Determination of the Accessibility of Acidic Oligosaccharide Sugar Chain to Blood-Brain Barrier Using Surface Plasmon Resonance

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There exist limitations in the detection of exogenous oligosaccharides due to their polydisperse and diversiform nature, and particularly the interference of endogenous glycosaminoglycans (GAGs). Herein, a surface plasmon resonance (SPR) assay for detecting acidic oligosaccharide sugar chain (AOSC), an anti-Alzheimer's drug candidate, in cerebrospinal fluid (CSF) was developed based on a carbohydrate antigen–antibody interaction. Rabbits were treated with AOSC intravenously and orally at 40 or 200 $mg \cdot kg^{-1}$, respectively. CSF samples were collected at given time points for quantitative determination of AOSC concentrations in the CSF using an SPR-based competitive inhibition assay, and the existence of AOSC in the CSF was indicated as a blood-brain barrier (BBB) accessibility index. AOSC concentration as low as 50 ppb (0.05 $\mu g \cdot ml^{-1}$) was detected in the CSF, with its peak value approaching 2.091 and 3.316 $\mu g \cdot ml^{-1}$ following intravenous and oral administration, respectively. This is the first time the capacity of AOSC to pass through the BBB has been confirmed using SPR-based competitive inhibition immunoassay. Importantly, the accessibility of AOSC to the BBB indicates AOSC has potential therapeutic value for treating neurodegenerative diseases, particular Alzheimer's disease.

Key words acidic oligosaccharide sugar chain; surface plasmon resonance; competitive inhibition assay

Although the exact pathogenesis of Alzheimer's disease (AD) remains to be fully defined, several pharmacological strategies for preventing and treating AD are under active investigation. More recently, drug design has targeted molecular events involved in the pathogenesis of AD including β-amyloid (Aβ) and neurofibrillary tangle formation.[1—3] In *in vitro* studies, Leveugle[1,4] has shown that heparin oligosaccharides (known as a type of glycosaminoglycans (GAGs)) pass through a blood-brain barrier (BBB) model in a molecular weight-dependent manner and inhibit amyloid β precursor protein processing, suggesting their potential effects against AD.

As an analogue of low molecular weight GAGs, acidic oligosaccharide sugar chain (AOSC), an anti-Alzheimer's disease drug candidate, is currently undergoing preclinical evaluation. AOSC bearing rich mannuronate blocks is an acidic marine-derived oligosaccharide extracted from the brown algae *Echlonia Kurome Okam* by enzymatic depolymerization and has an average molecular weight of about 1300 Da. We have demonstrated that AOSC can alleviate Alzheimer-type behavioral symptoms induced by scopolamine and Aβ1-40 in rodent models. The mechanisms of action underlying the cognition-improving activities of AOSC have been illustrated to be attributed to the inhibition of apoptosis, and thus, neurotoxicity *via* binding to Aβ peptide.[5,6] Given that AOSC has a promising therapeutic value for AD, the ability of AOSC to pass through the BBB becomes crucial for understanding its direct central nerve system (CNS) effects.

The detection of oligosaccharides has traditionally been performed on TLC, GC, HPLC, and capillary electrophoresis. However, there exist limitations due to the polydisperse and diversiform nature of oligosaccharides, and particularly the interference of endogenous GAGs. Moreover, these analytical methods require many clean up steps and are often time-consuming. Antibody-based methods have been developed as a favorable alternative for either the identification or quantification of oligosaccharides.[7] Antibodies are often used in the detection of oligosaccharides in immunoassays for these compounds,[8,9] and enzyme-linked immunosorbent assay (ELISA) is the most commonly used.

Recently, the surface plasmon resonance (SPR) biosensor assay has become increasingly recognized to be an accurate method for identifying the interaction between antibody and antigen.[10,11] Compared with conventional methods such as ELISA and fluorescence enzyme immunoassay,[12,13] it possesses several inherent advantages. A particular advantage of SPR assay is there is no need to label reactants, allowing 'real-time' detection of biomolecular interactions, which has attracted considerable attention due to its easy and specific recognition of antigen–antibody (antigen–antibody interaction). Other advantages are easy sample preparation, fully automated operation, short-time analysis, and small amount of sample consumption, which are of particular relevance when the interested components are difficult to generate.

Therefore, in this paper, based on specific monoclonal antibody raised against AOSC, we preferentially used the BIAcore SPR technique to detect whether AOSC exists in the cerebrospinal fluid (CSF) of New Zealand rabbits following both intravenous and oral administration. The results indicated that AOSC exhibited the capacity to cross the BBB, which provides a clue to understanding the therapeutic value of AOSC in AD.

