

Revisiting Boronate/Diol Complexation as a Double Stimulus-Responsive Bioconjugation

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ABSTRACT: This study presents a quantitative assessment of the complexation between boronic acids and diols as a reversible and double-stimulus (oxidation and acidification)-responsive bioconjugation reaction. First, by using a competition assay, we have evaluated the equilibrium constants (water, pH 7.4) of 34 boronate/diol pairs, using diols of both aliphatic and aromatic (catechols) nature; in general, catechols were characterized by constants 3 orders of magnitude higher than those of aliphatic diols. Second, we have demonstrated that successful complexation with diols generated in situ via enzymatic reactions, and the boronate complexation was also employed to calculate the Michaelis–Menten parameters for

two catechol-producing reactions: the demethylation of 3-methoxytyramine and the 2-hydroxylation of estradiol, respectively, mediated by P4502D6 and P4501A2. Third, we have prepared phenylboronic acid-functionalized hyaluronic acid (HA) and demonstrated the pH and H₂O₂-responsive character of the adducts that it formed with Alizarin Red S (ARS) used as a model catechol. The versatility and selectivity of the complexation and the mild character of the chemical species involved therefore make the boronate/catechol reaction an interesting candidate for bioconjugation purposes.

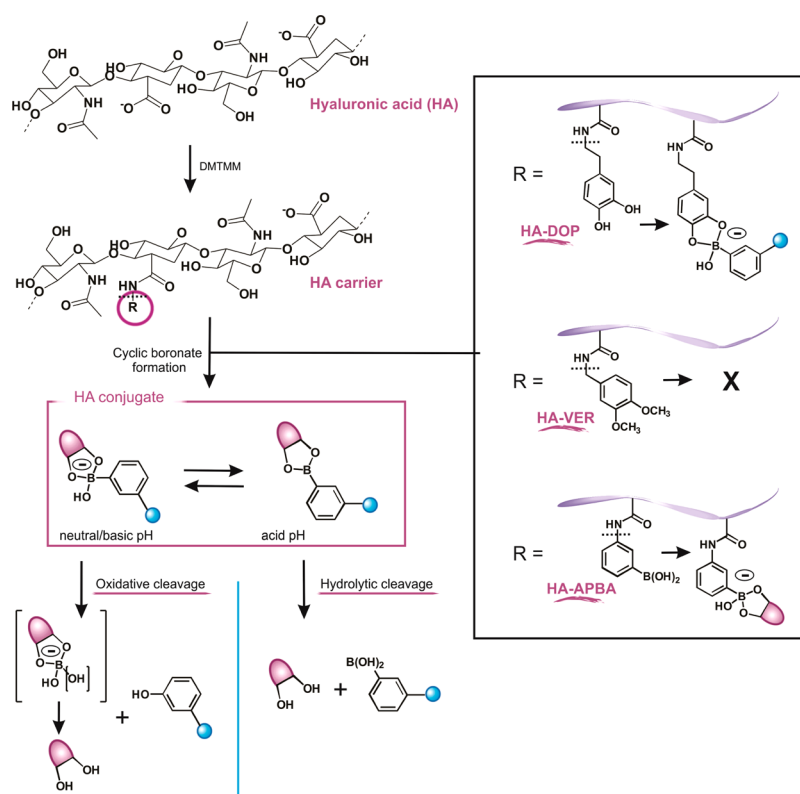
INTRODUCTION

The reaction between boronates and diols in aqueous solution is quantitative, selective, and orthogonal to the reactivity of a majority of biologically relevant functional groups,^{1,2} thus fulfilling the typical requirements of a bioconjugation reaction.³ Indeed, the boronate/diol complexation has been used to decorate the surface of living cells (their glycocalyx) with synthetic polymers,^{4,5} to selectively recognize saccharides in biological fluids,⁶ and for protein immobilization.^{7,8} The complexation between boronate-functionalized polymers and aliphatic⁹ or aromatic¹⁰ polyols has also been employed to prepare cross-linked colloids, or hydrogels that acquire self-healing properties from the reversibility of the reaction.¹¹ A review covering the various applications of boronic acid-containing polymers (and their diol complexes) has recently been published by Sumerlin¹² and provides an up-to-date overview of the field. We are specifically interested in applying this versatile bioconjugation to hyaluronic acid (HA, [Scheme 1](#)). HA is a glycosaminoglycan found in virtually all extracellular matrices,^{13–17} with a broad spectrum of FDA-approved applications as a biomaterial.^{18,19} HA conjugation with bioactive molecules is appealing, because it combines the biocompatibility, degradability, and nonimmunogenicity of HA,

the improvement in solubility and half-life of the coupled (pro)drug,^{20,21} and the possibility to target HA receptors such as CD44, which is overexpressed in several pathological conditions²² (e.g., tumor progression^{23–26} or inflammatory disorders^{27,28}). Following this approach, we have recently used boronic acid-bearing HA derivatives for the reversible bioconjugation and delivery (in a nanoparticle form) of a biocidal polyphenol, tannic acid.²⁹

Interestingly, boronic esters are also environmentally responsive, being cleavable under acidic and oxidizing conditions (bottom left in [Scheme 1](#)): (A) B–O bonds have a markedly different hydrolytic stability when involving tricoordinated boron atoms (at low pH, easily hydrolyzable) or quaternarized, e.g., by hydroxyl groups (at neutral or basic pH, more stable against hydrolysis).³⁰ This has often been exploited to release drugs at acidic pH^{10,31} or to design carriers that would disassemble under acidic condition allowing their cargo to be released.^{10,32} (B) C–B bonds can be easily cleaved by oxidants such as hydrogen peroxide,³³ a feature used in

Scheme 1. HA Structures Were Prepared via DMTMM-Mediated Amidation with Dopamine (DOP), Veratrylamine (VER: Negative, Nonboronate-Binding Control) And 3-Aminophenyl Boronic Acid (APBA)^a



^aDOP and APBA derivatives can complex boronate- and diol-containing molecules, respectively, and in both cases HA conjugates presenting cyclic boronic ester groups as side chains are produced. The latter bear a negative charge at neutral/basic pH due to quaternarization of boron by OH anions; at acid pH, their neutralization decreases the complex stability and thus determines the release of payloads via hydrolytic cleavage and production of a diol and a boronate. As a second destabilization mechanism, the lability of the carbon–boron bond in an oxidizing environment releases a phenol and a cyclic borate, the latter eventually releasing a diol.

boron organic chemistry since the 1950s^{34–36} and more recently investigated as a tool to deliver payloads under oxidative conditions (e.g., sites of inflammation).³⁷ Recent literature also offers examples of bioconjugates sensitive to the combination of acidic pH and reactive oxygen species (ROS), and used to image apoptotic phenomena.³⁸

In order for the boronate/diol conjugation to have practical applications, the advantages of its stimulus responsiveness must be accompanied by a good stability in the absence of the stimuli. Therefore, the quantitative evaluation of boronic ester stability becomes critical; for example, its dependency on the diol pK_a is well-known.³⁹ Here, we have focused on the comparative evaluation of the binding strength (pH = 7.4) of a library of diols (Scheme 2) when binding to 3-aminophenylboronic acid (APBA) as a low molecular weight ligand, as well as a functional side group in a macromolecular structure (HA). The diols encompass a number of different structures, including sugars, catechols with high (HA-dopamine and PEG-dopamine), and low molecular weight and negative controls (1,3- or methylated catechols). Some substrates were enzymatically generated in situ (dopamine, 2-hydroxyestradiol) and/or solubilized with the help of a surfactant or a molecular host (quercetin, 2-hydroxyestradiol).

