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Dendrimer-Like PEO Glycopolymers Exhibit Anti-Inflammatory Properties

Shyam M. Rele^{*}, Wanxing Cui, Lianchun Wang, Sijian Hou, Ginger Barr-Zarse, Daniel Tatton, Yves Gnanou, Jeffrey D. Esko, and Elliot L. Chaikof^{*}

Departments of Surgery and Biomedical Engineering, Emory University and School of Chemical and Biomolecular Engineering, Georgia Institute of Technology, Atlanta, Georgia 30322

Selectin-induced leukocyte rolling on endothelial surfaces is an essential step in mediating cellular adhesion thereby initiating the complex cascade of events leading to inflammatory and cell-mediated immune responses.¹ Characteristically, the adhesion cascade is facilitated by the interaction of selectins (L-, P-, E-) with O-glycosylated protein ligands (PSGL-1, CD34 and GlyCAM-1) that present carbohydrate epitopes containing sulfated derivatives of the tetrasaccharide sialyl Lewis x (Neu5Acα3Galβ4(Fucα3)GlcNAc-, sLe^x). Significant effort has been directed towards generating sLe^x mimetics (di-, tri-, tetrasaccharides) in the form of small molecules, polymers, liposomes, and protein conjugates, as competitive inhibitors of selectin-mediated binding events.² However, relatively weak affinity (K_d 0.1–5.0 mM), susceptibility to hydrolytic cleavage, potential antigenicity, and short circulating half-life, in addition to the absence of a convenient synthetic route are acknowledged limitations of sLe^x-derivatized bioconjugates. Thus, motivation exists to develop simpler therapeutic oligosaccharide analogs as selectin-binding antagonists, which structurally resemble naturally occurring saccharide arrays and exhibit multiple and cooperative receptor binding properties.

The simultaneous presentation of multiple copies of biorecognizable saccharide epitopes on an appropriate macromolecular scaffold creates a multivalent display that amplifies the affinity of glycoside-mediated receptor targeting.^{3,4} In this regard, well-defined branched polyethylene oxide (PEO) polymers of tailored size, shape, and geometry provide singularly useful scaffolds for in vivo blockade of selectin binding due to their defined molecular architecture, hydrophilicity, and availability of multiple surface reactive sites.⁵ Moreover, the branched polymer structure also provides a mechanism for controlling accessibility, mobility, density, and supramolecular organization of pendant sugar epitopes, as additional elements that may facilitate the design of optimal selectin-binding antagonists with defined circulating half-life. Herein, we report a simple strategy for the synthesis of 1st and 2nd generation dendrimer-like PEO glycopolymers bearing sulfated β-lactose as potential L-selectin inhibitors. Most current glycoconjugation strategies describe the use of an aliphatic or aromatic aglycone linker that carries an amine, thiol, thiourea, acid or active ester as the reactive moiety. Significantly, by using imidated lactose donors and a Schmidt glycosidation coupling protocol protecting group manipulations were minimized in the process of glycopolymer synthesis.

Well-defined three (M_n ~5200 mu) and four arm (M_n ~5100 mu) PEO stars (1st generation) carrying hydroxy end groups, were synthesized by anionic polymerization using “core-first” methodology, as previously reported.^{6a,b} In addition, a dendrimer-like second generation PEO star polymer (twelve arm) was synthesized based on a phosphazene core. This polymer consisted of a first generation of six PEO arms, produced by an “arm-first” strategy onto the phosphazene core, followed by a second generation of 12 hydroxy-terminated PEO branches

polymerized directly onto the original 6-arm core ($M_n \sim 52$ kD).^{6c} β -Lactose octaacetate was selectively brominated at the anomeric centre and subsequently activated to the imidate donor (**A**).

Glycosylations of hydroxy-terminated PEO star and dendrimer-like polymers by trichloroacetimidate glycosidation methodology, using $\text{BF}_3 \cdot \text{OEt}_2$ as a Lewis acid activator resulted in the covalent attachment of acetyl protected lactose residues in high yield. Zemplen conditions for deacetylation (NaOMe/MeOH) followed by lactose sulfation using an excess of SO_3 trimethylamine complex furnished target PEO glycoclusters carrying terminal sulfated oligosaccharides **1c**, **2c**, and **3c**. The efficiency, homogeneity, and degree of ligand (lactose) loading on the PEO polymers was estimated by ^1H NMR spectroscopy, as well as by mass estimates obtained by MALDI-TOF and laser light scattering. Moreover, FTIR and SDS-PAGE analysis provided additional evidence of sulfated lactose units (See supplementary information).

