Glycoconj J (2008) 25:521–530
DOI 10.1007/s10719-008-9105-0

Sequential synthesis of chondroitin oligosaccharides by immobilized chondroitin polymerase mutants

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Received: 2 November 2007 / Revised: 5 January 2008 / Accepted: 8 January 2008 / Published online: 5 February 2008

Abstract *Escherichia coli* strain K4 expresses a chondroitin (CH)-polymerizing enzyme (K4CP) that contains two glycosyltransferase active domains. K4CP alternately transfers glucuronic acid (GlcA) and *N*-acetyl-galactosamine (GalNAc) residues using UDP-GlcA and UDP-GalNAc donors to the nonreducing end of a CH chain acceptor. Here we generated two K4CP point mutants substituted at the UDP-sugar binding motif (DXD) in the glycosyltransferase active domains, which showed either glycosyltransferase activity of the intact domain and retained comparable activity after immobilization onto agarose beads. The mutant enzyme-immobilized beads exhibited an addition of GlcA or GalNAc to GalNAc or GlcA residue at the nonreducing end of CH oligosaccharides and sequentially elongated pyridylamine-conjugated CH (PA-CH) chain by the alternate use. The sequential elongation up to 16-mer was successfully achieved as assessed by fluorescent detection on a gel filtration chromatography and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and MALDI potential lift tandem TOF mass spectrometry (MALDI-LIFT-TOF/TOF MS/MS) analyses in the negative reflection mode. This method provides exactly defined CH oligosaccharide derivatives, which are useful for studies on glycosaminoglycan functions.

Keywords Chondroitin · Stepwise glycosylation · MALDI-TOF MS · MS/MS fragmentation · Pyridylamination

Abbreviations

CH	chondroitin
CS	chondroitin sulfate
GalNAc	*N*-acetyl-*D*-galactosamine
GlcA	*D*-glucuronic acid
GalNAc-T	GalNAc transferase
GlcA-T	GlcA transferase
K4CP	CH polymerase from *Escherichia coli* strain K4
MALDI-TOF MS	matrix-assisted laser desorption/ ionization and time-of-flight mass spectrometry
MALDI-LIFT-TOF/TOF MS/MS	matrix-assisted laser desorption/ ionization and potential lift tandem time-of-flight mass spectrometry
m/z	mass-per-charge ratio
PA	pyridylamine

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Introduction

Chondroitin (CH) is a major constituent of glycosaminoglycan (GAG). It is a linear polysaccharide chain with an alternating carbohydrate backbone of glucuronic acid (GlcA) and *N*-acetylgalactosamine (GalNAc), usually mod-

ified by sulfation and epimerization, and it is present as proteoglycan forms in the extracellular matrix and pericellular matrices of most animal species. GAG plays various biological roles in development, regeneration, infection, cytokine interaction, signal transduction, and morphogenesis [1–5]. Recent studies have accumulated lines of evidence to support that specific saccharide structures of GAGs have distinct functions. For example, structure-specific oligosaccharides of heparin/heparan sulfate bind different types of growth factors [6]. Hyaluronan oligosaccharides with several chain lengths demonstrated suppression of tumor proliferation, promotion of tumor cell migration, expression of heat shock protein, and induction of matrix metalloproteinase [7]. Oversulfated CH sulfate (CS) D-type [GlcA(2S)-GalNAc(6S)] oligosaccharides bind pleiotrophin [8] and E-type [GlcA-GalNAc(4, 6S)] oligosaccharides promote neurite outgrowth [9]. To investigate the association of the specific saccharide structure and length with biological function and in an attempt to utilize these oligosaccharides for medical applications, various GAG oligosaccharides have been used in biological and biochemical studies. However, most oligosaccharide samples used thus far are heterogeneous mixtures of different sizes and modifications since these oligosaccharides are prepared from natural GAG polymers. To obtain homogenous samples, chemical syntheses of CS tetrasaccharide [10] and hexasaccharide [11] have successfully been performed, but the large-scale preparation of these saccharides with longer carbohydrate chains remains impractical.

Escherichia coli strain K4 produces a capsule polysaccharide with CH backbone to prevent host immunological attacks [12], from which we previously performed molecular cloning of a CH polymerase (K4CP) [13]. This enzyme is a bifunctional glycosyltransferase containing GalNAc- and GlcA-transferase active sites (GalNAc-T and GlcA-T) at the N- and C-terminal regions of the protein, respectively. A similar bifunctional glycosyltransferase from *Pasteurella multocida* (pmHAS), which synthesizes hyaluronan polysaccharide, has been identified. In most of the glycosyltransferases, the DXD motif that binds UDP-sugar donor substrate is known to be essential for the enzyme activity. In fact, a point mutation at the DXD motif in one of the glycosyltransferase domains of pmHAS abrogates the corresponding glycosyltransferase activity while retaining the other glycosyltransferase activity [14]. By using agarose bead-immobilized point mutants of the respective glycosyltransferase domains, a stepwise synthesis of HA oligosaccharides has been achieved [15].

In this study, we generated two K4CP point mutants substituted at either DXD motif, thereby exhibiting individual glycosyltransferase activity, and developed a stepwise CH elongation method by using immobilized enzyme reactors of the mutants and pyridylaminated CH hexasaccharide (PA-CH6) as the initial acceptor. The PA-CH products obtained by sequential reactions were homogenous in size as assessed by MALDI-TOF MS and MALDI-LIFT-TOF/TOF MS/MS systems. These systems enable a large-scale preparation of CH derivatives with an expected length.

Materials and methods

Materials

CH polymer (a chemically desulfated derivative of CS-C from shark cartilage) was obtained from Seikagaku Corporation (Tokyo, Japan). UDP-GlcA, UDP-GalNAc, testicular hyaluronidase, and β-glucuronidase were purchased from Sigma-Aldrich (St. Louis, MO). Ni-NTA™ agarose and anti-tetra His antibody were from Qiagen (Hilden, Germany). QuikChange™ site-directed mutagenesis kit was obtained from Stratagene (La Jolla, CA). Superdex™ Peptide HR10/30 column, Superdex™ 30 HiLoad 16/60 column, Q-Sepharose ion exchange resin, NHS-activated Sepharose beads and ECL detection system were from GE Healthcare (Piscataway, NJ). A semi-quantitative SAX MAGNAM ion exchange column was purchased from Whatman (Clifton, NJ).

Preparation of CH oligosaccharides

CH oligosaccharides were prepared as described previously [16]. Briefly, for the preparation of even-numbered oligosaccharides, CH polymer was digested with hyaluronidase at 37°C. For the preparation of odd-numbered oligosaccharides, the hyaluronidase digests were further treated with β-glucuronidase at 37°C. These products were separated by chromatography on a Q-Sepharose ion exchange column with sodium chloride concentration gradient and a Superdex 30 gel filtration column in 0.2 M ammonium acetate buffer. Even-numbered oligosaccharides contain GlcA residues at the nonreducing termini and GalNAc residues at the reducing termini [17], while odd-numbered ones have GalNAc residues at nonreducing termini [18].

Preparation of PA-CH oligosaccharides

Pyridylamination of CH oligosaccharides (3-mer to 6-mer) was carried out by a slight modification of the method of Takagaki *et al*. [19]. Briefly, the purified CH oligosaccharide (1 μmol) solution in 0.2 ml water and 2-aminopyridine (1 mmol) solution in 300 μl of 3 M HCl (final pH 6.2) were mixed and added to a tube sealed with a screw cap. The tube was sealed and heated at 90°C for 2 h; sodium cyanoborohydride (12.8 μmol) dissolved in 40 μl water was then added to the mixture and it was heated at 90°C for 18 h. The PA-CH oligosaccharides were purified by chromatogra-

phy on a Superdex 30 gel filtration column in 0.2 M sodium acetate and a SAX ion exchange column with a linear gradient of 30~300 mM potassium dihydrogen phosphate.

Preparation of recombinant K4CP proteins with point mutations

DNA fragments for the truncated polypeptides were amplified by PCR using pTrcHis-*kfoC* [13] as the template, *Pfu* DNA polymerase and synthetic oligonucleotide primers corresponding to the K4CP protein. DNA constructs with point mutations were prepared using QuikChange™ site-directed mutagenesis kit with pTrcHis-*kfoC* DNA as the template according to the manufacturer's instructions. The oligonucleotide primers, each complementary to the opposite strands of the vector, for point mutation at DCD^{241} in the GalNAc-T active site to DCK^{241} (D241K) were as follows: 5′-GTTG CAATTCTGGATTGTAAGATGGCTCCGAACCCAC-3′ and 5′-GTGGGTTCGGAGCCATCTTACAATCCA GAATTGCAAC-3′. The oligonucleotide primers for point mutation at DSD^{521} in the GlcA-T active site to DSK^{521} (D521K) were as follows: 5′-AGGTCAGTTAGACTCTAA GGACTTTCTTGAACCAG-3′ and 5′-CTGGTTCAAG AAAGTCCTTAGAGTCTAACTGACCT-3′. The PCR products were purified using a gel extraction kit, digested with *Bam*HI and *Eco*RI, inserted into the pTrcHis expression vector, and transformed into *E. coli* strain TOP10.

Expression and purification of the mutant proteins were carried out as described previously [13]. Briefly, the cells transformed with the pTrcHis expression vectors containing the desired mutant were cultured in LB medium containing 100 μg/ml ampicillin at 37°C for 3 h. β-Isopropylthiogalactoside (final concentration 1 mM) was then added to the culture and the bacteria were further cultured at 37°C for 5 h. The expressed proteins were extracted from the bacterial suspensions by sonication and purified by Ni-NTA agarose column chromatography.

Measurements of enzyme activity

GalNAc-T, GlcA-T, and CH polymerase activities of the recombinant enzymes were measured using radioisotope donor substrates as described previously [16] with a slight modification. Briefly, for the assay of the polymerase activity, a 50-μl mixture containing 50 mM Tris–HCl (pH 7.2), 20 mM $MnCl_2$, 0.15 M NaCl, UDP-[^{3}H]GalNAc (3 nmol, 0.1 μCi) and UDP-GlcA (3 nmol) as the donor substrates and 0.1 nmol of CH hexasaccharide (CH6) as the acceptor substrate was incubated with the recombinant enzymes (0.75 μg) at 30°C for 30 min and then heated in boiling water for 1 min. For the assay of GalNAc-T activity, only UDP-[^{3}H]GalNAc (3 nmol, 0.1 μCi) was used as the donor substrate and 1 nmol of CH6 was used as the acceptor substrate. For the assay of GlcA-T activity, UDP-[^{14}C]GlcA (3 nmol, 0.1 μCi) and 1 nmol of CH pentasaccharide (CH5) were used. The radiolabeled saccharides were separated by a Superdex Peptide column and measured by liquid scintillation counter. The enzyme activities were determined by calculating the amount of the incorporated radioactive sugars. To measure the enzyme activities with longer CH chains, longer even-numbered (10~16-mer) oligosaccharides (CH10–16) and odd-numbered (9~15-mer) oligosaccharides (CH9–15) were used as acceptor substrates instead of CH6 and CH5, respectively.

Immobilization of the mutant enzymes

Immobilization of the mutant enzymes was carried out according to the manufacturer's instruction with a slight modification. Briefly, the purified mutant enzymes (100 μg protein) solution in PBS (500 μl) were mixed with NHS-conjugated Sepharose bead suspension (500 μl) and rotated at 4°C for 16 h. Residual activated esters of the resin were quenched with 10 μl of 1 M ethylamine for 2 h at 4°C. The enzyme-conjugated beads were stored at 4°C in PBS containing 20% glycerol. The activity of the immobilized enzymes was stable at least for 2 months at 4°C.

Stepwise synthesis of PA-CH oligosaccharides using the immobilized enzyme beads

PA-CH6 (100 nmol) as the initial acceptor substrate and UDP-GalNAc (200 nmol) donor were mixed with D521K-immobilized beads (100 μl) in 1 ml of reaction buffer containing of 50 mM Tris–HCl (pH 7.2), 20 mM $MnCl_2$ and 0.15 M NaCl. The suspension was vigorously mixed using a vortex (200 rpm) at 30°C for 1~2 h. To monitor the reaction, a small portion (2 μl) of the supernatant of the suspension was chromatographed on a Superdex Peptide column and the products were detected by fluorescence photometer FP2020 (JASCO, Tokyo, Japan) using excitation and emission wavelengths at 310 and 370 nm, respectively (Fig. 3). After confirmation of the completion of reaction, the reacted suspension was applied to Ultrafree centrifugal filter unit (0.45 μm pore, Millipore, Billeria, MA, USA) and centrifuged at 6,000 rpm for 1min. The filtrate separated from the enzyme beads contained PA-CH7.

The reacted solution containing PA-CH7 without purification was mixed with D241K-immobilized beads (100 μl) and UDP-GlcA (300 nmol). The suspension was vigorously mixed with vortex at 30°C, monitored to confirm the completion of sugar elongation, applied to a new filter unit, and centrifuged as described above. The filtrate containing PA-CH8 was separated from the enzyme beads and used without purification for further reaction with additional UDP-GalNAc (300 nmol) and the D241K-immobilized

beads (100 μl). These stepwise alternative elongation reactions using the two immobilized mutant beads were repeated ten times up to the synthesis of PA-CH16. The synthesized PA-CH oligosaccharides (7-mer to 16-mer) were purified by gel filtration on a Superdex 30 column.

Mass spectrometry analysis

Small portions of the purified PA-CH oligosaccharides (approximately 0.1 nmol in 10 μl water) were passed through a small column of Dowex 50W-X8 ion exchange resin (H^+ form) to remove cation adducts. The sample solutions (1 μl) were mixed with the same volume as the DHB matrix solution (10 mg/ml in 50% acetonitrile). An aliquot (1 μl) of the mixtures was applied to a target plate and air-dried at room temperature. Their molecular mass profiles were measured by AutoFlex MALDI-TOF and UltraFlex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) using an accelerated potential of 20 kV in the negative reflection mode. In the LIFT-MS/MS experiments, the precursor ions of the first MS were accelerated at 8 kV, and the fragment ions generated from these precursors were subsequently accelerated through 20 kV; MS/MS spectra were analyzed in the negative reflection mode.

SDS-PAGE and Western blotting

SDS-PAGE analysis of the proteins was carried out on a 10% gel by the method of Laemmli [20]. The proteins were detected by Coomassie brilliant blue staining. For Western blotting, the proteins on the SDS-PAGE gel were transferred onto a nitrocellulose membrane. The proteins on the membrane were then treated with anti-tetra-His antibody and detected with the ECL detection system. The protein content was determined using a micro BCA protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin as the standard.

Results and discussion

Generation of single-action point mutants

CH polymerase K4CP contains 686 amino acid residues and two glycosyltransferase domains; GalNAc-T and GlcA-T domains at the N- and C-terminal regions, respectively. Initially, we speculated that one domain acts independent of the other. When the domain $K4CP_{1–399}$ or $K4CP_{306–686}$ was expressed, neither of them exhibited glycosyltransferase activity (data not shown), suggesting that the rest of the protein region is required for either glycosyltransferase activity.

We then tested recombinant proteins with point mutation in the DXD motif of either domain. The expressed intact K4CP and two point mutants, D241K and D521K (Fig. 1a), were purified by Ni-NTA affinity chromatography yielding 1~2 mg of protein per liter of culture, and single bands were obtained at approximately 82 kDa by SDS-PAGE and

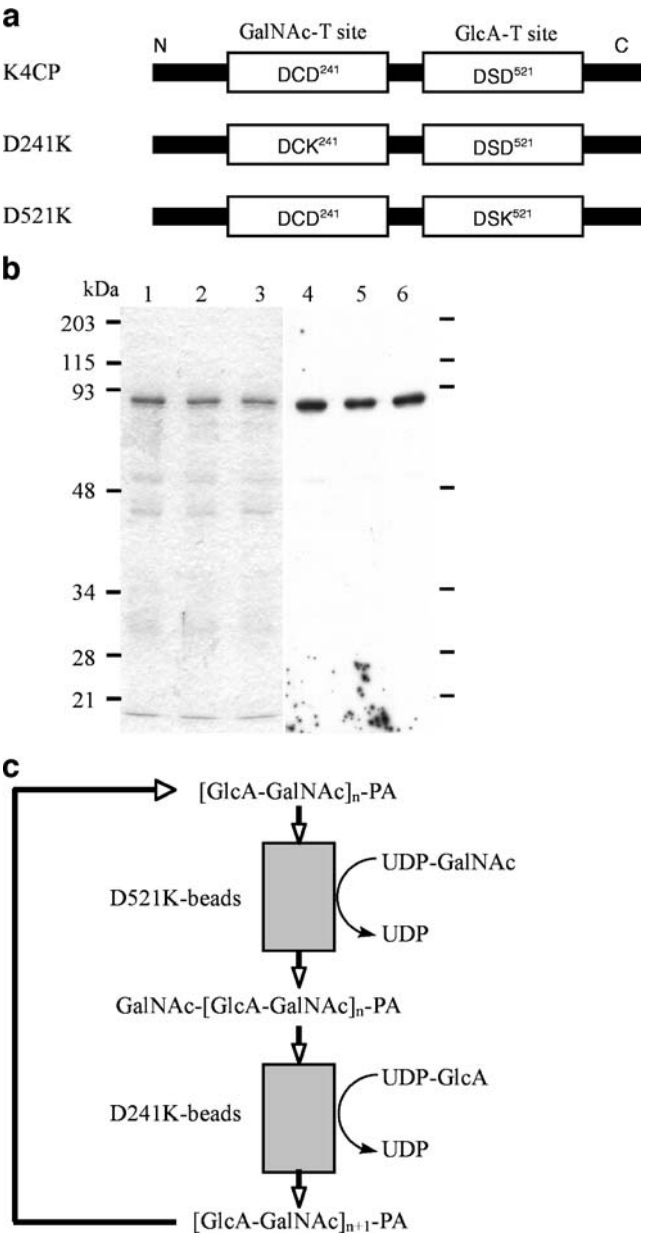

Fig. 1 **a** The structure of recombinant native K4CP, point mutant D241K at the N-terminal region, and point mutant D521K at the C-terminal region are shown. The putative GalNAc-T and GlcA-T domains are indicated as boxes. The mutated amino acid residues and their positions are indicated. **b** SDS-PAGE followed by Coomassie brilliant blue staining (*lanes 1*, *2*, *3*) and Western blotting using anti-His antibody (*lanes 4*, *5*, *6*). Native K4CP (*lanes 1* and *4*), D241K mutant (*lanes 2* and *5*), and D521K mutant (*lanes 3* and *5*) are detected as approximately 82 kDa proteins. Molecular standards are indicated at both sides. **c** The reaction profile of the stepwise syntheses of PA-CH oligosaccharides using K4CP mutant-immobilized beads

