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Novel Highly Sensitive Fluorogenic Probe for Imaging Glycoproteins and Mucine Activity in Live Cells in the Near Infrared Region

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Abstract

We introduce a novel fluorescent molecular probe able to detect glycoproteins, especially mucins, with high sensitivity and with a turn-on response along with a large Stokes shift (>130 nm), within the biologically active window. The probe contains an aminotricarbocyanine as the fluorescent reporter with a linked benzoboroxole as the recognition unit, which operates through a dynamic covalent reaction between the boronic hemiester residue of the receptor and *cis*-diols of the analyte. The superior selectivity of the probe is displayed by the labeling of mucins present in Calu-3 cells. The new benzoboroxole fluorescent derivative gathers together key properties to meet high rated molecular probes: specificity, excellent solubility in water and off-on near infrared emission. The probe we provide is expected to be an excellent tool for imaging intracellular mucin to evaluate mucus related diseases as well as a sensing strategy towards glycosylated structures with a high potential for theranostics approaches in biological samples.

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A large amount of research has been made by chemists over the lasts decades to provide fluorescent molecular probes for the specific and non-specific labeling of biomolecules escorting the rapid development of imaging techniques by means of fluorescence, from spectroscopy to nano/microscopy. Among the possible labeling targets present in a biomolecule structure to attach a reporter molecule, carbohydrate residues have been an elusive tag in probe design. This is due mainly to the structural similarity between carbohydrate monomers within a glycostructure which can be seen as a bunch of OH groups whose subtle structural and stereochemical differences make difficult a selective recognition process. In addition, in aqueous media, water molecules represent the main competitor of sugars for a given probe. In Nature, especially in mammals, only six carbohydrate monomers accounts for a diversity of *ca*. 10^{12} different oligosaccharides. Despite this discouraging fact, the challenge of carbohydrate recognition has been taken by different groups in the last three decades motorized by the fundamental role of saccharides and their conjugates in major biological processes. Following mainly supramolecular chemistry strategies to address this point, Davis´s group intensively developed a biomimetic approach based on non-covalent interactions.[1] On the other hand, a dynamic covalent strategy arising from the reversible reaction between boronic acids and *cis*-diols to give boronate complexes in a reversible way was also explored by different researchers.[2] Our group is particularly interested in this dynamic recognition event to achieve carbohydrate recognition in aqueous media focusing in the development of new molecular fluorescent probes for glycostructures, named boronolectins. Recently, we reported a novel benzoboronic acid fluorescent sensor with excellent performance for the bulk detection of glycoproteins in vitro and in live cells.[3] However, this benzoboronic acid probe requires a membrane permeation step and the affinity is not optimized unless a large concentration of carbohydrates is offered to the probe.

In order to overcome these limitations (high pKa, low affinity, need of permeation pre-treatment) we designed a near-infrared (NIR) fluorescent probe that contains benzoboroxole (also named benzoxaborole) as the recognition unit. We find essential to focus in the development of NIR fluorophores (650-900 nm) to preclude photodamage of biological samples, to accomplish deep tissue penetration and high signal-to-noise ratios by minimizing background autofluorescence.

Since the work of Hall *et al*.[4] benzoboroxole became an improved receptor for sugars showing high potential for different applications, from saccharide detection in vitro[5], materials science[6] to medicinal chemistry due to its bioactivity against different pathogens.[6-7] Our unique fluorescent boronolectin probe named **Cy-Bx** (**7**) is presented in this work as a powerful dye to label mucins in live cells with high sensitivity and selectivity. **Cy-Bx** achieved some of the high

rated requirements for a molecular probe to be used in biological systems: excellent water solubility, NIR fluorescence emission and fluorogenic response.

