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Synergic 'Click' Boronate/Thiosemicarbazone System for Fast and Irreversible Bioorthogonal Conjugation in Live Cells

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Synergic 'Click' Boronate/Thiosemicarbazone System for Fast and Irreversible Bioorthogonal Conjugation in Live Cells

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ABSTRACT: Fast, high-yielding and selective bioorthogonal 'click' reactions employing non-toxic reagents are in high demand for their great utility in the bioconjugation of biomolecules in live cells. Although a number of click reactions were developed for this purpose, many are associated with drawbacks and limitations that justify the development of alternative systems for both single- or dual-labeling applications. Recent reports highlighted the potential of boronic ester formation as a bioorthogonal click reaction between abiotic boronic acids and diols. Boronic ester formation is a fast dehydrative process, however it is intrinsically reversible in aqueous medium. We designed and optimized a synergic system based on two bifunctional reagents, a thiosemicarbazide-functionalized nopoldiol and an *ortho*-acetyl arylboronic acid. Both reagents were shown to be chemically stable and non-toxic to HEK293T cells at concentrations as high as 50 μM. The resulting boronate/thiosemicarbazone adduct is a medium-sized ring that forms rapidly and irreversibly without any catalyst at low μM concentrations, in neutral buffer, with a rate constant of 9 $M^{-1}s^{-1}$ as measured by NMR spectroscopy. Control experiments in the presence of competing boronic acids showed no cross-over side-products and confirmed the stability and lack of reversibility of the boronate/thiosemicarbazone conjugates. Formation of the conjugates is not affected by the presence of biological diols like fructose, glucose and catechol, and the thiosemicarbazide-functionalized nopoldiol is inert to aldehyde electrophiles of the sort found on protein-bound glyoxylyl units. The suitability of this system in the cell-surface labeling of live cells was demonstrated using a SNAP-tag approach to install the boronic acid reagent onto the extracellular domain of Beta-2 adrenergic receptor in HEK293T cells, followed by incubation with the optimal thiosemicarbazide-functionalized nopoldiol reagent labeled with fluorescein dye. Successful visualization by fluorescence microscopy was possible with a reagent concentration as low as 10 μM, thus confirming the potential of this system in biological applications.

INTRODUCTION

By exploiting reactions that are inert to native functional groups, bioorthogonal chemistry has become a powerful tool to chemically label biomolecules in living cells to understand the roles of these biomolecules within their native environment.[1-6] Bioorthogonal chemistry has also been used in other applications such as installing post-translational modifications of proteins[7] or constructing antibody drug conjugates.[8] 'Click' chemistry[9,10] is central to the development of bioorthogonal reactions because it exhibits fast reactivity in aqueous environment at low concentrations (μM), with high yields and selectivity without any side products.[1,11,12] Moreover, in view of dual-labeling applications, it is desirable for a click reaction to be orthogonal with other existing bioorthogonal reactions.[3,13] Even the most common bioorthogonal reactions display limitations such as slow rates, use of complex reagents, side-reactions or lack of mutual orthogonality due to possible cross reactivity.[2,4,13] In order to address these challenges, our group recently reported a novel bioorthogonal reaction based on a 'click' boronate formation, which enables fast dynamic ligation and high stability (k_{ON} = 7.7 ± 0.5 $M^{-1}s^{-1}$, K_{eq} = 12 x 10^4 M^{-1}) between nopoldiol derivatives and 2-methyl-5-carboxymethyl-phenylboronic acid (Figure 1A).[14] Although the stability of the resulting boronic ester is high, its reversibility may be detrimental in live cell imaging since washing steps to remove excess reagents might lead to undesirable cleavage.