Specifically, the relative intensities of the anomeric $\text{H}:\text{Ac}:\text{CH}_3$ signal ratio of 6:20.9:3 for the three arm (**1a**) derivative and an integration ratio of 7.95:27.97:8 for the four arm glycocluster **2a**, indicated complete glycosylation of the hydroxyl groups on the parent PEO precursor. The increase in molecular weight was further corroborated using MALDI-TOF (**1a**: 6899 mu, **2a**: 7524 mu) and LLS measurements confirming quantitative functionalization. Likewise, an NMR integration ratio of 3.9:13.7:3, as well as MALDI-TOF demonstrated a high degree of lactose conjugation (> 95%) onto the dendritic PEO scaffold of the 12-arm, 2nd generation, branched compound. Subsequent deprotection followed by sulfation produced a highly charged sulfated glycodendron **3c** (observed SELDI-TOF: 61.8 kD, expected value ~ 62 kD).

Recent investigations have demonstrated that heparin exhibits anti-inflammatory properties by mediating blockade of L- and P-selectins via sulfate dependant interactions.⁷ Moreover, in vitro observations have illustrated that sulfated esters promote selectin binding when appropriately orientated on a lactose core. For example, a sulfated lactose derivative (6,6'-disulfo lactose), lacking in fucose and sialic acid residues, was a superior to sLe^x as an in vitro inhibitor of L-selectin binding to GlyCAM-1.⁸ Since selectin-glycoligand binding is greatly amplified through multivalent presentation of oligosaccharide determinants, we explored the capacity of sulfated branched star and dendrimer-like glycopolymers to limit inflammatory responses in vivo via presumed selectin-dependant blockade. Acute peritoneal inflammation was induced in a mouse model by thioglycollate injection into the peritoneal cavity. Test compound potency was valence-dependant with glycopolymers **1c/2c** exhibiting little activity, while **3c** (0.5 mg/mouse IV) dramatically reduced neutrophil and macrophage recruitment by 86% and 60%, respectively (Fig. 4, $p < 0.05$). Although heparin inhibited inflammatory cell recruitment to a similar degree, concurrent anticoagulant effects limit heparin's clinical applicability. In contrast, **3c**, does not exhibit anti-thrombin activity (data not shown). The ability of **3c** to inhibit the adhesion of U937 lymphoma cells to immobilized P-, L- or E-selectins was examined, in order to determine whether the observed in vivo effect was mediated by presumed selectin blockade. Test compound **3c** selectively blocked the adhesion of U937 cells to L-selectin in a dose dependent manner ($\text{IC}_{50} = 2.4$ nM), but did not exhibit anti-P-selectin or E-selectin activity (Fig. 5). Therefore, **3c** is among the most potent L-selectin inhibitors yet reported.^{2a,8,9,10} Significantly, precedence exists for increased biological activity as a consequence of ligand presentation by dendrimeric scaffolds when compared to linear counterparts.^{2b} Despite significant anti-L-selectin activity, the observed in vivo activity was surprising, since it has been recently reported that heparin's anti-inflammatory activity in vivo is critically dependant on its ability to inhibit *both* L- and P-selectin mediated inflammatory cell adhesion.⁷ Indeed, a compound acting solely as a selective inhibitor of L-selectin is not anticipated to block in vivo leukocyte infiltration so completely. Thus, it is likely that **3c**, like

heparin, blocks chemokine binding to the endothelium, which would further limit leukocyte extravasation.

In summary, a new class of high molecular weight polysulfated PEO dendrimer-like glycopolymer has been synthesized by a combination of arm-first and core-first methodologies followed by trichloroacetimidate glycosidation as a facile bioconjugation strategy. This is the first report to describe the synthesis and biological evaluation of complex branched PEO heparinoid-mimics, which provides an easily accessible route to carbohydrate-based compounds with anti-inflammatory activity in vivo.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

I) Selectin blockade using polyvalent branched glycoconjugates. II) Glycosidation of terminal hydroxyls on a PEO scaffold using lactose imidate (**A**).

Figure 2. Saccharide-functionalized PEO star and 'dendrimer-like' polymers as selectin ligands.

Figure 3. 600 MHz ^1H NMR spectrum of **3a** (2nd generation, 12 end groups) recorded in CDCl_3 at 25 °C. Inset shows the MALDI-TOF spectra of the original hydroxyl-terminated phosphazene core-based PEO branched polymer and glycodendrimer **3a**, confirming a high degree of sugar conjugation.

Figure 4. Neutrophil and macrophage content in thioglycollate-induced peritoneal inflammation (n = 6). **A:** Neutrophil. **B:** Macrophage. Significant differences were observed in neutrophil and macrophage content in mice treated with heparin and **3c** compared with control mice that received saline ($p < 0.05$). Compounds **1c/2c** did not exhibit in vivo activity. Each bar represents the average value $\pm$ SD.

Figure 5. Competitive inhibition of U937 cell adhesion to immobilized A) L- selectin, B) P-selectin. Neither heparin nor **3c** exhibited inhibitory activity towards E-selectin. Inhibition was not observed in the absence of test compound or heparin. Data represents means of at least $n = 3$, $SD < 10\%$).