The receptor **5** was synthesized starting from benzoboroxole according to Scheme 1, by standard synthetic methods (section 2, Supporting Information). Compound **5** possess a primary amino terminal group, suitable to perform the radical nucleophilic aromatic substitution reaction S_{RN1} with chlorotricarbocyanine **6** at the *meso* position[8]. We chose compound **4** to explore the performance of the boronolectin *in vitro*. Similar to benzoboroxole (pKa 7.3), receptor **4** has a pKa of 6.9 that matches the physiological range. These values were determined spectroscopically and are in accordance with those determined by other methodologies[4a] (Figure S6). Benzoboroxole is more acidic than benzoboronic acids (pKa values in the range 9-10) and showed to have better affinity for pyranoses than for furanoses in neutral water, favouring the rehybrydization that follows the formation of the boronate complex.

Moreover, the fact that receptor **4** is slightly more acidic than benzoboroxole correlates with the observed affinity for tested sugars. Figure 1 shows the direct change in the emission of **4** at 325 nm upon binding of common monosaccharides (Figure S9). In general terms, it follows the affinity tendency of benzoboroxole (Figure S9, Table S1) with the highest K_a values computed for fructose and sorbitol (K_a 811 and 637 M^{-1} respectively) and moderate binding to glucose (177 M^{-1}), mannose (28 M^{-1}), galactose (58 M^{-1}) and fucose (44 M^{-1}).

The experiments were carried out in buffered solution (PBS, 0.1 M) at pH 7.4 without the need of using co-solvent. The boronolectin receptor (**4**) was also studied for the recognition of sugar related structures like adenosine triphosphate (ATP) and adenosine monophosphate (AMP) because of their high abundance in biological samples (*vide infra*). The affinity of **4** to these analytes was computed to be significantly higher than common monosaccharides (Fig. 1, Table S1). A cooperative effect for the binding involving the phosphate groups is probably the main reason for the observed boost in affinity.

Moving forward the synthesis of the fluorescent boronolectin **Cy-Bx** (**7**), BOC-deprotected receptor **5** was reacted with chlorotricarbocyanine **6** in mild conditions as previously described.[3, 9] **Cy-Bx** was obtained as a deep blue solid after chromatographic purification and it was completely characterized by spectrosocopic methods (Figures S3 and S4). In methanol, **Cy-Bx** shows a maximum at 650 nm (16724 M^{-1}) in the absorbance spectrum and at 780 nm in the emission spectrum (Figure S5, λ_{exc} 650 nm). These parameters remain practically unchanged in aqueous medium (PBS buffer pH 7.4, Figure S5). The decrease of extinction coefficient (□) and the quantum yield in aqueous medium is frequently observed in these cyanine dyes, due to the formation of aggregates. **Cy-Bx** has a large Stokes shift (>130 nm) as expected for

aminotricarbocyanines, an important optical feature for imaging fluorescent probes, as the interference from excitation light is minimized and the filter settings in the microscopic set up is optimized. It is worth to note that unlike our previous probe[3] **Cy-Bx** is not completely quenched in water, a fact probably related to the presence of the succinic-ethylendiamine linker between the fluorophore and the recognition unit, that contributes in reducing the formation of typical aggregates. Besides, the presence of amide groups confers an electronic configuration not suitable to intramolecular quenching processes such as electronic transfer.

The calculated pKa value for **Cy-Bx** by spectroscopic methods is 8.5 (Figure S7), more basic than that of the receptor **4**. This can be attributed to the presence of the secondary amine arised after the nucleophilic substitution of chlorine in the *meso* position of the heptamethine chain. We studied the behavior of the complexes between **Cy-Bx** and model monosaccharides within the pH range 3-12 (Figure S8). In all cases the pKa values of the complexes were more acidic than the free probe as a result of the binding process and the concomitant change in geometry of the boron center increasing the amount of boronate species in the complex.

Next we evaluated the performance of **Cy-Bx** to detect model glycoproteins *in vitro* (Figure 2).

