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# A comparison of glycans and polyglycans using solid-state NMR and X-ray powder diffraction

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**Abstract**

Individual polyglycans and their corresponding monomers have been studied separately for several decades. Attention has focused primarily on the modifications of these polyglycans instead of the simple relationship between the polyglycans themselves and their corresponding monomers. Two polyglycans, chitin and chitosan, were examined along with their respective monomeric units, *N*-acetyl-D-glucosamine (GlcNAc) and (+)D-glucosamine (GlcN) using solid-state proton decoupling Magic Angle Turning (MAT) techniques and X-Ray Powder Diffraction (XRPD). A down-field shift in isotropic $^{13}C$ chemical shifts was observed for both polymers in Cross Polarization/Magic Angle Spinning (CP/MAS) spectra. An explanation of misleading peak assignments in previous NMR studies for these polyglycans was determined by comparing sideband patterns of the polymers with their corresponding monomers generated in a 2D FIve $\pi$ REplicated Magic Angle Turning (FIREMAT) experiment processed by Technique for Importing Greater Evolution Resolution (TIGER). Structural changes in the crystalline framework were supported by XRPD diffraction data.


*Keywords:* Chitin; Chitosan; (+)D-glucosamine; *N*-acetyl-D-glucosamine; Sidebands; Crystalline; Amorphous; Peak assignment; CP/MAS; FIREMAT; TIGER; XRPD

## 1. Introduction

Crustaceans are marine creatures classified under Phylum *Arthropoda* in the Animal Kingdom (Animalia). Lobsters, crabs, prawns, and shrimp are commonly found members of this group. Substances found within the exoskeletons of these living creatures have made a variety of important contributions across a wide variety of applications in medicine, industry, and science. The main component of this skeletal material is chitin [1], $\beta(1\rightarrow4)$-amino-polysaccharide [2,3]. Chitin contains repeating *N*-acetyl-D-glucosamine (GlcNAc) units [4] and is the second most abundant biopolymer next to cellulose [5,6]. Chitin is a wound healing accelerator [7–9] activating macrophages to fight foreign bodies [10–14]. In addition to inflammation recovery, GlcNAc, like chitin, enhances immune function to reduce the proliferation of cancer cells and excessive growth of fiber corpuscle [15–17], activates detoxification of the liver and kidney [18], and alleviates joint pains from arthritis [19].

Chitin is known to be insoluble in almost all simple solvents eliminating the option of liquid state analysis and single-crystal X-ray crystallography. The amide group in chitin is a secondary amide. Removing the acetyl substituent from the amide group in chitin leaves an amine moiety in chitosan. This derivative, chitosan, can be dissolved in 1–4% acetic acid. Deacetylation reduces bulk within the polymeric chain in chitin, allowing chitosan to have more degrees of freedom in chemical structure and to be converted into a unique cationic polysaccharide. Chitosan contributes to many functions [20,21]. Its largest commercial use is as a weight-loss supplement, a major contributor to dietary market [22–24]. It also exhibits antimicrobial [25], anti-cancer [26], anti-aging [27], and antifungal properties [28]. It aids in cartilage replacement

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[29], tissue replenishment [30], and homeostatic osteostimulation [31]. Multichelation techniques are advanced in clinical usage [32], chromatography [33–35], electrochemistry [36], and ground water purification [37]. Chitosan is composed of D-glucosamine (GlcN) monomers [38] which, like chitosan, participate in many physiological functions such as defending against liver inflammation [39], fighting rheumatoid arthritis [40], and monitoring cell escalation [41].

Past studies have used the liquid shift to assign the solid shift for GlcNAc, however, the agreement between liquid and solid isotropic shifts are not ideal. The average solid shift for GlcNAc is about 2.22 ppm downfield from the corresponding liquid shift obtained from the Biological Magnetic Resonance (BMR) data base. When comparing the theoretical monomer shifts with the equivalent experimental shifts, the use of liquid shifts to assign the solid shifts generally has little merit. In the case of GlcN, an even larger discrepancy of about 20 ppm difference in shift assignment was observed in comparison with liquid shifts, which makes solid analysis more doubtful.

Previous NMR studies of chitin were evaluated based on NMR data under lower field conditions, usually in comparison with chemical shifts assignments established with similar systems such as glucose, chitosan, or different polymorphs of chitin [42–44]. Some were compared between solid and solution phases. The chitin in this study proved to be α-chitin, Peaks for C3 and C5 were difficult to separate [45,46] for shift assignment. For the last two decades, many attempts in assigning these two chemical shifts were made. Chemical shifts between C3 and C5 of GlcNAc are 1 ppm apart in both liquid and solid samples. The assignment of these two closely placed peaks has been contested for nearly two decades. Bundle et al. [47] in 1973 assigned $^{13}C$ chemical shift for the α- and β-GlcNAc with the observed chemical shifts of its analogous hexose. The assignments for the axial groups, however, were compared with cyclohexane, inositols, and aldohexoses, which were not directly measured from the monomers themselves. In 1981, Gagnaire in France assigned GlcNAc and its dimers as chitobiose in a complicated solvent system with deuterium labeled on C3 and C4. Gagnaire [48] claimed that C3 and C5 are superimposed. Tanner et al. [49] in 1989 first attempted to assign chitin chemical shifts from different sources using CP/MAS at 7.05 Tesla and found that C3 and C5 gave two partially resolved peaks for α-chitin but only a single broad, asymmetric peak for the β-polymorph with large differences in chemical shift. Tanner determined the spectra of α-chitin and the anhydrous form of β-polymorph are similar, but the use of C3 and C5 chemical shifts is not totally reliable. Detailed differences between α- and β-polymorphs were reported in the earlier literature [50,51]. Varum et al. [52] in 1990 attempted to assign chemical shifts for chitosan in solution, but their study was focused mainly on proton coupling. All these assignments are somewhat tentative.

Simple Solid-state NMR methods generally are not useful for this purpose due to the 1 ppm separation in isotropic chemical shifts between these two carbons. Slow spinning sideband patterns, however much like fingerprints, provide unique information for each carbon signal with respect to the influence of the neighboring atoms and define the uniqueness of each carbon shifts (i.e., no two identical sideband patterns are alike unless the two carbons are identical or indistinguishable) [53,54].

Kono et al. in 2004 addressed the differences between α- and β-polymorphs using solid phase INADEQUATE and HETCOR techniques [55]. Kono measured the scalar coupling between each carbon and determined that the shift assignment describes C4 downfield of C5 and C5 slightly downfield of C3 in α-chitin. With this direct coupling measurement, Kono et al. resolved the assignment problem for α-chitin. With this evidence and our FIREMAT results, we correct the shift assignments for C3, C4, and C5 in the monomer GlcNAc.

FIREMAT [56–58] is a unique technique to extract efficiently the sideband patterns and index them with the isotropic chemical shift simultaneously. More than 25 FIREMAT studies have been published in the past decade covering simple materials like anthracene aerosols [59,60], tobacco leaves [61], small peptides [62], and anti-bacterial compounds [63,64]. None encountered a molecular size (~94 kDa) comparable to chitin. The synergism between FIREMAT and XRPD techniques was applied in this study. FIREMAT successfully extracts sideband patterns for both polyglycans and allows clarification of the peak assignments in GlcNAc and GlcN. XRPD data also give supplementary support of systemic absence of peaks due to low angle scattered diffraction between parallel planes in the polyglycans. The optimized structure of GlcN supports previous literature concerning the presence of hydrogen bonding between C3 and C5 in chitosan, which because of overlapping isotropic peaks allows only one shift and one NMR sideband pattern for these two carbons in chitosan. The two monomers were also examined to help understand the inter-relationship between the polymers with an average chain of 15 or more units.

## 2. Materials

Chitin from shrimp shells and D-(+)-glucosamine hydrochloride (>99%) were purchased from Sigma-Aldrich Chemical, St. Louis, Missouri, USA. *N*-acetyl-D-glucosamine (>99%) was purchased from Fluka Chemical, Japan. All chemicals except chitosan from lobster shells were used as received.

Chitosan extracted from Cape lobster shells with a molecular weight of 94 kDa was received from Vaal University of Technology, South Africa. The extraction procedure was first described in detail by Muzzarelli et al. in the *Chitin Handbook* [13,65]. Chitosan was processed as received by crushing wet chitosan beads into a paste with an agate mortar and pestle. An aliquot of 300 μl each of