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Alizarin Red S. as a general optical reporter for studying the binding of boronic acids with carbohydrates

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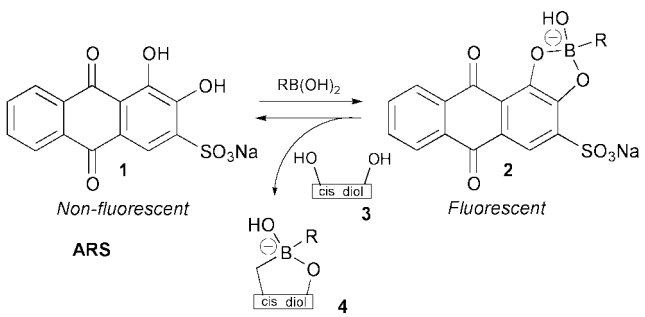
Alizarin Red S. displays a dramatic change in fluorescence intensity and color in response to the binding of a boronic acid and can be used as a general reporter for studying carbohydrate–boronic acid interactions, both quantitatively and qualitatively.

Boronic acids are known to bind diol moieties with high affinities through reversible boronate formation.[1] Consequently, boronic acid compounds have been widely used for the synthesis of artificial receptors for sugars with great success.[2–4] In addition, boronic acid compounds have been used for the development of protease inhibitors and other biologically important agents.[5,6] However, the examination of the binding of boronic acids to the target compounds is often difficult. Detection of boronate ester formation can sometimes be achieved by appending a fluorophore directly to the boronic acid.[4,7] In cases without a readily detectable and sensitive signal associated with the binding events, nuclear magnetic resonance, circular dichroism, and pH titration have been used for the examination of the binding.[1,8] In our research, we are interested in the design and synthesis of boronic acid compounds with highly selective affinities for saccharides,[9] especially certain cell surface carbohydrates. In such studies, the incorporation of fluorophores onto the boronic acid could greatly affect the binding affinity of the artificial receptor for the target sugar in a negative fashion, and could significantly increase the difficulty of the synthesis. Non-fluorescent assays using NMR and absorbance techniques often lack the desired sensitivity. Therefore, we wish to develop a general fluorescence assay method that allows us to monitor the binding of an unmodified boronic acid compound with its target carbohydrate.

Conceivably, the binding between boronic acids and their target carbohydrates can be monitored with the inclusion of a separate reporter fluorophore whose fluorescence is sensitive to the binding event. This type of fluorescence assay system requires two competing equilibria (Scheme 1). The first equilibrium, between the boronic acid and a fluorescent reporter compound (**1**), will be directly measured. To this end, we have worked on the synthesis of sensors that detect free boronic acids.[10] Alizarin Red S. (ARS) (**1**, Scheme 1) has been used as a reagent for the fluorimetric determination of boric acid concentrations.[11] Although the mechanism of the fluorescence increase was not elucidated, it is known that the active protons of hydroxyanthraquinones are responsible for a large fluorescence quenching.[12] Conceivably, binding of a boronic acid to the catechol diol of ARS would remove the active protons and, therefore, abolish the fluorescence quenching (**2**, Scheme 1). The addition of a carbohydrate (**3**) sets up a second equilibrium between the boronic acid and the diol moiety of the carbohydrate to give complex (**4**). This perturbs the reporter–boronic acid equilibrium, resulting in a change in fluorescence intensity. The fluorescence intensity changes in a three-component system can be used for the determination of binding constants using well-known literature procedures.[3,13,14]



Scheme 1 Competitive binding of a boronic acid with Alizarin Red S. and a *cis*-diol.

To test the feasibility of this assay method, we first examined the effect of added boronic acid on the spectroscopic and fluorescence properties of ARS in aqueous solution at physiological pH. ARS shows a color change, from deep red to yellow, and an increase in fluorescence intensity by 20–80 fold in the presence of a wide variety of arylboronic acids in aqueous solution at pH 7.4 (data not shown). All of the boronic/boric acids tested induced significant fluorescence increases, indicating the general applicability of this method. It should also be noted that ARS has reasonable water solubility, up to 10^{-3} M, which makes it a good candidate as a reporter compound in an aqueous assay.

In order to determine the applicability of using ARS for the determination of the binding between a boronic acid and a carbohydrate in a three-component system, we used fructose as a model compound. D-Fructose is known to bind to phenylboronic acid (PBA) with high affinity.[1] When D-fructose was added to the mixture of PBA and ARS, significant fluorescence intensity changes were observed. Titrating fructose into an aqueous solution of 10^{-4} M ARS and 10^{-3} M PBA caused an eight-fold drop in fluorescence intensity (at 100 mM), and a measurable change down to the 100 μM level (Fig. 1). The fructose–PBA complex forms immediately upon mixing, and can also be monitored by UV absorption and a corresponding change in solution color from yellow to red (Fig. 2).

The ARS system also allows the quantitative determination of association constants (K_a) of diol–boronic acid complexes.

