is the response at time t, in this case the fluorescence change measured by SPFS. R_{eqm} is the response at equilibrium for a given concentration of diol quencher in the binding solution.

A plot of k_{on} *vs.* concentration of quencher was linear and gave an association rate constant, k_a, of 4.6×10^3 mol^{-1} dm^3 s^{-1} (slope) and a dissociation rate constant, k_d, of 3.21×10^{-3} s^{-1} (intercept). A second value for k_d was obtained by directly fitting the fluorescence recovery upon rinsing, and was calculated to be 3.25×10^{-3} s^{-1} (fit shown in Fig. 5). The fluorescence signal from the surface bound FLAB was

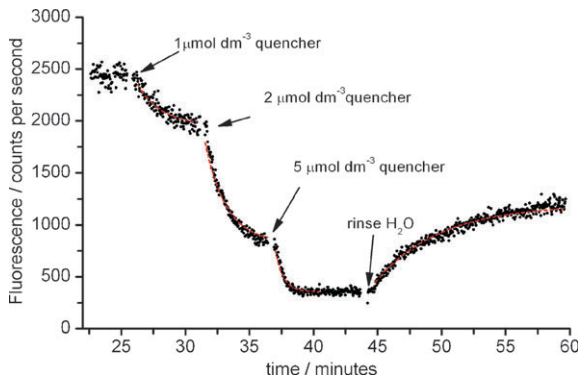

Fig. 5 The drop in surface enhanced fluorescence of FLAB when measuring at a fixed angle of incidence as increasing amounts of quencher were introduced into the flow cell. The data have been fitted (red lines) to the Langmuir model for adsorption and desorption, assuming simple bimolecular association/dissociation at the surface.

measured every 5 s, and a chopper was used to prevent the laser light from hitting the sample between measurements. Chopping the light reduced photobleaching of the surface-bound fluorophore, but it was impossible to eliminate it. As a result, the fluorescence recovery, and hence k_d, might be underestimated as the fluorescence signal may have also dropped due to photobleaching. The association equilibrium constant, K_a, and the disassociation equilibrium constant, K_d, were calculated to be 1.4×10^6 mol^{-1} dm^3 and 7×10^{-7} mol dm^{-3}, respectively.

$$R_t = R_{eqm}(1 - e^{-k_{on}t}) \quad (1)$$

$$k_{on} = k_a[\text{quencher}] - k_d \quad (2)$$

The results presented illustrate that it is possible to use a surface-bound fluorophore and to monitor diol binding. We suggest that this method may find applications in saccharide sensing. For this possibility to become a reality, reliable and broadly applicable saccharide quencher labelling techniques need to be more widely available. One such strategy under development in our laboratories is a reductive amination protocol akin to that used to label saccharides with fluorophores for electrophoresis.[11,22]

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Experimental

Brief preparative details for compounds **1–4** are provided.¶

Compound 1

Commercially available BHQ-3 NHS ester (Biosearch Technologies, Inc.) (1.623 mg, 2.05 μmol) was dissolved in dichloromethane (10 mL) and 3-aminopropane-1,2-diol (232 μL of a 1.06×10^{-2} mol dm^{-3} solution in acetonitrile, 2.46 μmol) added. The mixture was stirred overnight at room temperature under nitrogen, and the solvent and unreacted diol were removed *in vacuo*, thus providing compound **1** quantitatively. HRMS-ESI$^+$: Calculated for $C_{36}H_{42}N_7O_3$ $[M - PF_6]^+$ 620.3344, found: 620.3327.

Compound 2

D-Biotin (300 mg, 1.23 mmol) and *N*,*N*′-disuccinimidyl carbonate (DSC; 360 mg, 1.40 mmol) were dissolved in DMF (10 mL), and triethylamine (0.30 mL) was added. The solution was stirred at room temperature for 6 h under nitrogen and next added to a solution of hexamethylene diamine in DMF (1.35 g, 11.6 mmol). The mixture was then stirred overnight and diethyl ether added. The precipitate thus formed was collected by filtration, washed with diethyl ether and a little dichloromethane, and dried *in vacuo* to give a white solid of **2** (99%). HRMS-ESI$^+$: Calculated for $C_{16}H_{31}N_4O_2S$ $[M + H]^+$ 343.2162, found: 343.2148.

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Compound 3

Under a nitrogen atmosphere, a flask was charged with dried, powdered molecular sieves (3 Å), DMF (10 mL) and **2** (170 mg, 0.497 mmol). To the mixture were added 4-formyl phenylboronic acid (75 mg, 0.497 mmol) and pinacol (59 mg, 0.497 mmol); the mixture was next stirred under nitrogen for 16 h. Sodium triacetoxyborohydride (157 mg, 0.745 mmol) was then added, and the mixture was stirred for a further 24 h. The solution was neutralized by the addition of ethyl acetate, and the solvents were evaporated under a high vacuum to give 125 mg of crude white solid **3**.

Compound 4

Alexa Fluor® 647 NHS ester (Invitrogen) (1 mg, 0.8 mmol) was dissolved in DMF (1 mL), to which **3** (0.45 mL of a 1 mg mL^{-1} solution in DMF, 0.8 mmol) was added, followed by triethylamine (1 mL). The mixture was stirred overnight under nitrogen while being protected from light. Subsequent drying *in vacuo* delivered **4** as a blue solid.

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¶ Full experimental details will appear in a subsequent full paper. We are currently working on extending the scope of the sensor construct.

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