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Examining the effect of common nitrosating agents on chitosan using a glucosamine oligosaccharide model system

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ABSTRACT

Chitosan has received substantial attention as a biomaterial due to its unique properties. It has become increasingly common to derivatize chitosan to produce nitric oxide (NO)-releasing materials that exert various therapeutic effects through the action of NO. It is generally the case that these NO-releasing polymers are prepared by exposure to high-pressure NO or nitrosating agents like nitrous acid (HNO_2) or alkyl nitrites (RONO). In our study, mass spectrometry and spectroscopic methods demonstrate that both monomeric and oligomeric glucosamine experience chemical alteration after exposure to HNO_2-based nitrosating conditions from the literature. In polymeric chitosan, HNO_2-based nitrosating conditions were found to induce degradation through the formation of 2,5-anhydro-D-mannose and oligosaccharides. In contrast, the RONO *tert*-butyl nitrite and high-pressure NO were not found to significantly degrade or otherwise alter the structure of glucosamine or its oligomers, supporting the suitability of these approaches.

1. Introduction

Nitric oxide (NO) is ubiquitous in mammalian biochemistry, where it is produced by the vascular endothelium to regulate vasodilation, immune cells (*e.g.*, macrophages) as an antimicrobial agent, and nerve cells as a critical neurotransmitter (Moncada, Palmer, & Higgs, 1991). These physiological roles have led to the conscription of exogenous NO as a therapeutic agent. Because NO is highly reactive and exhibits a short half-life under physiological conditions, the use of more stable NO prodrugs has greatly facilitated the continuing development of NO-based therapies. These prodrugs typically take the form of molecules or specific functional groups that spontaneously decompose to form NO (Miller & Megson, 2007). In recent years, the fabrication of biomaterials ranging from bulk polymers to nanomaterials that exhibit NO-forming properties has received considerable attention (Wo, Brisbois, Bartlett, & Meyerhoff, 2016; Yu, Guowei, Liu, Ma, & Xue, 2018). Due to their natural origin and medically-useful properties, polysaccharides have been frequently modified to include NO donor moieties. As early as 1996, Smith et al. demonstrated that NO-forming *N*-diazeniumdiolate groups could be grafted to the branched polysaccharide dextran (consisting of α-1,6 and α-1,3-linked glucose units) (Smith et al., 1996). This principle was later applied to the glycosaminoglycan heparin, which is commonly used in medicine as an anticoagulant (Saavedra et al., 2000). More recently, chitosan (a naturally-derived copolymer consisting of β-1,4-linked D-glucosamine [GlcN] and *N*-acetyl-D-glucosamine [GlcNAc]) has been investigated as a platform for NO delivery applications. Lu et al. produced water-soluble NO-releasing chitosan oligosaccharides (COS) bearing *N*-diazeniumdiolate groups that were found to exhibit pronounced antibacterial effects against *Pseudomonas aeruginosa* (Lu, Slomberg, & Schoenfisch, 2014). It was subsequently demonstrated that the antibacterial properties of NO-releasing COS could be preserved when using the *S*-nitrosothiol functional group as an alternative NO donor (Lu, Anand, Hunter, Soto, & Schoenfisch, 2015). The versatility of chitosan as a biomaterial and the broad therapeutic potential of NO has led to the use of chitosan-derived NO-releasing materials for proposed applications that include disruption of bacterial biofilms (Reighard & Schoenfisch, 2015), transdermal delivery of NO (Pelegrino, Weller, Chen, Bernardes, & Seabra, 2017), treatment of Crohn's disease (Shah, Martinho, Socha, Pin Reis, & Gibhaud, 2015), wound-healing (Kim et al., 2015; Lutzke, Pegalajar-Jurado, Neufeld, & Reynolds, 2014), and prevention of platelet adhesion (Simon-Walker et al., 2017). In general, this category of material is intended to exert therapeutic effects through the spontaneous production of NO in a biological environment.