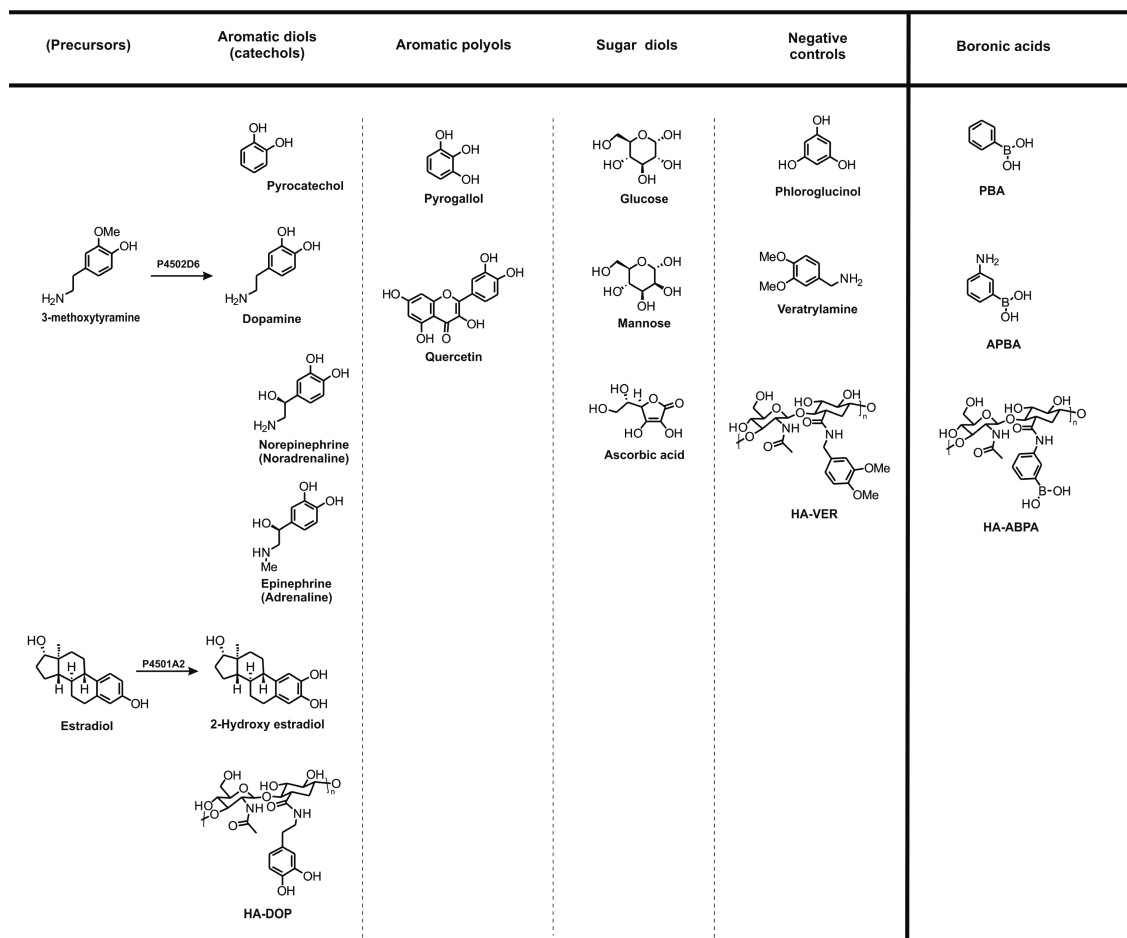
From the analytical point of view, the most popular assay employed in similar studies is based on the fluorescence of Alizarin Red S (ARS, a low pK_a anthraquinonic catechol), whose emission markedly increases when complexed to a bor(on)ic

acid. The affinity of a diol of interest and the bor(on)ic acid can be easily calculated through its competition with ARS and the correspondingly reduced emitted intensity.^{30,40} This approach, however, suffers from two drawbacks: First, the use of the Benesi–Hildebrand method, which for weak complexes (i.e., when the diol is a strong competitor such as a catechol) may produce large systematic errors.⁴¹ Second, fluorescence readings can be seriously affected by scattering, which means that complex dispersions can be a challenge. In this paper, to overcome the aforementioned limitations, we present a method based on the ARS hypsochromic shifts: the absorption spectrum of ARS shifts from red to yellow after binding boronic acids, which has often been used to qualitatively assess the formation of boronic esters.^{10,38,40}

RESULTS AND DISCUSSION

Preparation of Hyaluronic Acid Derivatives. In this study we have prepared and employed HA derivatives bearing 16–19 mol % of functional residues, and with a weight-average molecular weight in the range of 200–300 kDa (from SEC-MALLS measurements; see Supporting Information, Section 1SI, Additional materials and methods). HA was functionalized with amine-containing compounds using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) as a water-soluble activating agent (experimental procedure provided in the Supporting Information). DMTMM was preferred to the more popular *N*-(3-(dimethylamino)propyl)-

Scheme 2. Structures of Diols (Columns in the Left and in the Center) and Boronic Acids (Right Column) Used in This Study^a



^aDopamine was used in a free aromatic diol form, but was also generated in situ via enzymatic demethylation from 3-methoxytyramine; a second aromatic diol was prepared in situ, i.e. 2-hydroxyestradiol obtained via enzymatic oxidation of estradiol in cyclodextrin suspension. As negative controls, we have used compounds cannot bind (strongly) to boronic acids either because of the non-vicinal position (phloroglucinol) or the methylation of OH groups.

N' ethylcarbodiimide (EDC): the reaction product of the latter is both strongly hydrogen bonding and partially protonated at physiological pH, and therefore difficult to remove from HA in a quantitative fashion. While the size of HA with low and medium molecular weight ($M_w = 51$ and 210 kDa, respectively) did not show significant decrease following functionalization, degradation was observed when the starting material was high molecular weight HA (see Supporting Information, Table 1SI). The choice of the degree of derivatization was a compromise between high functionalization and retention of enzymatic degradability. Since mammalian hyaluronidases bind to HA utilizing its anionic carboxylic groups, with a binding region spanning at least a hexasaccharide sequence,⁴² the introduction of groups bearing no negative charge may alter its degradability, as happens for a number of HA derivatives such as the HYAFF family.^{43,44} However, although with a slower kinetics than the pristine HA, all the derivatives prepared in this study substantially maintained their enzymatic degradability (see Supporting Information, Figure 1SI A). The presence of boronic acid groups caused negligible cytotoxicity on two cellular models with $IC_{50} > 1$ wt % for both L929 fibroblasts and J774.2 macrophages (see Supporting Information, Figure 1SI B); veratrylamine still caused relatively low toxicity, with IC_{50} values in the range 5–10 mg/mL, but the free catechols of

HA-DOP caused the IC_{50} to drop in the proximity to 1–2 mg/mL in both cell lines.