We chose to evaluate the binding of the probe to mucin MUC-1 and asialofetuin (Afet) because these glycoproteins are considered reliable candidates as disease biomarkers. Asialofetuin expresses the oncofetal Thomson Friedenreich antigen (TF, Gal(β1–3)GalNAc), a cell surface biomarker for colon cancer and others[10] while MUC-1 is a mucine overexpressed in ovarian, breast, colon cancer among others.[11]

The boronolectin **Cy-Bx** also binds with good affinity to fetuin, the sialylated glycoform of asialofetuin (8% sialic acid). In all cases the binding was detectable at the nanomolar level yielding an off-on response in the near infrared region (780 nm), in buffered medium at pH 7.4 (Figure 2). When compared to the non-glycosylated control protein (streptavidin), an hypsochromic shift was observed for the labeled proteins of *ca*. 30 nm along with emission enhancement for all the glycoproteins (Figure S10) accounting for the binding event.

To study the performance of the novel boronolectin **Cy-Bx** as an imaging agent, we conducted labeling assays toward the mucin granules present in airway epithelial Calu-3 cells, which are related to human lung adenocarcinoma.[12] In goblet cells, mucin production entails several biological processes. The biosynthesis of glycosylated mucins requires transcription of a *MUC* gene to mRNAs that are translated in the rough endoplasmic reticulum (RER) into proteins that are post-translationally modified in the Golgi complex by glycosyltransferases. Secretory mucins are stored in granules and released at the apical surface.[13]

Imaging the secretion pathway of mucin granules and their kinetics in live cells has been an elusive task due to the lack of suitable dyes for monitoring this dynamic process. Recently, Shumilov *et al*. proposed the use of acridine orange at saturating levels to image the mucin secretion in ephithelial Calu-3 cells by means of total internal reflection fluorescence (TIRF) technique.[14] They proposed a two color excitation approach in the visible range by varying the concentration of the dye and making profit of its ability to interplay between monomers and aggregates of different color. This enabled the visualization of mucins focusing in the post-secretory swelling kinetics. This report is so far, the most accomplished example when mucin is the target. We expected our probe to be superior in this regard, since it can be excited and imaged within the near infrared range excluding autofluorescence interferences and background signals. Besides, **Cy-Bx** includes the benzoboroxol as a specific recognition unit for *cis*-diols motifs, introducing better selectivity. Moreover, it was demonstrated by Kreda *et al* that mucin granules constitutes a major source of nucleotide uptake/storage/release in the secretory pathway.[15] Given the observed affinity of the receptor to both, nucleotides and mucin (Figure 1) and mucin (Figure 2) **Cy-Bx** must be able to achieve both recognition events enhancing the efficiency of the binding.

Therefore, human lung adenocarcinoma Calu-3 cells were labeled by gently washing with PBS and incubated at 37ºC for 30 minutes in modified Tyrode's buffer containing 50 μM of the probe **Cy-Bx** (see section 7 in Supplementary Information for cell maintenance and differentiation details). In a first experiment we performed MUC5AC immunostaining simultaneously with **Cy-Bx** labeling in order to verify its specificity (Figure 3). In all experiments the incubation with the probe did not affect the cell morphology as compared to control cells.

Co-localization plot corroborates **Cy-Bx** specificity for mucin granules. The coincidence of both channels is indicated (Figure 3 E). In the same culture, some Calu-3 cells differentiate into goblet cell phenotype and are able to produce mucin, while others cells have the ability to polarize in an apical and basal surface.[16] As we mentioned before, mucin synthesis occurs in RER and afterwards they are modified in the Golgi complex producing perinuclear localization (Figure 3).

To further analyze secretory cells labeled with our probe, confocal z-stacks were performed. 3D reconstructions showed granules spread in cytoplasm as well as with membrane labeling. This result suggest that **Cy-Bx** not only is able to stain mucin granules but also labels mucin on cell surface (Figure 4 A and C). Orthogonal view shows that **Cy-Bx** endures inside the cells as granules can be observed in different levels without significant degradation of the probe and preserving a high quality of the signal (Figure 4 B). This is not a minor observation taking into

