Rationally Designed Peptidomimics Biosensor for Sialic acid on Cell Surfaces

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bEqual contribution.

1. General methods
2. Synthesis of Fmoc-biotin derivative
4. High performance liquid chromatography (HPLC) of peptidomimics
5. Microarray binding studies
6. Confocal imaging

1. General Methods

General Experimental Details. Commercial grade reagents and solvents were used without further purification except as indicated below. Deionized water was obtained from an inhouse purification system. Analytical thin layer chromatography (TLC) was performed on Merck silica gel 60 F254 plates (0.25 mm). Compounds were visualized by UV irradiation or dipping the plate in CAM solution followed by heating. Column chromatography was carried out using force flow of the indicated solvent on Fluka Kieselgel 60 (230–400 mesh). 1H and 13C NMR spectra were recorded Jeol 400 MHz (or 100 MHz for 13C ) using residual solvents signals as an internal reference (CDCl3 δ H, 7.26 ppm, δ c 77.3 ppm and CD3OH δ H 3.31 ppm, δc 49.0 ppm). The chemical shifts (δ) are reported in ppm and coupling constants (J) in Hz. DMEM media was
purchased from Invitrogen. HeLa, MCF-7, MDA-MB, and NIH-3T3 cells were obtained from the National Centre for Cell Science (NCCS), Pune. High resolution mass spectra (HRMS) were recorded with an Agilent 6210 ESI-TOF mass spectrometer. University of Pune, Zoology department microarray scanner facility was used to read the microarray plates. Biotinylated-SNA, FITC-conjugated ConA, PNA and UEA1 was purchased from vector labs. Energy-minimized structures were calculated by molecular mechanics using MM2 in CS Chem 3D.¹

2. Synthesis of Fmoc-biotin precursor.

Scheme S1. Synthesis of 4: (a) Boc₂O, DCM 0 °C-RT, 12 h, 80%; (b) Biotin, EDC, HOBT, DMF, 12 h, 80%; (c) 40%TFA, DCM, 2 h, 90%; FmocGlu(O'Bu)OH, EDC, HOBT, DMF, 12 h, 73%; (d) 25% TFA, DCM, 2h, 98%.

Synthesis of 1. Synthesis of mono Boc protected ethylene diamine was carried out as reported in the literature.²

Synthesis of 2. Biotin (1 g, 4.09 mmol), HOBT (0.66 g, 4.91 mmol) and EDCI (0.93 g, 4.91 mmol) were stir in anhydrous DMF at ice cold codition for 15 min. To this solution mono-Boc protected ethylene diamine (0.65 g, 4.09 mmol), dissolved in anhydrous DMF was slowly added. The corresponding reaction mixture was allowed to stir for 12 h. After completion of reaction (monitored by TLC) water (100 mL) was added and the aqueous layer was extracted with DCM (3 x 80 mL). The combined organic layer was washed with NaHCO₃ (50 mL) to remove the
unreacted free acid followed by washing with KHSO$_4$ (50 mL) to remove the unreacted free amine. The combined organic layer was washed with NaHCO$_3$ (50 mL) followed by water (3 x 50 mL) and brine. The organic layer was dried over anhydrous Na$_2$SO$_4$ and concentrated under vacuum. The crude product obtained was purified by silica gel chromatography (with 6% MeOH in DCM) to afford compound 2 as a white solid. Yield: 1.2 g, (80 %). Molecular Formula, C$_{17}$H$_{30}$N$_4$SO$_4$.

1H NMR (CD$_3$OD, 400 MHz) $\delta$H: 4.47 (dd, $J = 7.8$, 4.3 Hz, 1H), 4.28 (dd, $J = 7.9$, 4.5 Hz, 1H), 3.75-3.64 (m, 1H), 3.34 – 3.30 (m, 1H), 3.26 – 3.15 (m, 4H), 3.12 (t, $J = 6.0$ Hz, 2H), 2.95 – 2.86 (m, 1H), 2.68 (d, $J = 12.7$ Hz, 1H), 2.24 – 2.13 (m, 2H), 1.76 – 1.51 (m, 4H), 1.40  (s, 9H).

Synthesis of 3.

