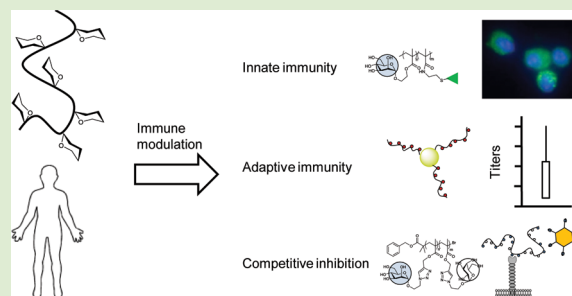


Carbohydrate-Based Polymers for Immune Modulation

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ABSTRACT: Carbohydrates play prominent roles in immune surveillance and response to infection. Multivalency, molecular weight control, and molecular architecture control are properties that polymer science is well suited to address. Each of these properties has been demonstrated to impact the biological interaction of carbohydrate-bearing chains with their binding partners. This viewpoint highlights synthetic advances and potential applications of carbohydrate-based polymers for immune modulation. It also offers future directions in polymer science necessary for carbohydrate polymers to fulfill their potential as immune modulators.



Biological polymers include DNA, RNA, proteins, and polysaccharides. DNA, RNA, and proteins are sequence-specific linear polymers with fixed molecular weights; they generally lack polydispersity in chain length, other than for different isoforms. In contrast, natural polysaccharides tend to be less exact in chain length (exhibit broader polydispersities), molecular architecture (incorporate branches), connectivity (multiple reactive sites on each glycoside), and sequence (i.e., glycosaminoglycans exhibit variations in patterns of sulfation). In terms of these structural properties, polysaccharides are conceptually similar to synthetic polymers. The complex nature of polysaccharides makes their precise synthesis difficult for chemists. However, polymer chemists are becoming adept at synthesizing glycomimetics which recapture many of the properties of naturally occurring polysaccharides. Polysaccharides play a myriad of roles in human health and disease; they play crucial roles in our immune system, including in pathogen recognition and viral entry. Polymers in general, and glycomimetics specifically, are therefore promising candidates for immune system modulation.

Immune system modulation induces, enhances, or suppresses the body's natural immune response toward a particular disease state. The polymer community has contributed significantly to recent advances in the field through the design of polymeric vehicles that improve or enhance the delivery of immunomodulatory signals to cells of the immune system. For example, lipid or polymer nanoparticles loaded with adjuvant drugs have been covalently conjugated to T cells to provide sustained stimulation to the lymphocyte.¹ In another example, tumor lysates were loaded into a polymer scaffold along with GM-CSF (a dendritic cell chemoattractant) and cytosine-guanosine oligonucleotides (a danger signal that would mimic a bacterial infection) to activate dendritic cells *in vivo* and generate antitumor immunity.² In both these cases, the primary immune modulatory component was a donor immune cell or tumor lysate, which have the drawbacks of any natural product: difficulty with the isolation, purification, and verification of the safety of products, batch to batch variability, limited supply, and

complexity in determining structure–property relationships of compounds.

As an alternative to their use as delivery vehicles for immune signals, biomimetic polymers can be used as the immunomodulatory cues themselves (synthetic antigens). Compared to immune cells, tumor lysates, or other natural extracts, synthetic antigens present far fewer concerns about their purity and availability. Additionally, synthetic antigens exhibit defined and reproducible structures so that mechanistic studies linking structure and activity can be conducted. Accordingly, many immune modulators are synthetic, such as polyinosinic:polycytidylic acid (analogues of double-stranded RNA present in viral genomes) or aminoalkyl glucosaminide 4-phosphates³ (analogues of bacterial lipopolysaccharides). Structures based on carbohydrates have also been used as immune modulators due to the prominent role carbohydrates play in immune recognition and response. This research path was pioneered with the use of bacterial conjugate vaccines derived from natural bacterial membrane polysaccharides. Along similar lines, tumor-associated carbohydrate antigens (TACA) have been investigated as targets for prophylactic or therapeutic vaccination against cancer.⁴ The first generation of these vaccines presented TACAs in their monomeric form,⁵ but later iterations used clustered antigens on a peptide backbone⁶ or dendrimers functionalized with exterior carbohydrates.⁷ This evolution of vaccine design toward larger multivalent structures⁸ reflects the need to account for carbohydrate heterogeneity on tumor cell surfaces, as well as the increased protein binding seen for structures with higher carbohydrate density and loading.⁹