consideration that although a fluorogenic response was observed for mucin in cuvette (Figure 2) the recognition of ATP or AMP *in vitro* correlates with a quenching process (Figure S11). Given the mentioned report of Kreda *et al* accounting for the heavy cargo of ATP and related molecules co-existing with mucin within granules, we notice that another effect is empowering the emissive signal. Such enhancing effect must be given by the inherent viscosity of the granules and mucin itself increasing the fluorescence quantum yield. To assess this fact was studied the change of the fluorescence intensity of **Cy-Bx** by monitoring the emission with viscosity rendering an enhancement of 90% in the signal at 755 nm (Figure S12). Moreover, the quenched complex of **Cy-Bx** with ATP showed an increase in intensity with higher amounts of glycerol in solution (Figure S13).

The images also allowed us to discriminate different morphologies of the granules in our cellular model. It is well known that mature mucin secretory granules are large, with a mean diameter of 1 μm.[17] Therefore, size variation correlates with the stage of maturation. Figure 5 shows different fields of a same culture. Large and small mucin granules are visible in secretory cells.

In summary, we accomplished the synthesis and application of a novel near infrared molecular probe **Cy-Bx** with high sensitivity for glycoproteins by combining a benzoboroxole fragment as the recognition moiety for carbohydrates with a tricarbocyanine fluorophore as the reporter.

Cy-Bx showed a fluorogenic response to the presence of model glycoproteins in cuvette with negligible non-specific binding. By means of a simple labeling protocol, the probe shows high specificity for mucin by imaging granules in Calu-3 cells, demonstrated by co-localization analysis in comparison to a specific antibody.

The versatility of the chemical modification of the tricarbocyanine structure over other NIR dyes is suitable for designing improved probes, including orthogonal functionalization. Having achieved higher demands for a fluorescent molecular probe regarding NIR emission, large Stokes shift and water solubility, we foresee **Cy-Bx** as an excellent bioanalytical probe for carbohydrate recognition, filling a vacancy in the fluorescence imaging tool-box.

Acknowledgements

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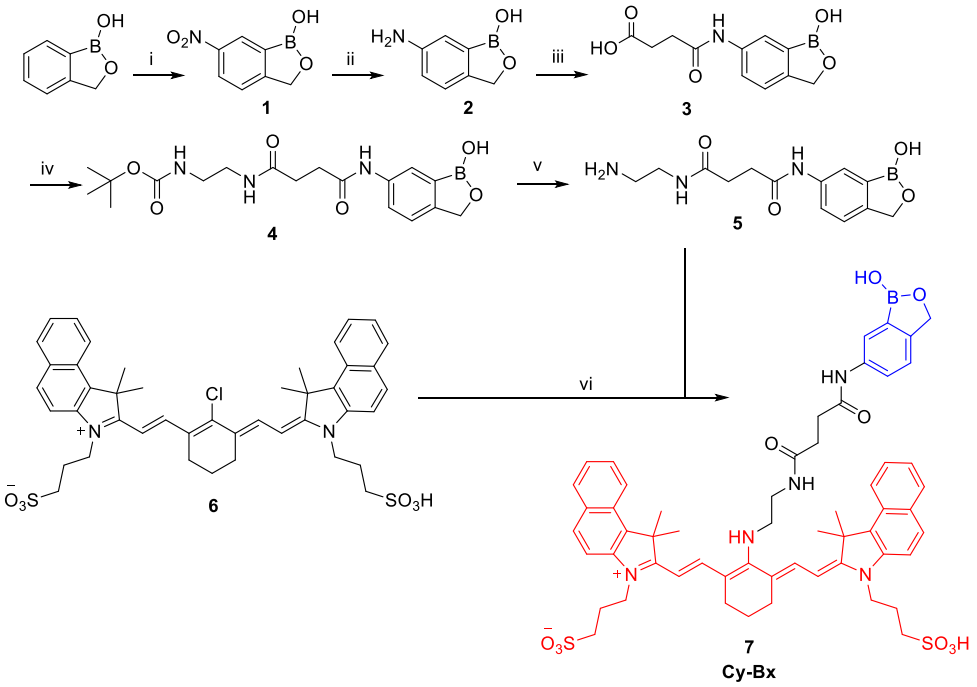

