Effects of Molecular Weight and Degree of Acetylation on the Release of Nitric Oxide from Chitosan-Nitric Oxide Adducts

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ABSTRACT: A series of chitosans with different molecular weights and degrees of acetylation (DAs) are reacted with nitric oxide (NO) to form $[NONO]^-$ groups. The effects of molecular weight and DA on NO release are investigated by Griess assay. Heterogeneous reaction of NH_2 groups of chitosan with NO was shown to be influenced greatly by the crystalline form of chitosan. Total NO release exhibited a bell-shaped distribution at different DAs ranging from 6 to 95%, peaking at about 50%. When DA is held constant, total NO release is directly proportional to the molecular weight. X-ray diffraction patterns indicate that the total NO release of chitosan-NO adducts is in general agreement with the intensity of reflections at low Bragg angles ($2\theta = 8.6°$–$11.1°$), and in turn, a relaxed hydration crystalline form and NO molecules could penetrate this crystal and react with the chitosan molecules. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 117: 2183–2188, 2010

Key words: chitosan; nitric oxide; diazeniumdiolates; nitric oxide release; crystal structures

INTRODUCTION

Nitric oxide (NO) has very important functions in biological systems. It is generated in various tissues from the amino acid L-arginine by different forms of nitric oxide synthase (NOS). Insufficient or excessive production of NO may cause many ailments including hypertension, chronic heart failure, and respiratory distress.[1,2]

To treat ailments due to subphysiological NO level, many new NO donors have been designed and synthesized. Diazeniumdiolate, i.e., a compound containing the anionic $[N(O)NO]^-$ functional group, is one of the most important groups of these NO donors (Fig. 1), typically synthesized by reactions of a nucleophile (secondary amines) with NO at elevated pressure.[3] They have a variety of physical forms and spontaneously generate NO under physiological conditions at different rates. However, some problems, such as toxicity and biocompatibility of the nucleophile, are usually associated with these types of NO donors. Some diazeniumdiolate carriers, such as diethylenetriamine, are found to be harmful for the body, and free polyethylenimine to induce widespread cell death.[4,5] It is therefore important to look to new materials as diazeniumdiolate carriers that degrade spontaneously to give harmless byproducts after releasing NO.

Bohl and West[6] have synthesized NO-releasing hydrogel materials using biocompatible polymers of poly(ethylene glycol) (PEG) or poly(vinyl alcohol) as carriers and poly(amino acid) (poly-L-lysine or L-cysteine) as the nontoxic nucleophile covalently bound to these polymers. These materials can form tissue coatings to provide local and sustained NO therapy following vascular or dermal injury.

Jun et al.[7] have incorporated a diazeniumdiolate-modified NO-producing peptide ($SGG[K[N(O)NO]^-]_4GGS$) into a polyurethane to improve the thromboresistance of this biocompatible polymer. The results show that NO production by polyurethane films occurred for $\sim$ 2 months under physiological conditions, inhibiting platelet adhesion and smooth muscle cell proliferation, while encouraging endothelialization and may be suitable as a candidate material for small-diameter vascular grafts.

Smith et al.[8] reported incorporation of the functional group $X[NONO]^-$ (where X is a nucleophile) into polymeric matrices that could be used for altering the time course of NO release and/or targeting it to tissues with which the polymers are in physical

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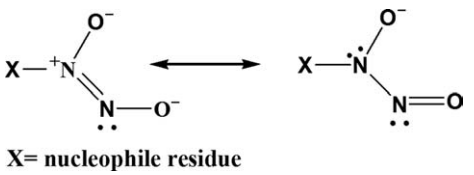

Figure 1 Resonance forms of the diazeniumdiolate group.

contact. Three structure types were investigated: (1) X[NONO]$^-$ noncovalently distributed throughout the polymeric matrix; (2) diazeniumdiolate groups covalently bound to pendent polymer sidechains; and (3) diazeniumdiolate groups covalently bound directly to the polymer backbone. PEG, polycaprolactone, dextran, and polyethylenimine were used as polymer matrices, and polyamines (DEA, Spermine, and DPTA) as the noncovalently bound nucleophiles. The results suggest that the polymers containing the [N(O)NO]$^-$ functional group may hold considerable promise for a variety of biomedical applications in which local delivery of NO is desired.