Boronic Acid/Diol Equilibrium Constants through a Spectral Shift Method. The UV–vis spectrum of ARS largely changes upon complexation with boronic acids: in water the wavelength of the absorption maximum of ARS shifts from 510 nm in the free state to 460 nm in the complexed form (Figure 1A); this red-to-yellow color change is accompanied by the development of fluorescence associated to the boronic ester and the two phenomena have been extensively reported in the literature.³⁰ Both fluorescence and spectral shifts are associated with the formation of the complex; thus, either of them can provide the boronate-bound/free ARS molar ratio, allowing the calculation of the corresponding equilibrium constants (see experimental section for a detailed description).

For a comparison of the two techniques, we have used the complexation of ARS to two model compounds: phenyl boronic acid (PBA) and 3-aminophenyl boronic acid (APBA). PBA allows a comparison to literature data, whereas APBA is a model for more functional boronic acids. From fluorescence, we have obtained $K_1^{\text{fluor}}(\text{PBA}) = 1648 \pm 97 \text{ M}^{-1}$, a value very similar to what obtained by Wang³⁰ using the same method (1300 M^{-1}), whereas the spectral shift method provided $K_1^{\text{shift}}(\text{PBA}) = 2357 \pm 172 \text{ M}^{-1}$. For the second

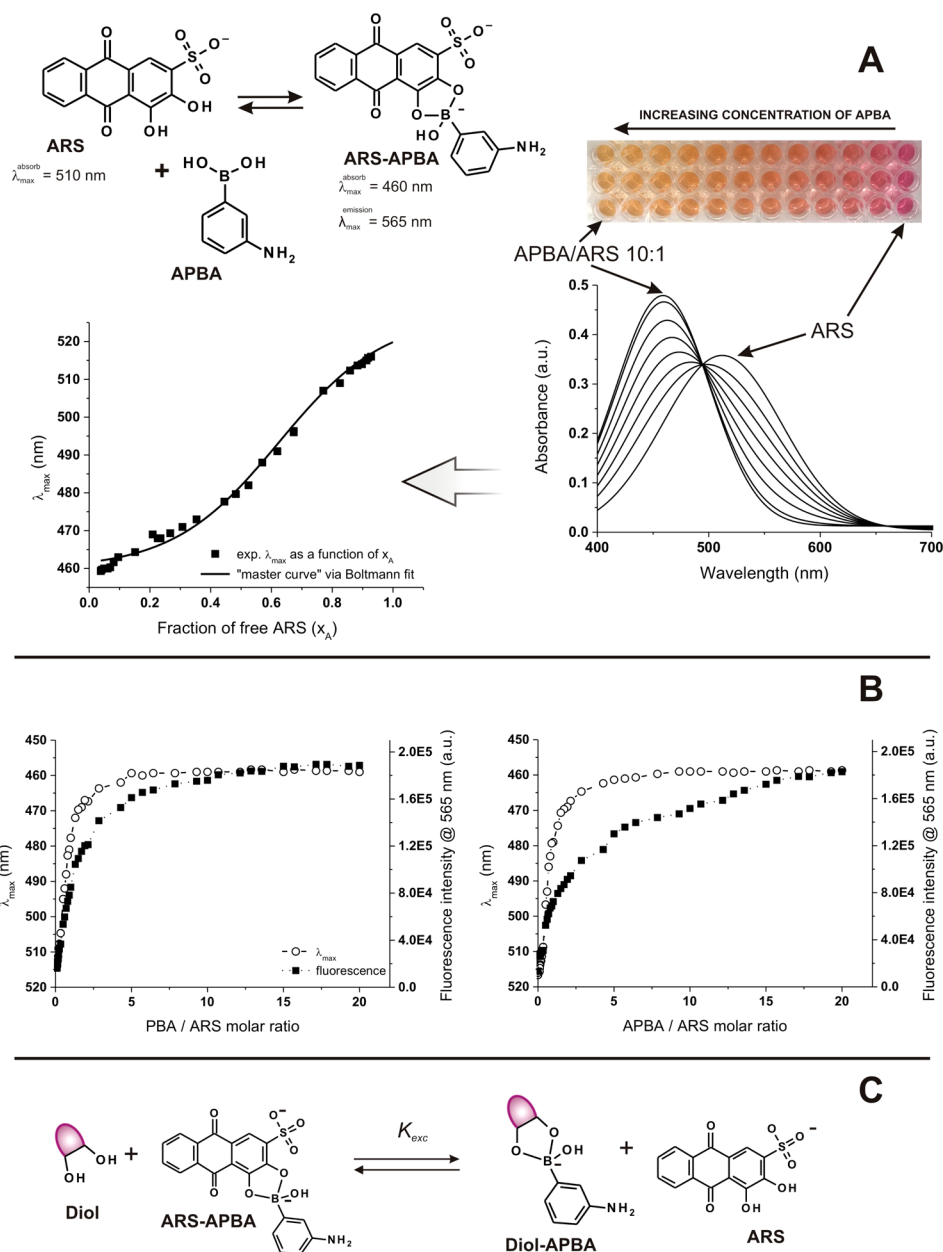


Figure 1. (A) Vis spectra of 0.15 mM ARS in PBS show a strong (up to 50 nm) hypsochromic shift with increasing content of the boronic acid APBA (graph on the right); the clear isosbestic point at 490 nm ensures the presence of only two species in the equilibrium: ARS and ARS-APBA. The λ_{\max} of the curves obtained as linear combinations of the spectra of ARS and of the ARS/APBA complex (see Supporting Information, Figure 2SI) has a sigmoidal dependence on x_A that can be fitted with a Boltzmann equation, generating a “master curve” that has an excellent agreement with the experimental data (graph on the left). (B) The shift in the absorption maximum and the fluorescence intensity provide comparable results, but in some cases fluorescence data are affected by additional phenomena. For example, while the two methods provide identical results for the complexation of ARS with phenyl boronic acid (PBA), the use of APBA (featuring an additional NH_2 group) causes a progressive increase of the fluorescence, possibly related to scattering; additionally, when using HA-APBA, the fluorescence intensity exhibited a clear time dependency (see Supporting Information, Figure 3SI). (C) Sketch of the competitive equilibria used to calculate the affinity of diols to APBA (or HA-APBA); considering $K_{\text{exc}} = \frac{K_{\text{diol}}}{K_{\text{ARS}}}$, from the knowledge of K_{ARS} , $[\text{ARS}]$, and $[\text{ARS-APBA}]$ it is possible to obtain K_{diol} (Table 1).

