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## A rationally designed peptidomimetic biosensor for sialic acid on cell surfaces†

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Cite this: *Chem. Commun.*, 2015, 51, 8112

Received 25th February 2015,  
Accepted 1st April 2015

DOI: 10.1039/c5cc01662b

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**We have developed peptidomimetic sialic acid (Sia) biosensors using boronic acid and arginine groups on the peptide backbone. The designed peptides were conjugated to fluorescent streptavidin via biotin enabling the optical labeling of cells. This approach provides unique opportunities to detect Sia composition on the cell surfaces and filopodia.**

Proteomic, genomic and glycomic changes occur during carcinogenesis resulting in an alteration of cellular features. The differences in the surface profiles of malignant and their non-malignant counterparts can serve as a molecular address for drug delivery to the desired cell types.<sup>1</sup> Over the last 30 years, dozens of tumor associated carbohydrate antigens (TACA) have been identified and most of these TACAs have sialic acids (Sias) as terminal sugars.<sup>2</sup> Nearly 50 different types of naturally occurring Sias have been identified. *N*-Acetylneuraminic acid (Neu5Ac) and its hydroxylated derivative, *N*-glycolylneuraminic acid (Neu5Gc), are the two predominant Sia forms in most mammals.<sup>3</sup> Interestingly, larger amounts of Neu5Gc were reported in human malignant tumors and in fetal tissues.<sup>4</sup> Furthermore, a significant difference exists between sialylation patterns and concentration in normal cells and their malignant counterparts. Abnormal Sia expression favours cancer metastasis by enhancing migration and tissue invasion.<sup>5</sup> It is therefore desirable to develop specific markers for sialic acid glycans.

Several biomacromolecules such as lectins, antibodies or nucleic acids have been used to bind Sias on cancer cells.<sup>6</sup> Phage display technology has also been used to develop short 12 or 15-mer peptides of the Sias binding site.<sup>7</sup> However, these biomolecules must overcome different drawbacks such as toxicity, selectivity, stability, immunogenicity and cost. Ideally, short peptidomimetics with high affinity and specificity could provide an alternative to lectins. In this context, the ability of boric and phenylboronic acid to interact with sugars has been

extensively reported.<sup>8</sup> Phenylboronic acid binds covalently to *cis* 1,2 or 1,3-diols and forms cyclic esters.<sup>9</sup> Hall *et al.* have demonstrated that benzoboroxole conjugated peptidomimetics can be used to target pyranoside sugars.<sup>10</sup> Wang *et al.* synthesized a large library of boronic acid modified DNAs to target glycoproteins.<sup>11</sup> Lavigne *et al.* used a boronic acid based peptide library to detect glycoproteins.<sup>12</sup> Levonis *et al.* have reported an artificial fluorescent boronate receptor for the detection of free sialic acid.<sup>13</sup> Gold coated surfaces,<sup>14</sup> glass beads,<sup>15</sup> graphene,<sup>16</sup> polymers,<sup>17</sup> lanthanide complexes<sup>18</sup> and quantum dots<sup>19</sup> were all used as templates to conjugate boronic acid derivatives to target cell surface Sias. However, interaction between phenylboronic acid and Sias in glycoconjugates is much weaker than free Neu5Ac, due to the presence of glycosidic linkage at the C2 position. To increase the avidity of specific peptide–sugar interactions multivalent guanidine or tryptophan moieties were incorporated as secondary ligation groups.<sup>20</sup> However, the selectivity and sensitivity of these molecules are still far lower compared to natural lectins. Herein, we propose to synthesize a new class of peptidomimetics based on a short hexapeptide model (SPYGRG), which has been reported to bind Sias containing glycoconjugates.<sup>21</sup> We have synthesized peptidomimetic analogs by incorporating phenylboronic acid (*meta* or *para*-orientation) residues into the EYGR tetrapeptide. Molecular modelling studies have revealed boronic acid and arginine groups in close proximity (Fig. S22, ESI†). We hypothesized that such close proximity could allow selective binding of phenylboronic acid with germinal diol function at C8 and C9 of Sia as well as electrostatic interactions between positively charged arginine and carboxylic acid residues of Sia. In addition, the tyrosine amino acid residue was expected to provide H-bonding and CH– $\pi$  interaction to stabilize the pyranoside ring of Sia. The binding affinity with different monosaccharides was achieved by microarray. Confocal imaging of cancer cell lines – HeLa (cervix) and MDA-MB-231 (breast) and normal cell line – NIH-3T3 (fibroblast) revealed the correlation of the binding pattern of peptidomimetics and Sambucus Nigra Lectin (SNA), a commercial sialic acid binding lectin.

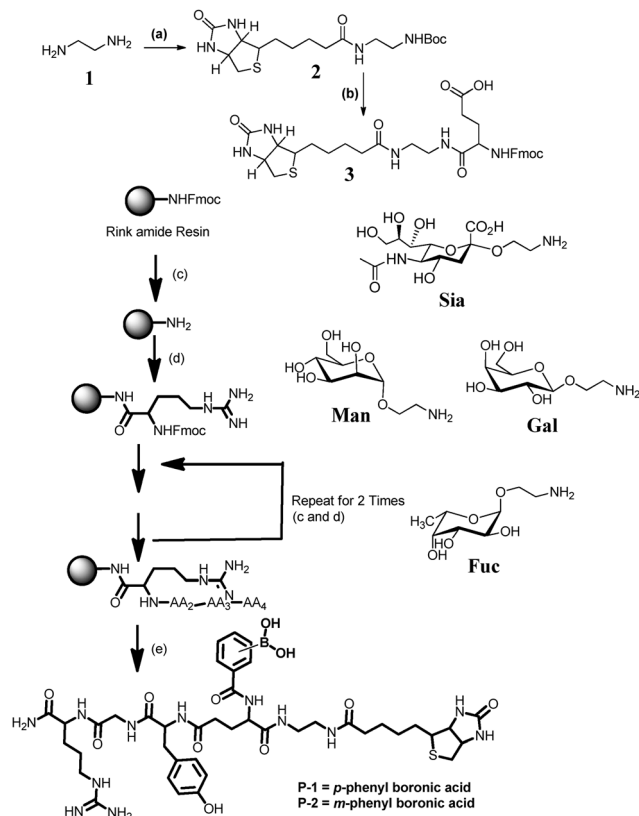
The synthesis of peptides **P-1** and **P-2** was done using Fmoc solid phase peptide synthesis on Rink amide resin (Fig. 1).

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c5cc01662b

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Both peptidomimetics are composed of the same amino acid sequence with a terminal biotin residue, however, they differ in the *m* or *p*-phenyl boronic acid residue. The biotin residue provided an additional slot for conjugation with fluorescent-streptavidin. **P-1** was synthesized by step-by-step coupling of biotin conjugated FmocGlu(O<sup>t</sup>Bu)OH (**3**), FmocGlyOH, FmocTyr(<sup>t</sup>Bu)OH and FmocArg(Pbf)OH amino acid residues. Finally, *m* or *p*-phenyl boronic acid was coupled to the glutamic acid residue to obtain the final peptides (Fig. 1). After cleavage from the resin, the peptides were purified by preparative HPLC and characterized by HRMS, <sup>1</sup>H and <sup>13</sup>C-NMR.

Next, we examined the interaction of the peptidomimetics with sugars. Four monosaccharides (Sia, Man, Gal, and Fuc) were printed in eight (50, 10, 5, 2, 1, 0.5, 0.2 and 0.1 μM) different concentrations on the *N*-hydroxysuccinimide derivatized surfaces. The resulting sugar arrays were treated with **P-1**, **P-2** and **SNA** in the presence of 0.05% Tween-20, followed by Cy3-streptavidin. Mean fluorescence intensities were determined by using data from five independent experiments. Results obtained from sugar array show that **P-1** displays a strong interaction with Sia and a weak interaction with Man, Gal and Fuc sugars (Fig. 2). To understand the molecular level details of these interactions, minimum energy structure was constructed using Sia–**P-1** conjugation (Fig. S23a and Table S2, ESI<sup>†</sup>).

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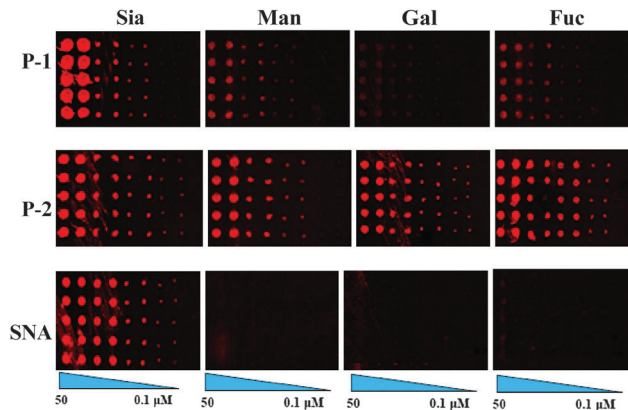


Fig. 2 Sugar microarray containing four monosaccharides with **P-1**, **P-2** and **SNA**.