Chitin is the second most abundant polysaccharide found in nature and chitosan is its N-deacetylated derivative. It is a biodegradable and biocompatible cationic polymer and can take different physical forms, such as films, fibers, powder, and solution. It has been widely used in many different biomedical applications. The primary amine group of chitosan is reactive toward alkylation to give a secondary amine. These secondary amine groups can act as a nucleophile and when NO is attached to it, it becomes an effective NO donor. Smith and Serhatkulu[9] have synthesized a series of chitosan derivatives, reacted them with NO to form many NO donors, and studied their different NO release rates and physical forms. The release of NO from all the synthesized chitosan derivatives corresponds to the low levels of NO. To increase NO capacity, water-soluble polyethyleneimine was grafted onto chitosan to obtain an insoluble carrier. These studies have three potential medical targets: wound healing, treatment of erectile dysfunction, and pulmonary hypertension.

It is known that the [NONO]$^-$ group binds to the nucleophile preferentially via a secondary amine.[10] The primary amine group that reacts with NO releases very low amounts of NO and it is also unstable.[11] However, Bohl and West and Jun et al. studies indicated that the primary amine of lysine can be reacted with NO to give a material capable of giving sustained NO release.[6,7] However, nothing that shows whether the NH_2 group of chitosans can be reacted with NO and form [NONO]$^-$ groups has yet been published. In this study, a series of chitosan-NO adducts with different molecular weights and degrees of acetylation (DAs) were synthesized, and the effect of molecular weight and DA on the release properties studied.

EXPERIMENTAL

Materials and instruments

Two commercial chitosan samples (Jinhu, China) were prepared from crab shell, with molecular weights of 1330 (M133) and 78 KDa (M78), and degrees of acetylation of 10 and 6%, respectively. Nitric oxide (gas, 99%) was purchased from Foshan Kodi Gas Chemical Industry, China. All other chemicals were of reagent grade and used without further purification. The instrument used for ultrasonic degradation was CQX25-06, by Branson Ultrasonics (Shanghai), China.

Ultrasonic degradation

Chitosan samples with the same degree of acetylation but different molecular weight were obtained by ultrasonic degradation.[12] Chitosan M133 was dissolved in acetic acid aqueous solution (5%, v/v) to a concentration of 1% and then ultrasonically degraded for various lengths of time (0–40 h) at 60°C. The degraded samples of chitosan were precipitated with 2.0*M* NaOH solution, washed with water until neutral, and then dried at 60°C under vacuum.

The DAs of these samples were determined by potentiometry.[13] The molecular weight of chitosans was determined by the capillary viscometry method using an Ubbelohde viscometer at 25°C.[14] The results are listed in Table I.

Preparation of chitosan with various degrees of acetylation

Chitosan samples with different DAs but with the same molecular weight were prepared by homogeneous reacetylation.[15] A commercial sample of chitosan M78 (DA6%) was dissolved in 0.1*M* CH_3COOH. The solution was passed through filter paper to remove insoluble material and gel particles, then precipitated by addition of 1*M* NaOH solution, washed with deionized water until neutral, and dried at 60°C under vacuum.

Aliquots (2 g) of the products were redissolved in 0.1*M* CH_3COOH (200 mL) and diluted with methanol (350 mL). Then to each solution was added, with vigorous stirring, a further 50 mL of methanol containing the amount of acetic anhydride required to meet the target level of N-acetylation. After standing

TABLE I
The Degrees of Acetylation and Molecular Weights of Chitosan Obtained by Ultrasonic Degradation

	Degree of acetylation (%)	Molecular weight (×104 Da)
M133	10	133
M129	12	129
M102	12	102
M61	11	61

at room temperature for 24 h, the N-acetylated products were precipitated by adding concentrated aqueous ammonia solution, filtered off, washed to neutral with 75% aqueous methanol, and then dried at 60°C under vacuum. The DAs of these products, as determined by potentiometry and FTIR spectroscopy,[16] are given in Table II.