equilibrium, we found $K_1^{\text{fluor}}(\text{APBA}) = 1349 \pm 73 \text{ M}^{-1}$ and $K_1^{\text{shift}}(\text{APBA}) = 2465 \pm 132 \text{ M}^{-1}$. The spectral shift method therefore provided higher equilibrium constants, as a graphical comparison may also suggest (Figure 1B: the empty circles reach a plateau before the black squares). This difference may stem from the higher sensitivity of fluorescence to factors such as aggregation and scattering; in fact, in some cases the fluorescence signal showed a significant decrease in intensity with time (see Supporting Information, Figure 3SI), which is

possibly due to self-quenching caused by the increased proximity of fluorophores in aggregates. In order to confirm the possibly higher accuracy of the spectral shift method, we have used a third technique to estimate the equilibrium constants for the two systems above; the values obtained via $^1\text{H NMR}$ $K_1^{\text{NMR}}(\text{PBA}) = 3100 \text{ M}^{-1}$ and $K_1^{\text{NMR}}(\text{APBA}) = 3600 \text{ M}^{-1}$; see Supporting Information, additional material and methods and Figure 4SI) were indeed closer, albeit 30–40% higher, to those obtained with the spectral shift method.

Table 1. Equilibrium Constants for the Complexation of Diols with APBA and HA-APBA at pH 7.4 in 0.1 M PBS Buffer^a

diol					
class	name	pK _a	K[APBA] (M ⁻¹)	K[HA-APBA] ^b (M ⁻¹)	optimal pH ^c
positive control	ARS	4	2465 ± 132	2550 ± 150 ^d	6.5
negative controls	Phloroglucinol	8.5 ⁴⁵	0	0	8.7
	Veratrylamine	=	0	0	=
	HA-VER	=	0	0	=
aromatic diols	Pyrocatechol	8.6 ⁴⁶	1490 ± 70	1844 ± 110	8.8
	Dopamine	8.9 ⁴⁷	1555 ± 66	1955 ± 117	8.9
	Dopamine from 3-methoxytyramine	8.9 ⁴⁷	1550 ± 73	1839 ± 110	8.9
	Epinephrine	8.6 ⁴⁸	1445 ± 65	1910 ± 114	8.7
	Norepinephrine	8.6 ⁴⁹	1545 ± 66	1969 ± 118	8.8
	2-hydroxyestradiol from estradiol	9.7 ^e	955 ± 38 ^f	1061 ± 67 ^f	9.4
aromatic polyols	Pyrogallol	9.1 ⁵⁰	1796 ± 78 ^g	2325 ± 140 ^g	9.0
	Quercetin	7.6 ⁵⁰	1240 ± 98 ^h	1312 ± 115 ^h	8.3
saccharidic diols	Mannose	12.1 ⁵¹	6 ± 1	6 ± 1	10.5
	Glucose	12.3 ⁵¹	4 ± 1	4 ± 1	10.6
	Ascorbic acid	4.2 ⁵²	6 ± 1	7 ± 1	6.5
polymeric diols	HA-DOP	(8.9) ^j	1440 ± 61	1717 ± 103	(8.9) ^j
	PEG-dopamine	(8.9) ^j	1025 ± 42	1432 ± 86	(8.9) ^j

^aSD are calculated over $n = 3$. ^bEquilibrium constants were measured by using HA-APBA(1100). ^cOptimal pH was calculated according to Yan et al.⁵³ as $\text{pH}_{\text{optimal}} = (\text{pK}_a(\text{boronic acid}) + \text{pK}_a(\text{diol})/2)$. The pK_a of HA-APBA is assumed to be in the region of 9, similarly to APBA. ^dThe values for the complexation of ARS with HA(235)-APBA or HA(35)-APBA are respectively 2605 ± 332 and $2525 \pm 213 \text{ M}^{-1}$. ^ePredicted via the ChemAxon pK_a calculator (<http://www.chemaxon.com/products/calculator-plugins/property-predictors/#pka>). ^fIn the presence of sulfonated β -cyclodextrin to solubilize the steroid. The end point of the enzymatic reaction was used to calculate the value of the equilibrium constant, assuming complete conversion of estradiol. ^gIn the presence of TCEP to avoid pyrogallol oxidation. ^hIn the presence of Tween 20 to solubilize quercetin. ⁱDue to the structural similarity, we estimate the pK_a of these catechols to be substantially analogous to that of dopamine.

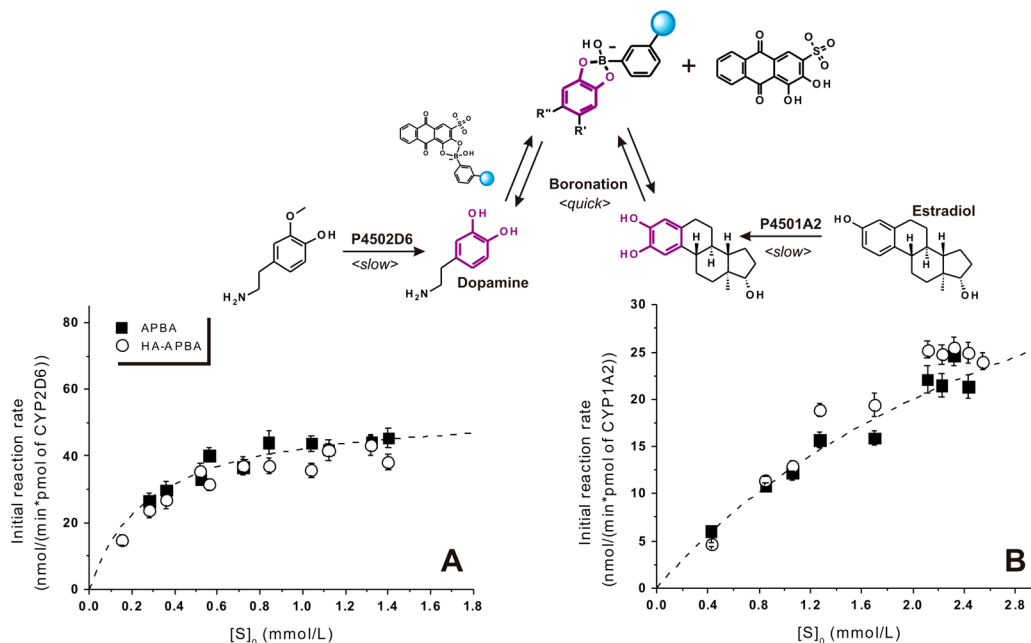


Figure 2. One-pot conjugation through in situ enzymatic generation of catechols and binding to boronic derivatives can be monitored using ARS as an efficient reporter system that allows us to extract information about enzymatic kinetics. The Michaelis–Menten plots in A and B are respectively obtained through the P450-mediated demethylation of 3-methoxytyramine to dopamine (A) and the hydroxylation of estradiol (B).

