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Valency and density matter: Deciphering impacts of immunogen structures on immune responses against a tumor associated carbohydrate antigen using synthetic glycopolymers

Qian Qin [a], Zhaojun Yin [a], Xuanjun Wu [a], Karen M. Haas [b], Xuefei Huang [a, *]

[a] Department of Chemistry, 578 S. Shaw Lane, Michigan State University, East Lansing, MI 48824, USA
[b] Departments of Microbiology and Immunology and Cancer Biology, Wake Forest School of Medicine, Winston-Salem, NC 27157, USA

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ABSTRACT

For successful carbohydrate based anti-cancer vaccines, it is critical that B cells are activated to secret antibodies targeting the tumor associated carbohydrate antigens (TACAs). Despite the availability of many TACA based constructs, systematic understanding of the effects of structural features on anti-glycan antibody responses is lacking. In this study, a series of defined synthetic glyco-polymers bearing a representative TACA, i.e., the Thomsen-nouveau (Tn) antigen, have been prepared to probe the induction of early B cell activation and antibody production via a T cell independent mechanism. Valency and density of the antigen in the polymers turned out to be critical. An average of greater than 6 Tn per chain was needed to induce antibody production. Glycopolymers with 40 antigens per chain and backbone molecular weight of 450 kDa gave the strongest stimulation to B cells *in vitro*, which correlated well with its *in vivo* activity. Deviations from the desired valency and density led to decreased antibody production or even antigen specific B cell non-responsiveness. These findings provide important insights on how to modulate anti-TACA immune responses facilitating the development of TACA based anti-cancer vaccines using glycopolymers.

1. Introduction

Tumor associated carbohydrate antigens (TACAs) such as glycolipids and glycoproteins are over-expressed on a wide range of cancers [1–4]. Clinical studies have shown that patients with higher levels of naturally generated anti-TACA antibodies are associated with better prognosis [5–8]. Moreover, a monoclonal antibody targeting the glycan structure of ganglioside GD2 has been approved by FDA as a first line therapy for pediatric patients with high-risk neuroblastoma validating TACAs as targets for vaccine development [9]. With the increasing appreciation of roles of TACAs, intensive efforts have been dedicated towards TACA based vaccine design [1–4]. To facilitate these efforts, a better understanding of how structures of TACA constructs influence antibody generation is much needed.

TACAs elicit humoral responses characteristic of T cell independent (TI) antigens [10], which primarily bind to antibody secreting B cells through B cell receptors (BCRs). BCRs initiate signaling across the cell membrane leading to either B cell activation or tolerance [11]. Antigen requirements for B cell activation and differentiation are complex [10,12–14]. Some monovalent antigens such as an ovalbumin peptide and hen egg lysozyme have been shown to be able to activate B cells [15–17]. For other antigens, multivalent constructs are needed to crosslink multiple BCRs with the number of antigens per construct (valency) needed varying significantly. Using nitro-iodophenol (NIP) as the hapten and polypeptide as the carrier, the Schamel group showed that constructs containing two to three NIPs per peptide could activate NIP specific B cells [18]. The spacing between the antigen (density) was not critical as dimers with NIP on adjacent amino acid residues activated B cells as well as a dimer containing NIPs separated by 24 amino acids. In contrast, Dintzis and coworkers showed with fluorescein or dinitrophenyl haptens, the constructs must exceed molecular mass of 100 kDa and 20 haptens per chain for induction of antibody secretion [19–21]. Above these threshold values, higher valency (126 antigens per chain) led to continual increase of B cell responses [19]. Using dinitrophenyl bearing ring-opening

* Corresponding author. Department of Chemistry, Michigan State University, 578 S. Shaw Lane, Room 426, East Lansing, MI 48824-1322, USA.
E-mail address: xuefei@chemistry.msu.edu (X. Huang).

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metathesis polymers, the Kiessling group showed that low valency polymer (10 mer) could activate B cells, although the high valency (500 mer) construct was much more effective in antibody induction [22]. These meticulous studies suggest the valency needed for B cell activation and antibody production is highly antigen dependent.

Compared to foreign TI antigens such as NIP and dinitrophenyl, the generation of TACA-specific antibody responses poses additional challenges. As they are self-antigens, most high affinity TACA specific B cells undergo cell death during conventional B cell development [23], resulting in limited frequency and functionality of these cells. Therefore, it is desirable to increase TACA specific B cell numbers and titers of anti-TACA antibodies through interactions with vaccine constructs.

Herein, to better understand how structures of TACA constructs impact B cell functions, we prepared a set of systematically varied synthetic glyco-polymers. The antigen valency and density of the constructs were found to significantly influence antibody generation. Decoding the key parameters eliciting TACA specific B cell activation vs non-responsiveness can have important implications for the establishment of effective TACA based anti-cancer vaccines.

2. Experimental section

2.1. General experimental procedures and methods for synthesis

All reactions were carried out under nitrogen with anhydrous solvents in flame-dried glassware, unless otherwise noted. Chemicals used were reagent grade as supplied except where noted. Compounds were visualized by UV light (254 nm) and by staining with a yellow solution containing $Ce(NH_4)_2(NO_3)_6$ (0.5 g) and $(NH_4)_6Mo_7O_{24}4H_2O$ (24.0 g) in 6% H_2SO_4 (500 mL). Flash column chromatography was performed on silica gel 60 (230–400 Mesh). NMR spectra were referenced using residual $CHCl_3$ (δ ^{1}H NMR 7.26 ppm), D_2O (δ ^{1}H NMR 4.79 ppm). The molecular weight and polydispersity of the polymers were determined by gel permeation chromatography (GPC) at 35 °C using two PLgel 10-μm mixed-B columns with DMF as the eluting solvent.

