Electrokinetic probes for single-step screening of polyol stereoisomers: the virtues of ternary boronate ester complex formation†

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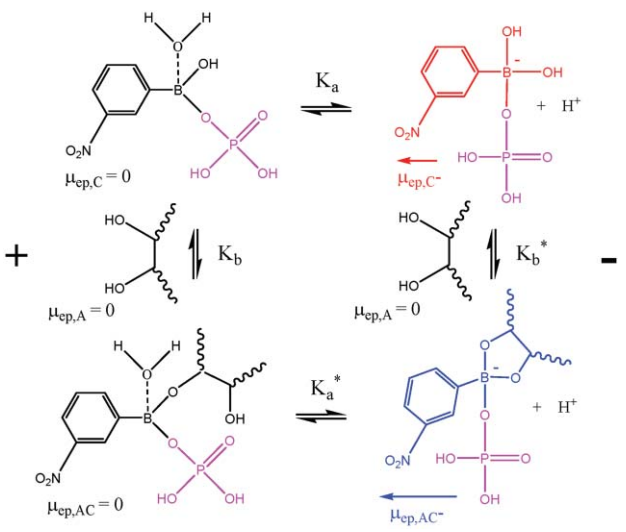
Electrokinetic probes based on the differential migration of ternary boronate ester complexes permit the selective analysis of micromolar levels of UV-transparent polyol stereoisomers in urine samples *via* dynamic complexation–capillary electrophoresis that is applicable to single-step screening of in-born errors of sugar metabolism, such as galactosemia.

The design of boronic acids as probes for carbohydrates has wide interest in the clinical diagnosis of disorders involving sugar metabolism. To date, several groups have developed various boronic acid structural motifs to enable selective binding of sugars using colorimetric or fluorescence detection.[1] Polyols or sugar alcohols represent important clinical target(s) that have been implicated in neuropathic complications with diabetes, as well as biomarkers for in-born errors of metabolism.[2] However, target selectivity and assay sensitivity have been major limitations of conventional boronic acid probes when applied to sub-mM detection of sugar stereoisomers in complex biological samples. Herein, we introduce a new strategy for enhancing target selectivity when using 3-nitrophenylboronic acid (NPBA) as an electrokinetic probe in dynamic complexation–capillary electrophoresis (DC–CE). Stereoselective resolution of polyols was achieved based on their differential electromigration behavior with NPBA in phosphate buffer. Direct photometric detection of μM levels of hexitol stereoisomers in urine samples was also realized *via in-situ* generation of a UV-active anionic ternary boronate ester complex. These features are relevant in newborn screening of galactosemia, where early detection of elevated urinary galactitol[2a,e] is crucial to prevent acute liver cirrhosis, neurological impairment and/or neonatal death, which can be treated by a lactose-free diet.[2d]

In this study, NPBA was selected as an electrokinetic probe because of its good water solubility, stronger acidity and useful UV spectral properties due to the presence of an electron-withdrawing nitro group relative to phenylboronic acid. In general, boronic acid derivatives with lower pK_a tend to exhibit higher affinity for sugars, although other variables can impact borate chelation, such as buffer pH, ionic strength and electrolyte type.[3] Scheme 1 depicts a dynamic complexation model involving reversible 1 : 1 covalent interactions of NPBA with neutral polyols in the presence of phosphate under an electric field. Note that several distinct equilibria occur in DC–CE such as the formation of trigonal and tetrahedral boronate ester complexes;[3] however, only species that are intrinsically charged contribute to changes in polyol apparent electrophoretic mobility (μ_{ep}^{A}), such as the NPBA–polyol–phosphate ternary complex.

The composition of the buffer can significantly influence the apparent acid dissociation constant (K_a) and binding constant (K_b) of boronic acids in free solution.[3] For instance, phosphate has been reported to function as both weak ligand[3b] and proton-exchange catalyst[3d] with boronic acid analogs. Scheme 1 highlights that ternary complex formation results in a larger negative complex mobility (μ_{ep,AC^-}) relative to free NPBA (μ_{ep,C^-}), which alters polyol migration behavior based on the affinity of NPBA–polyol interactions. Further evidence of ternary complex formation is provided by CE, UV and NMR experiments.†

The apparent mobility, acidity and spectral properties of NPBA were first characterized in phosphate buffer by CE and UV spectroscopy. Fig. 1(a) qualitatively depicts changes in the electromigration of NPBA with the presence of a 50-fold excess of sorbitol in phosphate buffer. It is clear that the apparent negative mobility (*i.e.*, migration time) of NPBA increases significantly with sorbitol chelation despite the increase in hydrodynamic size of the complex. This observation suggests that the fraction of ionization for the NPBA–sorbitol complex increases relative to free NPBA. Fig. 1(b) compares the pH-dependent μ_{ep}^{A}



Scheme 1 Dynamic complexation model depicting NPBA as an electrokinetic probe for polyols in phosphate buffer by DC–CE. An increase in complex mobility (μ_{ep,AC^-}, vector arrow) with UV spectral changes for the anionic ternary complex enables the direct analysis of multiple neutral UV-transparent polyol stereoisomers.