Due to its better stability, we have then employed the ARS spectral shifts for all further complexation studies. In particular, we have used the ARS as a reporter in competitive equilibria with other diols (Figure 1C; see Scheme 2 for the chemical structures of all diols used), estimating the binding constants of these diols to APBA and HA-APBA (Table 1). The reliability of the method was confirmed by using three negative controls: the dimethylated catechol of veratrylamine and the all-*meta*

aromatic hydroxy groups of phloroglucinol did not show any appreciable competition with ARS.

In accordance with the literature, the affinity for boronates of sugar diols was markedly lower than that of aromatic diols;³⁰ this is due to a combination of lower acidity (hence negligible deprotonation at physiological pH) and a less favorable conformation of the vicinal OH groups. The latter point explains why mannose—a *cis* diol—binds more strongly than

glucose, while both factors rationalize the higher binding of ascorbic acid.

On the other hand, all aromatic diols and polyols exhibited a similar affinity to APBA or HA-APBA, indicating a negligible influence of the nature, size, or polarity of the other parts of the molecule. The equilibrium constants for these compounds were lower than that of ARS, which is a likely consequence of their higher pK_a (= lower chance of quaternarization of the boronate ester).

It is noticeable that the macromolecular nature of HA-APBA did not appear to have any detrimental influence: HA-APBA with $\bar{M}_w = 50$ and 300 kDa had indistinguishable affinity for ARS (respectively, 2605 ± 332 and $2525 \pm 213 \text{ M}^{-1}$). Further, the binding of any diol was slightly but consistently higher for the polymeric HA-APBA than for the low molecular weight APBA; this slight increase is likely to stem from an avidity effect (= local concentration of boronates being higher than the macroscopic one), which would also explain the higher binding strength recorded for HA-DOP vs PEG-dopamine. It is, however, important to notice that the equilibrium constants recorded on macromolecular compounds are average values: the binding strength of HA-APBA with free boronates is likely much higher than that of the same polymer where the same groups are almost all bound to catechols.

Enzyme-Induced Bioconjugation. Catechols can be generated by a variety of enzymatic methods and we investigated whether these processes can be used for the in situ bioconjugation of the products. As examples, we have used two variants of cytochrome P450, CYP2D6 as a demethylase⁵⁴ and CYP1A2 as a hydroxylase,⁵⁵ to respectively produce dopamine from 3-methoxytyramine⁵⁶ and 2-hydroxyestradiol from estradiol⁵⁷ (Figure 2). These two processes can act as general models for the conjugation of in situ generated catechols, respectively, with a hydrophilic and a hydrophobic nature.

The constants for the dopamine-APBA/HA-APBA equilibria were measured previously and those obtained for enzymatically generated dopamine were indistinguishable (Table 1, compare the sixth and seventh entries), which confirmed both the efficiency in the demethylation of 3-methoxytyramine and the robustness of the analytical method. The conversion of estradiol into a catechol was performed in a more complex environment, since estradiol was solubilized in a sulfated β -cyclodextrin guest-host system. The constants that we recorded for the 2-hydroxyestradiol-APBA/HA-APBA equilibria were significantly lower than those shown by all other catechols; we ascribe this to both the more difficult accessibility of the catechol in the guest-host complex and above all to the electrostatic repulsion between the anionic groups on the cyclodextrin and on ARS.

Most interestingly, the spectral shift method also allowed us to follow the kinetics of the enzymatic reactions, by monitoring the ARS absorbance as a function of time (Figure 2A,B). By knowing the equilibrium constant of the catechol and under the assumption that the complexation is considerably quicker than the enzymatic conversions, it was then possible to estimate the kinetic parameters for both reactions (Table 2). For the production of dopamine, a direct comparison of our results with literature data is hardly possible, because this reaction has never been studied quantitatively; our data are broadly comparable to examples of CYP2D6-mediated demethylation of *o*-methylphenols, which however are largely variable (see Supporting Information Table 2SI) possibly as a result of the

large polymorphism of this enzyme.⁵⁸ The only noteworthy point is that our K_M values are rather high, suggesting a lower affinity of the enzyme for a substrate with an OH vicinal to the target methoxy group. A more quantitative comparison would appear to be possible for the 2-hydroxylation of estradiol, for which K_M and the enzymatic clearance appear to be respectively in the proximity of 20 μM and in the range 0.018–0.06 $\mu\text{L}/(\text{pmol P450}\cdot\text{min})$ (see Supporting Information Table 2SI; please note that the reactions are strongly NADPH-dependent and the K_M values vary considerably depending on the presence of the latter; see Supporting Information, Figure 6SI). We have observed a very comparable clearance value but a dramatically lower K_M ; this can be attributed to the charge and steric hindrance of the estradiol/cyclodextrin complex, but literature values often refer to oversaturated solutions (up to 200 μM with estradiol water solubility being 20 μM ⁵⁹) where phase separation may lead to an overestimated affinity of CYP1A2 for estradiol.

Release Behavior and Double Responsiveness of Boronate/Catechol Conjugates. Despite the relatively low values of the equilibrium constant at neutral pH, the complexation strength of aliphatic diols with boronates is still sufficiently high to allow, e.g., the determination of a variety of saccharidic structures.^{60,61} As reported above, the complexation of boronate/catechol couples is orders of magnitude more stable than that of aliphatic diols, therefore in principle amenable to bioconjugation; nevertheless, the equilibrium constants are not very large (around 10^3 M^{-1}), and they may not be able to guarantee stability against dilution, which is a common problem in the design of injectable pharmaceutical formulations. In this part of the work, we have focused on improving the stability of the structure through the use of a stoichiometric excess of boronates on the HA structure, i.e., by locally increasing the carrier avidity. Please note that, due to the higher local concentration of free boronates, the resulting binding of the catechols is considerably stronger than what could be assumed on the basis of the equilibrium constants presented in Table 1; while the latter were obtained using variable boronate/catechol ratios and therefore present an average binding strength, an excess of free boronates is expected to increase significantly (see, e.g., the difference between experimental data and theoretical values in Figure 3A).

The boronate/diol complexes are well-known to reverse by acidification,³⁰ although in a fashion dependent on the diol pK_a . Here, we have placed the solution of an HA-APBA/ARS conjugate at pH 7.4, 7.0, 6.0, and 5.0 in dialysis bags, measuring the release of ARS in a 20-fold larger volume of fluid (thereby at least partially evaluating the effect of dilution) as a function of time. The equilibrium is apparently reached after 24 h (Figure 3A), showing that at pH 7 about 30% of the diol was released, therefore showing a relatively good stability of the complex; on the other hand, at acidic pH the decomplexation was quantitative; importantly, the release kinetics shows an initial burst followed by a slower release, but this profile is likely greatly affected by the transfer through the dialysis membrane, and the actual release may be considerably more rapid. Importantly, the overall trend observed was in agreement with the data already reported,³⁰ emphasizing once more the little effect of the HA on the pH-induced release phenomenon.

