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Recognition of saccharides in the NIR region with a novel fluorogenic boronolectin: *in vitro* and live cell labeling†‡

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This work describes a novel mono-boronic acid derivative of a tricarbocyanine. The probe is a genuine near-infrared fluorescence emitter with improved properties such as a large Stokes shift, excellent water solubility and sensitive fluorogenicity upon binding to carbohydrates under physiological conditions.

Boronolectins are a group of synthetic lectins bearing a boronic acid fragment which serves as a receptor site for carbohydrates, due to the well-known interaction between boronic acids and vicinal diols.[1] A wide variety of boronic acid-based sensors displaying different architectures have been developed not only for sugar recognition but also for other molecules or ions of interest as well.[2] The issue concerning the low affinity has also been addressed by different groups following the multivalent strategy in the construction of scaffolds exposing multiple boronic acid residues available for the recognition event.[3] The performance of carbohydrate biosensors as components of several types of nanomaterials such as metal nanoparticles, carbon materials, quantum dots, magnetic nanoparticles and silica nanoparticles have been recently reviewed focusing on promising advances in different applications such as carbohydrate–lectin interactions and cancer cell detection.[4] This specific binding was also exploited in the design of different methodologies for the enrichment of glycoproteins and glycopeptides prompting the finding of new biomarkers.[5]

Regarding the application of luminescent boronolectins for monitoring sugars in a biological medium, it is mandatory to consider biocompatible properties in the structural design such as high water solubility, low cytotoxicity and long excitation and emission wavelengths. In particular, in the near infrared region (NIR) an improved signal is achieved due to the absence of autofluorescence of the biological species under study. Besides, it is highly desirable that the recognition event triggers an OFF–ON signal rather than an ON–OFF response. Among the fluorescent boronolectins available to date there are no examples of probes that fulfill these requirements without being part of more complex arrays, especially those concerning the water solubility and the emissive properties at the physiological pH. In 2012 Saito *et al.* reported a boronic acid-derived squaraine dye for on-column labeling of Gram-positive bacteria with excitation and emission maximum at 630 and 660 nm, respectively. This probe exhibits a high affinity for fructose when working at pH 10 due to its high pK_a value (>11).[6] It is well known that the boronic–diol binding shows increased affinities at pH values in the range 9–10[7] excluding the use of most of the existent probes under physiological conditions because of the low enhancement of the signal at pH 7.4.

We have previously reported the application of tricarbocyanine dyes for specific labeling and sensing.[8] These small molecular fluorophores are advantageous over other NIR dyes (BODIPYS, squaraine) due to their synthetic versatility, truly long excitation (650–760 nm) and emission wavelengths (>800 nm) and excellent water solubility. Moreover, a highly rated property of this probe is the large Stokes shift between excitation and emission (>100 nm) when it has an $-NH_2$ group in the *meso* position.

In this context we have designed a boronolectin derived from a tricarbocyanine combined with a boronic acid fragment linked by a piperazine unit. Keeping in mind a suitable electronic interplay between the boronic residue and the fluorophore, it was expected that a fluorogenic response would be achieved upon binding of the monosaccharide to the probe. This binding event changes the geometry of the boron center from trigonal to tetrahedral pivoting the OFF state to an emissive (ON) species.

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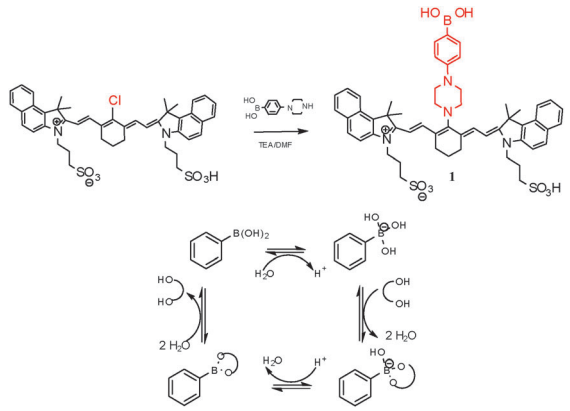
† This work is dedicated to the memory of Elizabeth A. Jares-Erijman.

‡ Electronic supplementary information (ESI) available: Reagents and instruments, synthetic and labeling procedures, additional spectroscopic data, ^{1}H, ^{13}CNMR, and HRMS. See DOI: 10.1039/c4cc10425k



Scheme 1 Synthesis of boronolectin **1** and the sensing mechanism.

We report here the synthesis and the photophysical properties of a novel NIR fluorogenic boronolectin (**1**) and its behaviour as a sensor for simple saccharides, glycoproteins and glycoconjugates in aqueous solution and in live cells. The synthesis of the probe was performed according to Scheme 1 by substitution of the chlorine atom by the boronic acid compound containing a piperazine unit. The reaction proceeds in moderate yields under mild conditions.

