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Carbohydrate Antigen Delivery by Water Soluble Copolymers as Potential Anti-cancer Vaccines

Qian Qin, Zhaojun Yin, Philip Bentley, Xuefei Huang*

Department of Chemistry, Chemistry Building, Room 426, 578 S. Shaw Lane, Michigan State University, East Lansing, MI 48824

Email: xuefei@chemistry.msu.edu

ABSTRACT
Tumor associated carbohydrate antigens (TACAs) are overexpressed on tumor cells, which renders them attractive targets for anti-cancer vaccines. To overcome the poor immunogenecity of TACAs, we designed a polymer platform for antigen presentation by co-delivering TACA and helper T (Th) cell epitope on the same chain. The block copolymer was synthesized by cyanoxyl-mediated free radical polymerization followed by conjugation with a TACA Tn antigen and a mouse Th-cell peptide epitope derived from poliovirus (PV) to afford the vaccine construct. The glycopolymer vaccine elicited an anti-Tn immune response with significant titers of IgG antibodies, which recognized Tn-expressing tumor cells.

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Water soluble polymers can deliver tumor associated carbohydrate antigens and generate significant titers of tumor cell binding IgG antibodies.
INTRODUCTION

The stimulation of immune systems through the use of a construct that can elicit a specific immune response against cancer is the basis of anti-cancer vaccines. Cancer cells often bear characteristic carbohydrate structures on their cell surface. These tumor associated carbohydrate antigens (TACAs) are shared by a variety of cancer cell types, which make them attractive for anti-cancer vaccine development. However, serious challenges exist in order to elicit powerful anti-TACA immunity. Direct vaccination with TACA alone typically can only induce weak activation of antibody secreting B cells with no cooperation from Th cells. As a result, the antibodies secreted are mainly the low affinity IgM type. Since T cells typically recognize peptide epitopes, conjugating TACA to a Th cell peptide epitope should allow the stimulation of both B cells and Th cells. The matched Th cells provide stimulatory signals that can induce the B cells to undergo isotype switching leading to high affinity IgG antibodies.

Many innovative carriers have been developed to co-deliver TACAs with Th epitopes. The most common type of carrier is immunogenic proteins such as keyhole limpet haemocyanin, tetanus toxoid, and Bacillus Calmette-Guerin. Other antigen presenting platforms include dendrimers, regioselectively addressable functionalized templates, nanomaterials, liposomes and proteoliposomes, polysaccharides and virus-like particles.

Polymers are a class of synthetic carrier that has multiple potential advantages for TACA delivery. A polymer chain can carry many TACA molecules, which can enhance the avidities between the antigen and B cell receptors (BCRs) through the polyvalency effect and lead to strong activation of B cells. Furthermore, Th epitopes can be introduced into the glycopolymer to potentiate Th cells generating a long lasting humoral immune response. Although synthetic glycopolymers have been utilized in a variety of applications including biosensing, delivery of therapeutic, modulation of natural killer cell function and cellular signaling, it is only recently that they have been explored as a TACA carrier. Herein, we present our results on using water soluble block copolymers as a platform to codeliver TACA and a Th epitope as a potential anti-cancer vaccine.

RESULTS AND DISCUSSION

We selected the cyanoxyl-mediated free radical polymerization method for polymer construction due to the mild reaction condition. In order to incorporate both TACAs and Th epitope, the copolymer was designed to contain a block with multiple ammonium moieties followed by a methyl ester block (see polymer 7 in Scheme 1). The polymerization was initiated by the treatment of aniline with sodium nitrite and fluoroboric acid, which was followed by the addition of a mixture of sodium cyanate, acrylamide and methacrylamide amine and heating at 50 °C for 40 hours leading to intermediate polymer 5 (Scheme 1). Subsequently, acrylamide and acrylamide methyl ester monomer were added to the reaction mixture with further heating for another 40 hours. The resulting mixture was dialyzed in water to obtain copolymer 7 in 50% yield. Based on integrations of 1H-NMR peaks from the polymers using the aromatic peaks from the terminal phenyl ring as the internal standard, there were on average 45 ammonium ion and 4 of methyl esters per polymer chain of 7. Gel permeation
chromatography analysis showed that polymer 7 has a molecular weight (Mn) of 13,800 with a polydispersity index of 1.14.