Table 1 Enzyme activities of the recombinant proteins of native K4CP and the point mutants

Enzymes	Activities (nmol min^{-1} mg^{-1} proteins[a])					
	GalNAc transferase		GlcA transferase		Polymerase	
	CH6	CH10–16	CH5	CH9–15	CH6	CH10–16
K4CP	5.19±1.30	4.30±0.91	6.94±2.38	7.73±2.12	5.12±1.65	5.99±1.33
D241K	N.D.	N.D.	3.69±0.82	3.71±0.93	N.D.	N.D.
D521K	4.43±0.93	4.06±1.06	N.D.	N.D.	N.D.[b]	N.D.[b]
D241K+D521K	4.83±1.16	4.45±1.13	3.18±0.75	3.10±0.81	1.80±0.80	2.01±0.87

N.D. Not detected
[a] Values are expressed as mean±S.D (n=3~6).
[b] These reactions showed only single sugar incorporation. *i.e.*, GalNAc-T activity.

Western blotting analysis (Fig. 1b). GalNAc-T, GlcA-T, and CH polymerase activities of the proteins were measured by radio-labeling methods. While intact K4CP showed both glycosyltransferase activities, the two point mutants in which one UDP-sugar binding motif (DXD) was mutated and the other was intact showed a glycosyltransferase activity of the corresponding intact active sites but lacked the activity of the mutated sites. Neither of the point mutants showed the CH polymerase activity, but the mixed enzymes of D241K and D521K exhibited polymerase activity (Table 1).

Stepwise elongation of PA-CH oligosaccharides using the immobilized mutant enzyme reactors

Pyridylamine (PA) conjugation at the reducing ends of sugar chains [21] is a useful fluorescent labeling method for structural analysis and metabolic studies of glycoconjugates. PA-conjugated sugars have been used for micro-scale analyses (sugar mapping) by HPLC [22] and frontal affinity chromatography [23] and recently for mass spectrometric analysis because of their high sensitivity. PA-conjugated oligosaccharides showed 100-fold improvement in sensitivity when compared to the corresponding underivatized

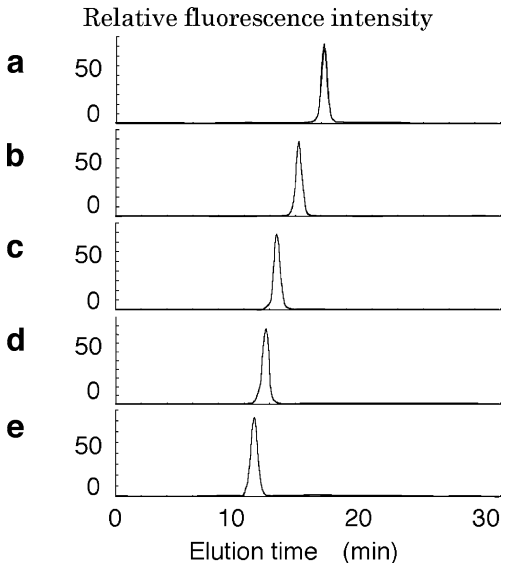

Fig. 2 Gel filtration chromatography of PA-CH oligosaccharides: Chromatographic profiles of **a** PA-CH3, **b** PA-CH6, **c** PA-CH9, **d** PA-CH12, and **e** PA-CH16 on a Superdex Peptide column are shown

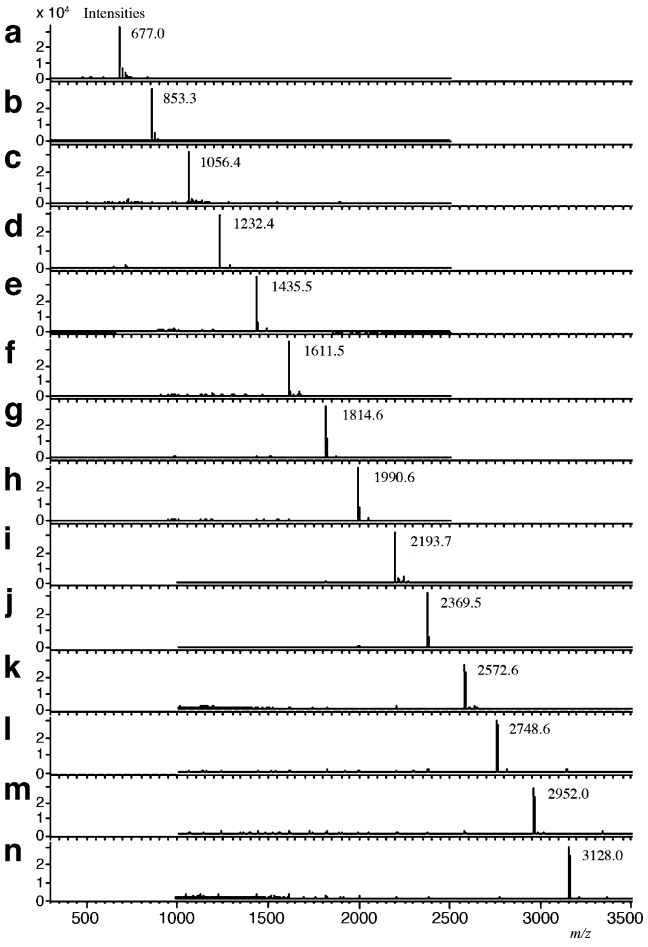

Fig. 3 MALDI-TOF mass spectra of PA-CH oligosaccharides: Synthesized **a** PA-CH3, **b** PA-CH4, **c** PA-CH5, **d** PA-CH6, **e** PA-CH7, **f** PA-CH8, **g** PA-CH9, **h** PA-CH10, **i** PA-CH11, **j** PA-CH12, **k** PA-CH13, **l** PA-CH14, **m** PA-CH15, and **n** PA-CH16 were subjected to the MALDI-TOF MS system

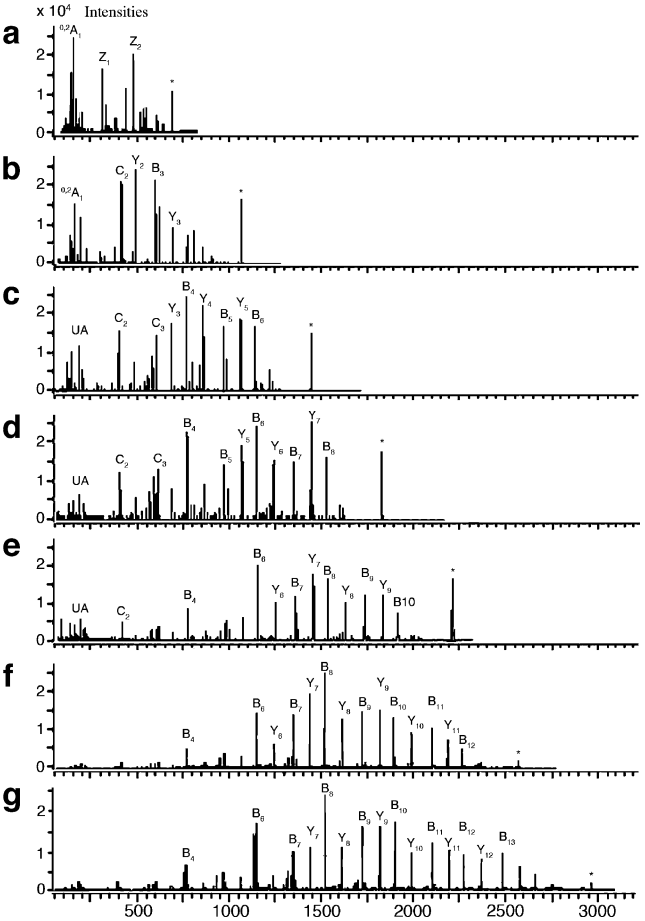

Fig. 4 MALDI-LIFT-TOF/TOF MS/MS fragment ions from the $[M–H]^-$ ions of odd-numbered PA-CH oligosaccharides: **a** PA-CH3, **b** PA-CH5, **c** PA-CH7, **d** PA-CH9, **e** PA-CH11, **f** PA-CH13, and **g** PA-CH15. *Asterisk* represents the precursor ion

oligosaccharides [24]. Pyridylamination is also utilized for disaccharide quantification of GAG [25] and investigation of nonreducing terminal structure of CS [26]. Thus we prepared small PA-CH oligosaccharides (3-mer to 6-mer) chemically by reductive pyridylamination of corresponding CH oligosaccharides and confirmed that these PA-CH oligosaccharides serve as acceptor substrates similar to aglycon-free CH oligosaccharide acceptors in the reactions with the soluble enzymes (data not shown).

We then immobilized the mutant enzymes onto agarose beads and compared the glycosyltransferase activities with those of the corresponding soluble enzymes. Both enzymes were trapped on 90% of the beads, and these immobilized enzymes exhibited comparable activities (data not shown). Based on these results, we attempted to establish a stepwise elongation of CH oligosaccharides by using the PA-conjugated acceptor substrates and the immobilized mutant enzyme reactors.

D241K-immobilized beads (100 μl gel suspension) completely transferred GlcA to 100 nmol of PA-CH5 with 300 nmol of UDP-GlcA at 30°C for 1 h, as assessed by fluorescent detection HPLC and MS analysis. The same volume of D521K-immobilized beads completely transferred GalNAc to 100 nmol of PA-CH6 with 300 nmol of UDP-GalNAc under the same conditions. The stepwise elongation of PA-CH oligosaccharides was initiated with PA-CH6 as the acceptor and D521K-immobilized beads, resulting in the production of PA-CH7. The filtrate of the reaction mixture containing PA-CH7 was used without any purification for PA-CH8 preparation by using D241K-immobilized beads. These stepwise reactions using two immobilized mutant enzyme reactors alternately were repeated ten times to produce PA-CH16 (Fig. 1c). The reactions performed using PA-CH3 and PA-CH4 as initial acceptors gave substantially lower yields (data not shown).

These PA-CH oligosaccharides obtained by the stepwise reactions were purified by gel filtration on a Superdex 30 HR16/60 column to remove residual small molecules such as nucleotide sugar donors, nucleotides, and salts. Figure 2 indicates the gel filtration profiles of PA-CH oligosacchar-

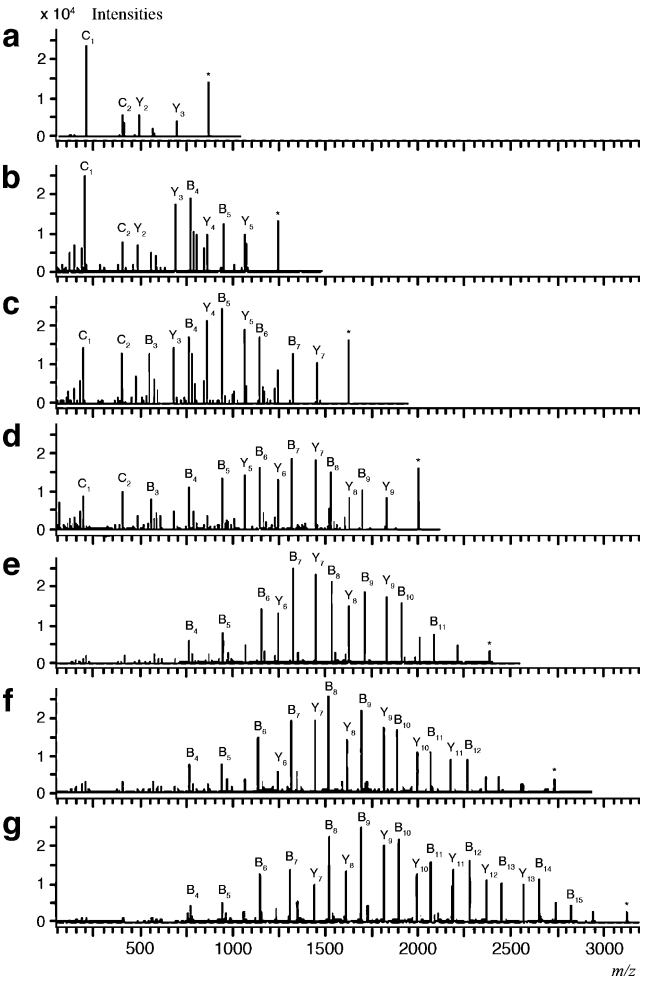

Fig. 5 MALDI-LIFT-TOF/TOF MS/MS fragment ions from the $[M–H]^-$ ions of even-numbered oligosaccharides derivatives: **a** PA-CH4, **b** PA-CH6, **c** PA-CH8, **d** PA-CH10, **e** PA-CH12, **f** PA-CH14, and **g** PA-CH16. *Asterisk* represents the precursor ion