Polymer science is well-suited to address the requirement for multivalent structures. While dendritic structures allow for sufficient multivalency, their synthesis is plagued by significant

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limitations such as the need for repeated protection/deprotection steps, low overall yields, and difficulty in fully characterizing higher generations. Synthetic carbohydrate-based polymers represent the most facile route toward creating well-defined, high-valency structures. Automated solid-phase synthesis¹⁰ allows for smaller synthetic polysaccharides (sugars connected through glycosidic bonds), and controlled polymerization techniques¹¹ allow for synthesis of glycopolymers (sugars connected through a synthetic backbone). Polymerization techniques, including controlled polymerization methods, offer the opportunity to introduce other structural variables such as branching or variations in monomer density or chain sequence into synthetic antigens, permitting the establishment of structure–bioactivity relationships and introducing the possibility of molecular-based attenuation.

These carbohydrate-containing polymers have immunotherapy applications beyond cancer vaccinations, including vaccination against bacterial pathogens, complement activation, macrophage targeting, viral inhibition, and transplant rejection. In this viewpoint, we provide an overview of the role glycopolymers have played in basic and clinical research and conclude with thoughts about future progress necessary to advance the field.

Innate immunity is the branch of the immune system that defends against infection through a nonspecific manner. It includes macrophages and other cells that engulf and destroy foreign material, as well as mechanisms such as inflammation, coagulation, and complement. Complement is a group of circulating blood proteins that when activated cause localized inflammation, phagocyte recruitment, pathogen opsonization, and lysis of the target cell. When working with implanted biomaterials or drug delivery devices, complement activation is an unwanted occurrence that leads to inflammation and poor patient prognoses. Materials used in these implants trigger the complement cascade (among other innate immunity reactions) through adsorption of plasma proteins (e.g., antibodies which lead to the classical pathway or C3 which leads to the alternative pathway). Poly(ethylene glycol) and other polymers are often added to implanted materials to decrease protein adsorption and thus shield against innate immunity reactions. Carbohydrates, due to their hydrophilicity and general biocompatibility, would also be a good material for complement passivation. In an example of this approach, Narain's group synthesized a hyperbranched glycopolymer containing pendant ring-opened glucose and lactose derivatives that did not trigger blood coagulation, platelet activation, or complement activation but did cause some cytotoxicity at high concentrations depending on the specific cell line (Figure 1a).¹²

Complement activation can also be manipulated through the mannose binding lectin pathway as a potential avenue for immunotherapy. In nature, the mannose binding lectin (MBL) pathway is activated by the binding of MBL to sugars such as mannose or *N*-acetylglucosamine that make up polysaccharides and glycoproteins of pathogens.¹³ Glycopolymers could serve as biomimetics of these pathogenic structures. The Haddleton group reported bovine serum albumin–mannose glycopolymer conjugates which bind MBL and result in deposition of complement protein C9 (Figure 1b).¹⁴ These polymers were synthesized from a thiol reactive initiator that could be conjugated to bovine serum albumin's free cysteine. In another example, we reported the synthesis of linear and branched mannose glycopolymers and found that interaction of MBL with mannose glycopolymers increases with multivalency,

