Carbohydrate Research 389 (2014) 165–173

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Sialo-CEST: chemical exchange saturation transfer NMR of oligo- and poly-sialic acids and the assignment of their hydroxyl groups using selective- and HSQC-TOCSY

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ARTICLE INFO

Article history:
Received 19 September 2013
Received in revised form 3 February 2014
Accepted 6 February 2014
Available online 18 February 2014

Keywords:
Sialic acid
Polysialic acid
Chemical exchange saturation transfer (CEST)
NMR
Exchange rate constants
Hydroxyl groups assignments

ABSTRACT

Chemical exchange saturation transfer (CEST) is an NMR method that takes advantage of proton exchange between solute and solvent molecules in dynamic equilibrium, enabling the detection of the solute NMR signals with enhanced sensitivity. Herein, we report that the hydroxyl groups in a naturally occurring polysaccharide, α-2,8 polysialic acid in aqueous solution, yield very significant CEST effects even at 37 °C where the resonances of the hydroxyl groups are not directly observed. We also report the assignments of the hydroxyl groups for the polymer and its oligomeric building blocks, from monomer to hexamer. We show that the same assignments can be made by either $^{1}H–^{1}H$ TOCSY methods or $^{1}H–^{13}C$ HSQC-TOCSY methods, to alleviate spectral overlap. Finally, we report the exchange rates of the OH groups with water and show how these rates can be used to select and fine-tune CEST effects.

1. Introduction

Chemical exchange saturation transfer (CEST) is an evolving method currently being used to obtain contrast in MRI. This technique enables the detection of endogenous as well as exogenous agents containing labile protons such as hydroxyl, amine, or amide protons with enhanced sensitivity. In this technique, a selective RF pulse saturates the exchangeable protons and the reduction of the water signal, caused by magnetization transfer, is monitored. Enhancement factors between 10^2 and 10^6 relative to the concentration of the molecules of interest have been reported for certain systems (for reviews see Refs. 1–3). The CEST approach has been used to image tissue pH,[4] map brain proteins through their –NH residues,[5] monitor glycogen concentration in the liver,[6] track glucose accumulation in tumors,[7,8] image myo-inositol and glutamic acid in the brain[9–11] and map a specific gene expression in vivo.[12] Using CEST we also imaged changes in glycosaminoglycan (GAG) levels in human joints in vivo following injury[13] followed by changes in the GAG contents in the nucleus pulposus part of intervertebral disks in vitro.[14] In this paper we demonstrate that CEST is a very sensitive method for the detection of polysialic acid and related oligomers.

Sialic acids (SA) are a family of nine carbon acidic monosaccharides that occur naturally at the end of sugar chains. They are attached to cell-surfaces and proteins as in glycoproteins, and peptides as in mucopolysaccharides. SAs are ubiquitous in nature, especially in mammals, playing a central role in many physiological and pathological processes.[15] The mammalian central nervous system has the highest concentration of SA, most of which is present in gangliosides and glycoproteins.[16] SA also plays a role in the regulation of immune response, and alteration in sialylation is associated with malignancy and metastatic phenotypes of various types of tumors.[17,18] For instance, it has been shown that SA is overexpressed in cells from colon cancer tissues, and its mucosal expression correlated to the metastatic stage.[19] Therefore, SA is an important molecular target for diagnostic and therapeutic approaches.[20]

SA can form linear homo-polymers (pSA) of various lengths, between 8 and 280 residues depending on the biological context, linked through α-2,8 and/or α-2,9 glycosidic bonds. Due to its location at cell surfaces, pSA plays a vital role in biological processes such as embryogenesis,[21] neural cell growth, differentiation, cell–cell interactions, and membrane transport.[22] pSA is also

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http://dx.doi.org/10.1016/j.carres.2014.02.008
0008-6215/

implicated in pathological processes such as tumor progression, especially nodal and distant metastases in lung cancer cells,[23,24] and is an important virulence factor of *Neisseria meningitidis* and *Escherichia coli* K1.[25,26] Recently the structure of pSA was determined in living bacteria by NMR spectroscopy.[27] SA has six exchangeable hydrogen atoms (Fig. 1A) in five hydroxyl groups and one NH group. In the case of pSA, nearly all the residues have only three hydroxyl groups since two are lost when the glycosidic linkage is formed (Fig. 1B). As we show in this article, the proton exchange of the hydroxyl groups makes CEST a sensitive method to detect SA and pSA in vivo. Furthermore, since pSA is present in so many contexts, CEST has great potential in cancer detection and tracking human development, because both involve changes in the quantities of pSA being expressed.

2. Materials and methods

SA (*N*-acetylneuraminic acid) was purchased from Sigma–Aldrich. α-2,8-linked sialic acid oligomers (one to six units) were purchased from Nacalai Inc. (San Diego, CA.).

pSA was obtained from a variant of *E. coli* K12, as previously reported.[28,29] The polymer we studied is a linear homo-polymer with a length varying from 170 to 280 SA residues, linked α-2,8 (Fig. 1B).

