Accurate non-invasive determination of pK_a of surface functionalised ion exchange monoliths using capacitively coupled contactless conductivity detection†

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The use of on-column capacitively coupled contactless conductivity detection for the accurate non-invasive visualisation of the pH dependence of covalently bound boronic acid groups within a monolithic polymeric capillary column is demonstrated.

The production of new monolithic stationary phases in capillary formats has generated considerable interest over the past decade, with the production of a variety of phases from ion exchange to affinity based monoliths. However, once the column has been produced, characterisation of the stationary phase within the column can be a significant challenge,[1] often requiring invasive and destructive measures for chemical and physical characterisation.[2] Capacitively coupled contactless conductivity detection (C^4D) has previously been reported to rapidly and non-invasively profile the spatial and temporal distribution of semi-permanently coated charged surfactants, along the length of a single ODS capillary monolith in capillary ion chromatography.[3,4] Later, the same procedure was further developed to allow for the evaluation of photografted ionisable species and proteins within polymeric capillary columns.[5] These studies have clearly demonstrated that C^4D is a useful tool for the non-invasive characterisation of capillary columns, particularly when charged functional groups have been either semi-permanently or covalently immobilised on polymer monoliths for ion-exchange or affinity chromatography applications.

The ability of boronic acids to form complexes with 1,2-*cis*-diol functionalities at high pH, with the subsequent dissociation of the complex at low pH has been utilised for the preferential capture of glycoprotein over non-glycosylated protein,[6] the separation of nucleosides[7] and for extraction of sugars.[8] The primary mechanism involves shifting the pH above 8.5, which promotes the conformational change of the boronic acid group from the planar to the tetrahedral form. In this form the boronic acid anion is well known to selectively retain molecules with 1,2- and 1,3-*cis*-vicinal diol moieties, as are commonly present in carbohydrates and the glycan region of glycoproteins, *via* the reversible formation of cyclic anionic esters.[6–8]

As illustrated in Fig. 1, the transition from the planar to the tetrahedral form is accompanied by a change in charge on the boronic acid functional group. It has been reported that that phenylboronic acids with lower pK_a's have higher affinities for diols at neutral pH,[9] and that the acid–base properties of bound ligands depends strongly on the type of matrix, spacer and coupling chemistry, which may result in very different affinity to biomolecules. Thus the accurate determination of pK_a is an important part of the characterisation of adsorbents for boronate affinity chromatography.

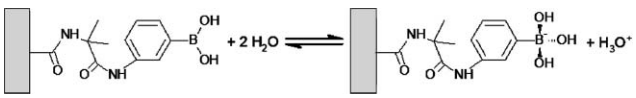

Fig. 1 Schematic representation of the transition of planar boronic acid to a tetrahedral boronic acid anion under high pH conditions.

In this communication, for the first time contactless conductivity detection is utilised to characterise a novel immobilisation strategy for *m*-aminophenyl boronic acid on a pre-formed polymeric monolith within a capillary column, and for the subsequent study of the pH modulated switching of the boronic acid groups from the planar to tetrahedral form. The ability to non-invasively characterise the pH dependence of immobilised boronic acid functionalities allows for a better understanding of the processes involved in its use as an affinity phase and the study has implications for the characterisation of any capillary monolithic column containing weakly acidic or basic functional groups resulting in a strongly pH-dependent ion-exchange capacity of the stationary phase.

Details of chemicals used for base monolith production and basic micro-LC instrumentation can be found in the ESI.† The detector used was a TraceDec capacitively coupled contactless conductivity detector (Innovative Sensor Technologies GmbH, Innsbruck, Austria) and was programmed with the following settings: frequency, 2× HIGH; voltage, −0 dB; gain, 50% and offset, 0. The monolithic column (fabricated within 100 μm id, 360 μm od fused silica capillary) was threaded through the C^4D cell, with the exact position of the cell along the length of the monolithic column being varied manually, using a graduated guide as a position indicator. Regression analysis of the results obtained were carried out using Sigma Plot v 8.1.