Chitosan is typically obtained from alkaline deacetylation of chitin, a biopolymer of GlcNAc that occurs as a structural component of the

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shells of marine crustaceans and fungal cell walls (Rinaudo, 2006). This deacetylation is rarely complete and yields a copolymer consisting predominantly of GlcN (> 50%) and GlcNAc that can be dissolved in aqueous acid and exhibits hemostatic, antimicrobial, and wound-healing properties (Croisier & Jérôme, 2013). These properties have been paired with the known physiological effects of NO to produce NO-releasing chitosan derivatives for therapeutic applications. The primary approach to the development of such materials involves the chemical derivatization of chitosan to include thiol or secondary amine groups, which be subsequently converted to *S*-nitrosothiols or *N*-diazeniumdiolates that generate gaseous NO in response to an appropriate trigger (*e.g.*, light or pH). In the specific case of thiol-bearing chitosan derivatives, treatment with HNO_2 may form *S*-nitrosothiols according to the equation RSH + HNO_2 → RSNO + H_2O (Dicks, Beloso, & Williams, 1997). Because chitosan is composed of structurally complex repeating units derived from both GlcN and GlcNAc, there is significant potential for undesirable reactions with other functional groups. For example, it is known that the reaction of HNO_2 with alcohols results in the formation of alkyl nitrites (Wang et al., 2002). More importantly, the reaction of HNO_2 with aliphatic primary amines results in the initial formation of a primary nitrosamine, followed by further reaction to yield an unstable aliphatic diazonium intermediate (Collins, 1971). In the case of GlcN, nitrogen gas is displaced from this intermediate by an internal rearrangement, resulting in ring-contraction and the formation of 2,5-anhydro-D-mannose (2,5-AM) (Collins, 1971). As an example, Claustre et al. (among others) previously prepared 2,5-AM by the deamination of GlcN hydrochloride using $NaNO_2$ and a strong cation-exchange resin (Claustre et al., 1999; Horton & Philips, 1973). It has been established by substantial prior work that this reactivity extends to polymers that include GlcN structural units, often inducing depolymerization (Conrad, 1995; Shively & Conrad, 1976).

In an idealized scenario, the preparation of an NO-releasing chitosan derivative is achieved through a sequence of reactions in which a precursor to an NO donor (a thiol in the case of *S*-nitrosothiols and secondary amine in the case of *N*-diazeniumdiolates) is grafted to the polysaccharide, followed by exposure of this conjugate to chemical agents that are intended to form an NO donor from the grafted functional group. However, the ability of the primary amine group of GlcN to react under such conditions renders the polymer susceptible to chemical alteration or degradation that may impact its proposed structure or function. Both nitrous acid (HNO_2) and alkyl nitrites (RONO) function as *nitrosating agents* (formal donors of NO^+) in solution that may effect the conversion of nucleophilic functional groups to nitrosated derivatives (Miller & Megson, 2007; Wang et al., 2002). This reactivity is often exploited in the synthesis of NO-releasing compounds such as *S*-nitrosothiols through treatment of thiol precursors with HNO_2. For instance, Lu et al. treated a solution of thiol-bearing chitosan oligosaccharides with sodium nitrite under acidic conditions to produce an *S*-nitrosated derivative (Lu et al., 2015). Simon-Walker et al. immersed titania nanotube arrays coated with a chitosan-thioglycolic acid conjugate in *tert*-butyl nitrite (*t*-BuONO) to impart NO release properties through the putative formation of *S*-nitrosothiol functional groups (Simon-Walker et al., 2017).