The oxidative cleavability of the boronate esters is based on cleavage of sp² carbon-boron bond,³⁷ and correspondingly increasing amounts of 3-aminophenol were produced when a APBA/ARS adduct was incubated with increasing amounts of

H₂O₂ (Figure 3B). It is noticeable that, although the oxidation of free catechols to *ortho*-quinones is also possible, no trace of ARS quinone were found, thus suggesting that catechols may be released as oxidation-protected boric acid complexes. This is an important point to ensure the stability of in principle oxidation-sensitive drugs in an oxidizing (e.g., inflamed) biological environment.

Having proven the principle of oxidative responsiveness, it is also important to note that this was not perfectly stoichiometric, and a certain excess of oxidant appeared necessary for a quantitative conversion of the complexes. For example, stoichiometric equivalence of H₂O₂ only corresponded to a 35% oxidation of the APBA/ARS, and higher excesses of oxidants were necessary for HA-APBA/ARS conjugates (50% release with 10 equiv). The apparently lower reactivity of the macromolecular complexes may actually stem from the presence of an excess of noncomplexed boronic acids (to boost the carrier avidity) that are likely easier to oxidize. However, this event corresponds to the release of boric acid, not of the drug.

CONCLUSION

Here we have presented a successful proof of principle for the use of boronic/diol complexation for the purpose of reversible and responsive bioconjugation. In this demonstration, the use of HA as a model platform offers a plethora of benefits, including targeting.

We have thus compared the performance of a boronic acid-containing HA derivative to that of the low molecular weight APBA in the complexation to a library of diols. Instead of detrimentally affecting the equilibrium constants (e.g., because of steric hindrance or electrostatic repulsion between anionic boronic esters), the use of HA-APBA slightly increased (5–15%) the average binding strength, which we attributed to the avidity of the polymeric structure.

It is noteworthy that we have adopted a modified analytical method for the evaluation of boronic/diols equilibria, which uses ARS as an absorbance reporter. In comparison to the more commonly used fluorescence tests (based on ARS emission), this method is specifically advantageous to study concentrated and possibly heterogeneous samples, allowing, for example, to monitor this “click” reaction in a self-emulsifying system (Tween 20 + quercetin) and during enzymatic kinetics, also performed on solubilized active principles (cyclodextrin + estradiol). Using this absorbance assay, we have also for the first time demonstrated that CYP2D6 is able to demethylate the monomethyl ether of dopamine (3-methoxytyramine). This finding could be important considering that one of the major drawbacks of Parkinson’s disease therapies based on the use of catecholamine drugs or pro-drugs (e.g., L-DOPA) is the inactivation of the exogenous catecholamines through methylation (operated by catechol *O*-methyltransferase, COMT).⁶²

In the conclusive part of the study, we have then demonstrated that the boronic acid-bearing HA derivatives allowed the responsive release of catechols in acidic or oxidative environment. Indirect evidence suggests that in the latter case the released catechols are not further oxidized to quinones possibly because they are released as boric acid complexes.

Since a slightly acidic pH and the presence of ROS are often associated with pathological states, we believe that this kind of HA bioconjugation could provide the means for a more efficient delivery due the combination of receptor (CD44) targeting and triggered (pH or H₂O₂) release.

EXPERIMENTAL PROCEDURES

The list of all reagents and the description of the synthetic procedures for all HA derivatives are reported in the Supporting Information, additional materials and methods.

If not otherwise stated, all solutions were prepared in 0.1 M PBS obtained by dissolving sodium dihydrogen orthophosphate dihydrate, disodium hydrogen orthophosphate dihydrate, and sodium chloride, supplied by BDH (U.K.), in concentrations, respectively, of 2.3 g/L, 11.8 g/L, and 9 g/L in water purified in a Milli-Q system (Millipore, U.K.).

Study of Boronate-Diol Equilibria. We have employed a BioTek Synergy 2 multimode microplate reader to record absorbance spectra (in the range $\lambda = 400\text{--}800$ nm) and fluorescence readings (filters at $\lambda_{\text{exc}} = 485 \pm 20$ nm and $\lambda_{\text{em}} = 620 \pm 40$ nm); temperature was kept at 25 °C, except for the spectra of enzymatic reactions which were recorded at 37 °C. All the spectra were corrected by subtracting the scattering component of the buffer solution using appropriate blanks.

In this analysis we have not differentiated between the equilibria leading to the formation of the trigonal or the tetragonal form of the diol-boronic esters. The binding constants provided for the diol-boronic equilibria are overall affinities at pH 7.4.

In this discussion we will identify ARS as *A*, the boronic acid partner as *B*, any competitive diol as *D*, the complex between *A* and *B* as *AB*, and the complex between *B* and *D* as *DB*.

Determination of ARS/Boronic Acid Binding Constants through Fluorescence. General Conditions. The experimental design developed by Springsteen et al.³⁰ was adapted to be performed in 96 well plates. Briefly, 125 μL ARS solution (1.8×10^{-5} M) were titrated with an equal volume a solution of PBA or APBA with concentrations ranging between 3.6×10^{-3} and 1.8×10^{-4} M. The plates were incubated 30 min in the plate reader at 25 °C and then the fluorescence intensities were recorded ($\lambda_{\text{exc}} = 485 \pm 20$ nm and $\lambda_{\text{em}} = 620 \pm 40$ nm).

Analytical Method Used for the Calculation of Binding Constants. The binding of ARS to PBA and APBA can be studied by recording the increase in fluorescence intensity of an ARS solution upon addition of the boronic acids, a method pioneered by Wang.⁴⁰ Fluorescence data were fitted according to the Benesi–Hildebrand method which can be used to measure the association constant (K_{eq}) of a reaction when two species are in equilibrium with their one-to-one complex.⁶³ The mathematical approach is based on the assumption that when either one of the reactants is present in molar amount higher than 10 equiv over the other reactant, its concentration can be considered constant. Under these conditions a set of data relating the recorded changes in fluorescence intensity (ΔI_f) at different initial concentrations of the boronic acid containing compound (C_B) can be fitted according to the following equation:

$$\frac{1}{\Delta I_f} = (\Delta k p_0 C_A K_{\text{eq}})^{-1} \cdot \frac{1}{C_B} + (\Delta k p_0 C_A)^{-1} \quad (1)$$

where C_A is the total concentration of ARS, and $\Delta k p_0$ is a constant derived from the quantum yield and instrumental parameters. Thus, by plotting $1/\Delta I_f$ vs $1/C_B$, it is possible to obtain K_{eq} dividing the intercept by the slope of the fitted line.

Determination of ARS/Boronic Acid Binding Constants through Hypsochromic Shifts. General Conditions. For binding experiments, 125 μL of a solution of APBA or PBA at a concentration ranging from 0.00 to 4.05 mM (at least 23

different concentrations were prepared) were mixed in each well of a 96 well plate with 125 μL of a solution 0.30 mM of ARS (final ARS concentration = 0.15 mM, total volume = 250 μL /well). The plate was incubated 30 min in the plate reader at 25 °C and then the absorbance readings were recorded between 400 and 800 nm (Figure 1A provides a picture of a typical plate during the assay).