Synthesis of chitosan-NO adducts

To synthesize the diazeniumdiolate derivatives, chitosan (0.6–0.7 g, 0.004 mol) was suspended in the solvent prepared from MeOH (50 mL) and NaOMe (1.3 g, 0.012 mol) in a molar ratio of $[Na^+]/[NH] = 3$. The high-pressure reactor was first flushed with nitrogen (N_2) and then degassed under vacuum. Fresh NO gas was introduced to the compounds at 80 psi for 7 days. After the reaction was complete, the reactor was again flushed with N_2. The products were filtered, washed with ether, and dried at room temperature under vacuum. They were stored in an airtight container in desiccators at −20°C.

UV spectroscopy

The product of chitosan-NO adducts (50 mg) was suspended in 5 mL of deoxygenated phosphate buffer saline (PBS) solution (pH 7.4) in a sealed centrifugal tube and kept at a 37°C water bath with continuous stirring. At appropriate time intervals, the solution was centrifuged for 10 min, and then 1 mL of the upper layer extracted and diluted with 2 mL of PBS solution. UV spectra of the solution were obtained with Lambda 20, Perkin-Elmer. The characteristic band of the $[NONO]^-$ functional should be observed at about 250 nm.

NO release study

An established method for NO detection is the colorimetric Griess reaction,[17] which entails measuring nitrite or nitrate content in PBS (0.2*M*, 5 mL) solution. Griess reagent I is a 0.1% β-naphthylethylenediamine dihydrochloride aqueous solution, and Griess reagent II is prepared from 1% sulfanilamide in 5% phosphoric acid. The standard curve was generated using 0–100 μ*M* sodium nitrite in PBS solution. About 5 mg of Chitosan-NO adduct was dispersed in 5 mL of PBS and stirred in a sealed centrifugal container at 37°C in a water bath. At appropriate time intervals, the solution was centifuged for 10 min, and then 1 mL of the upper layer was extracted (replenished with 1 mL of fresh PBS) and combined with Griess reagent I (1 mL) and II (1 mL). Then, the solution was incubated for 15 min at room temperature, protected from light. A purple/magenta color forms immediately. The maximum absorbance was read at 540 nm on a UV–vis spectrophotometer. The total amount of NO release was calculated as follows:

$$r_1 = C_1 \times 0.005$$
$$r_2 = C_2 \times 0.005 + C_1 \times 0.001$$
$$r_3 = C_3 \times 0.005 + C_2 \times 0.001 + C_1 \times 0.001$$
$$r_4 = C_4 \times 0.005 + C_3 \times 0.001 + C_2 \times 0.001 + C_1 \times 0.001$$
$$\ldots\ldots$$

where r_n stands for the amount of drug released, and C_n stands for the concentration of drug, during each time period.

Powder X-ray diffraction

The powder X-ray diffraction (XRD) measurements of chitosans were performed on an X-ray diffractometer (Model 6000, Shimadzu, Japan) with scanning range of $3° < 2\theta < 40°$ and scanning rate of 6°/min, using Cu Kα radiation.

RESULTS AND DISCUSSION

The synthesis and characterization of chitosan-NO adducts

Since Drago and Paulik[3] first reported N-bond diazeniumdiolate in 1960s, direct reaction of NO with an amine remains the only useful methods of preparation.[18]

TABLE II
DAs of Chitosan Samples After Reacetylation

	Degree of acetylation (%)
D6	6
D18	18
D29	29
D39	39
D52	52
D62	62
D75	75
D95	95

Scheme 1 Forming of diazeniumdiolate derivatives of chitosan

Four samples of chitosan with different molecular weights (Table I) and eight samples with different DAs (10–95%, shown in Table II) were reacted with NO gas under 80 psi to form diazeniumdiolate derivatives of chitosan (Scheme 1). A detailed discussion of the vibrational spectroscopy of an N-bound diazeniumdiolate has been presented.[19] IR spectra of the diazeniumdiolate group exhibit three characteristic bands of which two may be attributed to N—O stretching (1225–1210 and 1187–1155 cm^{-1}) and one to N—N stretching (1131–1129 cm^{-1}). They are however obscured by a strong broad band at 1100–1200 cm^{-1} attributable to chitosan itself, and one can tell only from UV spectra that synthesis of chitosan-NO adducts has been successful.