Table 2 Summary of the MALDI-TOF MS and the MALDI-LIFT-TOF/TOF MS/MS measurements for the PA-CH oligosaccharides

PA-CH	MALDI-TOF MS	MALDI-LIFT-TOF/TOF MS/MS	
Chain size	Molecular-related ion $[M–H]^-$ (monoisotopic, *m/z*)	Major fragment ions Glycosidic cleavages (*m/z*)	Cross-ring and secondary cleavages (*m/z*)
3-mer	677.0	B_1 201, C_1 219, Y_1 297, **Z_1 279**[a], **Z_2 455**	^{0,2}**A_1 119**, Z_2–CO_2 411
4-mer	853.3	B_2 377, B_3 553, **C_1 192**, **$C_2$395**, **Y_2 473**, Y_3 677, Z_2 455	
5-mer	1,056.4	B_1 202, **B_3 581**, **B_4 757**, **C_2 395**, **C_3 599**, Y_1 298, **Y_2 474**, **Y_3 677**, Z_1 280, Z_2 456, Z_4 835	^{0,2}**A_1 119**, **UA 175** Z_2–CO_2 412, **Z_4–CO_2 791**
6-mer	1,232.4	**B_1 174**, B_2 377, B_3 553, **B_4 756**, **B_5 932**, **C_1 192**, **C_2 395**, C_3 571, **C_4 774**, Y_1 297, **Y_2 473**, **Y_3 676**,**Y_4 852**, **Y_5 1056**, Z_1 279, Z_2 455, **Z_4 834**	**Z_4–CO_2 790**
7-mer	1,435.5	B_1 202, **B_3 581**, **B_4 757**, **B_5 960**, **B_6 1136**, **C_2 396**, **C_3 599**, C_4 775, **C_5 978**, Y_1 298, **Y_2 474**, **Y_3 677**, **Y_4 853**, **Y_5 1056**, Y_6 1232, Z_1 280, Z_2 456 **Z_4 835**, **Z_6 1215**	$^{0,2}A_1$ 119, **UA 175**, **193** Z_2–CO_2 412,**Z_{42} 791** Z_6–CO_2 1170
8-mer	1,611.5	**B_1 174**, B_2 377, **B_3 554**, **B_4 757**, **B_5 933**, **B_6 1136**, **B_7 1312**, **C_1 192**, **C_2 395**, **C_3 572**, **C_4 775**, C_5 951, C_6 1154, Y_1 297, **Y_2 474**, **Y_3 677**, **Y_4 853**, **Y_5 1056**, **Y_6 1232**, **Y_7 1436**, Z_1 279, Z_2 456, **Z_4 835**, Z_6 1214	**Z_4–CO_2 791**
9-mer	1,814.6	B_2 377, **B_3 580**, **B_4 756**, **B_5 959**, **B_6 1336**, **B_7 1339**, **B_8 1515**, **C_2 395**, **C_3 598**, C_4 774, **C_5 977**, C_6 1153, C_7 1356, **Y_2 473**, **Y_3 676**, **Y_4 852**, **Y_5 1055**, **Y_6 1232**, **Y_7 1435**, Y8 1610, Z_2 455, Z_4 834, Z_6 1214, Z_8 1593	**UA 172**, 190 Z_4–CO_2 790
10-mer	1,990.6	**B_1 174**, B_2 378, **B_3 553**, **B_4 757**, **B_5 933**, **B_6 1137**, **B_7 1313**, **B_8 1515**, **B_9 1691**, **C_1 192**, **C_2 396**, C_3 572, C_4 775, C_5 951, C_6 1154, C_8 1533, Y_2 473, **Y_3 677**, **Y_4 853**, **Y_5 1057**, **Y_6 1233**, **Y_7 1436**, **Y_8 1611**, **Y_9 1814**, Z_2 456, Z_4 835, Z_6 1215, Z_8 1593	Z_4–CO_2 791
11-mer	2,193.7	B_3 580, **B_4 756**, B_5 960, **B_6 1136**, **B_7 1339**, **B_8 1515**, **B_9 1718**, **B_{10} 1894**, **C_2 395**, C_3 598, C_4 774, C_5 978, C_6 1154, C_7 1357, Y_2 473, Y_3 676, Y_4 852, **Y_5 1056**, **Y_6 1232**, **Y_7 1435**, **Y_8 1611**, **Y_9 1814**, Y_{10} 1990, Z_6 1214, Z_8 1593, Z_{10} 1972	**UA 174**, 192, C_5–CO_2 932
12-mer	2,369.5	B_1 174, B_2 377, B_3 553, **B_4 756**, **B_5 932**, **B_6 1135**, **B_7 1311**, **B_8 1515**, **B_9 1691**, **B_{10} 1894**, **B_{11} 2071**, C_1 192, C_2 395, C_3 571, C_4 775, C_5 950, C_6 1153, C_7 1329, C_8 1533, C_{10} 1912, Y_1 298, Y_2 473, Y_3 677, Y_4 852, Y_5 1055, **Y_6 1231**, **Y_7 1435**, **Y_8 1611**, **Y_9 1814**, Y_{10} 1991, Y_{11} 2194, Z_4 834, Z_6 1213, Z_{10} 1973	Z_4–CO_2 791, Z_6–CO_2 1169 Z_8–CO_2 1551
13-mer	2,572.6	B_1 201, B_2 377, B_3 580, **B_4 756**, B_5 959, **B_6 1135**, **B_7 1339**, **B_8 1515**, **B_9 1718**, **B_{10} 1894**, **B_{11} 2098**, B_{12} 2274, C_2 395, C_3 598, C_4 774, C_5 977, C_6 1153, C_7 1357, C_8 1533, C_9 1736, Y_1 279,Y_2 473, Y_3 676, Y_4 852,Y_5 1055, **Y_6 1231**, **Y_7 1435**, **Y_8 1611**, **Y_9 1814**, **Y_{10} 1990**, **Y_{11} 2193**, Y_{12} 2370, Z_4 834, Z_6 1213, Z_8 1593, Z_{10} 1972, Z_{12} 2352	UA 174, 192, Z_4–CO_2 790, Z_8–CO_2 1549,
14-mer	2,748.6	B_1 174, B_2 377, B_3 553, **B_4 756**, **B_5 932**, **B_6 1135**, **B_7 1311**, **B_8 1514**, **B_9 1691**, **B_{10} 1894**, **B_{11} 2070**, **B_{12} 2274**, B_{13} 2450, C_1 192, C_2 395, C_3 571, C_4 774, C_5 950, C_6 1153, C_7 1329, C_8 1533, C_9 1709, C_{10} 1912, Y_2 473, Y3 676, Y_4 852, Y_5 1055, **Y_6 1231**, **Y_7 1435**, **Y_8 1611**, **Y_9 1814**, **Y_{10} 1990**, **Y_{11} 2194**, Y_{12} 2369, Y_{13} 2573, Z_4 834, Z_6 1213, Z_8 1593, Z_{10} 1972,	Z_8–CO_2 1550
15-mer	2,952.0	B_1 201, B_2 378, B_3 580, **B_4 756**, B_5 959, **B_6 1135**, **B_7 1338**, **B_8 1514**, **B_9 1718**, **B_{10} 1894**, **B_{11} 2097**, **B_{12} 2273**, **B_{13} 2476**, B_{14} 2653, C_2 395, C_3 598, C_4 774, C_5 977, C_6 1152, C_7 1356, C_8 1533, C_9 1736, C_{10} 1912, C_{11} 2115, Y_1 299, Y_2 473, Y_3 676, Y_4 852, Y_5 1055, Y_6 1231, **Y_7 1434**, **Y_8 1611**, **Y_9 1814**, Y_{10} 1990, **Y_{11} 2193**, Y_{12} 2369, Y_{13} 2573, Y_{41} 2749, Z_4 834, Z_6 1213, Z_8 1592, Z_{10} 1972, Z_{12} 2351, Z_{14} 2731	UA 174, 192
16-mer	3,128.0	B_1 174, B_2 377, B_3 553, B_4 756, **B_5 932**, **B_6 1135**, **B_7 1311**, **B_8 1514**, **B_9 1691**, **B_{10} 1894**, **B_{11} 2070**, **B_{12} 2273**, **B_{13} 2449**, **B_{14} 2653**, B_{15} 2829, C_1 192, C_2 395, C_3 571, C_4 774, C_5 950, **C_6 1153**, C_7 1329, C_8 1532, C_{10} 1912, C_{11} 2097, C_{12} 2291, Y_2 473, Y_3 676, Y_4 852, Y_5 1055, Y_6 1231, **Y_7 1434**, **Y8 1611**, **Y_9 1814**, **Y_{10} 1990**, **Y_{11} 2193**, **Y_{12} 2369**, **Y_{13} 2573**, Y_{14} 2749, Y_{15} 2952, Z_4 834, Z_6 1213, Z_7 1418, Z_8 1593, Z_{10} 1972, Z_{14} 2731	Z_4–CO_2 790 Z_7–CO_2 1374