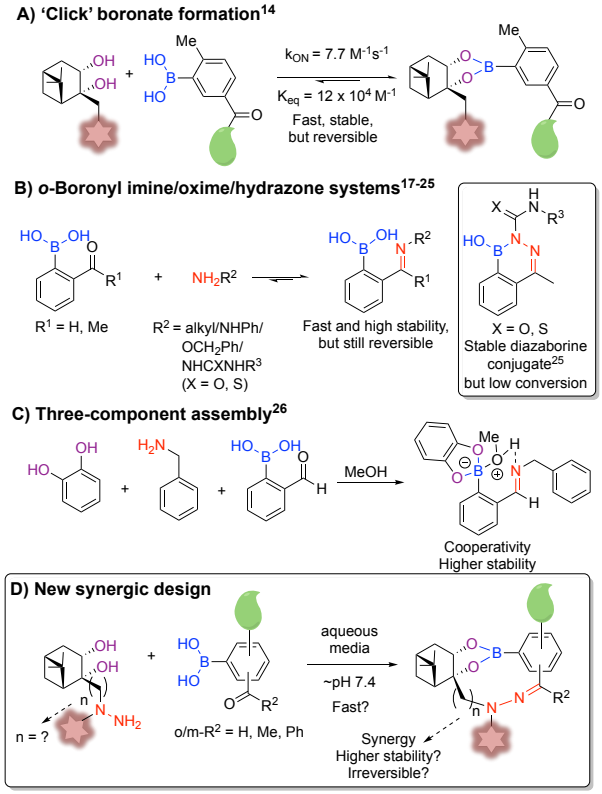


Figure 1. A) Previously reported 'click' boronate formation system. B) Enhancement in the rate and stability of the imine/hydrazone/oxime ligation in the presence of a boronyl unit in *ortho*-position. C) Positive cooperativity and higher stability of a three-component amine/boronic acid/catechol system. D) New synergic design based on the optimal placement of a hydrazine or (thio)semicarbazide on the nopol diol reagent, along with a carbonyl handle (*ortho* or *meta*) on the arylboronic acid.

Therefore, our ongoing goal is to improve the stability of this system and ideally achieve a fast and irreversible boronate click reaction. We hypothesized that such an advance could be achieved by exploiting a second, synergic interaction.

The most suitable additional covalent interaction must exhibit bioorthogonality and fast reactivity. Moreover, its reactive groups must be small, non-cytotoxic, stable and easy to install. To this end, imine, hydrazone and oxime ligations attracted our attention. Hydrazone and oxime ligations have found many applications in bioorthogonal chemistry. Those methods, however, tend to suffer from slow rates at neutral pH and can also exhibit reversibility.[1,15,16] Fortunately, recent reports indicate that the presence of a boronic acid *ortho* to an aromatic aldehyde or acetylketone improves both the ligation rate and stability of the resulting imine, hydrazone and oxime product due to a dative B–N bond (Figure 1B).[17-20] For example; Gois and co-workers initially reported an iminoboronate formation between 2-formylbenzeneboronic acid or 2-acetylbenzeneboronic acid (0.16 M) and lysine (0.16 M) in aqueous media with 61 and 71% yield, respectively (K_{eq} = 10 mM).[17] Gillingham and co-workers designed a rapid alkoxy-iminoboronate condensation between 2-formylbenzeneboronic acid and *O*-benzylhydroxylamine at neutral pH (k_1 = ~11 x 10^3 $M^{-1}s^{-1}$, k_{-1} = ~4.2 x 10^{-5} s^{-1}, K_{eq} = ~2.6 x 10^8 M^{-1}).[19] Meanwhile, Gao and co-workers demonstrated a rapid hydrazone ligation between phenyl hydrazine and 2-acetylbenzeneboronic acid.[18] Unfortunately, the reversibility of these reactions may limit their application in living cells. Bane and Gillingham recently disclosed the formation of 1,2-dihydro-1-hydroxy-2,3,1-benzodiazaborine as a stable product of click condensation between 2-formylbenzeneboronic acid and phenyl hydrazine.[21,22] Phenyl hydrazine is, however, susceptible to oxidation, and it exhibits cytotoxicity. Furthermore, the bioorthogonality of 2-formylbenzeneboronic acid is problematic due to its high reactivity towards *N*-terminal cysteines.[23,24] In this regard, Gao and co-workers demonstrated a new bioorthogonal process where a benign and stable (thio)semicarbazide forms a stable diazaborine conjugate with 2-acetylbenzeneboronic acid (10 minutes, maximum 60% conversion at 50 μM concentration for semicarbazide, maximum 27% conversion for thiosemicarbazide).[25] According to the authors, this reaction stalls before completion likely due to the interaction of the product with the reactants. Remarkably, Anslyn and James reported a stable three-component assembly with 2-formylbenzeneboronic acid, catechol and benzylamine[26] or *N*-hydroxylamines.[27] The authors performed a structural and thermodynamic analysis of *ortho*-iminophenylboronate ester formation in a protic solvent, and they found that initial binding of either benzylamine or catechol to 2-formylbenzeneboronic acid improves the complexation of catechol and benzylamine, respectively (Figure 1C).[26] In other words, binding of these two molecules to 2-formylbenzeneboronic acid becomes cooperative in a protic solvent.

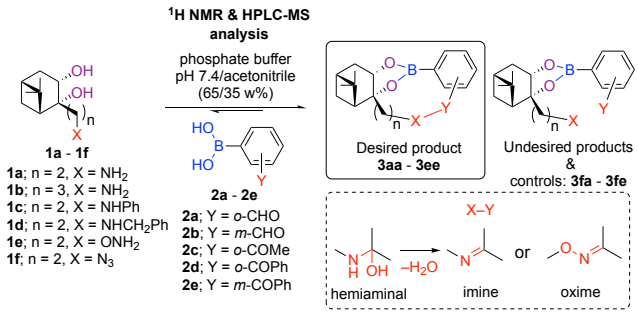
Altogether, despite these advances, the current systems suffer from issues such as stability and toxicity of phenyl hydrazine, incomplete conversion to the diazaborine product or the lack of bioorthogonality of 2-formylbenzeneboronic acid. Inspired by the iminoboronate concept of Anslyn and James, synergy was sought in our design based on the judicious placement of a hydrazine or (thio)semicarbazide unit on a bifunctional nopol diol reagent, along with a carbonyl handle (*ortho* or *meta*) on the arylboronic acid (Figure 1D). This design was put to the test as a potentially bioorthogonal, fast and irreversible click reaction system with stable and benign reagents.