UV spectroscopy is the best method for characterization of *N*-diazeniumdiolates. UV spectra of diazeniumdiolates measured in basic solution typically show an absorption maximum at 250 nm with absorbances in the range from 7.2 to 9.4 Mm^{-1}.[18] The UV spectra of chitosan-NO adducts (D52, water-soluble) were taken in 0.2*M* PBS solution (pH 7.4) and 0.1*M* sodium hydroxide solution, respectively, giving similar results (Fig. 2). A characteristic absorption at 253 nm indicates the presence of an $[NONO]^-$ group. Absorption intensity decreases with time in PBS solution but remains constant in NaOH solution, consistent with the properties of diazeniumdiolates that the adduct decomposes to regenerate its precursors (nucleophile and NO) at a rate that increases as pH decreases.[20]

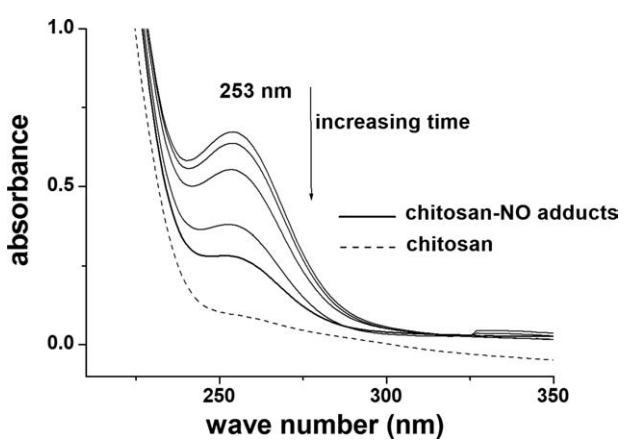

Figure 2 UV spectra of chitosan-NO adducts in PBS solution (0.2*M*, pH 7.4).

Effect of degree of acetylation on release of chitosan-NO adducts

The release of NO from the chitosan-NO adducts was determined using Griess assay in PBS (pH 7.4) at 37°C. The NO release profiles of chitosans with different DAs, ranging from 6 to 95%, are shown in Figure 3. A burst release occurs for all the chitosan-NO adducts and the half-lives of the NO release were less than 5 min. The bell-shaped graph shows that the total release of NO reaches a maximum at around 50% acetylation. The results suggest that 50% acetylation gives the best binding performance. It is well known that 50% acetylated chitosan can be soluble in water. This may be attributed to random distribution of N-acetyl groups disrupting the regularity of the crystal structure, resulting in an increase of amorphous regions. To confirm this, the XRD measurements of chitosans with different DAs were carried out (Fig. 4), and the relationship between peak intensities of the reacetylated chitosans at low Bragg angles and DAs is shown in Figure 5.

The crystal structure of chitosan has been studied extensively. Six polymorphs have been proposed for chitosan: tendon chitosan, annealed, I-2, L-2, form I, and form II.[21] As shown in Figure 4, the original chitosan shows the strongest reflection at $2\theta = 20°$, coinciding with the pattern of the form II crystal. With increasing acetylation, the intensity of

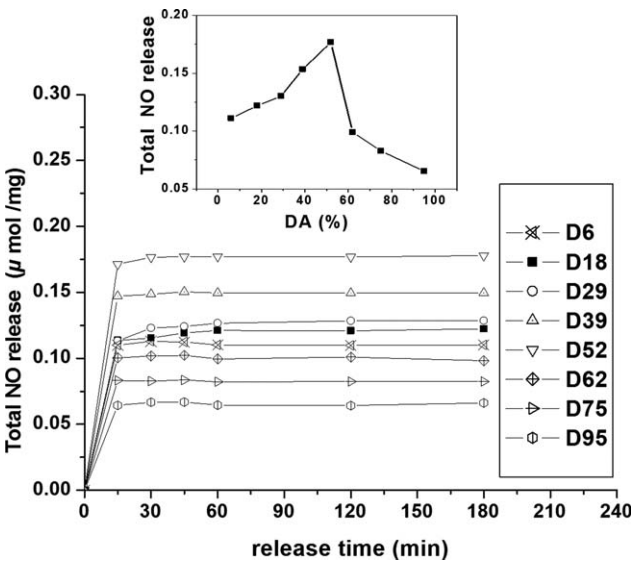

Figure 3 release profiles for chitosan with different degrees of acetylation in PBS (pH 7.4) at 37°C.

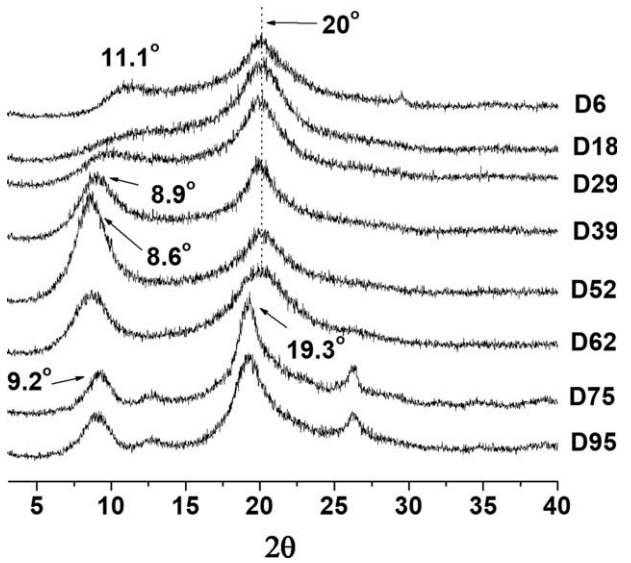

Figure 4 X-ray diffraction patterns of chitosan with different degrees of acetylation.

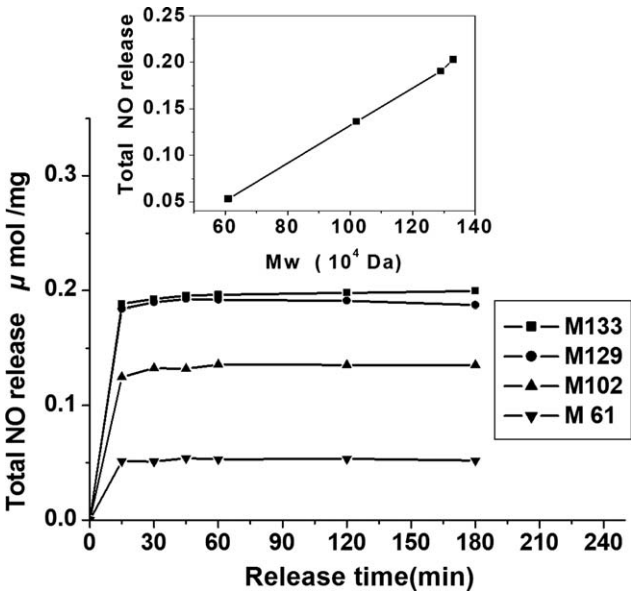

Figure 6 NO release profiles for chitosan with different molecular weights in PBS (pH 7.4) at 37°C.

reflection at low Bragg angles 2θ = 11.1°–8.6° first increases remarkably and then decreases slightly. There is an inverse change of the reflection at about 2θ = 20°. For chitosan D52, the strongest reflection appears at 2θ = 8.6°, indicating that a crystalline form transition occurs with the increase of DA. The "form II" crystal with constrained chain conformation converted to "form I" that has a more extended chain structure. When the degree of acetylation is more than 75%, the "form I" crystal converts to a crystalline form of chitin, which is insoluble in any aqueous acid solution and difficult to biodegrade.