The detailed experimental procedure and characterization data are provided in the ESI.‡

The compound is stable for long storage periods having precaution in stock concentrations (*ca.* mM) to avoid the formation of typical aggregates.

Probe **1** has two absorption maxima in water: at 711 nm (ε 25 297 M^{-1} cm^{-1}) and 879 nm (ε 22 860 M^{-1} cm^{-1}) in water and an emission maximum at 820 nm in methanol. When dissolved in buffer PBS the emission is diminished to almost zero (Fig. S1 in the ESI‡). The pK_a of the probe is 10.80, showing an increase of the intensity at 820 nm that correlates with increasing pH.

The response of the probe to simple saccharides was first investigated. In every experiment, a 2.8 μM solution of the probe in PBS buffer, pH 7.4 (absorbance <0.04) was titrated with the corresponding sugar up to a large excess of the monosaccharide (*ca.* 300 mM). At this point, the effect of the pH variation on the intensity at 820 nm was evaluated for each complex in order to explore the modulation of the emission with the acidity of the medium. As expected, we observed in every case, a turn-ON of the emission at 820 nm when increasing the pH. This behaviour is similar to that described by Zhang *et al.* for carbazole-based boronic acids sensors where an increase of the intensity is obtained from acidic/neutral to basic solutions.[9] This response is ascribed to a process named *d*-PET (photoinduced electron transfer) where the fluorophore acts like the electron donor. However, this is a venture proposal in our case since further experiments should be performed to really determine which process is operating. The affinity of the boronic residue to the diol moiety of the sugar also increased concomitantly. The magnitude of the enhancement is modulated by the pH of the medium reflecting the selectivity

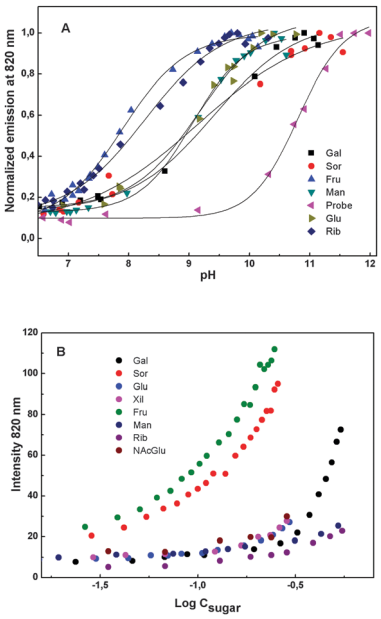


Fig. 1 (A) Variation of the intensity of the complex **1**-sugar with pH; (B) change in emission of **1** upon cumulative additions of sugar at pH 7.4. λ_{exc} 720 nm.

toward each monosaccharide at different proton concentrations (Fig. 1A).

At pH 7.4 the probe **1** has a strong affinity towards fructose and sorbitol with a detection wavelength of 820 nm, the longest reported to date for diol binding at the physiological pH. The relative order of fluorescence emission enhancement for the saccharides of interest at pH 7.4 was: fru ∼ sor > xil ∼ gal glu ∼ man ∼ NAcGlu (Fig. 1B). However, from Fig. 1A it can be seen that large spectral changes can be observed by changing pH in one unit, displaying selectivity toward ribose and fructose in the range 7.8–8.3. The binding constants for fructose and sorbitol at pH 7.4 were calculated by fitting the fluorescence intensity *versus* the concentration of the saccharide (see ESI‡) according to a model previously reported.[10] The values are 3.3 and 1.5 M^{-1} for sorbitol and fructose, respectively, falling within the expected range at this pH. For comparison the K_d of a similar probe developed by the group of Saito[6] at this pH is 5.75 M^{-1}.

Despite the small affinity constant values, even at low concentrations of saccharides the probe yields an acceptable emissive OFF–ON signal for practical purposes in aqueous experiments at the physiological pH. To date, most of the available synthetic boronolectins show moderate fluorescence enhancement in aqueous solution demanding significant amounts of a co-solvent (DMSO, MeOH, EtOH) to accomplish homogeneous solutions.

