Architecture Effects on L-selectin Shedding Induced by Polypeptide-based Multivalent Ligands

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Supporting Information

Polypeptide Expression and Purification. Polypeptides 17-H-3, 17-H-6, 35-H-6 and 35-RC-6 (sequences are listed in Table 1) were expressed from E. coli strain BL21(DE3)-pLysS transformed with expression plasmids pET28b- JS1-A3, pET28b-JS1-A6, pET19b-RF1-B6 or pET28b-JS1-35-RC-6,1,2 in 2 × YT media, and induced by IPTG as reported previously.3,4 After cultured on agar plates containing appropriate antibiotics overnight, single colonies of E. coli cells containing transformed plasmids were grown in 25 mL 2 × YT media (16g tryptone, 10g yeast extract and 5g NaCl per liter) with appropriate antibiotics, ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL) for 35-H-6, while kanamycin (25 μg/mL) and chloramphenicol (34 μg/mL) for 17-H-3,17-H-6 and 35-RC-6, respectively. The cell cultures were incubated overnight at 37 °C with shaking. Into a flask with 500 mL 2 × YT media, 10 mL of an overnight culture was inoculated. The optical densities (O.D.) of cultures were monitored and IPTG (final concentration of 0.4 mM) was added at O.D. = 0.6 to induce protein expression.5,6 Cells were harvested 4 hrs later via centrifugation (10 000 rpm, 20 min), and the cell pellets were resuspended in buffer B (8 M urea, 100 mM NaH$_2$PO$_4$, 10 mM Tris•Cl, pH = 8.0), with 4 mL of buffer B for 1 g of cell pellets, and frozen at -20 °C. After being thawed, the cell suspensions then were lysed via sonication, and the cell lysates were collected via centrifugation (15 000 rpm, 20 min) for further purification. The target polypeptides were purified from the cell lysate via immobilized Ni-NTA affinity chromatography with stepwise pH gradient elution under denaturing conditions.4 The resins were washed with urea buffer at pH 6.3 and the polypeptides were eluted with buffers with lower pH (pH 5.9 and 4.5). Eluted polypeptides were dialyzed against deionized water at 4 °C for 3 days (3 changes of water per day), and the dialysates were lyophilized to yield 15-20 mg of the purified target polypeptides per liter of culture. The protein purification was monitored with SDS-PAGE and HPLC and the composition confirmed by amino acid analysis (AAA).

Calculation of mean residue ellipticity $[\theta]_{MRE}$. The mean residue ellipticity $[\theta]_{MRE}$ (degrees cm$^2$/dmol$^{-1}$) was calculated via use of the concentration, molecular weight of the samples, number of residues and cell path length with the equation shown below, where $\theta_{RAW}$ (degrees) is the raw ellipticity, $M_r$ (Da) is protein molecular weight, $l$ (cm) is pathlength of light, $c$ (mg/mL) is sample concentrations, and $n$ is the number of amino acids in the protein.7

$$[\theta]_{MRE} = \frac{\theta_{RAW} \times M_r}{10 \times l \times c \times n}$$

Synthesis and characterization of N-(ε-aminocaproyl)-3,6-disulfo-β-D-galactosylamine. During sulfation of N-(ε-Fmoc-aminocaproyl)-β-D-galactosylamine, mono-, di- and tri-sulfated products can be obtained and the ratios of them vary from different reactions. These fractions bind to DEAE column and elute at different concentration of buffer containing 1 M NaCl. Shown in Figure S1 as an example, N-(ε-aminocaproyl)-3,6-disulfo-β-D-galactosylamine elutes at 54% buffer containing 1 M NaCl, while the mono- and tri-sulfated products elute at lower and higher percentage of buffer B. The deprotection of Fmoc group was conducted in DMF with 20% piperidine. The product N-(ε-aminocaproyl)-3,6-disulfo-β-D-galactosylamine was obtained by precipitation in cold ethyl ether. The mass spectrum confirmed the molecular weight is 452 with electrospray ionization mass spectroscopy (ESI-MS) with negative detection mode, shown in Figure S2. The peak at 925 g/mol shown in Figure S2 was isolated and analyzed with MS/MS method (tandem MS) with higher collision energy; in this spectrum there is a peak at 452 g/mol, indicating that the peak at 925 g/mol in Figure S2 is from dimers or weak-bound clusters formed in sample solution or during electrospray ionization, and does not arise from higher molecular weight impurities.
Figure S1 FPLC chromatogram of raw products obtained in sulfation of N-(6-Fmoc-aminocaproyl)-β-D-galactosylamine in 50mM Tris buffer pH 8.0 with 1M NaCl