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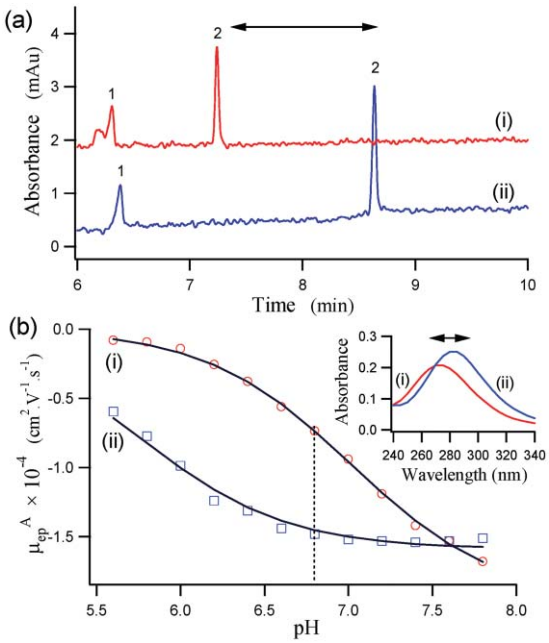

Fig. 1 (a) Electropherograms depicting the increase in μ_{ep}^{A} of the NPBA–sorbitol complex (ii) relative to free NPBA (i) where (1) is resorcinol as the neutral EOF marker and (2) NPBA. Conditions: 27 mM phosphate, pH 6.8 with (i) 0 and (ii) 5 mM sorbitol; voltage: 30 kV, capillary length: 86 cm, temperature: 25 °C; UV at 280 nm; sample injection: 3 s using 100 μM resorcinol and NPBA. (b) Overlay mobility plots for (i) NPBA and (ii) NPBA–sorbitol complex as a function of buffer pH for the pK_a and pK_a* determination. Inset of (b) highlights a 10 nm red-shift with absorbance enhancement at 280 nm for (ii) NPBA–sorbitol complex relative to (i) free NPBA, where $\Delta\varepsilon$ = 520 M^{-1} cm^{-1} at 280 nm.

changes of NPBA with 0 or 5 mM sorbitol, which allowed for assessment of the apparent pK_a of NPBA and NPBA–sorbitol species by non-linear regression.† It was determined that sorbitol complexation with NPBA reduced its apparent pK_a by 1.2 pH units from pK_a = (7.0 ± 0.2) to pK_a* = (5.8 ± 0.4), which resulted in longer migration times for the ternary complex. A similar enhancement in the acidification of phenylboronic acid–diol complexes has also been reported by other groups,[3] which has been used as a mode for detection.[1b] NPBA chelation with sorbitol also induced a significant bathochromic shift with hyperchromic effect as shown in the inset of Fig. 1(b), which provided a simple mechanism for the direct photometric detection of UV-transparent polyols. For instance, there was a red-shift of about 10 nm for the NPBA–sorbitol complex relative to free NPBA along with an increase in its molar absorptivity ($\Delta\varepsilon$) of 520 M^{-1} cm^{-1} at 280 nm. It was observed that the magnitude of the spectral changes for NPBA was dependent on the concentrations of both sorbitol and phosphate in solution, which influence the fraction of ternary complex formed.

Fig. 2 depicts a series of electropherograms at increasing [NPBA] that highlights the analysis of a 100 μM polyol mixture with UV detection at 280 nm. All samples also contained catechol and resorcinol, which were used as an internal standard and neutral electroosmotic flow marker (EOF), respectively. At 0 mM NPBA, all analytes are neutral and co-migrate with the EOF; however, only catechol and resorcinol are responsive at this wavelength. Fig. 2(ii) demonstrates that the addition of 3 mM

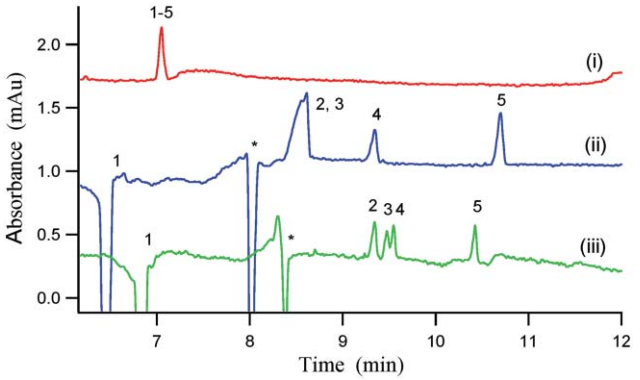

Fig. 2 Electropherogram overlay depicting the impact of increasing [NPBA] in phosphate for single-step analysis of polyols by DC–CE. Buffer conditions: 27 mM phosphate, pH 6.8 with (i) 0, (ii) 3 and (iii) 12 mM NPBA. All other conditions as in Fig. 1. Peak numbering in plots corresponds to 100 μM of (1) resorcinol–EOF, (2) mannitol, (3) galactitol, (4) sorbitol and (5) catechol, where * represents the NPBA system peak.

NPBA in phosphate buffer resulted in the separation and detection of anionic ternary complexes at longer migration times except resorcinol due to its 1,3-dihydroxy configuration. All polyols were partially resolved when using 12 mM NPBA as depicted in Fig. 2(iii). The use of NPBA generated two large negative system peaks in all electropherograms, the first one corresponding to the EOF and the second zone associated with free NPBA due to the injection of a UV-transparent water plug in the sample. A 2-level/3-factor central composite design was also performed in this study, which revealed that 27 mM phosphate, pH 6.8 at 25 °C was the optimum condition to achieve maximum polyol resolution. Overall, buffer pH was the most significant variable impacting polyol resolution, whereas phosphate concentration had a major influence on polyol absorbance response.†

To date, the majority of sugar sensors based on boronic acids have focused on enhancing binding affinity while tuning selectivity for specific targets, including polyols.[1d] However, DC–CE provides a thermodynamic and electrokinetic mechanism for high efficiency speciation of polyol stereoisomers using boronic acids in free solution. The latter effect is based on differences in μ_{ep,AC^-}, which is reflected by the distinct conformational size of the complex. Table 1 summarizes K_b* and μ_{ep,AC^-} parameters for polyols under optimum buffer conditions.† It is apparent that sorbitol has about a 2.5-fold greater affinity for NPBA relative to either mannitol or galactitol despite minor differences in their stereochemistry. Interestingly, the apparent binding affinity of mannitol was measured to be slightly greater than that of galactitol despite its shorter migration time. The anomalous mannitol–galactitol migration order in Fig. 2 is explained by differences in μ_{ep,AC^-}, where galactitol has a larger μ_{ep,AC^-} relative to mannitol despite its weaker affinity to NPBA. Thus, at high [NPBA] near complex saturation, μ_{ep,AC^-} override minor differences in K_b*, such that galactitol migrates after mannitol. The large μ_{ep,AC^-} of galactitol relative to other hexitols suggests a more compact ternary complex with a smaller hydrodynamic size. The temperature-dependence of NPBA–polyol interactions revealed that the driving force for complexation was enthalpy-driven ($\Delta H^\circ < 0$), with NPBA–sorbitol chelation providing the greatest exothermic and lowest entropy change indicative of tridentate complexation behavior.[1f] In

Table 1 Thermodynamic and electrokinetic factors influencing polyol analyses by DC–CE with NPBA in 27 mM phosphate, pH 6.8

Polyol	K_b*[a]/M^{-1}	ΔH°[b]/kJ mol^{-1}	$T\Delta S^{\circ}$[b]/kJ mol^{-1}	$\mu_{ep,AC^-} \times 10^{-4}$[a]/$cm^2$ V^{-1} s^{-1}
Mannitol	(497 ± 17)	−(10.4 ± 2.3)	(5.1 ± 2.2)	−(1.68 ± 0.02)
Galactitol	(453 ± 13)	−(10.5 ± 2.5)	(4.8 ± 2.4)	−(1.77 ± 0.02)
Sorbitol	(1255 ± 46)	−(19.4 ± 2.6)	−(1.6 ± 2.6)	−(1.72 ± 0.01)

[a] Parameters determined at 25 °C with error ±1σ, where the average mobility of free 3-NPBA was −(7.36 ± 0.02) × 10^{-5} cm^2 V^{-1} s^{-1}. [b] Thermodynamic parameters were derived from double reciprocal binding isotherms (n = 3) at five temperatures ranging from 20–40 °C.