2.1. Extraction of pSA from bacterial cell paste

A 10 g sample of *E. coli* K12[30] bacterial paste was mashed with a spatula, while 150 mL of a filtered (0.22 μm Stericup® filter, Millipore Corporation, Billerica, MA), 1 M calcium chloride solution was added to the paste drop-wise over the course of one hour. The homogeneous mixture was then centrifuged at 7500×*g* and 4 °C[30] for 30 min and the cell debris pellet was discarded. The supernatant was adjusted to 25% (v/v) ethanol and stirred on ice for 30 min to precipitate nucleic acids. The resulting mixture was centrifuged at 7500×*g* and 4 °C for 30 min, and the pellet was discarded. The clear supernatant was adjusted to 80% (v/v) ethanol, mixed, and stored at −20 °C freezer for one hour to precipitate crude polysaccharides. The appearance of a cloudy white solid marked the precipitation of pSA material; if the solution remained clear, 1 M $CaCl_2$ was added dropwise (to induce salting-out). The mixture was then centrifuged in a Corex tube at 7500×*g* and 4 °C for 30 min, and the alcohol supernatant was discarded. The crude polysaccharide pellets were combined and dissolved in 40 mL of filtered 10% (v/v) saturated sodium acetate solution and placed on ice. An equal volume of buffered phenol solution (3.57 g phenol/1 mL 10% (v/v) saturated sodium acetate solution) was added for extraction of protein material, and the mixture was stirred for 30 min on ice. The resulting emulsion was resolved by centrifugation at 7500×*g* and 4 °C for 30 min, yielding a biphasic mixture. The upper aqueous layer containing the pSA was carefully decanted by vacuum aspiration. Remaining nucleic acids and proteins were removed by at least one additional cycle of 25% (v/v) ethanol precipitation, followed by re-precipitation of pSA material at 80% (v/v) ethanol, and phenol extraction, as described above. The whiter, purified pSA was dissolved in 10 mL of filtered water and dialyzed against filtered water overnight (Fisher, Pittsburgh, PA; MWCO = 12,000–14,000 kDa). The dialysate was ultracentrifuged at 170,000×*g* and 4 °C for 90 min, and the supernatant was decanted into a pre-weighed conical tube. The solution was shell-frozen on dry ice and then lyophilized to yield 100–300 mg of pSA.

All NMR measurements were performed on Bruker 500 MHz DRX, and Avance III spectrometers.

2.2. CEST measurements

CEST spectra were recorded in aqueous solutions containing 10% D_2O after detuning of the probes to avoid radiation damping. B_1 fields were in the range of 0.6–3.0 μT (25–125 Hz), irradiation time 3 s, and relaxation delay of 7 s (except for amide proton measurements where irradiation time of 10 s was used). The read pulse was in most cases a hard pulse, but using a selective pulse instead gave the same results.

The %CEST was quantified by measuring the asymmetry in the magnetization transfer ratio (MTR_{asym}) according to the following equation:

$$MTR_{asym} = [M(-\omega) - M(\omega)]/M(-\omega)$$

where $M(-\omega)$ and $M(\omega)$ are the water signal intensities with saturation at $-\omega$ and ω from the water signal, respectively.

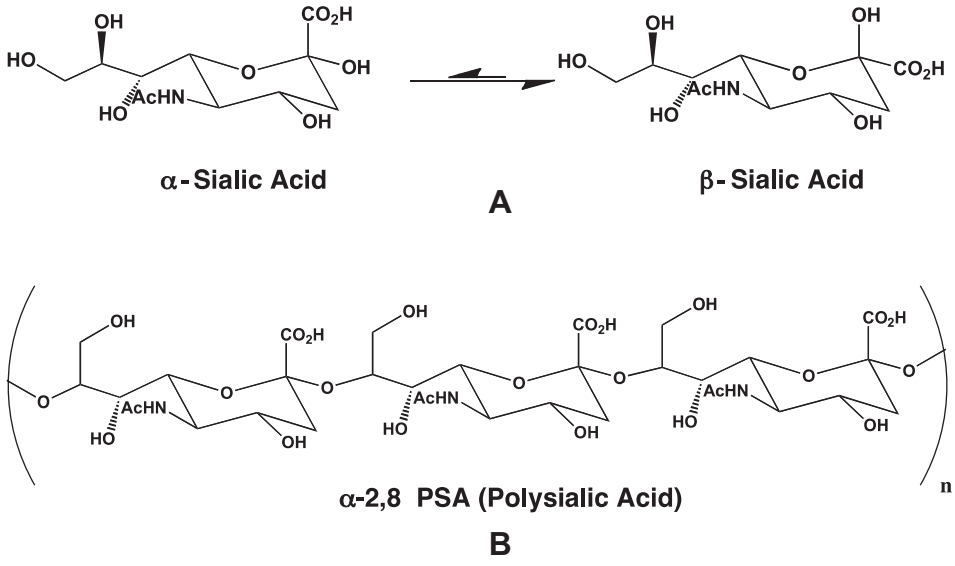

Figure 1. (A) Structure of sialic acid (SA) monomer showing that the monomer is present in dynamic equilibrium between the β and α anomers in a 94:6 ratio, respectively. (B) α-2,8 Linked polymer (pSA), the structure of the subject of the present study, α-2,8 linked pSA.