[a] Fragment ions having higher intensity (more than 20% of the highest fragment peak in each spectrum) are indicated by bold letters.

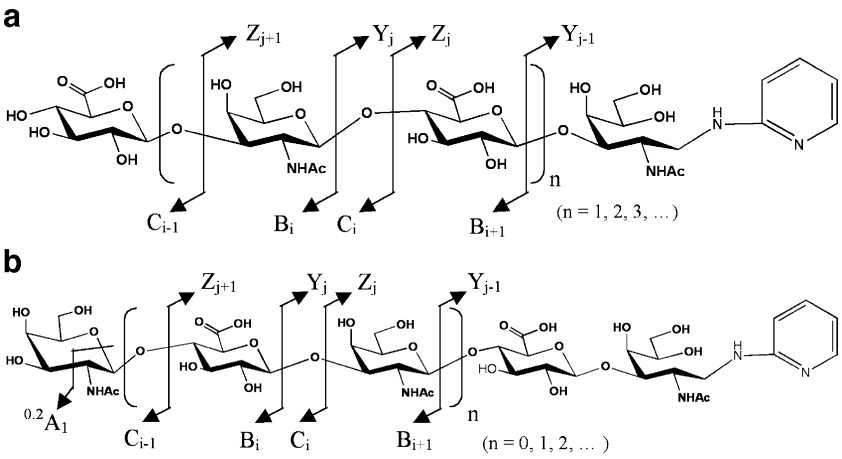

Fig. 6 MS/MS fragmentations of PA-CH oligosaccharides: **a** Glycosidic cleavage sites of the even-numbered PA-CH oligosaccharides, **b** glycosidic cleavage sites and cross-ring cleavage site of the odd-numbered PA-CH oligosaccharides

ides synthesized by the chemical and enzymatic methods. All the synthesized oligosaccharide derivatives were observed as single symmetrical peaks. When the reactions were not completed, the peak was split or the shape of the peak was distorted.

MALDI-TOF MS analysis of the PA-CH oligosaccharides

The chemically and enzymatically synthesized PA-CH oligosaccharides (3-mer to 16-mer) were analyzed by MALDI-TOF MS spectrometer in the negative reflection mode using DHB as the matrix. Their main ion peaks were identified as deprotonated molecular ions ($[M–H]^-$) calculated for desired PA-CH oligosaccharides, together with some sodium salt adduct ions observed at lower intensities. The ion peaks of the PA-CH odd-numbered and even-numbered oligosaccharides were observed as alternately added mass units of GalNAc residue (203 Da) and GlcA residue (176 Da) onto the nonreducing end of their precursor PA-CH odd-numbered and even-numbered oligosaccharide acceptors, respectively (Fig. 3). Ion-source decay (ISD) fragment ions that often appeared in the spectra of underivatized GAG oligosaccharides [27] were rarely observed in PA-CH oligosaccharides, since the laser power required for ionization of PA-CH oligosaccharides was substantially lower than that for ionization of the corresponding underivatized CH oligosaccharides,.