To test the efficiency of TACA delivery, a representative TACA, i.e., the Tn antigen was introduced into the polymer. The Tn antigen, found over-expressed on a variety of cancer cell surface including 90% of breast cancer carcinoma, is an appealing target for TACA based anti-cancer vaccine development.44-46 A flexible amide linker was designed to conjugate Tn with the polymer to avoid potential humoral responses to the linker.47 In order to accomplish this, Tn derivative 8 in the form of N-hydroxysuccinimide (NHS) activated ester was synthesized48 and linked with the amines in polymer 7 promoted by N,N-diisopropyl ethyl amine (DIPEA) leading to glycopolymer 9 in 65% yield (Scheme 2). On average, 31 copies of Tn were introduced per chain based on 1H-NMR analysis. Polymer 9 was then treated with LiOH to hydrolyze all the methyl esters, which was supported by 1H-NMR analysis showing the complete disappearance of the methyl groups. The resulting polymer was functionalized with pyridyl disulfide 10, which then reacted with a cysteine modified oligopeptide from polio virus (PV) to introduce the helper T cell epitope26,49 through the formation of disulfide bonds. The number of PV peptide per glycopolymer 13 was determined to be 2 peptides per chain by cleaving the disulfide linkage between the peptide and the glycopolymer followed by HPLC quantification. As a control, polymer 14 was synthesized by capping the amine groups of polymer 7 with methoxyacetic acid, which was followed by introduction of two PV peptides per chain utilizing a similar protocol as in the construction of polymer 13.
With the peptidic glyco-copolymer 13 in hand, its ability to elicit antibodies was evaluated. Mice were injected with glycopolymer 13 (4 µg Tn per dose) subcutaneously with Freund's adjuvant (CFA) followed by two booster injections at intervals of two weeks (days 14 and 28). Sera were drawn from mice one week after the final injection (day 35). The control group was vaccinated with polymer 14 following an identical protocol. To test anti-Tn antibody levels in sera, enzyme linked immunosorbent assay (ELISA) was performed using Tn functionalized bovine serum albumin (BSA) immobilized on microtiter plates. Analysis showed that the main antibodies induced by 13 were the IgG type with an IgG titer of 4,432 (Figure 1a. For IgM titers, see figure S2). In comparison, the sera from mice receiving control polymer 14 contained extremely low titers of anti-Tn IgG antibodies (mean titer = 100) (Figure 1a). The fact that high titers of IgG antibodies were induced by 13 implies the Tn-specific B cells have undergone isotype switching. Another important characteristic of a successful vaccine is the maintenance of immune responses. To evaluate this, mice immunized with 13 were bled on day 89. ELISA analysis showed that the anti-Tn IgG titers remained at a similar level (Figure 1a, mean titer = 4,369) suggesting long lasting humoral immunity was generated.
Figure. 1. a) Anti-Tn IgG titers on days 0, 35 and 89 from mice immunized with glyco-polymer 13 and the anti-Tn IgG titers on day 35 from mice receiving the control polymer 14. b) Anti-polymer backbone IgG titers on days 0 and 35 from mice immunized with glyco-polymer 13 and anti-polymer backbone IgG titers on day 35 from mice receiving control polymer 14. c) Flow cytometry analysis of Jurkat cell binding by IgG antibodies in sera from representative mice immunized with glycopolymer 13 (orange curve) and control polymer 14 (blue curve). The shaded curve was from pre-immune serum binding with Jurkat cells.

For many TACA constructs with highly immunogenic protein carriers, antibodies specific against the carrier are induced as well, the titers of which can be hundreds times higher than that against the desired TACA.\textsuperscript{47, 50} The strong anti-carrier responses can potentially interfere with the generation of glycan specific antibodies due to antigen competition.\textsuperscript{51-53} The antibodies generated against the polymer backbone were analyzed. As shown in Figure 1b, immunization with glycopolymer 13 or control polymer 14 elicited similar amounts of anti-polymer IgG antibodies with titers around 700 as compared to a titer of 400 pre-immunization. The relatively low anti-polymer titers induced suggest the polymer backbone most likely does not compete significantly for B cell interactions.

As ELISA tests antibody binding to an artificial BSA-Tn construct, it is important to determine whether the antibodies elicited can recognize Tn expressed in its native environment, i.e., cancer cells. Jurkat cells are known to express large amounts of Tn antigen on their surfaces.\textsuperscript{54} The post-immune serum from mice immunized with glycopolymer 13 exhibited significant binding with Jurkat cells, while those from the control polymer did not react with the cells (Figure 1c).

In conclusion, a fully synthetic glycopolymer vaccine incorporating multiple Tn antigen and Th cell peptide epitope has been prepared, which elicited significant and long-lasting anti-Tn IgG antibody titers. The antibodies recognized Tn antigens on tumor cells. Compared with other delivery platforms such as virus like particles,\textsuperscript{29, 30, 48} the anti-Tn antibody titers generated by the glycopolymer constructs were modest. However, the polymer platform offers great flexibilities to adjust antigen densities and valency as well as the ratio of TACA vs Th epitope. In addition, the immunogenicity of the polymer backbone is not high, which likely will not compete significantly with the desired TACA for B cell activation. These attributes bode well for further optimization of the
glycopolymer construct to enhance the humoral responses against the TACAs.

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Electronic supplementary information (ESI) available: Full experimental details including: chemical synthesis, NMR spectra and immunological analysis. See DOI: XXXX
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