Compound 2 (1.3 g, 3.4 mmol) was dissolved in dichloromethane/trifluoroacetic acid (3:1, 10 mL) and stirred at room temperature for 2 h. The solvent was evaporated in vacuo, the residue was re-dissolved in anhydrous DMF (20 mL) and the pH adjusted to 8 using triethylamine. FmocGlc(O'Bu)OH (1 g, 2.3 mmol), HOBT (0.47 g, 3.5 mmol) and EDCI (0.67 g, 3.5 mmol) were added to the above mixture and stirred for 12 h at rt. Finally, the mixture was concentrated in vacuo and purified by flash chromatography to afford 3 as a thick liquid Yield: 1.58 g, (73 %). Molecular Formula, C$_{36}$H$_{47}$N$_5$SO$_7$. [Mass: calculated 693.3196, observed (M + H$^+$) 694.3271].

1H NMR (CD$_3$OD, 400 MHz) $\delta$H: 7.77 (d, $J = 7.5$ Hz, 2H), 7.64 (t, $J = 6.5$ Hz, 2H), 7.36 (t, $J = 7.4$ Hz, 2H), 7.33 – 7.25 (m, 2H), 4.47 – 4.29 (m, 3H), 4.21 (t, $J = 6.2$ Hz, 2H), 4.02 (dd, $J = 9.2$, 5.1 Hz, 1H), 3.34 – 3.31 (m, 1H), 3.26 – 3.21 (m, 2H), 3.14 – 3.06 (m, 1H), 2.85 (dd, $J = 12.7$, 4.9 Hz, 1H), 2.64 (d, $J = 12.7$ Hz, 1H), 2.28 (t, $J = 7.5$ Hz, 2H), 2.20 – 2.09 (m, 2H), 2.08 – 1.97 (m, 1H), 1.81 (dt, $J = 21.2$, 7.2 Hz, 1H), 1.71 – 1.47 (m, 4H), 1.42 (s, 9H), 1.39 – 1.28 (m, 2H).

13C NMR (100 MHz, Methanol D4) $\delta$C: 175.06, 173.21, 172.45, 164.73, 165.05, 143.59, 141.19, 127.50, 126.87, 124.92, 119.63, 80.50, 66.64, 61.87, 60.28, 55.54, 54.66, 53.46, 39.68, 38.81, 38.56, 35.48, 31.34, 28.33, 28.07, 27.03, 25.37.

Synthesis of 4.

Compound 3 (1 g, 1.4 mmol) was dissolved in dichloromethane/trifluoroacetic acid (4:1, 10 mL) and stirred at room temperature for 2 h. The solvent was evaporated in vacuo, the residue was coevaporated with DCM.

3. Solid state synthesis of P-1 and P-2 peptide.
The readily available Rink amide resin with loading value 0.5-0.6 mmol/g was used and standard Fmoc chemistry was employed. The resin bound Fmoc group was first deprotected with 20% piperidine in DMF and the coupling reactions were carried out using \textit{in situ} active ester method, using HBTU as a coupling reagent and HOBT as a recemization suppresser and DIPEA as a catalyst. All the materials used were of peptide synthesis grade (Sigma-Aldrich) and was used without further purification. Analytical grade DMF was purchased from Merck (India) and was distilled over P$_2$O$_5$ under vacuum at 45$^\circ$C, stored over 4Å molecular sieves for 2 days before using for peptide synthesis.

(a) Synthesis protocol for solid phase synthesis

The resin was pre-swollen overnight and the following steps were performed for each cycle.

- Wash with DMF 4 x 5 mL.
- 20% piperidine in DMF 2 x 5 mL (15 min for each) for deprotection of Fmoc group.
- Wash with DMF 3 x 5 mL, MeOH 3 x 5 mL and DCM with 3 x 5 mL.
- Test for complete deprotection (chloranil test).
- Coupling reaction with amino acid, DIPEA, HOBT and HBTU (3 eq.) in DMF (1 mL).
- Repeat of the coupling reaction in NMP for better yield.
- Test for completion of coupling reaction (chloranil test).

This cycle was repeated for every amino acid.

General procedure for Fmoc deprotection

20% piperidine in DMF was added to the resin and the reaction mixture was kept for 15 min, drained and the piperidine treatment was repeated 3 times. Finally the resin was washed with DMF (3x), MeOH (3x) and DCM (3x).

(a) General procedure for peptide couplings on Rink Amide Resin

Fmoc-AA-OH (3 eq), HBTU (3 eq) and HOBT (3 eq) dissolved in DMF/NMP followed by DIPEA (7-8 eq) were added to the amino-functionalized resin in DMF. The mixture was kept for 2 h and last 5 min bubbled with N$_2$ and washed with DMF (3x), MeOH (3x) and DCM (3x). The loading value for peptide synthesis is taken as 0.5~0.6. The coupling reaction was repeated in NMP for better yield.