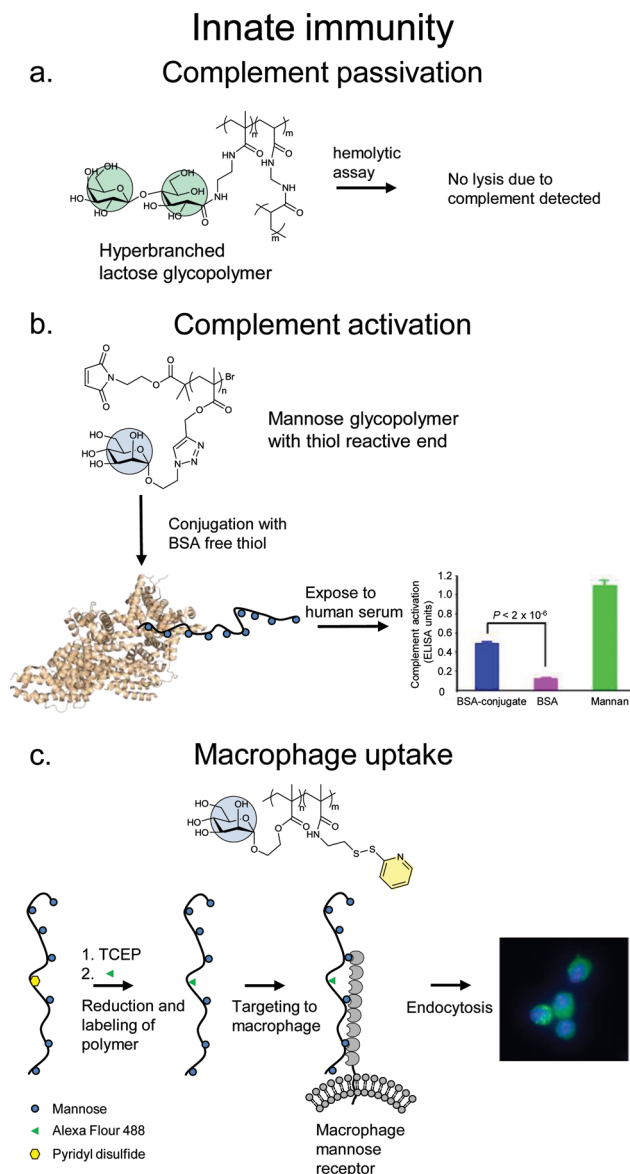


Figure 1. Glycopolymers have been used for modulation of innate immunity. Complement has been (a) passivated through the use of glycopolymers with pendant lactose and (b) activated through a mannose glycopolymer–bovine serum albumin conjugate. Adapted from ref 14 with permission from ACS Publications. (c) Fluorescent dye has been delivered to macrophages through the binding and subsequent endocytosis of fluorescent mannose glycopolymers by the macrophage mannose receptor. Adapted from ref 16 with permission from Elsevier.

branching density, and presence of mannose content at the branch point.¹⁵ Glycomimetic polymers that have been shown to induce complement not only help in establishing structure–property relationships for sugar–lectin interactions but also may find application as adjuvants to activate the immune system or as immunotherapeutic agents to induce lysis of undesirable cells.

Like the MBL pathway of complement, macrophages are also sensitive to mannose through the macrophage mannose receptor. This receptor is an attractive target for specifically targeting and delivering a therapeutic through a glycopolymer–drug conjugate. To demonstrate this concept, mannose, *N*-acetylglucosamine, or galactose-derived monomers were

copolymerized through RAFT with pyridyl disulfide containing monomers.¹⁶ The glycopolymers with mannose or *N*-acetylglucosamine exhibited increased uptake by macrophages compared to galactose-containing glycopolymers as determined by fluorescence uptake both *in vitro* and *in vivo*. Although not explored in this work, the pyridyl disulfide functional group on their polymers could be reacted with thiol-containing drugs or biomolecules for further delivery applications.

Adaptive immunity, in contrast to innate immunity, provides specific long-term defense against infection. Long-term defense is provided by immunological memory cells created from B and T lymphocyte activation against infection. This immune memory can be artificially formed by vaccinating with antigens that mimic the presence of a pathogen. Current vaccines against microbial infections such as bacterial meningitis are commonly manufactured by isolating the polysaccharide bacterial antigen followed by conjugation of the antigen to an immunogenic carrier protein that can generate long-lived immunity. The extraction of these natural polysaccharide antigens is an expensive process that can result in batch to batch variability.¹⁷ In response to the drawbacks of using naturally extracted antigens, a lower cost partially synthetic conjugate vaccine against *Haemophilus influenzae* type b (Hib) has been developed consisting of a synthetic polysaccharide antigen (polyribosylribitol phosphate connected through glycosidic bonds) conjugated to tetanus toxoid. The vaccine triggers long-term antibody protection against Hib and is now part of Cuba's vaccination program.^{18,19}