To simplify the docking studies, the phenylboronic acid residue was covalently conjugated to C8 and C9 of Sia. As expected, *p*-orientation of the boronic acid residue provided enough flexibility to accommodate ionic interaction between positively charged arginine and negatively charged carboxylic acid residues of Sia, resulting in selective binding. Despite high reactivity between phenylboronic acid and *cis*-1,2 or 1,3 diols of other sugars, the differential labeling with other sugars is likely related to the slow reactivity (Fig. S24 and Table S3, ESI<sup>†</sup>). In the case of the **P-2** peptide, microarray studies showed an identical binding pattern with all sugars. Molecular modelling of the **P-2**–sialic acid complex clearly showed close proximity between arginine–phenylboronic acid ester at the same time when the carboxylic acid residue of the Sia ligand is far away from the arginine residue (Fig. S23b, ESI<sup>†</sup>). A similar experiment with **SNA** showed selective binding to Sia compared to other sugars. These results indicated that **P-1** might be a better candidate to sense sialic acid compared to **P-2** analogs.

Microarray results can also be utilized qualitatively and quantitatively for the analysis of specific interactions and it is considered as a powerful method for the rapid and simultaneous measurement of dissociation constants ( $K_d$ ).<sup>22</sup> Thus, sugar microarrays were utilized to determine  $K_d$  values for peptidomimic–Sia interaction. The Sia ligand was imprinted eight times with different concentrations on *N*-hydroxysuccinimide derivatized surfaces, and the peptide microarrays were then probed with four concentrations each of **P-1** and **P-2** peptides with eight different concentrations of sugars, and the  $K_d$  values were determined (Table 1 and Table S1 and Fig. S7 to S12, ESI<sup>†</sup>). The results showed that most of the curves fit well to one site binding. The observations showed that both the peptides **P-1** and **P-2** were bound to Sia with sub-micromolar  $K_d$  values and display a very weak binding with other sugars. Moreover,

Table 1  $K_d$  values of **P-1** and **P-2** with different sugar substrates

Sugar	<b>P-1</b> (μM)	<b>P-2</b> (μM)
Sialic acid (Sia)	0.0754	0.0786
Mannose (Man)	10.23	0.104
Galactose (Gal)	2030	0.0753
Fucose (Fuc)	10.56	0.127

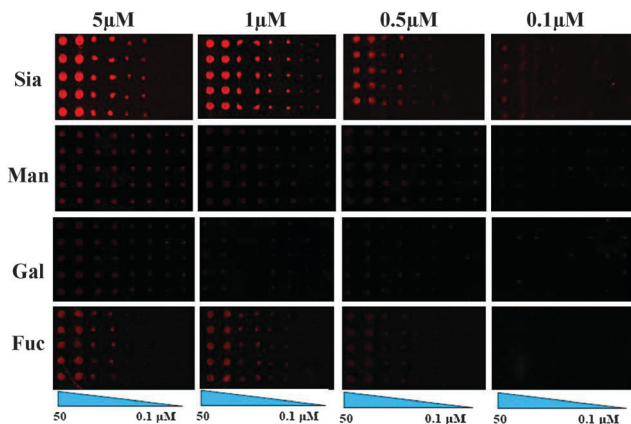


Fig. 3 Fluorescence image of four different concentrations of **P-1** peptides with eight different concentrations of sialic acid (Sia), fucose (Fuc), galactose (Gal) and mannose (Man).

Sia-**P-1** was relatively strongly bound compared to Sia-**P-2** with a  $K_d$  value close to **SNA** (Fig. 3).

Using microscopic imaging, **P-1** was evaluated for binding with Sias on normal and cancer cell surfaces, which are known to express different levels of Sias on the cell membrane. In the first step, **P-1** was conjugated with FITC-streptavidin (**F-S**) by mixing the two in PBS pH 7.4 for 2 h at RT. As can be seen from confocal images, both cancer cells and normal cells were visibly stained after 30 min incubation with the **P-1(F-S)** conjugate (Fig. 4 and Fig. S13, S15 and S17, ESI<sup>†</sup>).