RESULTS AND DISCUSSION

Optimization of nopol diol and boronic acid derivatives

Nopol diol derivatives (**1a-1e**) with two different spacers (n = 2, 3) and functional groups (amine/*O*-hydroxylamine) were designed and synthesized to test the desired conjugation with selected boronic acids (**2a-2e**). Not surprisingly, *o*-substituted aryl boronic acids (**2a**, **2c**, **2d**) provided the desired product with full conversion while *m*-substituted aryl boronic acids **2b** and **2e** could only form the boronate product in moderate yield, without any imine/oxime being formed (see Table S1).

Table 1. Results of initial conjugation attempts of diol derivatives (1a-1f) with aryl boronic acids (2a-2e)



Entry (**1a-f**)	Boronic acid	Product (2 h) (1 mM) **3**/**1**[a]	Product (24 h) (1 mM) **3**/**1**[a]	Imine/oxime formation?
1 (**1a**)	**2a**	**3aa**/**3aa•H_2O**/**1a**: 11/67/22	71/21/8	Yes[b, c]
2 (**1a**)	**2c**	**3ac**/**1a**: 43/57	43/57	Yes [b]
3 (**1b**)	**2a**	**3ba**/**1b**: 79/21	79/21	No
4 (**1c**)	**2a**	**3ca**/**1c**: 68/32[d]	68/32[d]	No
5 (**1d**)	**2a**	**3da**/**1d**: 86/14[d]	86/14[d]	No
6 (**1e**)	**2a**	**3ea**/**1e**: 100/0	100/0	Yes[b]
7 (**1e**)	**2c**	**3ec**/**1e**: 100/0	100/0	Yes[b]
8 (**1e**)	**2d**	**3ed**/**1e**: 100/0	100/0	Yes[b]
9 (**1f**)	**2a**	**3fa**/**1f**: 73/27	73/27	No

[a] The % product (boronate/imine/oxime) conversion was determined by ^{1}H NMR.

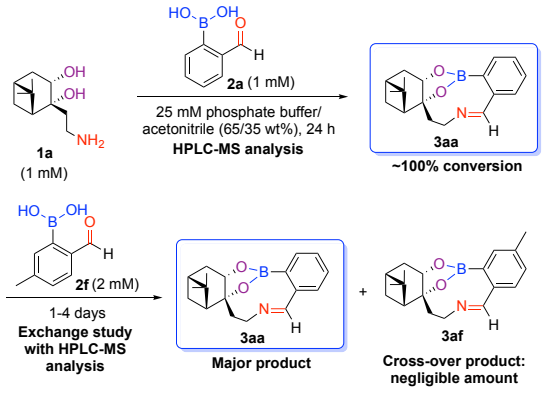
[b] Imine/oxime formation was also confirmed by HPLC-MS.

[c] Hemiaminal slowly converted into imine over time.

[d] Because of insufficient solubility at 1.0 mM, the reaction was performed at 0.5 mM.

Boronic acid **2a** and diol **1a** could initially form the medium-sized ring hemiaminal **3aa•H_2O**, which, according to the diagnostic –HC=N– resonance in ^{1}H NMR, slowly converted into imine **3aa** over 24 h in 1.0 mM concentration at rt (Table 1, entry 1). A comparison of **3aa** with **3fa** indicated that the presence of a second interaction (i.e., imine formation) clearly improved the conversion from 73 to 92% (Table 1, entry 1 and/vs 9). Moreover, boronic acid **2c** also formed an imine with **1a** albeit, with a lower conversion (43%) even after 24 h (Table 1, entry 2). On the other hand, **1b** with an additional –CH_2 spacer unit underwent only boronate formation with **2a**, with no imine or hemiaminal formation observed (Table 1, entry 3, also see Table S1). This result clearly indicates that **1a**, unlike **1b**, features the right geometry and size that favor both boronate and imine formation. Diol **1a** contains a simple primary amine, which could be further functionalized to include a reporter tag but the resulting secondary amines would render iminium formation more difficult. On the other hand, hemiaminal formation may still occur, and hopefully, be sufficient to form a stable conjugate. Thus, **1c** and **1d** were tested, however no hemiaminal formation was observed as evidenced by the lack of the corresponding –RC**H**(OH)(NHR') signal at ~6 ppm. Only boronate formation was observed in these cases (Table 1, entry 4, 5). Next, *O*-hydroxylamine **1e** was designed and tested with **2a**, **2c** and **2d**. All of these boronic acids formed the desired boronate-oxime conjugate, in a mixture of two oxime E/Z isomers, with full conversion in a very short time (Table 1, entry 6, 7, 8 and Table S1).