Analytical Methods Used for the Calculation of Binding Constants. When using boronic acids such as APBA or PBA (B) that lack a significant absorption in the visible or near UV spectral region, the only absorbing species are ARS (A) and the ARS-boronic ester (AB). The spectra of ARS alone and of its complex, obtained from ARS in the presence of a large excess of boronic acid containing compound, can be accurately fitted with Gaussian models (see Supporting Information, Figure 2SIA), it is possible to recreate the absorption spectrum of any mixture of ARS with its boronic ester, expressing it as a linear combination of the spectra of the two pure substances (see Supporting Information, Figure 2SIB)

$$\text{Abs} = x_A C_A \varepsilon_1 e^{[-2(\lambda - \lambda_{\text{max}1})/\omega_1]^2} + (1 - x_A) C_A \varepsilon_2 e^{[-2(\lambda - \lambda_{\text{max}2})/\omega_2]^2} \quad (2)$$

where the absorbance Abs depends on the molar concentrations of the two products (expressed as the molar fraction x_A times the total concentration of ARS, (C_A), and on parameters obtained from the spectra of the two pure products: $\lambda_{\text{max}1}$ and $\lambda_{\text{max}2}$, ε_1 and ε_2 , ω_1 and ω_2 , which are, respectively, the wavelengths of the maxima of the spectra of ARS and of its boronic ester, the corresponding extinction coefficients and parameters related to the bandwidth. By plotting the wavelength of the absorbance maxima of these calculated spectra vs x_A one obtains a sigmoidal graph (Figure 2, right). This graph allows one to relate the location of the maximum absorption for a given ARS/boronated compound mixture to the fraction of free ARS in that mixture (x_A). By knowing x_A as a function of the constant of the equilibrium between ARS and APBA or PBA (K_1) and of the initial concentrations of the reactants, C_A and C_B

$$K_1 = \frac{[AB]}{[A][B]} = \frac{[AB]}{(C_A - [AB])(C_B - [AB])} \quad (3)$$

$$[AB] = \frac{C_A + C_B + \frac{1}{K_1} - \sqrt{\left(C_A + C_B + \frac{1}{K_1}\right)^2 - 4C_A C_B}}{2} \quad (4)$$

$$\begin{aligned} x_A &= 1 - \frac{[AB]}{C_A} \\ &= 1 - \frac{C_A + C_B + \frac{1}{K_1} - \sqrt{\left(C_A + C_B + \frac{1}{K_1}\right)^2 - 4C_A C_B}}{2C_A} \end{aligned} \quad (5)$$

Using a fixed concentration C_A (0.15 mM) and varying C_B (between 0.00 and 2.03 mM) it is possible to obtain a set of x_A values, where $x_A = f(C_B)$. Equation 5 can be edited as a user defined function in data analysis software (Origin 8.5.1), allowing one to calculate K_1 as a fittable parameter.

Determination of Diol/Boronic Acid Binding Constants through Competitive Binding with ARS. General Conditions. For competitive binding experiments in each well of a 96 well

plate 50 μL of a 0.75 mM ARS solution and 50 μL of a 0.75 mM APBA solution were mixed; the color of the solution changed from red to orange. Separately, diol solutions with concentrations ranging between 0.13 and 250.00 mM were prepared (corresponding to ABPA/diol molar ratios ranging from 1:0.5 to 1:1000). 150 μL of each diol solution (final ARS and APBA concentration = 0.15 mM, total volume = 250 μL /well) were added, producing a clear orange to red chromatic change at high ABPA/diol molar ratios. The absorbance readings were then recorded as previously described.

The general procedure was modified for the following cases:

Reactions involving veratrylamine and phloroglucinol: Due to their limited solubility in water, the highest APBA/diol molar ratio was 1:100.

Reactions involving pyrogallol: Owing to the quick oxidation of this electron-rich polyol, TCEP was added to the diol stock solution in a molar ratio pyrogallol/TCEP 1:1.5. Due to acidity of TCEP, a few drops of 1 M NaOH were added to bring the pH back to 7.4.

Reaction involving ascorbic acid: Due to the acidic nature of this diol, the pH of the final solution in 0.1 M PBS was raised to 7.4 by adding few drops of 1 M NaOH.

Reaction involving quercetin: Owing to the very poor solubility of quercetin in water environment, Tween 20 (a surfactant widely employed in bioassays and pharmaceutical formulations) was used. For quercetin concentrations up to 1 mM a 1:1 Tween 20/quercetin molar ratio was sufficient to completely solubilize it in 0.1 M PBS buffer; in fact, quercetin UV-vis absorbance reached a plateau and did not increase with larger amounts of surfactant. The highest concentration of Tween 20 was shown to be limited by its ability to hamper the binding reaction: a maximum of 8% w/w of Tween 20 can be used without affecting the reaction kinetics (data not shown).

Reactions involving polymeric species: The amount of polymer was calculated on the basis of its number-average degree of functionalization. HA derivatives were dissolved and kept in solution for 12 h prior to the addition of any reagent. Finally, TCEP was added to the solutions when polymers functionalized with dopamine were involved (dopamine/TCEP molar ratio = 1:1.5) to prevent oxidation.

Calculation of Binding Constants. The gradual addition of a diol (D) to an ARS boronic ester determines an increasing bathochromic shift due to the production of free ARS. The corresponding competitive equilibrium $AB + D \rightleftharpoons DB + A$ is governed by a constant K which can be expressed as the ratio of the two equilibrium constants for the formation of individual boronic esters, AB and DB, but can also be expressed as a function of the concentration of free ARS as the only variable (see eqs 6 and 7).

$$K_{\text{exc}} = \frac{[A][DB]}{[AB][D]} = \frac{[A][B]}{[AB]} \cdot \frac{[DB]}{[B][D]} = \frac{K_{\text{diol}}}{K_{\text{ARS}}} \quad (6)$$

$$\begin{aligned} K_{\text{exc}} &= \frac{[A][DB]}{[AB][D]} \\ &= \frac{[A](C_B - [AB])}{(C_A - [A])[D]} \\ &= \frac{[A](C_B - C_A + [A])}{(C_A - [A])(C_D - C_B + (C_A - [A]))} \end{aligned} \quad (7)$$

For each given amount of diol, one obtains a λ_{max} value from which it is then possible to calculate the fraction of free ARS

($[A] = x_A \cdot C_A$), as shown in the previous section. From each C_D value is possible to obtain K_{exc} and, finally, its value multiplied by K_{ARS} is the equilibrium constant of interest, K_{diol} . The values of K_{diol} were averaged through a range of diol concentrations corresponding to ABPA/diol molar ratio from 1:0.5 to 1:1000.