Scheme 1. Synthesis of the new probe **Cy-Bx** (**7**). i) HNO_3, -45ºC, 84%; ii) H_2, Pd/C, THF, 7h, 90%; iii) succinic anhydride, EtOH, 75ºC, 10h, 80%; iv) DCC/NHS, EDA-BOC, TEA, DMF, 62%; v)TFA, DCM; vi) DMF, TEA, 12h, 60%

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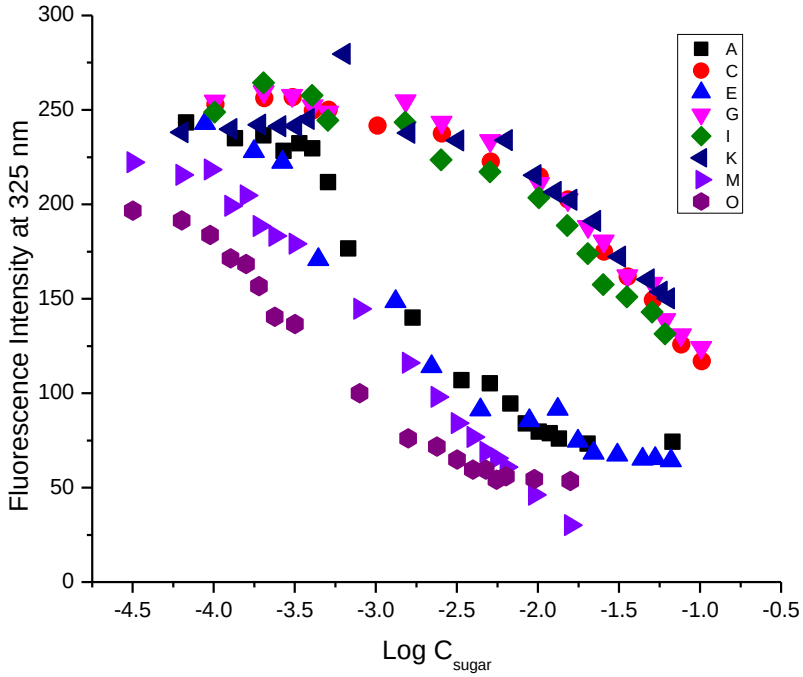

Figure 1. Binding of receptor **4** (3.10^{-4} M) to model monosaccharides and nucleotides in buffer PBS 0.1 M pH 7.4, monitored by the change in the emission at 325 nm (λ_{exc} 250 nm).

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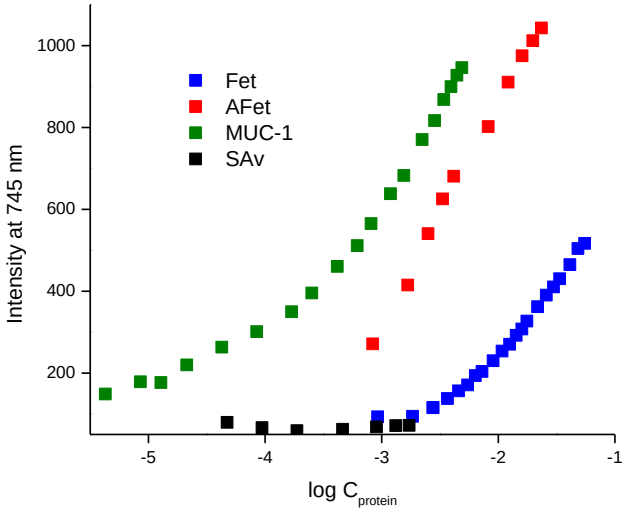