Scheme 1. Stability test of 3aa *via* a cross-over experiment



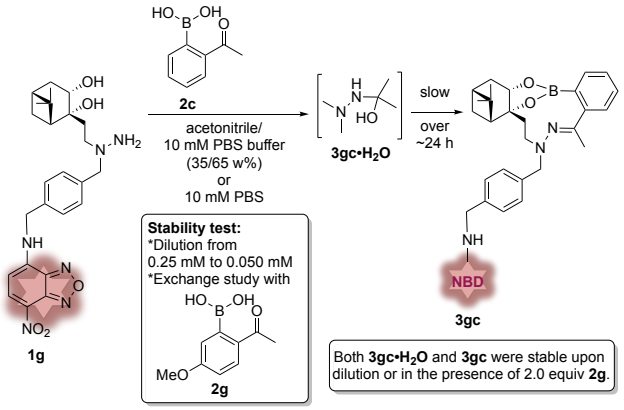
Meanwhile, the stability of **3aa** was assessed by a cross-over experiment where arylboronic acid **2f** (2.0 equiv) was introduced into a buffered solution of **3aa** and the reaction mixture was monitored *via* HPLC-MS analysis after 1 and 4 days (Scheme 1). Satisfactorily, the major product **3aa** was preserved and a negligible proportion of exchange product **3af** was observed (Scheme 1, see also Figure S8-9). This small amount of **3af** may be due to a small amount of unreacted **1a** in the reaction mixture rather than exchange of 'parts' released from the hydrolysis of **3aa**.

These initial studies led us to choose the most suitable spacer length for the ideal diol as n = 2 due to its stability as well as its relatively simple synthesis. Regarding the choice of boronic acid, **2a** was no longer considered due to its reported side reactivity with *N*-terminal cysteines.[23,24] Moreover, because **2c** exhibits lower hydrophobicity than **2d**, **2c** was selected as the optimal boronic acid.

Design of fluorescently labeled nopol diol derivative with hydrazine/(thio)semicarbazide unit.

Based on the efficiency of oxime derivative **1e**, our attention switched to the synthesis of a diol derivative with a disubstituted hydrazine unit. Thus, a nopol diol derivative **1g** with a NBD dye was designed and synthesized (Table 2). A benzyl linker was employed for the NBD dye since the reactivity of benzyl bromide towards the *in situ* synthesized nopol diol hydrazine is higher than alkyl bromides. Even though the reaction provided high conversion and diol **1g** preserved the fluorophore's intensity, it exhibited low stability and its purification led to partial decomposition. Nonetheless, a small amount of **1g** could still be isolated to test its conjugation with **2c** *via* HPLC-MS analysis. The conjugation reaction again proceeded through initial boronate formation **3gc•H_2O** followed by slow conversion into a hydrazone **3gc** with two E/Z isomers (Table 2, entry 1-4, Figure S11-14,18). After 24 h, a precipitate in PBS buffer was observed likely due to the low solubility of **3gc** (Table 2, entry 1, 2). In order to test the stability of the conjugates **3gc•H_2O** and **3gc**, the reaction mixture was subjected to either dilution or introduction of another boronic acid, **2g**, after 4 h. Satisfactorily, both **3gc•H_2O** and **3gc** preserved their stability under these conditions (Table 2 and Figure S16). Intermediate **3gc•H_2O** was found to be a stable conjugate that transforms slowly into **3gc**. The approximate rate constant was estimated as ~8 $M^{-1}s^{-1}$ for the formation of **3gc•H_2O** based on the reaction's half-life as monitored by HPLC-MS analysis (Table 2).

Table 2. Results of the aqueous conjugation reaction between 1g and 2c, and the stability test of conjugate 3gc•H_2O and 3gc *via* HPLC-MS analysis



Entry	Conc. of reagents (mM)	% Proportions of **1g**/**3gc•H_2O**/**3gc**			
		10 min	40 min	70 min	24 h
1	0.025[a]	83/12/5	72/18/10	65/22/13	precipitate
2	0.050[a]	71/23/6	54/36/10	49/40/11	precipitate
3	0.050[b]	62/31/7	45/45/10	41/53/12	-
4	0.25[c]	37/37/26	30/22/48	25/15/60	8/4/88

[a] 10 mM PBS buffer was used.