However, GlcN units in chitosan may undergo undesirable reactions in the presence of HNO_2/RONO that result in significant structural alteration. The *N*-nitrosation of aliphatic primary amines by HNO_2 is known to result in deamination through diazotization and subsequent displacement of N_2 (Collins, 1971). This reactivity occurs in the Demjanov and Tiffeneau-Demjanov rearrangements and has been clearly established to occur in the case of glucosamine, where it results in the formation of 2,5-AM (Plutschack, McQuade, Valenti, & Seeberger, 2013; Claustre, Bringaud, Azema, Baron, Perie, & Willson, 1999), as shown in Fig. 1A. The deamination of chitosan itself using HNO_2 is accompanied by depolymerization and the production of 2,5-AM in a manner analogous to the reaction of monomeric GlcN (Horton & Philips, 1973). The ability of HNO_2 to initiate the deamination and depolymerization of polysaccharides is not confined to chitosan, and also occurs in the case of heparin and other polymers that contain GlcN units (Shively and Conrad, 1976; Vilar et al., 1997; Conrad, 1995). Other functional groups present in chitosan may also react in the presence of nitrosating agents, including alcohols (Doyle, Terpstra, Pickering, & LePoire, 1983) and amides (Lobl, 1972).

It is evident that exposure of chitosan to nitrosating agents is not chemically innocuous, and may result in depolymerization or other structural alterations. Curiously, this phenomenon has been largely ignored during the development of NO-releasing materials derived from chitosan. NO-releasing chitosan derivatives are rarely subjected to rigorous analytical scrutiny following their synthesis, and it remains unclear if the original structure and behavior of the polysaccharide is retained, yielding the possibility of chemical modifications to its structure such as those shown in Fig. 1B. This work seeks to elucidate the structural changes that may be induced in chitosan following exposure to common conditions used to produce NO-releasing derivatives. The chief structural constituents of chitosan (GlcN and GlcNAc), a COS model system, and chitosan itself were variously exposed to HNO_2, *t*-BuONO, and gaseous NO to replicate typical literature protocols for the synthesis of *S*-nitrosothiols and *N*-diazeniumdiolates, the most frequent NO donors grafted to chitosan. The products were characterized through a combination of ^1H and ^{13}C nuclear magnetic resonance (NMR), attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR), and time-of-flight mass spectrometry (MS).

2. Materials and methods

2.1. Materials

Chitosan oligosaccharides (COS, < 3000 MW, 93% deacetylated) were obtained from Carbosynth (Compton, Berkshire, UK). Chitosan (PROTASAN UP B 90/20, 80 kDa MW, 96% deacetylated) was obtained from NovaMatrix (Sandvika, Norway). D-Glucosamine hydrochloride (99.9%) was obtained from Calbiochem (Darmstadt, DE). *N*-Acetyl-D-glucosamine (< 98.0%) was obtained from TCI (Portland, OR). Sodium methoxide (98%) and *tert*-butyl nitrite (*t*-BuONO, 90%) were obtained from Alfa Aesar (Haverhill, MA). Anhydrous ACS grade diethyl ether, ACS grade sodium nitrite (97.0%), concentrated hydrochloric acid, HPLC grade methanol, and LC–MS grade water were obtained from EMD Millipore (Burlington, MA). Anhydrous ethanol was obtained from Pharmco (Brookfield, CT).

2.2. Spectroscopic characterization

Infrared (IR) spectra were collected using attenuated total reflectance (ATR) on a Nicolet 6700 FT-IR spectrometer (Thermo Electron Corp., Madison, WI, USA) fitted with a Smart iTR ATR sampling accessory and a ZnSe crystal plate. Nuclear magnetic resonance (NMR) spectra (^1H) were acquired using a Bruker Avance Neo 400 spectrometer. Deuterium oxide (D_2O) was used to dissolve GlcN, GlcNAc, and COS samples, while a 1 M solution of deuterium chloride in D_2O (DCl/D_2O) was generally used to dissolve chitosan. Samples were dissolved at a concentration of either 50 mg mL^{-1} (COS and chitosan) or 100 mg mL^{-1} (GlcN and GlcNAc) and compounds that experience pronounced mutarotation were allowed to equilibrate for 1 day prior to analysis.

2.3. Mass spectrometry

Mass spectra were acquired using an Agilent 6224 time-of-flight mass spectrometer (Agilent, Palo Alto, CA, USA) equipped with a dual electrospray ion source. Samples were prepared at a concentration of ~1 mg ml^{-1} in LC/MS grade water. The solutions were injected directly into the source without a pre-separation step at a flow rate of 0.220 mL/min using an Agilent 1260 high-performance quaternary