Enzymatic Reactions. General Conditions. Typical experiments were performed in 96 well plates at 37 °C sealed with the help of PCR plate adhesive seals to prevent evaporation. The plates were prepared by mixing ARS and APBA as previously (50 μL of a 0.75 mM ARS solution and 50 μL of a 0.75 mM APBA solution). Then, 100 μL of diol was added followed by 50 μL of the enzyme working solution. The diol and the enzyme working solutions were prepared as follows:

Demethylation of 3-methoxytyramine: The cytochrome P450 variant 2D6 can remove the methyl group from 3-methoxytyramine producing dopamine. 3-Methoxytyramine was solubilized in PBS at concentrations ranging from 0.19 mM to 1.22 mM (final APBA/3-methoxytyramine molar ratio of 1:0.5 to 1:3.2). The solution of CYP2D6 was prepared by diluting 1:50 a 500 pmol/mL solution of enzyme with a 8.3 mg/mL (10 mM on anhydrous basis) solution of NADPH in 0.1 M PBS.

Hydroxylation of estradiol: Estradiol can bind to boronic acid only upon the introduction of a second alcoholic function; this reaction can be carried out by cytochrome P450 variant 1A2. Estradiol (0.9 mg/mL, 3.38 mM) was solubilized in PBS by the addition of β -cyclodextrin sulfobutyl ether (7.3 mg/mL, 1:1 molar ratio). This solution was diluted to obtain diol concentrations ranging from 0.08 to 3.38 mM (final APBA/estradiol molar ratio of 1:0.1 to 1:4.5). The CYP1A2 solution was prepared by diluting 1:25 a 1000 pmol/mL solution of enzyme with a 10.0 mg/mL (12 mM on anhydrous basis) solution of NADPH in 0.1 M PBS.

Analytical Methods Used for the Calculation of Binding Constants. All enzymatic tests were based on the in situ production of a diol in the presence of the ARS/APBA complex. With increasing production of the catechol, the ARS UV-vis band undergoes a time- and concentration-dependent bathochromic shift. The dependence on time provides information about the kinetics of the enzymatic conversion; the dependence on the concentration of the precursor provides information about the binding constant of the diol. In this part of the study we have always assumed that the enzymatic conversion is the rate-determining step of the test, i.e., the competitive equilibrium is established in an “instantaneous” fashion as soon as new diol is produced. This assumption is reasonable, because the diol-boronic equilibria are established in a matter of seconds, while tenths of minutes are required to obtain quantitative yields in diols. We have also assumed that complete substrate conversion was obtained when the bathochromic shifts reached their asymptotic values; in this way it is possible to replace C_D in eq 7 with the initial substrate concentration ($[S_0]$), therefore allowing the calculation of the diol-boronic equilibrium constant K_{diol} .

It is then possible (see Supporting Information, Section 3SI Additional information about enzymatic reaction experiments) to use K_{diol} to express the substrate concentration ($[S]$) as a function of the molar fraction of free ARS (x_A), which can be calculated from the bathochromic shift; from the time dependence of $[S]$ it is possible to calculate the initial rate of the enzymatic reaction. In order to reduce the experimental error, this was done by fitting the time-dependent data as an exponential decay ($[S] = [S_0] \cdot e^{-kt}$). The initial reaction rate v

was then plotted vs $[S_0]$ and fitted using the usual Michaelis-Menten relationship $v = \frac{v_{\text{max}}[S]}{K_M + [S]}$

pH-Induced Release. Four 1 mL Spectra/Por Float-A-Lyzer (MWCO = 10 kDa) were filled with 1 mL of a solution containing 20 mg of HA-APBA (corresponding to 8.5 μmol of boronic acid) and 0.5 mg of ARS (1.5 μmol) in 0.1 M phosphate buffer at pH 6.0, 7.0, 7.4, and 0.1 M acetate buffer at pH 5.0 respectively. The Float-A-Lyzer was then immersed in 19 mL of the same buffers and incubated at 37 °C. 150 μL aliquots were collected at different time points ($n = 3$ for each sample) and the fluorescence intensities were recorded in a 96 well plate (filters at $\lambda_{\text{exc}} = 485 \pm 20$ nm and $\lambda_{\text{em}} = 620 \pm 40$ nm) and compared with a calibration curve of ARS in the same buffer to measure the amount of ARS released.

H₂O₂-Induced Release. Oxidation of APBA/ARS. Eleven wells of a 96 well plate were filled each with 50 μL of a 2.9 mM solution of ARS followed by 50 μL of a 2.9 mM solution of APBA; after 1 min 50 μL aliquots of hydrogen peroxide with concentrations ranging from 30 μM to 150 mM were added and the plate was incubated at 37 °C for 2 h. Finally, 50 μL aliquots of a 0.2 M Na₂SO₃ were added to quench the unreacted H₂O₂ and the samples were analyzed through HPLC. All the solutions were prepared in 0.1 M phosphate buffer at pH 7.4. Samples were analyzed via reverse phase HPLC on a Agilent 1100 series HPLC equipped with analytical (5 μm particle size, 4.6 \times 150 mm) Zorbax Eclipse XDB-C18 column and a Laserchrom S3210 UV/vis detector set at 254 nm. The analysis was performed at 1 mL/min in a mixed eluent composed of 10 mM phosphate buffer at pH 7.4 and acetonitrile with the following gradients: from 10% to 30% acetonitrile, min 0–6 min; from 30% to 80% acetonitrile, min 6–12; from 80% to 10% acetonitrile, min 12 to 15.

Oxidation of HA-APBA/ARS. 300 μL of a 5.0 mM ARS solution (1.5 μmol) was added to 1.7 mL of a 10 mg/mL solution of HA-APBA (corresponding to 7.2 μmol of boronic acid), both solutions in PBS at pH 7.4. 200 μL aliquots of the mixture were placed in Amicon Ultra-0.5 Centrifugal Filters with 3 kDa MWCO (Millipore) followed by 200 μL of 0.0073, 0.073, 0.73, and 7.3 mM H₂O₂ (0.01, 0.1, 1, and 10 equiv with respect to ARS or 0.0025, 0.25, 0.25, and 2.5 equiv with respect to boronates) in PBS. The samples were incubated for 2 h at 37 °C, and then free ARS was recovered via centrifugation (13 000 rpm for 30 min) and analyzed via the HPLC method described earlier.

ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.7b00080.

Additional materials and methods, binding studies, additional information about enzymatic reaction experiments (PDF)

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors acknowledge financial support from the EU FP7 project UniVax (grant number 601738) and from the Marie Curie IAPP Replixcel (grant number 251420), and from the AstraZeneca/University of Manchester cofunding of the NorthWest Centre of Advanced Drug Delivery (NoWCADD). The authors are indebted to Novozymes for the donation of all HA samples used in this study and want also to thank Dr. Elita Montanari for the helpful discussions.

ABBREVIATIONS

ARS, alizarin red S; APBA, 3-aminophenylboronic acid hydrochloride; PBA, phenylboronic acid; DOP, dopamine hydrochloride; NADPH, β -nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate; CYP2D6, human cytochrome P450 2D6 yeast reductase; CYP1A2, recombinant human cytochrome P450 1A2; HA, hyaluronic acid; PEG, poly(ethylene glycol); PEG-DOP, monomethoxy poly(ethylene glycol); RHAMM, hyaluronan-mediated motility receptor; GlcNAc, N-acetyl glucosamine; GlcA, glucuronic acid; ROS, reactive oxygen species

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