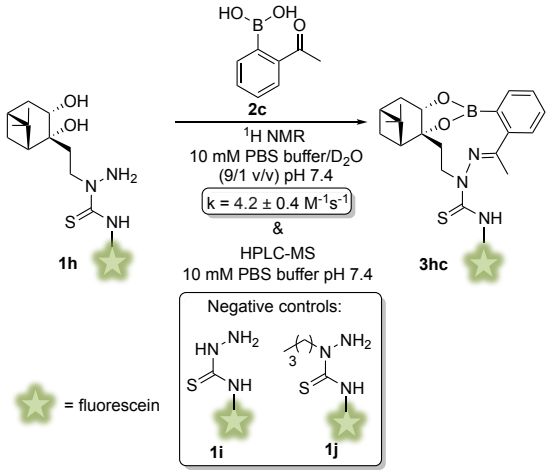
[b] 10 mM ammonium acetate buffer was used.

[c] Acetonitrile/10 mM PBS buffer (35/65 w%) was used.

In order to improve the chemical stability of the diol reagent, a (thio)semicarbazide unit, which was recently shown to be more stable and safer than phenyl hydrazine,[25] was chosen in the design and synthesis of diol reagent **1h**. This reagent was expected to be more stable, more reactive towards keto boronic acids like **2c**, and to afford high conversion[25] because the expected product, **3hc**, would not exhibit any inhibitory interaction with the starting materials that could stall the reaction. Moreover, **1h** could be synthesized easily with commercially available fluorescein isothiocyanate and nopol diol hydrazine, which was easily prepared *in situ* from nopol diol bromide and hydrazine monohydrate, as the precursors. As expected, the integrity of diol **1h** is intact even after one week according to ^{1}H-NMR (Figure S20B).

With the more stable diol reagent **1h**, in hand, the conjugation reaction was examined *via* both ^{1}H NMR, HPLC-MS and high resolution ESI-MS analyses (Figure S20-23,30,35). The reaction appears to proceed through concomitant formation of the boronate and thiosemicarbazone, with full conversion achieved within 3.5 hours at 50 µM, while negative controls **1i** and **1j** provide diazaborine (27% yield) and no thiosemicarbazone ligation, respectively (Table 3, entry 1-3). These results support the expectation that initial boronate formation is critical to promote the thiosemicarbazone formation, which occurs second, by intramolecular fashion.

Table 3. Results of the aqueous conjugation reaction of 1h, and negative controls 1i and 1j, with 2c



Entry	Conc. of reagents (mM)	% Proportions of **1**/**3**					
		10 min	30 min	60 min	120 min	3.5 h	24 h
1 (**1h**)	0.050[a]	90/10	86/14	64/36	35/65	0/100	0/100
2 (**1i**)	0.050[a]	73/27 (maximal conversion)					
3 (**1j**)	0.050[a]	100/0 (no product)					
4 (**1h**)	0.25[b]	89/11	81/19	62/38	47/53	-	0/100
5 (**1i**)	0.25[b]	72/28	64/36	-	64/36	-	64/36

[a] 10 mM PBS buffer/D_2O (9/1 v/v) was used.

[b] ACN-d_3/50 mM D_2O phosphate buffer (35/65 w%) was used.

In further experimentation, the stability of conjugate **3hc** was tested (Figure 2). Firstly, an equimolar concentration of **1h** and **2c** was incubated for one day, and the reaction mixture was subjected to dilutions. Conjugate **3hc** stayed intact even after 24 hours (Figure 2, S28B, S31B-C). Subsequently, another boronic acid such as **2g** or even the more reactive **2a** was introduced. No breakdown of **3hc** occurred since no cross-over products of these competitors were observed (Figure 2, S28C, S31D-E). Finally, subjecting **3hc** to either acidic (pH 3) or basic (pH 9) solutions did not affect its integrity significantly (Figure 2, S31F-G).

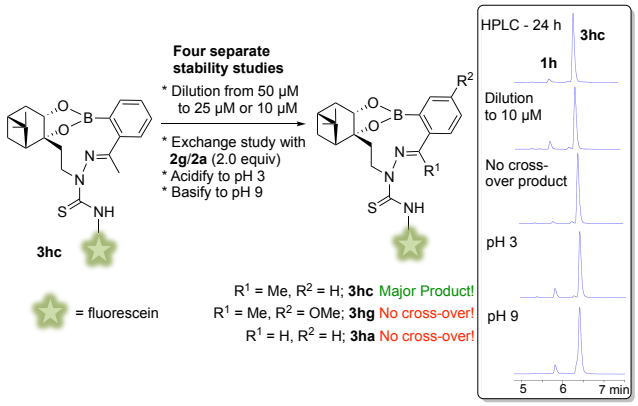
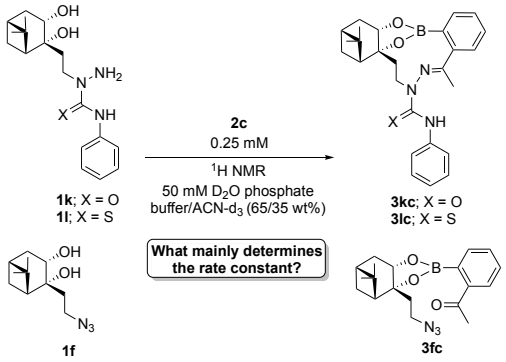


Figure 2. Studies on the stability of **3hc** *via* HPLC-MS and ^{1}H NMR.