2.2. Synthesis of polymer 4

A solution of *p*-nitro aniline **1** (13.8 mg, 0.1 mmol), sodium nitrite (8.3 mg, 0.12 mmol), and a 50% aqueous fluoroboric acid solution (18.7 μL, 0.15 mmol) in water was cooled to 0 °C for 30 min. At this time, sodium cyanate (6.5 mg, 0.1 mmol), monomer acrylic acid **3** (2 mL, 0.029 mol) dissolved in water (2 mL) were added. The reaction mixture was heated to 70 °C overnight. The polymer was dialyzed against water and then lyophilized (90% yield). ^{1}H NMR: δ 1.25–2.00 (br m, aliphatic **H**, from C**H**$_2$ of polymer backbone), δ 2.00–2.50 (br s, aliphatic **H**, from C**H** of polymer backbone), δ 7.30 (br s, aromatic **H**), δ 8.10 (br s, aromatic **H**). Peak integration was normalized to the small aromatic peaks at ~7.30 ppm (2 protons) and ~8.10 ppm (2 protons). Based on the integration ratio, there were an average of 180 acrylic acid units per chain (Mn ~ 12.9 kDa). To analyze the polymer by GPC, polymer **4** was converted to its methyl ester form by the following process. Polymer **4** (10 mg) was dissolved in MeOH: toluene (1:1, 1 mL) followed by drop wise addition of $TMSCHN_2$ (0.1 mL). The reaction mixture was stirred overnight and all the solvent was evaporated. The methylated polymer **4** was analyzed by GPC, which gave PDI of 1.22. Based on the molecular weight determined from GPC of the methylated polymer, the Mn of polymer **4** was determined to be 13.4 kDa.

2.3. Synthesis of polymer 8

All solutions used for synthesis of polymer **8** were deoxygenated with three freeze-pump-thaw cycles and the reaction was performed under nitrogen atmosphere. Monomer ^{t}butyl acrylate **6** (10 g), initiator methyl 2-bromopropionate **5** (0.016 mL), catalysts CuBr (0.0201 g) and $CuBr_2$ (0.78 mg) and ligand PMDETA (0.03 mL) in THF (3 mL) were mixed in 500:1:1:0.05:1.05 ratio. Catalysts were measured into a dry round bottom flask, and the system was purged of O_2 by pumping N_2 for 20 min. Distilled monomer was added by syringe and needle, and purged with N_2 for 10 min followed by addition of the ligand and the initiator. The reaction mixture was stirred at 60 °C for 24 h. The reaction was quenched by THF, and catalysts were removed by silica gel column using THF as solvent. ^{t}Bu protected polymer **8** was purified by precipitating with a CH_3OH:H_2O (50:50) solution. The purification process was repeated three times and the final polymer was dried under vacuum. ^{1}H NMR: δ 1.30–1.80 (br m, aliphatic **H** from C**H**$_2$ of polymer backbone and C**H**$(CH_3)_3$), δ 2.10–2.25 (br m, aliphatic **H**, from C**H** of polymer backbone). ^{t}Bu protected polymer **8** was dissolved in DCM and treated for 4 h with trifluoroacetic acid (TFA) at 3:1 molar ratio of TFA: monomer. The solid was separated and washed multiple times with diethyl ether. The hydrolysis was repeated three times. The final product polymer **8** was dried under vacuum (85% yield). ^{1}H NMR: δ 1.45–1.80 (br m, aliphatic **H** from C**H**$_2$ of polymer backbone), δ 2.20–2.30 (br s, aliphatic **H**, from C**H** of polymer backbone). GPC analysis gave Mn of 46 kDa with a PDI value of 1.57.

2.4. Synthesis of glycopolymer

For **450k-10**, polymer **450k** (10 mg) was dissolved in 1 mL anhydrous DMF. Tn-NH_2 (0.1 mg, 10 eq), DIPEA (48 μL, 2eq), TSTU (50 mg, 1.2 eq) were added. The reaction mixture was stirred overnight, and the final glycopolymer was purified by a LH-20 column using water as the solvent. The number of Tn/chain was determined by anthrone-sulfuric acid assay [24] using GalNAc as standards. All the other glycopolymers were synthesized and characterized in a similar way.

2.5. Cell viability

CytoTox 96 Non-Radioactive Cytotoxicity Assay of Promega (Madison, WI) was used for cell viability assay. EA.hy 926 cells were incubated with 450k-40 polymers at Tn concentrations ranging from 0 to 84 μM. 450k polymers were also tested with the same polymer concentration as the 450k-40. After incubation for 48 h, 50 μL of cell culture medium from each well was transferred to a fresh 96-well flat clear bottom plate, followed by addition of 50 μL of the Cyto Tox 96 Reagent to each well. The plate was incubated for 30 min at room temperature. Next, 50 μL of Stop Solution was added, and absorbance at 490 nm was measured. Each experiment was performed in triplicate. The average values of the culture medium background were subtracted from all values of experimental wells. For maximum lactate dehydrogenase (LDH) release control, 20 μL of 10× Lysis Solution was added 45 min before adding the CytoTox 96 Reagent.

Percentage of survival = [1−OD490(experimental)/OD490(control)]*100%

2.6. Cell isolation and culture

Splenic cells were harvested from C57BL/6 mice. Red blood cells