MALDI-LIFT-TOF/TOF MS/MS analysis of the PA-CH oligosaccharides

The MALDI-LIFT-TOF/TOF MS/MS analyses of the PA-CH oligosaccharides (3-mer to 16-mer) demonstrated extensive fragment ion peaks. Figures 4 and 5 show the MS/MS fragmentations of odd-numbered and even-numbered PA-CH oligosaccharides, respectively, and Table 2 summarizes the *m/z* data of their ion peaks. The spectra demonstrated highly regulated glycosidic cleavage fragments, indicating simple alternating sequence consisting of hexuronic acid and *N*-acetyl hexosamine. The nomenclature of the carbohydrate fragments in the MS/MS of glycoconjugates was defined according to Domon and Costello [28]. Figure 6 shows the cleavage sites of the PA-CH oligosaccharides in the MS/MS analysis. B_i and C_i represent fragment ions containing nonreducing ends. Y_j and Z_j labels designate the glycosidic cleavage fragments containing the reducing sugar units and aglycon. Subscripts i and j indicate the sugar number of the fragment moieties in the linear saccharide chain. The fragmentation pattern of glycosylation cleavage can be expressed by the following simple formulas.

m/z values of the nonreducing terminal fragments from the oligosaccharide ions

$$m/z(\mathrm{B_i}) = 203 \times k + 176 \times l - 2$$

$$m/z(\mathrm{C_i}) = 203 \times k + 176 \times l + 16$$

m/z values of the reducing terminal fragments containing PA residue

$$m/z(\mathrm{Y_j}) = 203 \times k + 176 \times l + 94$$

$$m/z(\mathrm{Z_j}) = 203 \times k + 176 \times l + 94 - 16 - 2$$

In the formulas, symbols k and l represent the number of GalNAc and GlcA residues in the fragment ions, and the numbers 94, 203, 176, and 16 represent the mass units of PA, GalNAc, GlcA, and oxygen (O) residues, respectively. The number "−2" designates deprotonation (−2H) accompanied with cleavage of the glycosidic bond because proton transfer occurs at Z and B fragment formation in the negative ion mode (mass shift rule) [29].

The *m/z* values observed for fragment ions between C and Y fragments having the same sugar length from saturated free oligosaccharide of nonsulfated GAG and for fragment ions between C and Z fragments from unsaturated free oligosaccharides were the same [27, 30]. All the observed fragments from PA-CH oligosaccharides were distinguished from each other due to the presence of aglycon PA at their reducing ends. This is an advantage of PA-conjugated derivatives for the determination of the oligosaccharide structure, in addition to increased sensitivity of the MS system.

Y and B fragment ions showed higher intensities than Z and C fragment ions in the MS/MS spectra of relatively large oligosaccharides (>5-mer). This fragmentation pattern of *N*-acetylheparosan oligosaccharides consisting of repeating disaccharide units (–4GlcA β1–4 GlcNAc α1–) was observed due to alternate B/C and Y/Z fragmentations [27]. Configuration of *N*-acetylhexosamine residue (GalNAc and GlcNAc) and glycosidic linkages (α or β, and 1–3 or 1–4 bonds) might contribute to the difference in the fragmentation patterns between CH and *N*-acetylheparosan. Ion peaks of lower mass C fragments (C_1, C_2, and C_3) showed large intensities in the relatively smaller PA-CH oligosaccharides (<11-mer). Even-numbered Z fragments (*e.g.* Z_4, Z_6,…) cleaved at the C4 site of GlcA and their decarboxyl ions (Z–CO_2) were often observed as small peaks, whereas odd-numbered Z fragments (*e.g.* Z_3, Z_5,…) that cleaved at the C3 site of GalNAc residue were rarely observed (Table 2).

In addition, $^{0,2}A_1$ cross-ring cleavage fragment was observed in the spectra of odd-numbered (GalNAc nonreducing end) and relatively small oligosaccharides (3-mer, 5-mer, and 7-mer). A uronic acid (UA) fragment was observed in the spectra of odd-numbered oligosaccharides. This suggests that GlcA located inside was liberated by cleavage at both reducing and nonreducing sites of the GlcA residue (secondary generated fragment) since GlcA is not present at either chain terminus in the odd-numbered saccharides (Fig. 6). The ion peaks discriminated as C_1 and B_1 fragments in the even-numbered saccharides might include the secondary generated UA fragments. PA-CH3, which was the shortest chain in this study, fragmented to yield Z_2, Z_1, and $^{0,2}A_1$ ions with relatively high intensity.

As mentioned above, all the oligosaccharide derivatives were precisely identified as single negative deprotonated molecular ions ($[M–H]^-$) at a high sensitivity in the MS system. Most of the MS/MS fragment ions were assigned to glycosidic cleavages, showing highly regular patterns in the extended sugar length. The tandem MS fragmentations of the PA-CH oligosaccharides directly reflected the alternating repeat sequences, thereby clearly differentiating reducing and nonreducing terminal cleavages. Recently the MS/MS analytical system for glycoconjugates has been remarkably improved, and this system will soon enable structural studies of more heterogenic GAG polysaccharides.

In this study, we developed a stepwise synthetic method of CH oligosaccharides by using two point mutants of K4CP. Compared with the preparation of GAG oligosaccharides via degradation of native GAG polysaccharides [8, 31], organic synthesis [10, 11], and enzymatic synthesis using glycosyltransferases [16] and transglycosylation reaction [32], this stepwise method enables the preparation of demanded length of monodisperse oligosaccharide derivatives. In our system, the reaction process is quite simple and the reacted solution can be readily used for the next elongation step without purification and deprotection. Most of the aglycon molecules, including *p*-nitrophenol, biotin, peptides, and lipids as well as PA, can be used for the enzymatic elongation. This method would provide exactly defined oligosaccharides, which are useful for identification of epitope sequences recognized by GAG-binding molecules. A homogenous CS oligosaccharide in length and sulfation can be prepared through additional modifications. CH and CS oligosaccharide derivatives prepared by this method would be useful for biological, biochemical, and medical studies to elucidate the function of CS.

Acknowledgements We are grateful to Hiroshi Maeda and Yuniko Shibata, Seikagaku Corporation for kindly providing desulfated CH. We thank Minoru Fukayama, Aichi Medical University and Takashi Nirasawa and Nobuyuki Shimura, Bruker Daltonics for assistance in MALDI-TOF MS and MALDI-LIFT-TOF/TOF MS/MS analyses. This work was supported by a special research fund from Seikagaku Corporation by the New Energy and Industrial Technology Development Organization (NEDO) and by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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