Immunogenicity may also be induced by using shorter di- or trisaccharide chains. However, when low molecular weight haptens are used, the resulting immune response is low in mice. To address this issue, one group attempted to cluster the haptens through a glycopolymer approach. Mannan trisaccharides from the *Candida albicans* cell wall were conjugated pendant to a poly(acrylamide) backbone, which itself was conjugated to an immunogenic carrier protein.²⁰ The glycopolymer conjugate with 33 trisaccharides per protein induces a more robust immune response than compared to a tetanus toxoid conjugate with 8–12 trisaccharides (Figure 2a). All mice generated antibodies specific to the mannan trisaccharide, but some did not generate antibodies against the fungal cell wall.

While most other saccharide-based vaccines incorporate natural protein content (in addition to the synthetic saccharide antigen), one vaccine incorporates both synthetic antigen and synthetic carrier components.²¹ A glycopolymer with pendant *N*-acetylglucosamines (GalNAc) was conjugated to gold nanoparticles as a mimic of cancer cell surfaces (Figure 2b). This monosaccharide, when presented as an α linkage to serine or threonine residues, is known as the Tn antigen and is associated with cancer and other disorders. The glycopolymer–nanoparticle conjugate successfully generates antibodies against the Tn antigen that were also cross-reactive with natural mucins displaying the antigen. In comparison, free polymers without nanoparticle content have low titer counts. The higher density of polymer generated from the nanoparticle surface can serve as a better mimic of cancer cell surfaces, indicating that 3D display of antigens is important, in addition to their molecular structure.

Competitive inhibition of pathogen binding to a host cell is a common strategy used to prevent disease. One common example of this strategy with a long history is neuraminidase inhibition, which uses sialic acid analogues to bind neuraminidase, a protein necessary for viral escape from the

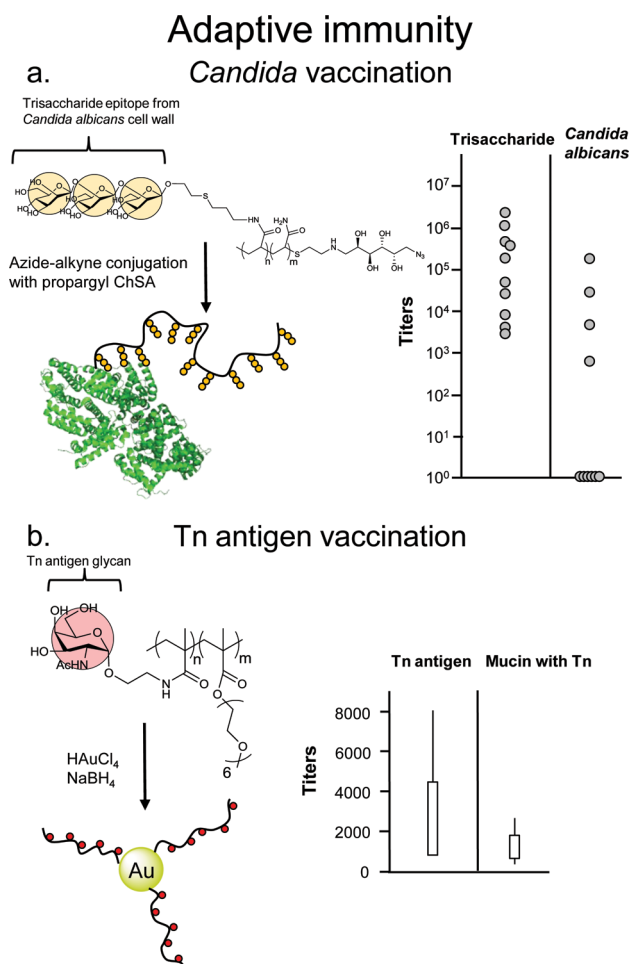


Figure 2. (a) Glycopolymer with pendant mannan trisaccharides from *Candida albicans* with an immunogenic carrier protein successfully induced antibodies against the mannan trisaccharides and *Candida albicans* cell wall. Adapted from ref 20 with permission from ACS Publications. (b) A fully synthetic vaccine consisting of gold nanoparticle decorated with *N*-acetylglucosamine (cancer associated Tn antigen) glycopolymers generated antibodies for the Tn antigen and a natural glycoprotein. Adapted from ref 21 with permission from ACS Publications.