MATERIALS AND METHODS

Materials AOSC was obtained from the Marine Drug and Food Institute, Ocean University of China. The compound is a white powder and is stored at room temperature. Specific monoclonal antibody raised against AOSC was generated in our lab.

A BIAcore X biosensor and CM5 biosensor chip were purchased from Amersham Pharmacia (Biacore AB, Uppsala, Sweden). Sulfo-*N*-hydroxysulfosuccinimide (NHS)-biotin and streptavidin were provided by Sigma (St. Louis, MO,

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U.S.A.).

Female Balb/c mice weighing 21.0±1.0 g and New Zealand rabbits weighing 2.5±1.0 kg were purchased from Shandong Experimental Animal Center (Certificate number: 20031108, Jinan, China), and housed in a room maintained at 23 °C with an alternating 12 h light–dark cycle. Mice or rabbits were randomly allowed free access to food and water, except during the learning test. All experiments were performed according to ethical guidelines on animal care.

Preparation of Specific Monoclonal Antibody against AOSC The immunogen of AOSC-bovine serum albumin (BSA) conjugate was prepared by a reductive amination method. The reducing terminus of AOSC has a reactive aldehyde group by which AOSC can be coupled to amino groups of BSA in the presence of $NaBH_3CN$.[14] Quantitative protein (based on A280 and a BSA standard curve) and carbohydrate (determined by phenol-sulfuric acid reaction) analysis showed that the conjugate contained carbohydrate and BSA in an approximate 7 : 1 molar ratio. AOSC-ovalbumin (OVA) conjugate was prepared by the same method.[15]

An AOSC-BSA-immunized mouse showing the highest antibody (Ab) titer was chosen to prepare hybridoma against AOSC. The fusion was performed for 3 d following the last intravenous injections. Stimulated spleen cells from the immunized mice were fused with NS-1 myeloma cells in a ratio of 5 : 1 in 50% (w/v) PEG-4000. Putative hybrids that resulted from hypoxanthine/aminopterin/thymidine selection were screened against AOSC-OVA by ELISA. Those producing an Ab of interest were cloned three times by limiting dilution to ensure stability and clonality. Finally, a monoclonal antibody (mAb), G3C5E8, specific against AOSC was obtained. The DNA content of mAb was determined by flow cytometry (Vantage, Becton Dickinson, U.S.A.). The Ig subclass of the object mAb (G3C5E8) was clarified as IgG1(κ) with supernatants using a mouse monoclonal antibody isotyping kit manufactured by Roche Diagnostics (Indianapolis, U.S.A.). By SPR assay, mAb (G3C5E8) showed no reaction with carrier proteins and little cross-reactivities with self glycosaminoglycans. Clones were expanded as ascites by intraperitoneal injection of 10^6 hybridoma cells in Balb/c mice 10—14 d following intraperitoneal treatment with 0.5 ml of 2,6,10,14-tetramethy1-pentadecane (pristane). Ascitic fluid was tapped 7—14 d postinjection. The mAb was purified with a Sepharose CL-4B-Protein G column (Amersham Pharmacia).

SPR Assay The SPR-based competitive inhibition immunoassay was conducted on a BIAcore biosensor instrument. For this purpose, AOSC was biotinylated at the reducing end, and the flow cell of a CM5 sensor chip was activated with streptavidin.[16] Biotinylated AOSC was allowed to react with the streptavidin-coated sensor chip. Briefly, 5 μmol AOSC was dissolved in 30 μl of deionized distilled water, followed by the simultaneous addition of 5 μmol *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide and 5 μmol NHS. The mixture was incubated at room temperature overnight. AOSC was immobilized on the CM5 sensor chip surface at 25 °C with a constant flow rate of 5 μl/min HBS-EP buffer (HBS-EP buffer: 0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% polysorbate 20 (v/v), pH 7.4). To assess real-time binding activity, the concomitant presence of anti-AOSC monoclonal antibody and AOSC or CSF samples containing AOSC were injected over the sensor chip surface, followed by 5 min washing with HBS-EP buffer. The sensor chip surface was regenerated using 60 μl of 2 M NaCl. All binding experiments were performed at 25 °C with a constant flow rate of 5 μl/min HBS-EP. To correct for non-specific binding and bulk refractive index change, a blank channel (FC2) without AOSC was employed as a control for each experiment. Sensorgrams for all binding interactions were recorded in real-time and analyzed after subtracting it from the blank channel. Changes in mass due to the binding response were recorded as resonance units (RU).[17]

CSF Sampling Healthy rabbits were anaesthetized with 3% pentobarbital and treated with a single dose of AOSC at a concentration of 40 $mg \cdot kg^{-1}$ or 200 $mg \cdot kg^{-1}$ following intravenous and oral administration, respectively. CSF samples (0.1 ml) were drawn from the cisterna magna.

