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Determination of the distributions of degrees of acetylation of chitosan

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ABSTRACT

Chitosan is often characterized by its average degree of acetylation. To increase chitosan's use in various industries, a more thorough characterization is necessary as the acetylation of chitosan affects properties such as dissolution and mechanical properties of chitosan films. Despite the poor solubility of chitosan, free solution capillary electrophoresis (CE) allows a robust separation of chitosan by the degree of acetylation. The distribution of degrees of acetylation of various chitosan samples was characterized through their distributions of electrophoretic mobilities. These distributions can be obtained easily and with high precision. The heterogeneity of the chitosan chains in terms of acetylation was characterized through the dispersity of the electrophoretic mobility distributions obtained. The relationship between the number-average degree of acetylation obtained by solid-state NMR spectroscopy and the weight-average electrophoretic mobilities was established. The distribution of degrees of acetylation was determined using capillary electrophoresis in the critical conditions (CE-CC).

1. Introduction

Chitosan is a polysaccharide derived from the *N*-deacetylation of chitin. Chitin is an abundant polysaccharide and it naturally occurs in the shells of arthropods such as shrimps, crabs and the cell walls of yeasts [1]. The molecular structure of the polysaccharide chitosan includes varying proportions of D-glucosamine and *N*-acetyl-D-glucosamine units (Fig. 1). Chitosan has several desirable properties that allowed it to become a significant area of research: it is biocompatible, biodegradable, antimicrobial and antifungal [2,3]. In characterizing chitosan samples, a properly established structure-property relationship is required to assist in tailoring individual samples for specific uses [4,5]. Therefore, accurately characterizing the supramolecular structure of chitosan is essential to understanding its properties. However, due to an incomplete understanding of chitosan's complex structure, several limitations exist in its characterization.

The degree of acetylation (*DA*) is defined as the fraction of *N*-acetyl-D-glucosamine units. The distributions of *DA*s correspond to the relative amount of chitosan macromolecules having a given *DA* plotted against *DA*. The existence of this distribution means that chitosan chains having different degrees of acetylation are present in a given sample. Although it has been well documented that a distribution of *DA*s exists (not all chitosan chains have the same *DA*), this is often overlooked [6,7]. Due to its natural origin and the variation in processing conditions, chitosan can have broad distributions of *DA*s. The existence and importance of the distributions of the *DA*s has been revealed through a coupling of size-exclusion chromatography (SEC) separation with ^{1}H NMR spectroscopy detection; however, the distributions still have not been determined [6]. SEC [8], gradient SEC [9] and gradient liquid adsorption chromatography [10] have been used to determine chemical composition against molar mass (named "chemical composition distribution"), as well as distributions of composition in some cases [10,11], for various copolymers, but not for chitosan. In addition, the

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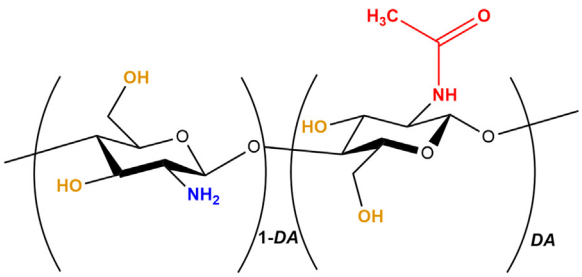

Fig. 1. Chemical structure of chitosan (of degree of acetylation *DA*) and of chitin (for *DA* = 1).

distribution of compositions (or distribution of *DA*s for chitosan) have never been determined. The presence of a distribution of *DA*s can be attributed to the production of chitosan from chitin. Chitin exists in 3 forms, α, β and γ [12]. The α and β forms vary in reactivity due to their structure. The α form is strongly stabilized by intra- and inter-sheet hydrogen bonds conversely to the β form which does not exhibit hydrogen bonding between successive chains. This results in β chitin being more soluble [13] as well as reactive [14]; however, the α form is more industrially available due to its natural abundance. Thus, heterogeneity is influenced by the structure of the chitin used in the production of chitosan.

The treatment of chitin to produce chitosan can also be a homogeneous or a heterogeneous process. The heterogeneous method was shown to be more effective in the deacetylation and is therefore more researched and used. A study in the heterogeneous deacetylation of both α and β chitin showed that in the deacetylation process two domains of deacetylation existed. There was also differences between α and β chitin which could be attributed to the structure of the α form preventing the accessibility of certain sites [15]. It was also identified that homogeneous deacetylation would increase the chance of a random distribution whereas heterogeneous deacetylation could cause blocks of acetylated/deacetylated units [16]. Block deacetylation is then attributed to the starting materials crystallinity in which the more amorphous region had the reactive acetylated sites more readily available and allowed a random distribution of acetylation [15].