Table 4. Examination of the reactivity of 1k, 1l and control 1f with 2c to identify the rate determining step

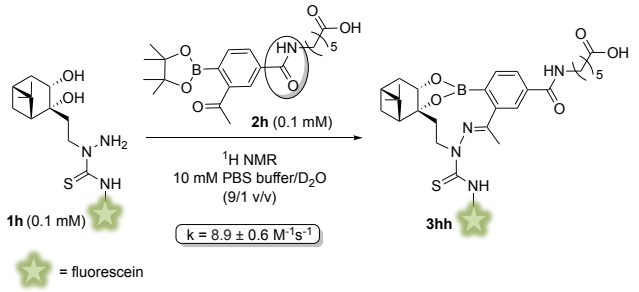


Entry	% Proportions of **1**/**3**							
	10 min	30 min	60 min	90 min	120 min	3.0 h	3.5 h	24 h
1 (**1k**)	82/18	70/30	55/45	49/51	47/53	-	38/62	0/100
2 (**1l**)	90/10	71/29	53/47	44/56	38/62	30/70	-	0/100
3 (**1f**)	82/18	67/33	58/42	54/46	52/48	49/51	-	47/53

Using ^{1}H NMR methodology, the overall rate constant for the formation of **3hc** in 10 mM PBS buffer was measured as 4.2 ± 0.4 $M^{-1}s^{-1}$, which is in agreement with our previous study on rate constant measurements of nopol diol boronate formation (Figure S24-25).[14] It is important to note that the reaction rate was lower in higher buffer concentration (50 mM),

which is likely due to the competitive effect of salts in boronate formation (Table 3, entry 1 vs 4).[28] Moreover, negative control **1j** without 1,2 *cis* diol gave no conversion (Table 3, entry 3). Thus boronate formation is essential in this system, acting as the overall rate-determining step preceding a fast intramolecular thiosemicarbazone formation. To understand the reaction mechanism further, the reactivity of diols **1k**, **1l** and **1f** with **2c** was monitored *via* ^{1}H NMR analysis. The results indicate that both **1k** and **1l** behave similarly and reach 100% conversion with the help of the synergic (thio)semicarbazone interaction. In contrast, control diol **1f** afforded a conversion similar to **1k** and **1l** in the first 60 min and then remained in equilibrium (53%) as expected (Table 4, entry 1-3). Hence, the conversion in this system is determined mainly by boronate formation. According to our previous study on nopol diol boronate formation,[14] the reactivity of this conjugation system could be improved *via* the placement of *e*-withdrawing substituents on **2c**. Therefore, a conjugatable *o*-acetylphenylpinacol boronate with a *p*-amide group, **2h**, was designed and synthesized. As predicted, the rate constant increased to 8.9 ± 0.6 $M^{-1}s^{-1}$ (Scheme 2, Figure S26-27). Not surprisingly, the presence of a pinacol protecting group in **2h** did not impede the formation of conjugate **3hh**.[26,29]

Scheme 2. Rate constant for the reaction between 1h and 2h as measured by ^{1}H NMR analysis



Studies to assess the bioorthogonality of the boronate/thiosemicarbazone system.

More importantly, the effect of biological polyols on the reaction progress was assessed using competition assays monitored by HPLC-MS analysis. Diol **1h** was allowed to form the irreversible conjugate **3hc**/**3hh** with **2c**/**2h** in the presence of a mixture of biocompetitors such as glucose (8 mM), fructose (300 μM) or catechol (0.1 μM) at concentrations higher than those found in the blood stream (Figure 3).[30,31] Remarkably, the formation of the desired conjugates **3hc**/**3hh** was not affected as similar conversion was obtained both in the absence and the presence of biological polyols (Figure S40,41,43). As expected, higher conversion was observed for **3hh** when compared to **3hc** due to the higher reactivity of boronic acid **2h** (Figure 3).

Apart from biological polyols, a recent report[32,33] highlighted the presence of protein-bound electrophiles caused by pyruvoyl (ketone) and glyoxylyl (aldehyde) electrophilic post-translational modifications in human cells (HEK293T cells and the human breast cancer cell line MDA-MB-231). Thus, in order to confirm the inertness and bioorthogonality of reagent **1h** and its thiosemicarbazide unit towards these electrophiles, especially the reactive glyoxylyl aldehydes, a readily available model aldehyde **2i**[34] (0.50 mM) was allowed to react with **1h**/**1i**/**1m** (0.10/0.10/0.25 mM) in the presence of a catalyst, *p*-phenylenediamine[35] (10 mM) (Figure 4). As the reactions were performed at neutral pH, the catalyst *p*-phenylenediamine[35] was required. These reactions were monitored by HPLC-MS and the analysis showed that **1h** is unreactive towards **2i** even in the presence of the catalyst while both **1i** and **1m** afforded the expected thiosemicarbazone and hydrazone adducts, **3ii** and **3mi**, respectively (Figure 4, S44).