Establishment of Analytical Methods. Calibration Curve 0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 $\mu g \cdot ml^{-1}$ standard solutions of AOSC were prepared from its standard stock solution with CSF of rabbits as solvent. A mixture of 15 μl of anti-AOSC-antibody and 15 μl of each tested analyte was injected over the AOSC-immobilized chip surface, followed by 2 min washing with HBS-EP buffer. The sensor chip surface was then regenerated by 30 μl of 2 M NaCl. The RU of each analyte was recorded. The inhibition rate of each sample was calculated according to the following formula: $(RU_0-RU)/RU_0$. All experiments were carried out in triplicate.

Recovery Test 0.05, 1.0 and 6.0 $\mu g \cdot ml^{-1}$ solutions of AOSC with CSF as solvent were prepared, and RU was determined as described above. Five samples of each were prepared.

Assay Precision The intraday and interday precisions were determined using three concentrations of AOSC (0.05, 1.0 and 6.0 $\mu g \cdot ml^{-1}$). The procedure was performed five times for each concentration.

Determination of CSF Concentration of AOSC after a Single Intravenous Dose CSF samples were obtained at 0, 5, and 30 min, and 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 h after intravenous injection of AOSC at the dosage of 40 $mg \cdot kg^{-1}$. The RU of each sample was determined by SPR-based BIAcore inhibition immunoassay. The inhibition rate $((RU_0-RU)/RU_0)$ of each sample was used to calculate the concentrations of AOSC from its corresponding regression equation.

Determination of CSF Concentration of AOSC after a Single Oral Dose CSF samples were obtained at 0, 5, and 30 min, and 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 h after oral administration of AOSC at the dosage of 200 $mg \cdot kg^{-1}$. The RU of each sample was determined by SPR-based BIAcore inhibition immunoassay. The inhibition rate $((RU_0-RU)/RU_0)$ of each sample was used to calculate the concentrations of AOSC from its corresponding regression equation.

Statistical Analysis Data are expressed as the means± standard deviation (S.D.) or standard error (S.E.) as indicated.

RESULTS

Method Validation Quantitative determination of AOSC in the CSF samples was carried out. The calibration curve is shown in Fig. 1. The longitudinal axis is the inhibi-

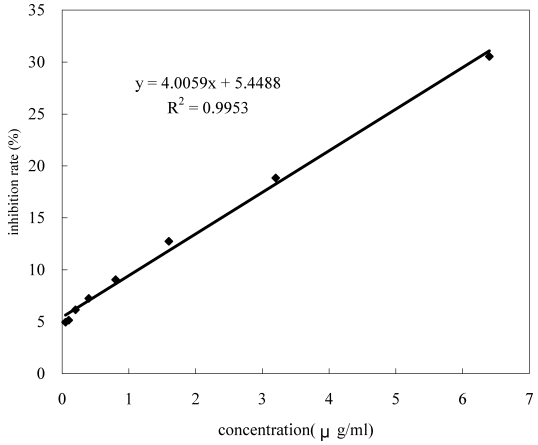

Fig. 1. Calibration Curve of SPR-Based Inhibition Immunoassay for Determination of the Optimal Range of Detection of AOSC with Antibody Concentration of 50 $\mu g \cdot ml^{-1}$

The linear range of detection was found to be between 0.05 and 6.4 $\mu g \cdot ml^{-1}$.

Table 1. Intra-Day Assay Precision and Accuracy (n=5)

Nominal concentration ($\mu g \cdot ml^{-1}$)	Calculated concentration ($\mu g \cdot ml^{-1}$, mean±S.D.)	Coefficient of variation (%CV)
0.05	0.048±0.005	10.059
1.00	0.982±0.082	8.309
6.00	5.961±0.186	3.124

Table 2. Inter-Day Assay Precision and Accuracy (n=5)

Nominal concentration ($\mu g \cdot ml^{-1}$)	Calculated concentration ($\mu g \cdot ml^{-1}$, mean±S.D.)	Coefficient of variation (%CV)
0.05	0.051±0.005	10.535
1.00	1.009±0.099	9.773
6.00	5.949±0.245	4.111

Table 3. Recovery and Precision (n=5)

Nominal concentration ($\mu g \cdot ml^{-1}$)	Calculated concentration ($\mu g \cdot ml^{-1}$, mean±S.D.)	Recovery (mean±S.D.) (%)	Coefficient of variation (%CV)
0.05	0.051±0.007	101.533±13.020	12.823
1.00	0.967±0.095	96.693±9.452	9.775
6.00	5.940±0.296	99.0±4.927	4.977

tion rate of each concentration of AOSC, while the horizontal axis is the concentration of AOSC. The regression equation was calculated as Y=4.0059x+5.4488 (R^2=0.9953), suggesting a good linear correlation between the concentration of AOSC and inhibition rate of AOSC. The calibration curve was linear and ranged from 0.05 to 6.4 $\mu g \cdot ml^{-1}$. The lowest sensitivity of this assay was 0.05 $\mu g \cdot ml^{-1}$.