Batch titrations of free aminophenylboronic acid (APBA) were performed using a 1 mg mL^{-1} APBA solution with

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0.2 M NaOH. Preparation of butyl methacrylate monoliths and photografting of two isolated and spatially distinct zones of vinyl azlactone (4,4-dimethyl-2-vinylazlactone) (VAL) was carried out as described by Connolly *et al*.[5] Full details are provided as ESI.† Prior to the photografting of VAL, the column was rendered hydrophilic by the photografting of polyethylene glycol methacrylate onto the surface of the monolith using the two-step photografting procedure described by Stachowiak *et al.*[10] (for full details see ESI†). After two 10 mm zones of VAL (separated by 20 mm) had been photografted onto the PEGMA-grafted butyl methacrylate monolith, a solution of 1 mg mL^{-1} *m*-aminophenylboronic acid (APBA) in water was passed through the column at a flow rate of 1 μL min^{-1} for 3 h. The column was then washed with water and any unreacted VAL groups were subsequently reacted with 1 M ethanolamine to avoid subsequent unwanted immobilisation of buffers with free amine groups such as histidine. The column was then thoroughly washed with water before introduction of a range of buffer solutions of varying pH, for on-column titration of the boronic acid groups. While pumping each buffer through the functionalised monolith at 1 μL min^{-1}, the detection cell was physically scanned across the length of the column at millimeter increments to allow the detection of localised regions of charge within the monolithic stationary phase. The conductivity value at each millimetre location along the column was recorded. The buffers used were chosen, where possible, to have low conductivity and compatibility with bio-molecules for future work. The buffers ranging from pH 4 to 10.2 were 5 mM acetate (pH 4), 5 mM acetate (pH 4.9), 5 mM MES/histidine (pH 6.1), 10 mM TES/MES (pH 6.8), 5 mM TES/4 mM ethanolamine (pH 8.1), 2.5 mM TES/3 mM ethanolamine (pH 9.0) and 1 mM ethanolamine (pH 10.2). Each buffer was passed through the column and allowed to equilibrate over 1 h before any readings were taken. Calculation of ΔResponse was carried out by using the average of 3 baseline points immediately before and after the respective zone ($n = 6$), followed by subtraction of this baseline conductivity reading from that recorded at the maximum zone height. In this way background conductivity from the base monolith and the various buffer solutions was subtracted from the conductivity resulting from the immobilised boronic acid. To investigate the reproducibility of the data collected, triplicate profiles for pH 9 were collected, and the average %RSD was found to be <1%.

The profiles collected showed a slight difference between the two zones of APBA covalently bound to the column, reflecting the reproducibility of the photografting process, with zone 2 having a higher response than zone 1 (Fig. 2). Over the pH range it was found that the ΔResponse of the APBA zones changed from *ca.* 40 to 430 and *ca.* 50 to 550, for zones 1 and 2, respectively. When the ΔResponse was plotted against pH, the immobilised boronic acid zones displayed the expected S-shape curves (see Fig. 3(a)). When a sigmoidal regression was carried out on the data collected for the two APBA zones the R^2 values returned were 0.999. In Fig. 2 it can be seen that the low pH buffers resulted in a low response for the APBA zones, but as the pH was increased (>8.5) the response from the zones increased dramatically. As previously reported,[4] the "form" of the monolith surface functionalities can be determined *via* C^4D, here the change in response is due to the change in the

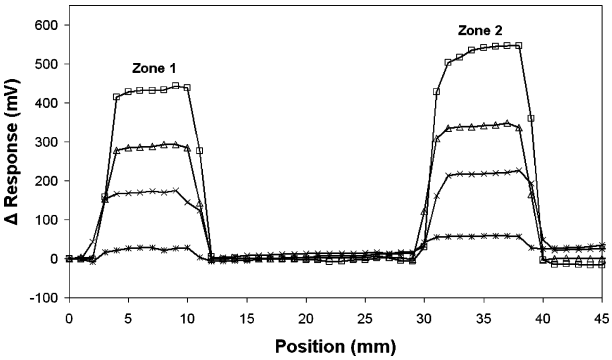

Fig. 2 Column profile showing APBA zones at (*) pH 4.0, (×) pH 6.1, (△) pH 6.8 and (□) pH 10.2.