(b) General procedure for cleavage of peptides from the solid support
The dry peptide-resin (20 mg) was taken in round-bottomed flask to which of 95% TFA in H₂O and Triisopropylsilane (as scavengers, 2-3 drops) were added. The resulting mixture was kept for 2 h by gentle shaking. The mixture was filtered through a sintered funnel and the resin was washed with 3 x 5 mL of above solution. The filtrate was collected in pear shape round-bottom flak and evaporated under reduced pressure. The resin was washed with MeOH (3 X 5 mL) and the washings were evaporated to dryness. The residue obtained was dissolved in anhydrous methanol (0.1 mL) and to it anhydrous diethyl ether (4 x 1.5 mL) was added. The off-white precipitate obtained was centrifuged. The precipitation procedure was repeated twice to obtained peptide as a colourless powder.
Scheme S2. Solid phase peptide synthesis of P-1 and P-2.

4. HPLC Characterization.

Peptides P-1 and P-2 were purified by semi preparative HPLC on reversed phase column using Waters 600 device equipped with a 2998-Photodiode array detector (PDA). The solvent system was 0.1% TFA in acetonitrile:water (5:95) - solution A and 0.1% TFA in acetonitrile:water (50:50) - solution B. A gradient of 0-100% A-B (in 30 min) and a flow rate of 3 mL/min were
used and the eluting solution was monitored at 220 nm. The fractions corresponding to the emergence of the peptide were collected and concentrated using a speed vacuum device to afford the neat peptides. The purity of the final products were further analyzed by analytical HPLC using a gradient of 0 to 100% B in 20 min at a flow rate of 1.5 mL/min. The peptides were found to be more than 95% pure.

HPLC chromatogram for Peptide **P-1**

HPLC chromatogram for Peptide **P-2**
**Peptide P-1**

$^1$H NMR (500 MHz, D$_2$O): 7.86 (d, $J = 7.6$Hz, 2H), 7.78 (d, $J = 7.6$Hz, 2H), 7.06 (d, $J = 8.2$Hz, 2H), 6.75 (d, $J = 8.2$Hz, 2H), 4.50-4.44 (m, 2H), 4.35-4.32 (m, 1H), 4.26-4.21(m, 2H), 3.84-3.70 (m, 2H), 3.45-3.40 (m, 1H), 3.37-3.32 (m, 1H), 3.29-3.24 (m, 2H), 3.10-3.07 (m, 3H), 3.03-2.99 (m, 1H), 2.89-2.84 (m, 2H), 2.68 (d, $J=10$Hz, 1H), 2.44-2.32 (m, 2H), 2.10-2.04 (m, 3H), 2.01-1.94 (m, 1H), 1.85-1.79 (m, 1H), 1.70-1.63 (m, 1H), 1.58-1.50 (m, 2H), 1.49-1.30 (m, 4H), 1.18-1.13 (m, 2H).

$^{13}$C NMR (500 MHz, D$_2$O): 177.02, 176.15, 175.00, 174.14, 173.57, 171.21, 170.05, 165.23, 156.60, 154.47, 134.42, 130.00, 130.41, 128.04, 128.61, 115.38, 61.85, 60.16, 55.54, 55.19, 54.10, 52.92, 42.47, 40.42, 39.66, 38.82, 38.45, 35.99, 35.52, 31.59, 28.03, 27.83, 27.46, 26.63, 25.07, 24.31.

**Peptide P-2**

$^1$H NMR (500 MHz, D$_2$O): 8.12 (s, 1H), 7.95 (d, $J = 8.2$Hz, 1H), 7.85 (d, $J = 8.2$Hz, 1H), 7.54 (t, $J = 10$Hz, 1H), 7.06 (d, $J = 8.2$Hz, 2H), 6.75 (d, $J = 8.2$Hz, 2H), 4.49-4.45 (m, 2H), 4.34-4.32 (m, 1H), 4.25-4.21(m, 2H), 3.83-3.70 (m, 2H), 3.45-3.40 (m, 1H), 3.37-3.32 (m, 1H), 3.29-3.24 (m, 2H), 3.10-3.07 (m, 3H), 3.01-2.99 (m, 1H), 2.88-2.83 (m, 2H), 2.68 (d, $J=10$Hz, 1H), 2.44-2.32 (m, 2H), 2.10-2.04 (m, 3H), 2.01-1.94 (m, 1H), 1.85-1.79 (m, 1H), 1.70-1.63 (m, 1H), 1.58-1.50 (m, 2H), 1.49-1.30 (m, 4H), 1.18-1.13 (m, 2H).
$^{13}$C NMR (500 MHz, D$_2$O): 177.00, 176.14, 174.98, 174.14, 173.62, 171.20, 170.29, 166.22, 163.07, 162.79, 156.69, 154.45, 137.76, 132.44, 130.38, 129.60, 128.45, 128.02, 115.38, 61.87, 60.18, 55.50, 55.17, 54.08, 52.92, 42.47, 40.42, 39.64, 38.88, 38.44, 35.96, 35.50, 31.57, 28.03, 27.82, 27.46, 26.66, 25.06, 24.30.