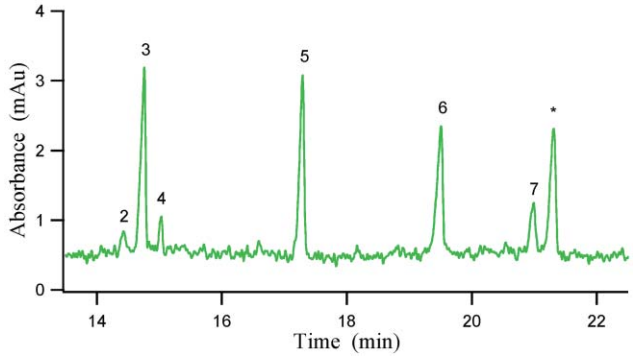

Fig. 3 Electropherogram demonstrating single-step screening of urinary polyols after 10-fold dilution in de-ionized water by DC–CE. Spiked healthy urine sample represents a simulated polyol profile associated with galactosemia with elevated galactitol, 3 (400 μM) and galactonic acid, 6 (100 μM) in the presence of other polyols (40 μM) and catechol as IS (200 μM). Optimum buffer conditions: 27 mM phosphate, 15 mM NPBA, 1.5 mM $MgCl_2$, pH 6.8. All other conditions are similar to Fig. 1, except peak numbering, where (6) is galactonic acid, (7) gluconic acid and (*) endogenous uric acid.

contrast, mannitol and galactitol had similar enthalpy yet large positive entropy changes. Thus, electrokinetic factors (*i.e.*, μ_{ep,AC^-}) played a dominant role underlying the mechanism for resolution of mannitol–galactitol, unlike galactitol–sorbitol, where thermodynamic (*i.e.*, ΔH°) factors had a much more decisive impact.

Fig. 3 demonstrates single-step screening of micromolar levels of polyols in human urine samples, including the secondary polyol metabolites, galactonic acid (6) and gluconic acid (7). Recently, a new method for the diagnosis of galactosemia by stable isotope dilution GC–MS[4b] revealed that galactosemic patients had elevated levels of urinary galactitol and galactonic acid present in about a 4 : 1 concentration ratio, unlike healthy controls. The major disadvantage of GC–MS is the time-consuming off-line sample preparation steps, which include urease treatment to remove excess uric acid, chemical derivatization for improved volatility of polar polyols, as well as lyophilization and/or liquid extraction sample workup.[2a,4a–c] LC–MS[4d,e] and ion-exchange with pulsed amperometric detection[4f] can reduce the extent of sample cleanup, but often do not provide adequate resolution of hexitol stereoisomers.[4d] In contrast, Fig. 3 highlights direct analysis of urinary polyol stereoisomers that can reliably quantify elevated galactitol and galactonate levels (≈ 0.1–2 mM) associated with galactosemia[2a,4b] in the presence of uric acid without interferences. The detection limit (S/N ≈ 3) was determined to be about 20 μM for polyols by DC–CE, whereas the average polyol recovery (n = 10) of a 100 μM spiked urine sample was about 94%.† Method reproducibility (n = 10) for spiked urine samples was also assessed in terms of relative peak area and migration time, which provided good precision as reflected by an average CV of 5.2 and 0.3%, respectively.†

Electrokinetic probes based on the differential migration of ternary boronate ester complexes offer a simple format for polyol screening, which can be applied for early detection of galactosemia, as well as other disorders of sugar metabolism. Although borate is a widely used alkaline buffer and chelating agent in CE, to the best of our knowledge this is the first report of using phenylboronic acid analogs as probes for the resolution and direct photometric detection of sugars. Notably, our studies revealed the essential role that phosphate plays in enhancing NPBA absorbance response for polyol detection at low micromolar levels.† For instance, the application of other common anionic electrolytes, including carbonate and MES, did not generate a similar UV response for polyols compared to phosphate. Further studies are needed to better understand the role that specific electrolyte conditions can play in improving molecular recognition processes of boronic acids for saccharides. In summary, DC–CE offers a promising microseparation platform that can greatly expand the performance of boronic acids for stereoselective analysis of multiple polyol metabolites in biofluids without sample pretreatment. Future work will focus on improving method sensitivity for sub-μM detection of other classes of sugars using fluorescent boronic acid analogs as electrokinetic probes.

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