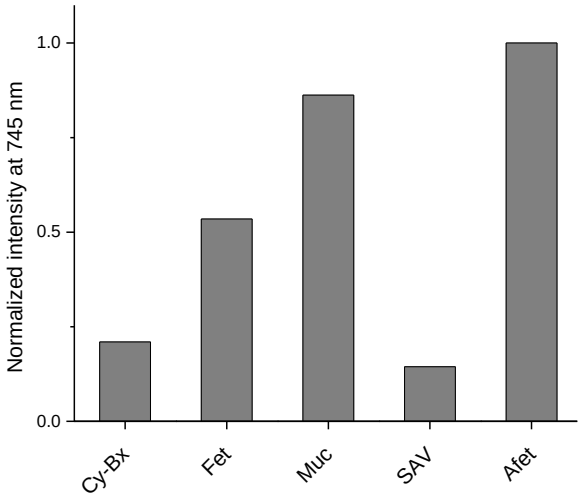

Figure 2. Fluorogenic response of boronolectin **Cy-Bx** 2.7 μM to model glycoproteins in buffer PBS 0.01 M, pH 7.4, λ_{exc} 650 nm.

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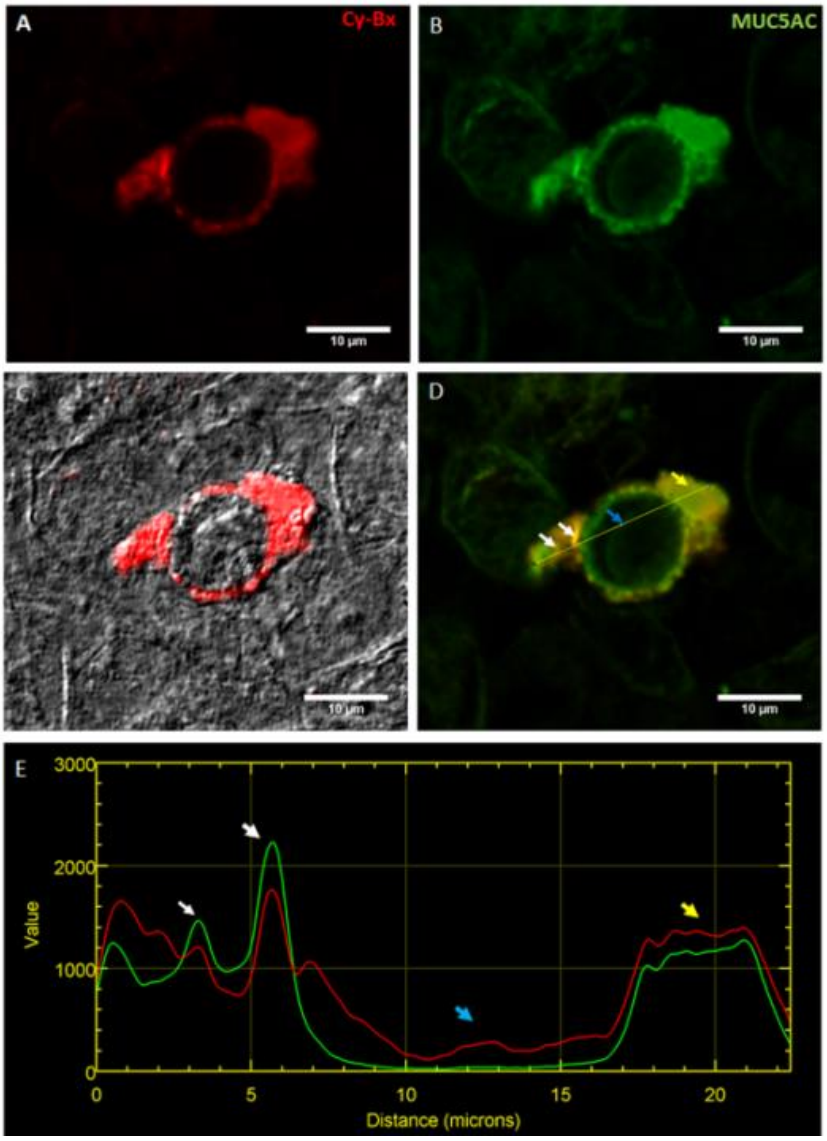