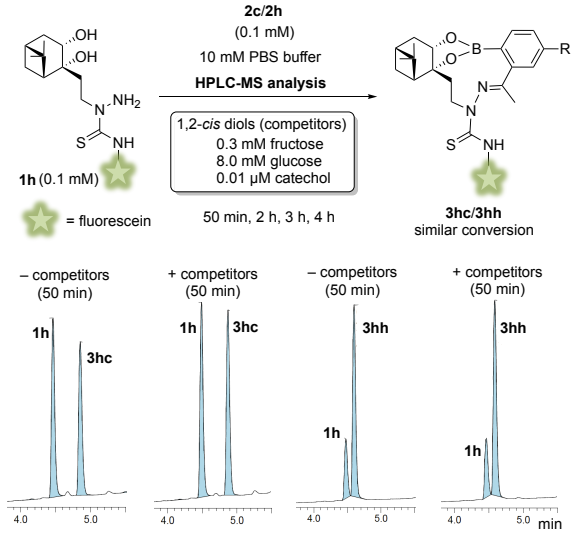


Figure 3. Competitive effect of biological polyols on the formation of conjugates **3hc**/**3hh**.

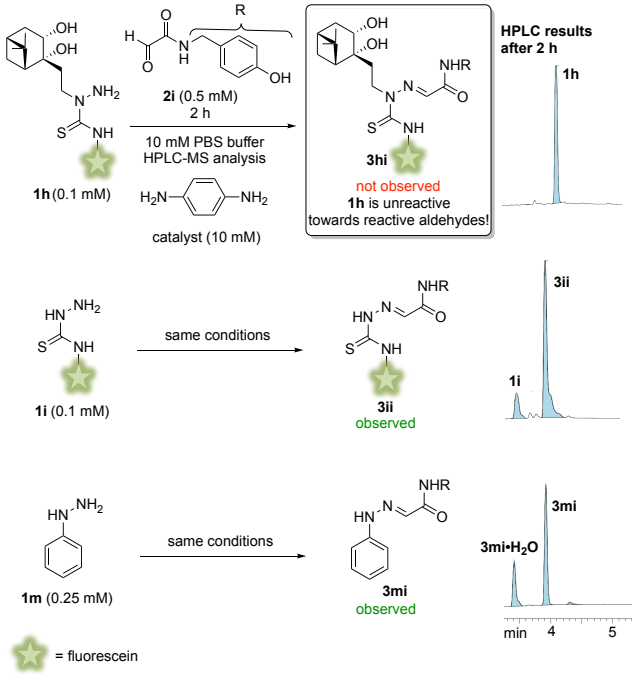


Figure 4. Study to confirm the inertness and bioorthogonality of diol **1h** towards model aldehyde **2i**, a mimic of protein-bound glyoxylyl unit.

Cytotoxicity and live cell labeling studies.

With these promising results in hand, the cytotoxicity of bioorthogonal handles **1l**, **2c** and **2h** was examined. These reagents were found to be benign towards HEK293T cells

over 18 hours at 50 and 25 μM concentrations (Figure 5), an outcome consistent with Gao's recent report.[25]

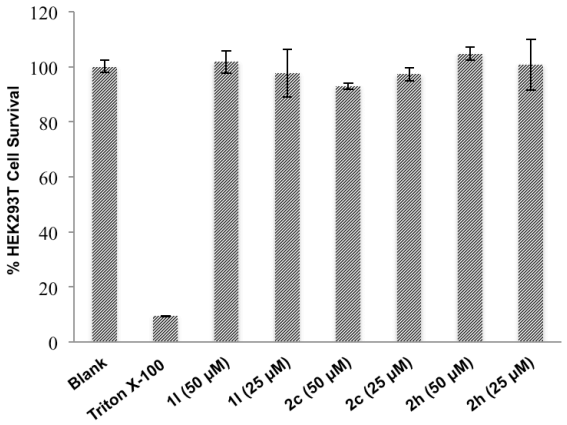


Figure 5. Cytotoxicity results of bioorthogonal handles **1l**, **2c** and **2h** and on HEK293T cells. Blank is DMSO, and Triton X-100 was used as a positive control.