Based on the morphology of the cells, **P-1** appears decorated on the cell surfaces and was found to internalize to some extent (Fig. S14, S16 and S18, ESI<sup>†</sup>). These results correlate with that of Liu *et al.* obtained with QD-phenylboronic acid conjugates.<sup>23</sup> To further validate that the staining observed was indeed due to the specific recognition between Sia and **P-1**, Sias were chopped off from the HeLa cell surfaces by treating them with sialidase enzyme for 30 min. It was clearly evident that the removal of Sias effectively inhibited **P-1** binding on the cell surfaces (Fig. S20, ESI<sup>†</sup>). A similar

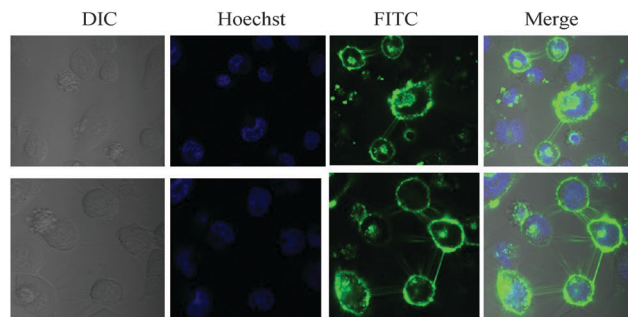


Fig. 5 Fluorescence images of filopodia in HeLa cells with **P-1**.

Sia distribution pattern was also revealed with Sia-specific FITC labelled **SNA** lectin (Fig. S19, ESI<sup>†</sup>). Also, Sia composition on normal and cancer cell types was evaluated by measuring the average fluorescence intensity. As expected **P-1(F-S)** conjugate treated HeLa cells showed 35–40% strong fluorescence response compared to that of normal (NIH-3T3) cells (Fig. S25, ESI<sup>†</sup>). In contrast, MDA-MB-231 showed nearly 36–42% less fluorescence response compared to HeLa. A similar experiment with FITC-conjugated **SNA** revealed identical results. Accordingly, we suspect that breast cancer cells might express less Sias compared to cervix or may express Sias in other linkage forms, which might be difficult to identify with the **SNA** and/or **P-1** peptide.

A close examination of **P-1** and **SNA** stained HeLa showed that both conjugates also stained filopodia (Fig. 5 and Fig. S19b, ESI<sup>†</sup>), the connecting networks between the cells.<sup>24</sup> To further confirm that the staining of filopodia is due to specific Sia glycan, we performed confocal imaging with FITC conjugated Concanavalin A, peanut agglutinin (PNA) and Ulex europaeus agglutinin 1 (UEA1), where Concanavalin A binds to all D-mannose and D-glucose structures, PNA is specific to T-antigen, a galactosyl ( $\beta$ 1–3) *N*-acetylgalactosamine structure, and UEA1 is specific to the  $\alpha$ -linked L-fucose residue. HeLa cells were treated with all these lectins separately and imaged. Interestingly, HeLa displayed a high concentration of mannose terminated glycans and a small quantity of fucose, galactose or galactosamine terminal sugars. However, none of them stained the filopodia component, indicating the presence of Sia glycans in the filopodia region (Fig. S21, ESI<sup>†</sup>). In the case of MDA-MB-231, the filopodia level was very less to get distinct staining (Fig. S17, ESI<sup>†</sup>).

In summary, we have shown how a short peptidomimetic can be applied to diagnose cell surface Sias. Microarray and confocal imaging studies clearly showed selective interactions between **P-1** and Sias. Our results clearly show the presence of Sias on filopodia, which is involved in cell motility, proliferation and cell–cell interactions. In a more general perspective, the ease and low cost of synthesis make **P-1** an ideal alternative substrate for **SNA** lectin to analyze complex glycocalyx machinery.

R. K thanks IISER Pune, Indo-German (DST-MPG) program and UGC, India, for financial support.

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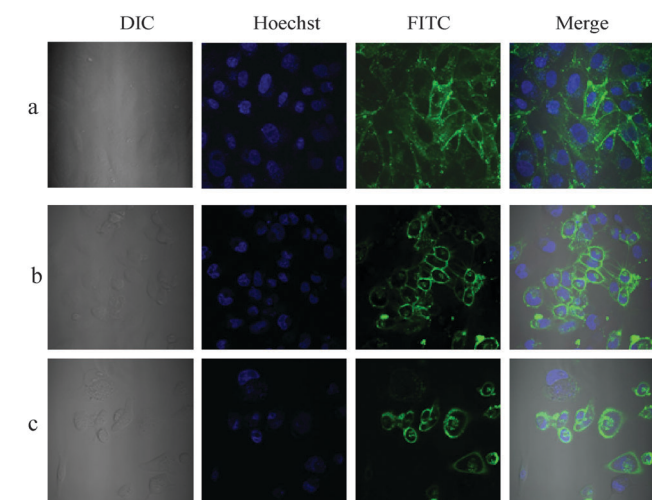


Fig. 4 Fluorescence images of different cell lines with **P-1**: (a) NIH-3T3; (b) HeLa; (c) MDA-MB-231.



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