host cell. Since viral entry, replication, and release are all controlled in part by binding of carbohydrates, glycopolymers can also be used to competitively inhibit binding between pathogens and host cells. Sialic acid containing glycopolymers have been synthesized that can disrupt influenza virus binding with sialoglycoproteins on host cells. Inhibitors containing sialyl oligosaccharides have been synthesized with poly(acrylamide),²² poly(acrylic acid),²³ poly(glutamic acid),²⁴ and poly(styrene) backbones.²⁵

More recently, glycopolymers have been investigated to prevent entrance of HIV to the immune system (Figure 3). One contributing path to HIV infection is thought to be through dendritic cell capture of the virus followed by transport to lymph nodes and subsequent infection of CD4⁺ T cells. HIV capture is mediated by the binding of HIV envelope glycoprotein gp120 with dendritic cell associated lectin DC-SIGN.²⁶ One strategy has been to disrupt the interaction of gp120 with immune proteins to prevent transmission. A great deal of work has focused on inhibitors that bind and block gp120 directly,²⁷ but one proposed strategy targets DC-SIGN

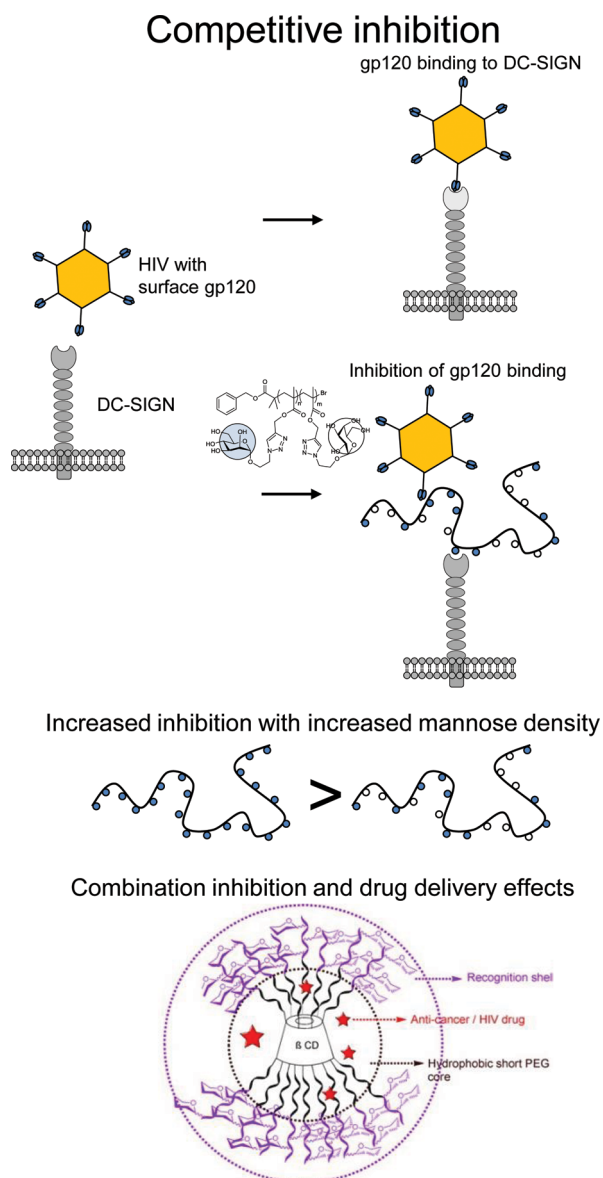


Figure 3. Dendritic cell associated lectin DC-SIGN is a contributing route for HIV infection. Mannose glycopolymers have been shown to competitively inhibit binding of HIV gp120 and internalization of the virus.^{31–33} Polymers with increased mannose density inhibit binding of gp120 more. More complex star polymers from a cyclodextrin core have been introduced to encapsulate an anti-HIV drug. Adapted from ref 33 with permission from ACS Publications.

through the use of mannose clusters,²⁸ other small molecules,²⁹ and mannose-based dendrimers.³⁰ This strategy binds the body's own immune system proteins, so it may be most useful as a prophylactic to prevent infection after an initial exposure to HIV rather than as a long-term therapeutic.