The coefficients of variation (CV) of intraday and interday assays were detected to assess the precision and recovery of the analytical method. The CV of the two assays, listed in Tables 1 and 2, were found to be reproducible. The recovery assay was performed according to a routine method. The data are presented in Table 3.

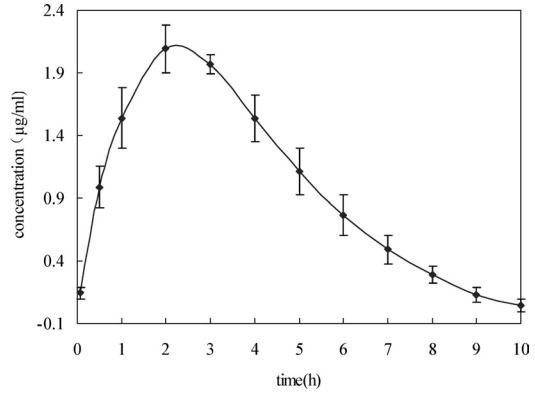

Fig. 2. CSF Concentrations of AOSC after a Single Intravenous Administration of AOSC at Dose of 40 $mg \cdot kg^{-1}$

AOSC concentrations in the CSF were determined using the calibration curve of SPR-based inhibition immunoassay. Data represent means±S.E.M. (n=5).

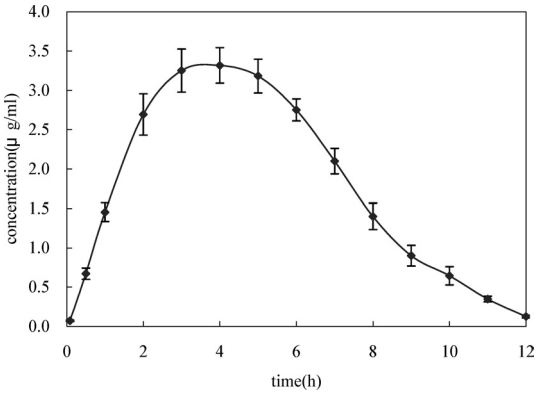

Fig. 3. CSF Concentrations of AOSC after a Single Oral Administration of AOSC at Dose of 200 $mg \cdot kg^{-1}$

AOSC concentrations in the CSF were determined using the calibration curve of SPR-based inhibition immunoassay. All data represent means±S.E.M. (n=5).

Determination of CSF Concentrations of AOSC after a Single Intravenous Injection Intravenous injection of AOSC (40 $mg \cdot kg^{-1}$) resulted in a significantly higher level in the CSF, with the peak concentration approaching 2.091 $\mu g \cdot ml^{-1}$ (Fig. 2).

Determination of CSF Concentrations of AOSC after a Single Oral Administration AOSC administered orally at 200 $mg \cdot kg^{-1}$ resulted in a significantly higher level in the CSF, as was observed in the intravenously-treated group, with the peak concentration being 3.316 $\mu g \cdot ml^{-1}$ (Fig. 3).

DISCUSSION

It is well known that a method for measuring the concentrations of exogenous sugar chains in the CSF should be sensitive, reproducible, accurate, and precise. In this paper, a highly sensitive, reusable SPR sensor chip assay for the determination of AOSC based on a competitive inhibition method was established. This SPR-based bioassay to conduct AOSC concentrations in the CSF can yield the lowest sensitivity equivalent to 0.05 $\mu g \cdot ml^{-1}$, with good linearity that ranged from 0.05 to 6.4 $\mu g \cdot ml^{-1}$. Importantly, the intraday and interday coefficients of variation (CV) were lower than 15%. These findings strongly suggest that this is a sensitive and feasible method for detecting AOSC in the CSF. Collec-

tively, the rapid and accurate determination of AOSC based on carbohydrate antigen–antibody recognition, with good stability and reproducibility using SPR biosensor chip, together with the reusability of the SPR sensor chip, suggests the SPR assay has potential value in measuring the concentration of any exogenous carbohydrates in body fluids, including CSF. The technique may also be accepted as a useful tool for evaluating pharmacokinetic profiles.

We have demonstrated that AOSC exists in the CSF following both oral and intravenous administration, indicating that AOSC is capable of passing through the BBB. The permeability of AOSC through the BBB gives us a good understanding of the *in vivo* potential antagonizing potency of AOSC against neuropathological changes induced by Alzheimer's disease.

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