5. Microarray studies.

For the synthesis of Sia, Man, Gal and Fuc previously reported protocol was used.$^{3,4}$

N-hydroxyl succinimide coated microarray plate was obtained from Arrayit company. Monosaccharides Sia, Man, Gal and Fuc monosaccharides at different concentrated (50, 10, 5, 2, 1, 0.5, 0.2 and 0.1 µM) were imprinted of volume 1 µL with five replicates vertically places in each sub-array. After 1 h incubation, slides were washed with blocking buffer containing (ethanol amine (10 mM) in PBS (PH 7.8, 0.01 mM) for 1 h and washed several times with PBS (0.05% Tween-20). After drying, slides were incubated with 100 µM of peptide (P-1/P-2) (volume 1 nL) for 2 h followed by 0.1 µg of streptavidin-Cy3. In case of SNA, 0.1 µg (7.5 µM) of SNA in PBS (PH 7.8, 0.01 mM) was added and incubated for 1 h. Finally, the images were obtained using Array Worx microarray reader exciting 550 nm and emission 570 nm.
Figure S1. Microarray profile: Sia, Man, Gal and Fuc with P-1, P-2 and SNA lectin. Sia (1, 3, 5); Man(2, 4, 6); Fuc (7, 9, 11); Gal(8, 10, 12)

Determination of $K_d$ of P-1/P-2 peptide Protocol.

$N$-hydroxyl succinimide coated microarray plate was obtained from Arrayit company and sialic acid at different concentrated of (50, 10, 5, 2, 1, 0.5, 0.2 and 0.1 µM) were imprinted with five replicates. After 1h incubation, slides were washed with blocking buffer. Slides were imprinted with P-1/P-2 peptide at different concentration (5000, 1000, 500, 100 nM) Followed by streptavidin-Cy3 (0.1 µg). Finally, slides were washed with PBS (0.05% Tween-20). The images were obtained using Array Worx microarray reader exciting 550 nm and emission 570 nm $K_d$ was calculated by equation 1. $F_{max}$ represent maximum fluorescent intensity and [P] is the total peptide concentration.
Figure S2. Sia binding affinity with different concentration of P-1 peptide.

Figure S3. Sia binding affinity with different concentration of SNA lectin.

\[ F = \frac{F_{\text{max}} [P]}{[P] + K_d} \]  
\[ \text{------} \]  
\[ \text{(1)} \]
**Figure S4.** Fuc binding affinity with different concentration of P-1 peptide.

**Figure S5.** Man binding affinity with different concentration of P-1 peptide.

**Figure S6.** Gal binding affinity with different concentration of P-1 peptide.
Figure S7. Binding curve of P-1 with different concentration of Sia.

Figure S8. Binding curve of P-1 with different concentration of Man.

Figure S9. Binding curve of P-1 with different concentration of Fuc.
Figure S10. Binding curve of P-1 with different concentration of Gal.

Figure S11. Binding curve of P-1 with different concentration of sugars.

Figure S12. Binding curve of P-2 with different concentration of sugars.
<table>
<thead>
<tr>
<th>Sugar</th>
<th>P-1 (µM)</th>
<th>P-2 (µM)</th>
<th>SNA (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sialic acid (Sia)</td>
<td>0.0754</td>
<td>0.0786</td>
<td>0.0705</td>
</tr>
<tr>
<td>Mannose (Man)</td>
<td>10.23</td>
<td>0.104</td>
<td>-</td>
</tr>
<tr>
<td>Galactose (Gal)</td>
<td>2030</td>
<td>0.0753</td>
<td>-</td>
</tr>
<tr>
<td>Fucose (Fuc)</td>
<td>10.56</td>
<td>0.127</td>
<td></td>
</tr>
</tbody>
</table>

*Table S1.* $K_d$ values of P-1, P-2 and SNA with different sugar substrates.
6. **Streptavidin-Peptidomimic conjugations.**

Streptavidin-Peptidomimic complexation was performed by using standard streptavidin-biotin conjugation. Briefly, **P-1** or **P-2** (300 µM) and streptavidin (10 ug/ml) were dissolved in PBS (pH 7.4) and vertex together for 10 min at RT. The complex was kept as such for another 2 h at RT and used as such for biological studies.