Figure S2 ESI-MS spectrum of N-(6-aminocaproyl)-3,6-disulfo-β-D-galactosylamine. Shown in Figure S3 is the 1H NMR spectrum of N-(6-aminocaproyl)-3,6-disulfo-β-D-galactosylamine. The chemical shift of the anomeric proton of the sulfated galactopyranosides in 1H NMR varies for molecules with different sulfated groups, due to the electro field influences from bulky negatively charged sulfated groups at different positions, which was used to confirm the composition of the product. As shown in Figure S3, the chemical shift of anomeric proton is at approximately 4.98 ppm for the N-(6-aminocaproyl)-3,6-disulfo-β-D-galactosylamine; this proton appears upfield, with the same J-coupling constant, for the mono-sulfated and non-sulfated molecules (at about 4.86 and 4.82 ppm, respectively). The chemical shifts of other protons from the sugar and the linker arm are assigned as shown in Figure S3. The peaks at 1.1 and 2.8 ppm are the chemical shifts of protons of residual TEAB molecules ((C2H5)3N+).
NMR spectra of glycopolypeptides. 

$^1$H NMR spectra of glycopolypeptides in D$_2$O were obtained to confirm the composition and determine the number of ligands coupled to the polypeptide backbones. NMR spectra of SulfoCap 17-H-6 are shown in Figure S4 as an example. After conjugation to the polypeptide scaffold, the anomeric proton on the galactopyranoside retains the same chemical shift as in the monovalent galactopyranoside. The protons on the linker close to amine group shift due to amide bond formation, serving as further confirmation of scaffold modification. In the spectra of SulfoCap 17-H-6, protons of amino acid residues from polypeptide backbones have chemical shifts at 1.2-1.7 ppm ($\beta$ protons of Ala), 1.8-2.1 ppm ($\beta$ protons of Glu, Gln, Met and Ile), 2.1-2.6 ppm ($\gamma$ protons of Glu, Gln and Met), 2.8-3.2 ppm ($\beta$ protons of Tyr and His), 3.7-3.9 ppm ($\alpha$ protons of Gly, $\beta$ protons of Ser), and 3.9-4.2 ppm ($\alpha$ protons of Ala, Glu, Gln and Ile). The degree of functionality can be obtained from the $^1$H NMR characterization based on the integration of the anomeric proton on the saccharide ligands and the comparison to the integration of the $\beta$- and $\gamma$-protons of the glutamic acid and glutamine residues of the polypeptide, which appear at 2.2 and 2.4 ppm. Similar functionalities can be obtained based on the integration of the anomeric proton on the saccharide ligands compared to that of the $\beta$- protons of the alanine or tyrosine and histidine residues of the polypeptide, which appear at 1.3 and 3.0 ppm, respectively. The glycopolypeptides are of approximately 70-85% functionality, as characterized via $^1$H NMR spectroscopy. Although lower than 100%, these functionalization values represent nearly complete functionalization, with 5 out of 6 sites modified, indicating the utility of these scaffolds for evaluation of the impact of glycopolymer architecture on the L-selectin shedding event.
Figure S4: $^1$H NMR spectrum of SulfoCap 17-H-6 in D$_2$O. (The structures of the most abundant amino acid residues in the sequence are shown here and the actual sequence is listed in Table 1)

Non-linear regression equation for ELISA data fitting. The sL-selectin ELISA data was plotted and fitted by a nonlinear regression equation as previously described. In the equation, $x$ is the log value of sample concentrations on the saccharide basis, $y$ is the normalized soluble L-selectin concentration compared to that of PMA induced L-selectin shedding, $A1$ and $A2$ are the lower and upper limits of the dose response curves that obtained from fitting, $x0$ is the concentration of the mid-point of the fitted curves (at a $y$-value at the mid-point between the lower and upper baselines), and $P$ is the Hill coefficient, which is related to the binding cooperativity. The EC$_{100}$ values were estimated from the fitted curves, and represent the concentration of ligand, on a saccharide basis, that induces 100% of PMA induced L-selectin shedding.

$$y = A1 + \frac{A2 - A1}{1 + 10^{(\log x0 - x)P}}$$

References: