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# Characterization of conformational epitope of alginate-derived polymannuronates by surface plasmon resonance

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**Abstract**

In the present study, we developed a mAb to alginate-derived polymannuronates (ADPM) and examined the antigenic epitopes using surface plasmon resonance (SPR) in conjunction with a large panel of oligomannuronate probes. We found that tetrasaccharide is the minimum-binding unit, and that increases in chain length from the tetrasaccharide to the heptsaccharide further enhance monovalent binding. A sharp increase in affinity was observed when increasing from the octasaccharide to the cosasaccharide, which is due to a further enhancement of the individual antigenic epitope combined with multivalency. Kinetic binding studies further suggested that the conformational epitope is discontinuous and infrequent and that a C6-carboxyl group is important in maintaining the conformational epitope. Moreover, CD analysis revealed there were conformational structures in epitopes. The data support our hypothesis that the conformational epitope for the mAb may be an extended helical segment of ADPM. ADPM exists mainly in linear form, but it can infrequently and spontaneously form extended helices. Although helices are not prevalent in ADPM, the immune system preferentially selects these conformational epitopes because they are unique. Together, our results indicate that the antigenic epitopes in β-D-mannuronates are conformational and require C6-carboxyl groups.


*Keywords:* Polymannuronates; Epitope; Monoclonal antibody; Surface plasmon resonance; Circular dichroism

## 1. Introduction

Alginate-derived polymannuronate (ADPM) is a polysaccharide generated from brown alginate by acidic hydrolysis. This polysaccharide is composed of β-D-mannuronic acid linked by $\beta_{1-4}$ bonds, and contains negatively charged side groups. Our previous studies have showed that ADPM protects cells from $A\beta_{1-40}$- and $H_2O_2$-induced neurotoxicity in vitro [1]. Further insights into the neuroprotection in vivo are being undertaken in our lab now, which preliminarily displayed the therapeutic potential. Therefore, an understanding of the accessibility to the blood–brain barrier (BBB) and thus its pharmacokinetic profiles becomes crucial for its potential CNS effects. The pharmacokinetic profiles of polysaccharides have traditionally been performed on thin-layer chromatography (TLC), gas chromatography (GC), high performance liquid chromatography (HPLC) and capillary electrophoresis (CE). However, there exist limitations due to the polydisperse and diversiform nature of polysaccharides, and particularly the interference of endogenous glycosaminoglycans (GAGs). Moreover, these analytical methods

*Abbreviations:* ADPG, alginate-derived polyguluronates; ADPM, alginate-derived polymannuronates; ADPM-BSA, conjugate of ADPM and BSA; ADPM-OVA, conjugate of ADPM and OVA; BBB, blood–brain barrier; CSA, chondroitin sulfate A; CSC, chondroitin sulfate C; DS, dermatan sulfate; FCM, flow cytometry; HA, hyaluronic acid; HBS-EP, 0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% polysorbate 20 [v/v]; HS, heparan sulfate; 4-mer*, tetramannuronate with a carboxyl group at terminus; OVA, ovalbumin; SPR, surface plasmon resonance.

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require many clean up steps and often time-consuming. Over the years, antibody-based methods have been developed as a favorable alternative for either the identification or quantification of polysaccharides. Currently, the availability of rapid and simple surface plasmon resonance (SPR)-based biosensor assay significantly promises the efficiency of analysis [2–4]. A particular advantage of SPR method attracts considerable attention due to its real-time, free-label and low-sample consumption and detailed information on the nature of antigen–antibody interactions [5].

In this paper, using SPR assay we demonstrated that the epitope for mAb G3C5E8 is length- and conformation-dependent, with a crucial requirement for carboxyl groups at the C6 position. In addition, a tetrasaccharide is a minimum requirement for binding, and the binding affinity rose with further increases in the polysaccharide length. Notably, specific mAb for ADPM has been developed and successfully used to evaluate the pharmacokinetic profiles of ADPM in rats. In view of this notion, the detailed understanding of the characteristics of ADPM specific mAb, in particular epitope mapping analysis should help to further instruct its application in clinical pharmacokinetic study and some other correlated biological researches.

## 2. Materials and methods

### 2.1. Materials

ADPM (~ 8000 Da), alginate-derived polyguluronate (ADPG ~ 8000 Da), and tetramannuronate with a carboxyl group at terminus (4-mer*) were provided by the Marine Drug & Food Institute, Ocean University of China (Qingdao, China). Sodium cyanoborohydride ($NaBH_3CN$), mannan, bovine serum albumin (BSA), ovalbumin (OVA), gelatin, horseradish peroxidase conjugated goat anti-mouse IgG (H+L), 3,3′,5,5′-tetramethylbenzidine, heparin, heparan sulfate (HS), dermatan sulfate (DS), chondroitin sulfate A (CSA), chondroitin sulfate C (CSC), hyaluronic acid (HA), biotin, and streptavidin were all purchased from Sigma (St. Louis, MO, USA). Flat-bottomed polystyrene microplates were purchased from Costar (NY, USA). Balb/c mice were obtained from the Animal Center of Shandong University, China. The Rainbow ELISA reader was purchased from Tecan (Durham, Austria). The microtiter plate washer was purchased from Bio-Rad (Hercules, California, USA). Carboxymethylated dextran sensor chips (CM5) were purchased from Amersham Pharmacia (Uppsala, Sweden).

### 2.2. Generation of anti-ADPM mAb

ADPM-BSA conjugate was prepared by reductive amination. The reducing end of ADPM has a reactive aldehyde group that can be coupled to amino groups of BSA in the presence of $NaBH_3CN$ [6,7]. Quantitative protein (based on $A_{280}$ and a BSA standard curve) and carbohydrate (determined by phenol–sulfuric acid reaction) analysis showed that the conjugate contained carbohydrate and BSA in an approximately 2:1 molar ratio. The ADPM-OVA conjugate was prepared in the same fashion.

An ADPM-BSA-immunized mouse showing the highest antibody titer was chosen to prepare a hybridoma against ADPM. The hybridoma fusion was performed 3 days after the last intravenous injection. Stimulated spleen cells from the immunized mice were fused with NS-1 myeloma cells at a ratio of 5:1 in 50% (w/v) PEG-4000. Putative hybrids resulting from hypoxanthine/aminopterin/thymidine selection were screened by ELISA against ADPM-OVA. Those producing the antibody of interest were cloned three times by limiting dilution to ensure stability and clonality. Finally, we obtained clone G3C5E8, which produced a mAb specific for ADPM. The DNA content of clone G3C5E8 was determined by flow cytometry (FCM) (Vantage, Becton Dickinson, USA). The Ig subclass was determined with a mouse monoclonal antibody isotyping kit (Roche Diagnostics, Indianapolis, USA). The crossreactivities with carrier proteins and (self) glycosaminoglycans, such as heparin, HS, DS, CSA, CSC, HA, were evaluated with SPR. Clones were expanded as ascites by intraperitoneal injection of $10^6$ hybridoma cells in Balb/c mice 10–14 days after intraperitoneal treatment with 0.5 ml 2,6,10,14-tetramethyl-pentadecane (pristane). Ascites fluid was collected 7–14 days after injection, and the mAb was purified using a Sepharose CL-4B-Protein G column (Amersham Pharmacia). Fab of mAb G3C5E8 was carefully prepared using an "ImmunoPure Fab Preparation Kit" (Pierce, Rockford, USA), following the established protocol [8].

### 2.3. Flow cytometric analysis

Followed by washing with 0.02 M PBS (pH 7.4), spleen cells ($10^6$) from a Balb/c mouse, NS-1 myeloma cells, and G3C5E8 hybridoma cells were labeled with 200 μl of 50 μg $ml^{-1}$ propidium iodicle for 30 min at 4 °C in the dark. After filtration, the relative DNA contents of the three cell types, represented by the average fluorescence intensity of $10^4$ cells in G0/G1 period, were detected by FCM.

### 2.4. Preparation of different sized oligomannuronates

ADPM was dissolved in distilled water at a final concentration of 0.1% and mixed with 0.5 mM hydrogen peroxide and 40 μM $Fe^{2+}$ (Fenton's reagent). The solution was incubated at 37 °C with stirring for 10 h. After adding $Na_2S_2O_3$ to terminate the degradation, the insoluble particles were removed by filtration. The oligomannuronate mixture was concentrated by rotary evaporation, desalted by gel chromatography in 0.2 M $NH_4HCO_3$ with a Sephadex G-15 column (1.6 × 170 cm; Amersham Biosciences, Uppsala, Sweden), and lyophilized.

A portion of the oligomannuronate mixture was purified with a Bio-gel P-4 (superfine) column (1.6 × 170 cm). The column was eluted at 3 ml $h^{-1}$ with 0.5 M $NH_4HCO_3$ at room