7. **Confocal laser scanning microscope images.**

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Growth Media</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HeLa</strong></td>
<td>Cells were grown at 37 °C and 5% CO₂ in DMEM medium containing 10% fetal bovine serum and 0.1% streptomycin.</td>
</tr>
<tr>
<td><strong>NIH-3T3</strong></td>
<td>Cells were grown at 37 °C and 5% CO₂ in DMEM medium containing 10% fetal bovine serum and 0.1% streptomycin</td>
</tr>
<tr>
<td><strong>MDA-MB</strong></td>
<td>Cells were grown at 37 °C and 5% CO₂ DMEM medium containing 10% fetal bovine serum and 0.1% streptomycin</td>
</tr>
</tbody>
</table>

All cells were detached by treating with 0.05% trypsin/EDTA solution. FITC-conjugated **P-1**, **P-2** and **FITC-SNA** were feeded for 2 h and cells were washed twice with PBS and fixed with 500 µL paraformaldehyde (3.7% in PBS, pH = 7.4) by incubating for 10 min at 4 °C. The paraformaldehyde was aspirated and cells were washed with PBS to remove the excess of paraformaldehyde. The cells were then treated with 2 µg mL⁻¹ Hoechst 33342 to stain nuclei. Then cells were washed three times with PBS buffer and mounted on a glass slide using 5 µL antifed-mounting medium. The fluorescence of Hoechst 33342 and FITC complexes were excited with an argon laser at 405 nm and 450 nm, and the emission was collected through 403–452 nm and 500-530 nm filters, respectively.
Figure S13. Fluorescence images of NIH-3T3 cell type with P-1 after 30 mins incubation

Figure S14. Fluorescence images of NIH-3T3 cell type with P-1 after 120 mins incubation
Figure S15. Fluorescence images of HeLa cell type with P-1 after 30 mins incubation

Figure S16. Fluorescence images of HeLa cell type with P-1 after 120 mins incubation
**Figure S17.** Fluorescence images of MDA-MB-231 cell type with **P-1** after 30 mins incubation

**Figure S18.** Fluorescence images of MDA-MB-231 cell type with **P-1** after 120 mins incubation
**Figure S19.** Fluorescent images of different cell lines with SNA: (a) NIH-3T3; (b) HeLa; (c) MDA-MB-231
**Figure S20.** Fluorescence images of sialidase treated HeLa cells with (a) P-1 and (b) SNA after 30 mins incubation.

**Figure S21.** Fluorescence images of HeLa cell type with (a) ConA, (b) PNA and (c) UEA1 after 30 mins incubation.
Figure S22. MM2 calculation and Minimum energy structure of (a) $P-1$ and (b) $P-2$ peptides.

Figure S23. MM2 calculation and Minimum energy structures of (a) $P-1$-sialic acid and (b) $P-2$-sialic acid.
<table>
<thead>
<tr>
<th>Energy profile</th>
<th>P-1</th>
<th>P-2</th>
<th>P-1-Sialic acid</th>
<th>P-2-sialic acid</th>
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<tr>
<td>Torsion</td>
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<td>Non-1,4 VDW</td>
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<tr>
<td>Dipole/Dipole</td>
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</tbody>
</table>

*Table S2.* MM2 minimization energy profile.

*Figure S24.* MM2 calculation and minimum energy structure of (a) P-1-Man; (b) P-1-Gal; (c) P-2-Man and (d) P-2-Gal
<table>
<thead>
<tr>
<th>Energy profile</th>
<th>P-1-Man (Kcal/mol)</th>
<th>P-2-Man (Kcal/mol)</th>
<th>P-1-Gal (Kcal/mol)</th>
<th>P-2-Gal (Kcal/mol)</th>
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<td>51.3343</td>
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<td>205.1441</td>
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*Table S3.* MM2 minimization energy profile.
**Figure S25.** Determination of Sia composition on cell surface by measuring the average fluorescence intensity.
SPECTRA
Reference