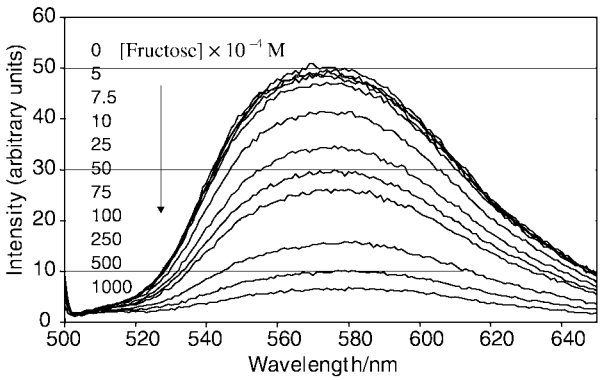


Fig. 1 Titration of fructose into a solution of ARS (10^{-4} M) and PBA (10^{-3} M). Fluorescence decreases with added fructose (0 through 0.1 M), aqueous 0.1 M phosphate buffer, pH 7.4, Exc. λ = 495 nm.

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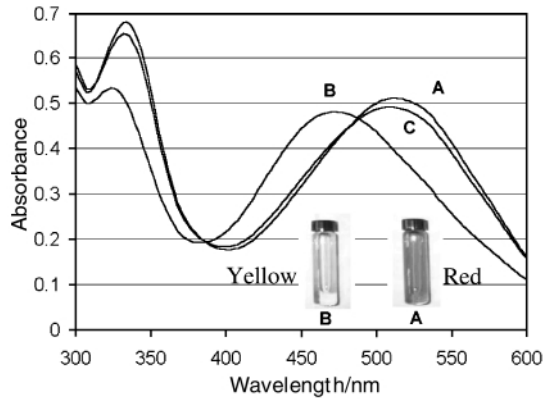

Fig. 2 A, Absorbance of ARS at 10^{-4} M in pH 7.4, 0.1 M phosphate buffer. B, ARS at 10^{-4} M with PBA at 10^{-3} M. C, Alizarin Red S. at 10^{-4} M with PBA at 10^{-3} M and fructose at 10^{-1} M.

Monitoring the fluorescence of ARS at different concentrations of boronic acid allows the calculation of the K_a of the boronic acid–ARS complex by the Benesi–Hildebrand method.[15] The boronic acid–diol association constant can then be measured in a separate titration with all three components using well-established literature procedures.[14] Using the ARS system, we have determined the binding constant between PBA and sialic acid (K_a, 21; pH 7.4 in 0.1 M phosphate buffer), one of the carbohydrates that we are interested in making a specific sensor for because of its important roles in biological systems.

In order to gain a greater understanding of the causes of the fluorescence change, the pH profile of the PBA–ARS complex was studied (Fig. 3). The profile shows that the largest response to PBA is centered on pH 7. The shape of the profile is likely a result of both the stability of the ARS–PBA complex, and the intrinsic fluorescence of the three ionic forms of ARS (pK_a = 5.3 and 10.9 by UV titration). It is well known that boronate ester complexes have decreased stability under acidic conditions.[16] This results in a high concentration of the quenched, free form of the dye at low pH (< 5). At medium pH (5–9), the boronate ester becomes stable, and therefore the quenching

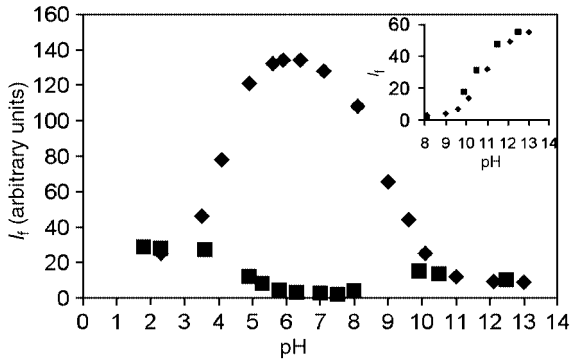

Fig. 3 (■)–pH titration of the fluorescence intensity (I_f) of ARS (10^{-4} M). (◆)–ARS (10^{-4} M) in the presence of PBA (10^{-2} M). Em. λ = 565 nm, Exc., λ = 495 nm, 0.1 M phosphate buffer. Inset–Em. λ = 633 nm, Exc. λ = 600 nm.

active protons are removed from the system. Association constants determined using the Benesi–Hildebrand method support this view (K_a = 190 at pH 4.6, and 1100 at pH 7.4). At high pH, there is also a considerable drop-off in the stability of the ester (K_a = 274 at pH 9.1). Although the uncomplexed dianion form has no active protons, there is a shift in fluorescence to longer wavelength (Fig. 3 inset), resulting in a sharp decrease in intensity at the shorter excitation and emission wavelengths (Fig. 3). Methylation of the 2′-hydroxyl of ARS causes an increase in the intrinsic fluorescence of the molecule, but removes virtually all response to phenylboronic acid at neutral pH (data not shown).

The absorption shift to lower wavelength upon complexation with phenylboronic acid (Fig. 2) is likely due to a decreased electron density on the ARS oxygens caused by the electron deficient boron. Acetyl and benzoyl substitution on hydroxy-anthraquinones results in similar shifts.[17]

ARS can be used as a general fluorescent reporter for studying the binding events between boronic acids and carbohydrates. It needs to be emphasized that there is no intrinsic specificity to the ARS system, and this is vital for its application as a general fluorescent reporter. There are many examples in the literature of boronic acids designed to specifically bind a particular analyte.[18,19] The ARS system will allow researchers a general and sensitive method to study the binding events of any boronic acid–diol interaction both quantitatively and qualitatively. This will aid in the further development of carbohydrate sensors and other biologically important boronic acid compounds.

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