Finally, in order to demonstrate the viability of this irreversible boronate/thiosemicarbazone system in live-cell imaging, a SNAP-tag approach was employed to install boronic acid **2j** on the cell membrane of transiently transfected HEK293T cells with the pSNAP$_f$-ADRβ2 plasmid (Figure 6A). Boronic acid **2j** (10 μM) was secured on the cell membrane of HEK293T cells through its reaction with the SNAP$_f$-Beta-2 adrenergic receptor. Diol **1h** (10/30 μM) or controls **1i**/**1j** (30 μM) were then introduced into the cell media and incubated for 0.5 h or 2.5 h at 37 °C. In parallel, cells without **2j** were incubated with diol **1h** (30 μM) as a negative control. The cells were washed and imaged on a total internal reflection fluorescence (TIRF) microscope. Whilst controls showed no labeling in 2.5 h (Figure 6B i,ii,iii), a clear cell surface imaging was observed for the HEK293T cells incubated with both diol **1h** and **2j** even after only in 0.5 h (Figure 6B iv). Moreover, a vivid image was possible even when using a low 10 μM concentration of **1h** (Figure 6B v).

CONCLUSION

In summary, we have successfully developed an irreversible nopol diol boronate bioconjugation system *via* a synergic thiosemicarbazone interaction that provides a stable medium-sized ring product of double condensation. This binary boronate reagent was demonstrated to be efficient (~9 $M^{-1}s^{-1}$), robust, compatible with biological polyols and also unreactive towards possible electrophiles found in human cells. Moreover, it was successfully utilized in low micromolar concentration for site selective protein labeling on mammalian HEK293T cells. Therefore, this irreversible boronate/thiosemicarbazone synergic system is a valuable addition to the bioorthogonal chemistry toolbox to enable the study of complex biological processes.

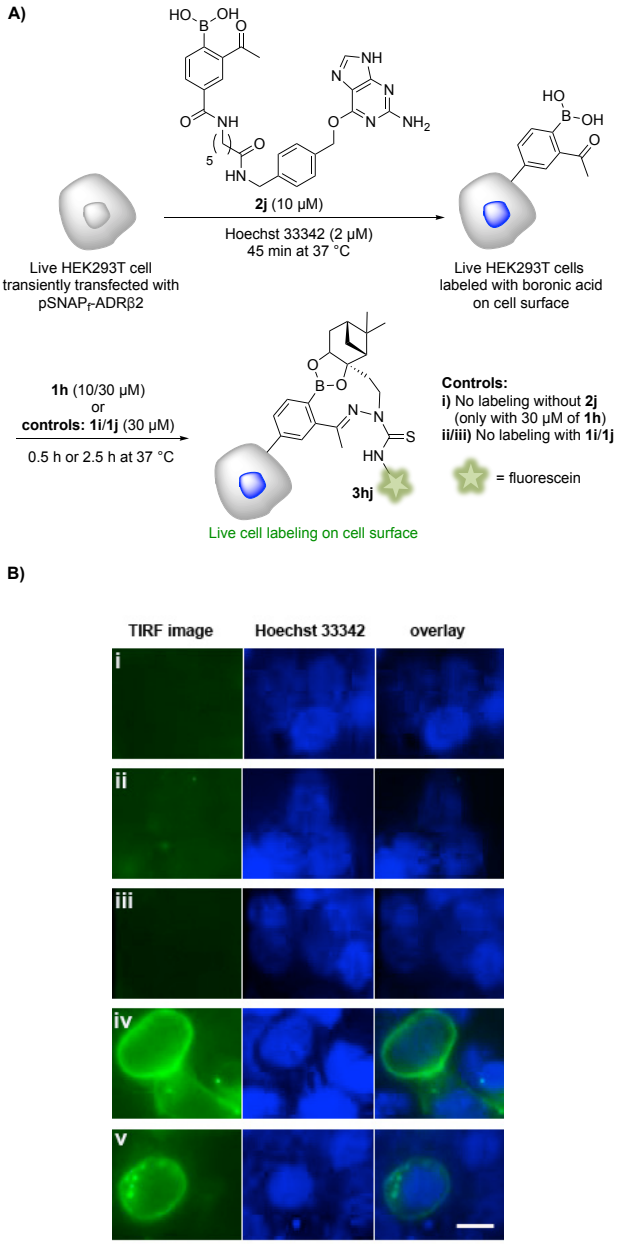


Figure 6. Imaging of boronic acid **2j** surface labeled HEK293T live cells with diol **1h**. A) Scheme of labeling procedure. B) Imaging results; i) HEK293T live cells not incubated with **2j**, but only incubated with **1h** (30 μM) in 2.5 h. ii) HEK293T live cells incubated with both **2j** (10 μM) and **1i** (30 μM) in 2.5 h. iii) HEK293T live cells incubated with both **2j** (10 μM) and **1j** (30 μM) in 2.5 h. iv) HEK293T live cells incubated with both **2j** (10 μM) and **1h** (30 μM) in 0.5 h. v) HEK293T live cells incubated with both **2j** (10 μM) and **1h** (10 μM) in 2.5 h. Scale bar corresponds to 10 μm. Hoechst 33342 was used for live cell nuclear staining.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental details, kinetic plots, spectroscopic data for new compounds, and additional images including Figures S1–S45 and Table S1.

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Author Contributions

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript.

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SYNOPSIS TOC (Word Style “SN_Synopsis_TOC”).