Methods used to characterize chitosan by its average degree of acetylation/deacetylation previously include FTIR [17], Raman [18] and NMR spectroscopy [7,19–21]. To determine the distribution of *DA*s, we require a method to identify the average degree of acetylation and a separation technique to identify the distribution of *DA*s. The most widely used method to separate polymers, especially polysaccharides, is size-exclusion chromatography (SEC) [22]. SEC separates polymers by their size (hydrodynamic volume) [23]. For chitosan this depends on both the molar mass and the degree of acetylation. Further, SEC analysis of chitosan has been plagued with aggregation [19]. Separation by composition is possible using liquid chromatography in the critical conditions [24] however, this can be extremely tedious and problematic [25] and is quasi-exclusively applied to organic systems, and not aqueous systems as used for chitosan. For this reason we propose to use free solution capillary electrophoresis (CE) for the analysis of chitosan by its degree of acetylation and its distributions [26,27].

CE has been proven to effectively separate the polysaccharide pectin by composition. Several studies reported the separation of pectin by its degree of substitution (*DS*, which may include esterification) [28]. Other research involved the use of capillary electrophoresis to determine the *DS* of caboxymethylcellulose [29]. Further, CE was proven to be a robust, reproducible method in the detection of impurities in the negatively charged biomolecule heparin [30]. The conditions of separation fall above the Manning condensation; however, separation is still possible although with a low selectivity. The separation of heparin is therefore very similar to that of chitosan. The ability to separate polymers by their composition independently from their molar mass is what differentiates CE from the aforementioned methods and makes it particularly appropriate for our study. It has been proven in the study of polylysine in which the electrophoretic mobility did not vary for a degree of polymerization above 4 [31]. This method is described as "in the critical conditions" (not referring to the separation mechanism but to the absence of separation by molar mass) and has been reviewed recently [26]. CE has also previously been used in chitosan analysis including both native [7,32] and modified chitosan [33].

To allow meaningful distributions to be obtained, the raw data, UV absorption against migration time, first needs to be converted into a distribution of electrophoretic mobilities, [34] and finally into chemical composition distributions. Different expressions of the dispersity of this distribution have recently been developed [27]. The dispersities determined from these different expressions are compared in this work in the case of chitosan. Analysis of the distributions of electrophoretic mobilities of cationic copolymers reveals information regarding their heterogeneity of composition. Understanding the heterogeneity of composition allows for property-structure relationships to be established. Expressions for the composition distributions were also established and tested for some block copolymers [27]. In this study, composition distributions for chitosan (under the form of distribution of *DA*s), or any statistical copolymer, are obtained for the first time.

2. Experimental section

2.1. Materials

Chitosan powders were purchased from Sigma-Aldrich, Castle Hill, Australia and from AK Biotech LTD, Jinan, China (Table S1). Samples were prepared at 1 g L^{-1}. Orthophosphoric acid (85%) and boric acid were purchased from BDH AnalR, Merck Pty Ltd. Acetic acid (AcOH, glacial, 99%) and hydrochloric acid (32%) were purchased from Unilab. Poly(diallyldimethyl ammonium chloride) (PDADMAC 20% in H_2O), alginic acid sodium salt, poly(allylamine hydrochloride) (PAlAm), sodium hydroxide pellets, lithium hydroxide, sodium chloride, hexaamminecobalt(III) chloride (≥99.5%), dimethyl sulfoxide (DMSO, ≥99.5%) and adamantane (99%) were purchased from Sigma-Aldrich. Sodium dihydrogen orthophosphate was purchased from Univar. Three ^{13}C singly labeled alanines were purchased from Cambridge Isotope Laboratories. All water used in this study was of Milli-Q quality. Sodium borate buffer (75 mM) was prepared from 0.5 M boric acid in Milli-Q water, titrated to pH 9.20 with 10 M sodium hydroxide, and diluted with Milli-Q water. Sodium phosphate buffer (100 mM) was prepared from 0.5 M sodium dihydrogen phosphate, titrated with phosphoric acid, and diluted with Milli-Q water. Lithium phosphate buffer (100 mM) was prepared from 85% orthophosphoric acid, titrated to pH 2 with 10 M LiOH and diluted with Milli-Q water. Lithium phosphate, sodium borate and sodium phosphate buffers were sonicated for 5 min and filtered with a Millex GP polyethersulfone (PES) syringe filter (0.22 μm) before use.

2.2. Methods

2.2.1. Capillary electrophoresis

Free solution capillary electrophoresis (CE) was carried out using an Agilent 7100 CE (Agilent Technologies, Waldbronn, Germany) instrument equipped with a diode array detector, contactless conductivity detector (TraceDeC, Innovative Sensor Technologies GmbH, Austria) and external circulating bath with MX temperature controller (Polyscience, USA). Polyimide-coated