Glycopolymers with pendant mannose residues have also been used to block DC-SIGN binding sites.^{31,32} Surface plasmon resonance showed that glycopolymers with higher densities of mannose had higher affinity interaction with DC-SIGN, although gp120 still exhibits the highest affinity. The technology has been developed further to include polymer with star architectures emanating from a hydrophobic cyclodextrin core that could also encapsulate anti-HIV drugs.³³

Competitive inhibition can also be used to protect xenograft transplants from rejection due to xenoreactive antibodies. One

of these antibodies binds to a disaccharide, Gal α 1–3Gal. Poly(styrene-*co*-maleic acid) was grafted with the disaccharide, and the resulting glycopolymers were used to inhibit binding of the anti-Gal antibody with pig endothelial cells.³⁴ This competitive binding successfully protected the pig cells from the cytotoxic effects of human serum. The authors envision using the glycopolymers as immunosuppressant drugs to prevent xenograft transplant rejection by clearing anti-Gal xenoreactive antibodies from plasma.

Further progress in polymer science is required for glycopolymers to fulfill their potential as immune system modulators. New synthetic techniques are needed for control over architecture in terms of branching, sequence control, and 3D display of glycans to have a better understanding of structure–property relationships between glycopolymers and immune system proteins. The relationship between saccharide identity, multivalency, and bioactivity is well understood, but there are clearly other factors determining protein interaction. For example, natural β -glucans have different immunomodulation potencies depending on their branching complexity.³⁵ We have synthesized branched glycopolymers¹⁵ that incorporate saccharide at the branch point to more fully capture the structure of natural polysaccharides. These polymers were inspired by previous branched polymers described by Müller³⁶ and Perrier.³⁷ Further advances can include incorporating disaccharide units at the branch point and further exploring lectin binding behavior with branched structures.

The importance of sequence control is illuminated by strategies used for presenting cancer antigens. A “unimolecular multivalent” structure displaying multiple cancer antigens on one synthetic backbone would provide broad spectrum immunization against heterogeneous cancers.³⁸ A logical next step would be to create multivalent as well as multiblock designs where several cancer antigens are displayed either as statistical or block mixtures. Monomer sequence control has been reported with multiblock copolymers³⁹ and gradient/block copolymers.⁴⁰ Haddleton has shown sequence control of glycomonomers but could not conclude any preference of lectin for monomer sequence; nevertheless, the authors believed further studies are warranted.³²

Additionally, protein–polymer conjugates must be synthesized to be able to fully capture immunization responses. Work by Matyjaszewski^{41–43} and Maynard^{44,45} has advanced the field for both grafting-to and grafting-from⁴⁶ techniques. Most examples of glycopolymers–protein conjugates graft an existing glycopolymers chain to the protein,^{20,31,47} while we have shown direct glycopolymers polymerization from protein macroinitiators through nonspecific modification of amino acids.⁴⁸ Better methods for specifically controlling the placement of the polymer chain, regardless of grafting technique, are required to create protein–polymer conjugates with uniform structures that can also maintain enzymatic and/or signaling activity.

Our study with glycopolymers–protein conjugates has shown remarkable enhancement in binding of an immune system protein due to 3D presentation of the carbohydrate chains. This is consistent with the observation that linking antigens to a gold nanoparticle to better replicate how they are displayed on the surface of cancer cells enhances their ability to cause antibody generation. While the general effects of carbohydrate number and density on carbohydrate–protein interactions are understood, there is less known about the synergistic role of multiple carbohydrate chains displayed in 3D around one central structure. Hyperbranched polymers,¹⁵ nanogels,⁴⁹ and nano-

particles⁵⁰ have shown an enhanced desired biological effect due to their 3D display of saccharides, but more systematic studies will be required to separate the effects of saccharide identity, density, and number from that of the 3D presentation. We are currently developing methods to create self-assembled nanoparticles that can answer these questions.⁵¹

We hope that with these advances synthetic carbohydrate structures, particularly glycopolymers, can have a dramatic impact on basic research for elucidating disease states and as potential therapies.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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