Due to the well-established biocompatible properties of probe **1** regarding NIR emission and water solubility, we explored the sensing of model glycoproteins such as fetuin (Fet), asialofetuin (AFet), avidin (Av) and mucin (MUC) in comparison with non-glycosylated proteins like streptavidin (SAv) and human serum albumin (HSA). The response of the probe upon binding

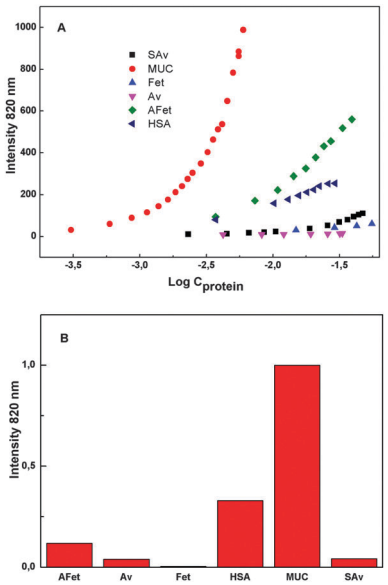


Fig. 2 Changes in the emission of boronolectin **1** 2.8 μM at 820 nm (λ_{exc} 720 nm) upon binding of (glyco)proteins in buffer PBS pH 7.4.

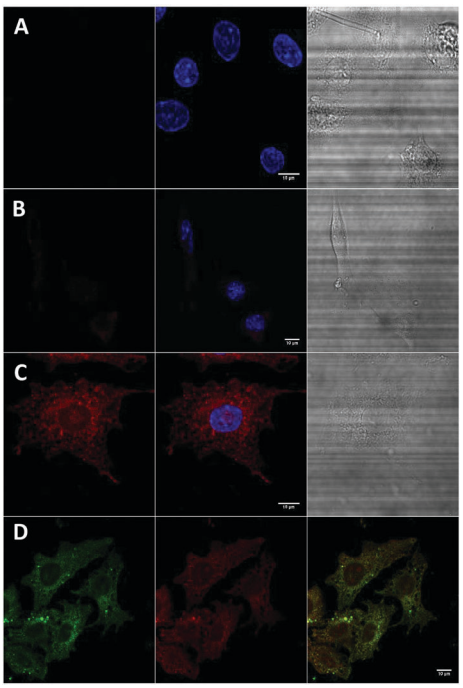


Fig. 3 Fluorescence imaging of live MCF-10 cells: (A) incubated with DAPI: exc 637 nm (left), merge exc 405/637 nm (middle), DIC image (right) (B) incubated with probe **1** (50 μM) and DAPI: exc 637 nm (left), merge exc 405/637 nm (middle), DIC image (right) (C) pre-incubated with boroxole 50 mM then with probe **1** (50 μM) and DAPI: exc 637 nm (left), merge exc 405/637 nm (middle), DIC image (right) (D) pre-incubated with boroxole 50 mM then with probe **1** (50 μM), washed and incubated with ER tracker Blue-white DPX (1 μM): exc 405 nm (left), exc 637 nm (middle), merged image (right). After incubation cells were fixed with PFA 4% (except D).

is reflected in Fig. 2. A very sensitive and enhanced response is achieved in the case of mucin, boosting the emission to a detectable level at a concentration of 300 nM. Mucine is a strongly glycosylated protein that owes more than half of its molecular weight to its glycostructure.[11] In the case of fetuin a very low increase of emission was observed which is correlated with the amount of sialic acid present on the structure (8%).[12] Asialofetuin shares exactly the same structure but lacks the sialic acid component suggesting a key role for binding of this saccharide, an aspect that was also regarded by others in related systems.[13]

Previous studies devoted to investigating the interaction of boronic acids with sialic acid showed the instability of this complex at neutral to basic pH values.[14]

On the other hand, the response of the probe to avidin was seen to be low, given its degree of glycosylation (*ca.* 10%). This could be ascribed to significant electrostatic repulsion between the protein and the probe since the isoelectric point of avidin is 10.5 and the pK_a of the probe is 10.8 yielding a net positive charge on both the ligand and the receptor at pH 7.4. As shown in Fig. 2 the probe also turns ON in response to non-glycosylated proteins (HSA and SAv), accounting for the extent of non-specific labeling. The boronic acid group could interact to some extent with nucleophilic aminoacid residues[15] as well as other secondary interactions (mainly electrostatic) that contribute to the enhancement of the affinity. In the case of HSA, it is reported by the provider that the sample could suffer from post-translational modifications such as glycosylation which would contribute in this case to the enhanced signal.