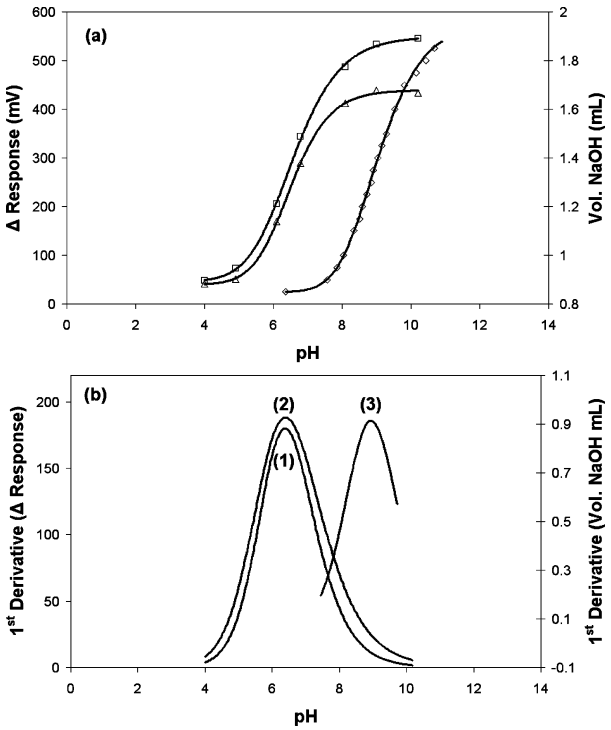

Fig. 3 (a) Change in response (ΔResponse) of APBA groups with pH, (△) zone 1 and (□) zone 2 and (◇) titration data for free APBA; (b) derivative analysis of (1) zone 1 and (2) zone 2 on column and (3) titration of APBA in solution.

conformation of the boronic acid groups from the planar form to the tetrahedral form. The classical pH ranges used with boronic acids (covalently bound to agarose gel) has been >8.5 for the tetrahedral form and pH 4.5 for the planar form. The results obtained here show that the maximum response obtained for the APBA region was reached at *ca.* pH 9.0 (with little change between this and pH 10.2), indicating that the groups have fully converted to the tetrahedral form. The minimum response was obtained at pH below 4.9 with little change between pH 4.9 and 4, indicating that the APBA groups are in the neutral planar form. The pH characteristic of the covalently bound APBA groups has shown good agreement with those used in classical boronic acid affinity chromatography for the purification of glycoprotein.[6,11,12]

From Fig. 3(a) and 3(b) it is possible to accurately determine the pK_a of the region with covalently bound APBA. Using first derivative analysis of the regression obtained the pK_a of the two APBA zones was found to be 6.4 (Fig. 3(b)). Previous studies have calculated the pK_a of APBA based molecules immobilised onto microspheres consisting of styrene, butyl acrylate, and *m*-acrylamidophenylboronic acid (synthesised from APBA), where the phenylboronic acid phase was found to have a pK_a of 7 (by bulk titration of modified microspheres). The deviation from the reference pK_a of 8.77 was attributed to a disturbance of the dissociation behavior of the phenylboronic acid due to being immobilised onto a polymer surface.[12,13] Here the titration of APBA in free solution resulted in a pK_a of 8.9 (Fig. 3(b)), which is in good agreement with litrature values.[14,15] It should also be noted that a 4-APBA attachment to a hydrophilic polymer matrix performed *via* a carbamoyl linkage, of similar composition to the above VAL linker, caused a significant drop in the pK_a value of phenylboronic acid to 7.8.[16] In this work it follows that as the phenylboronic acid is immobilised onto the methacrylate/polyethylene glycol monolithic stationary phase, the pK_a of the acid has been similarly perturbed.

This communication shows how the physio–chemical properties of polymeric monolithic stationary phases modified with acidic or basic functional groups can be characterised using C^4D. The ability to rapidly and non-invasively study the pH behaviour of covalently bound groups, together with the visualisation of the magnitude and longitudinal homogeneity of the functionalised zone, leads to a better understanding of how such phases work and opens up the possibility of using C^4D for investigation into other significant physio–chemical properties (*e.g.* p*I* and binding co-efficients for immobilised proteins).

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