Figure 3. Cy-Bx labeled mucin granules. Calu-3 cells mucin granules were labeled with **Cy-Bx** and MUC5AC immunostaining (A and B). Merge of confocal microscopy images of the differential interference contrast (DIC) and **Cy-Bx** (C). Merge of **Cy-Bx** and MUC5AC. Yellow line points out pixels to co-localization analysis (D). Fluorescence intensity versus distance shows of **Cy-Bx** and MUC5AC co-localization. White arrows mark coincident intensity among red and green channel; light blue arrow marks minimal fluorescence intensity of both cannels. Yellow arrow shows co-localization of red and green channels in another cell region (E). Images were taken with an objective Oil 60X PLAPON NA=1.42. Co-localization was analyzed employing FIJI (ImageJ) software.

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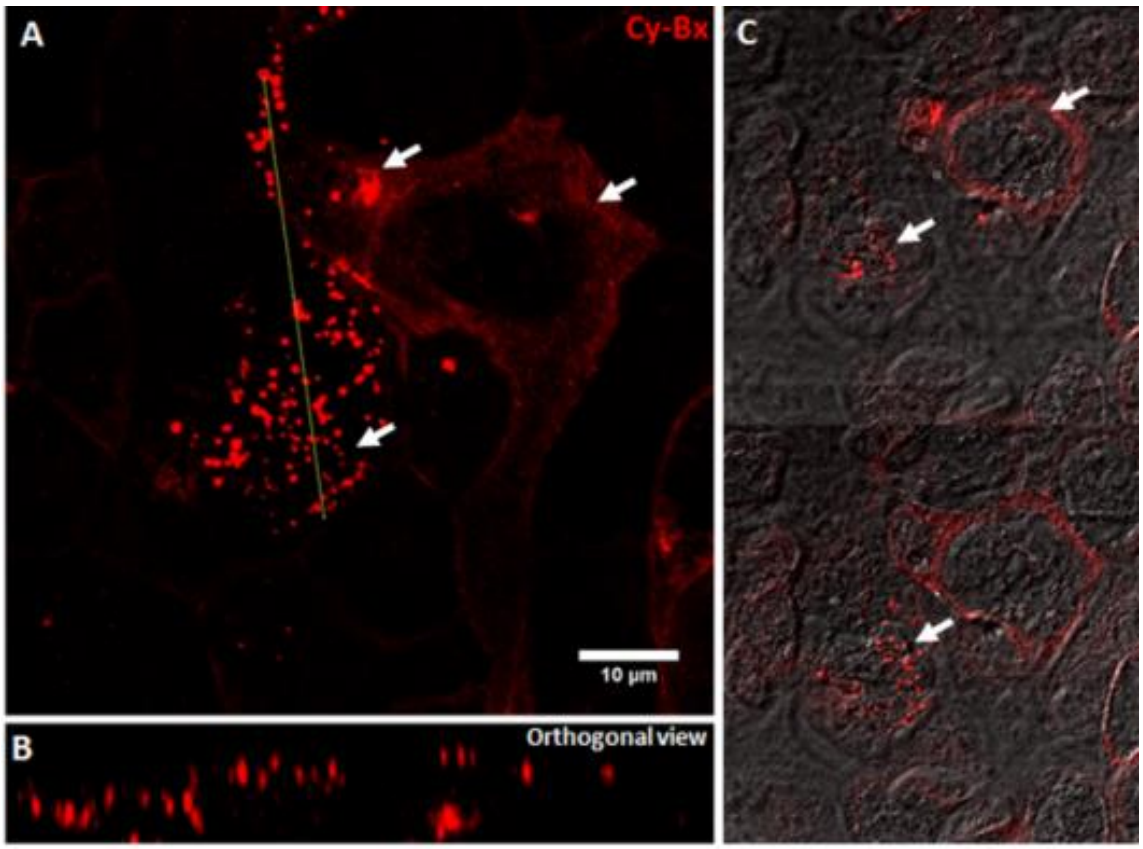

Figure 4. Cy-Bx labeled granules in cytoplasm and secreted mucin. 3D-reconstruction of confocal Z-stacks shows a Calu-3 cell with mucin granules throughout the entire cytoplasm. (**A**)Yellow line was drawn for the subsequent analysis of the granules. White arrows points out mucin granules and possibly secreted mucin localized on cellular membrane. (**B**) Orthogonal view shows granules localization inside the cell. (**C**) merge among DIC and **Cy-Bx** labeled cells. 3D reconstruction and orthogonal view were performed employing FIJI (ImageJ) software.