In order to assess this boronolectin for imaging applications, we investigated its ability to label glycoconjugates in live cultured cells. MCF-10 cells were incubated at 37 °C for 30 minutes in modified Tyrode's buffer containing 50 μM of the probe **1**, then washed three times before fixation and imaging with a confocal microscope (Fig. 3). We observed that probe **1** lights up the endoplasmic reticulum (ER) and Golgi apparatus matching the subcellular localization of an endoplasmic reticulum stain (ER Tracker™ Blue-white DPX). It is well known that glycosylation of proteins occurs in these organelles.[16]

An interesting finding is that it was possible to image these structures, when the cells were pre-incubated with boroxole, a NIR optically inactive boronic hemiester, prior to the incubation with boronolectin **1**. Otherwise, the signal was negligible. At first, some sort of chemical activation favouring the binding of **1** to the carbohydrates by means of boroxole was hypothesized. However, no enhancement of the binding of **1** to galactose, as the model saccharide, was observed in the presence of boroxole *in vitro* (data not shown). Then we turned to the possibility that somehow boroxole was affecting the permeability of the probe. To test this possibility we treated methanol fixed cells with boronolectin **1** without pre-incubation with boroxole achieving similar labelling to that in live cells (see Fig. S5, ESI‡). It has been previously reported that the cell uptake of boronic acid derivatives is not favoured due to the interactions with cell surface carbohydrates.[17] In our case, the absence of signal at this cell location could be a consequence of the low affinity of the probe toward the sugars that are predominant at the cell surface and the substantial difference in saccharide concentration, lower at the cell surface than at the RE.

We also found that acidic incubation (pH 4) of the cells in the presence of probe **1** with or without pre-treatment with

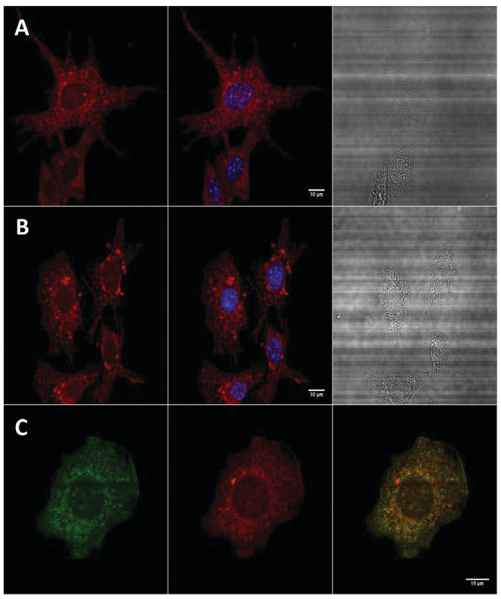


Fig. 4 MCF-10 cells incubated with: (A) probe **1** (50 μM) and DAPI: exc 637 nm (left), merged image (middle), DIC image (right); (B) pre-incubated with boroxole 50 mM then with probe **1** (50 μM) and DAPI: exc 637 nm (left), merged image (middle), DIC image (right); (C) incubated with probe **1** (50 μM), washed and then with ER-tracker Blue-white DPX (1 μM): exc 405 nm (left), 637 nm (middle), merged image (right). For all images: incubation time 30 min, 37 °C, at pH 4 (acetate buffer).

boroxole also promotes the entrance of the dye with a strong labeling of the RE system, co-localizing in good agreement with the ER tracker (Fig. 4). This result indicates that acid incubation also favours the permeability of the probe.

In all experiments the incubation with the probe did not affect the cell morphology as compared to control cells. The images displayed a homogeneous staining of endomembranous structures with the complete absence of background emission favoured by the NIR signal within a relatively short incubation time (30 minutes). Additionally, a negligible signal was observed when the probe was incubated with dopamine prior to cell labeling, which binds with strong affinity to boronic residues, reflecting a low degree of non-specific interactions (see Fig. S6, ESI‡).

In summary, we have synthesized a novel long wavelength fluorescent probe with a strong and sensitive OFF–ON response toward saccharides, going from simple sugars to oligosaccharide components of glycoproteins and glycoconjugates in live cells. The detection was achieved at the physiological pH in solution as well as in live cultured cells. For common monosaccharides a moderate selectivity toward fructose and sorbitol at pH 7.4 was observed. The selectivity toward different sugars can be modulated by the pH of the medium. Boronolectin **1** binds strongly to glycosylated proteins in solution at the nanomolar level and is able to differentiate with good selectivity among glycoproteins with different sialylation degrees. The boronolectin **1** performs excellent recognition of glycosylated structures within cultured cells both under fixed and *in vivo* conditions preserving cell viability. The versatility of the synthesis is compatible with orthogonal functionalization methodologies envisioning the immobilization of the probe in surfaces or nanostructures. To our knowledge, our probe shows the longest fluorescent emission reported upon sugar interaction with a maximum at 820 nm following excitation at 720 nm or 637 nm with a sensitive signal in aqueous medium avoiding the use of co-solvents and yielding a truly fluorogenic response. This outstanding NIR-boronolectin is a candidate probe to detect glycan abundance in cells or tissues, among other bioanalytical applications.

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