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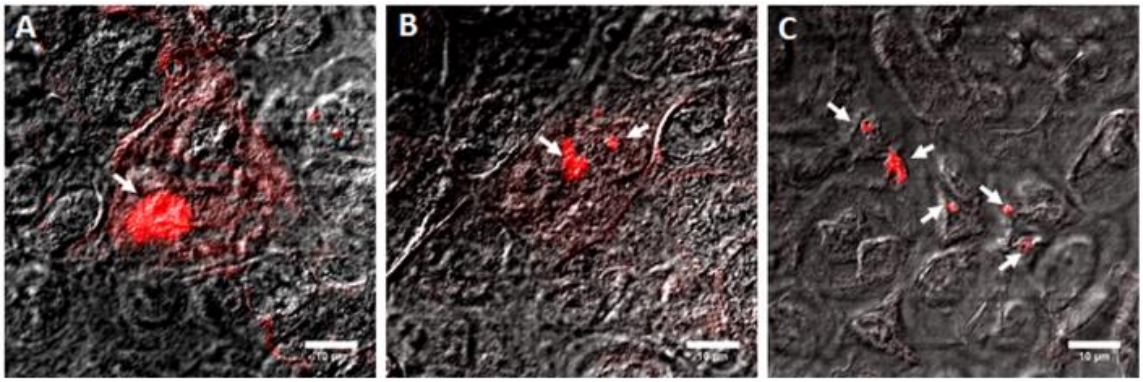

Figure 5. Cy-Bx labeled mucin granules with different morphologies. Merge of DIC and **Cy-Bx** images show different stages of mucin granules in the same cell culture (**A**, **B** and **C**). Images analysis was performed employing FIJI (ImageJ) software.

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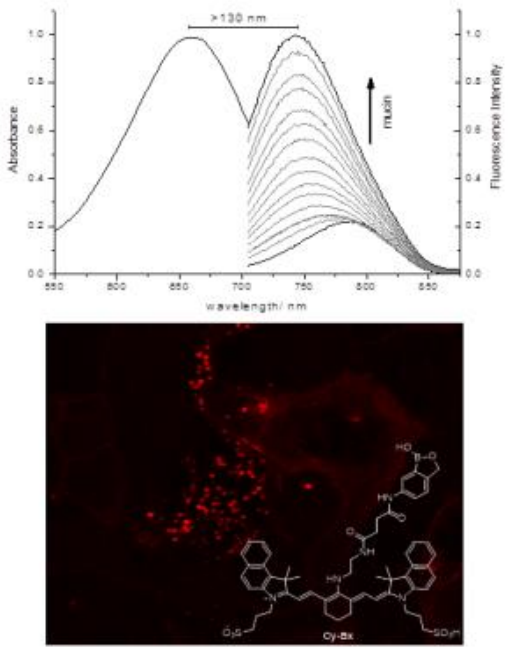
Table of contents

Boronolectin based Near-IR fluorescent probe: a benzoboroxol-tricarbocyanine based probe with large Stokes shift, fluorogenic response and high selectivity for glycoproteins was developed. The performance of the dye was demonstrated in live epithelial cells by imaging mucin granules. It was possible to detect differences in morphology and localization of mucin within the cells. The novel probe is expected to be a useful tool for bioanalytical imaging of glycostructures.



Keywords

boronolectin; cyanine; glycoprotein; fluorescent probes; dyes/pigments