It is apparent that the total NO release of chitosan-NO adducts is in generally agreement with the intensity of reflections at low Bragg angles (2θ = 8.6°–11.1°), which denote that a relaxed hydration crystalline form and NO molecules could penetrate this crystal and react with the NH_2 groups of chitosan molecular chains in this crystalline region.

Effect of molecular weight on the release of chitosan-NO adducts

The release profiles for different molecular weight chitosan-NO adducts are illustrated in Figure 6. A burst release occurs for all the chitosan-NO adducts, and the half-lives of the NO release were less than 5 min, and the total NO release increased linearly with the molecular weight. An XRD measurement of chitosans with different molecular weights but with the same degree of acetylation is shown in Figure 7. With the decrease in molecular weight, the intensity

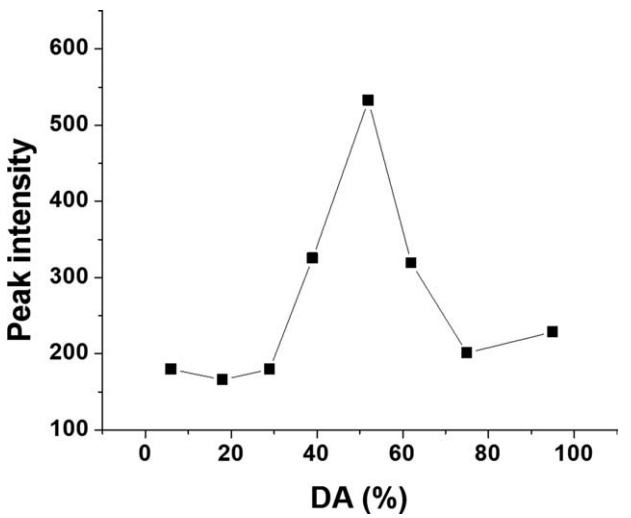

Figure 5 The relationship between peaks intensities of the reacetylated chitosans at low Bragg angles and DAs.

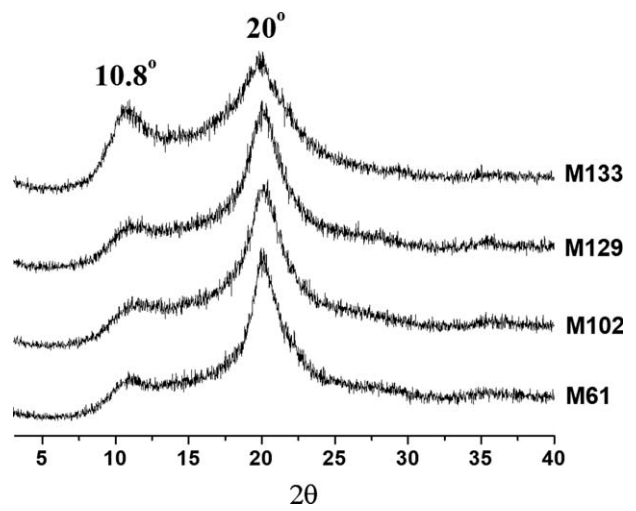

Figure 7 X-ray diffraction patterns of chitosan with different molecular weights.

of reflection at $2\theta = 10.8^\circ$ decreased, whereas the strongest reflection at $2\theta = 20^\circ$ became sharper. It is indicated that with the decrease of molecular weight, the larger *d*-spacing unit cells were destroyed and the regularity of the low *d*-spacing structure increased and chitosan molecules preferred to develop a more compact crystalline form. The NO molecules could not penetrate this crystal and react with the chitosan molecules, which resulted in the decrease of the total NO release. Therefore, the total NO release of chitosan-NO adducts is in generally agreement with the intensity of relaxed hydration crystalline form at low Bragg angles ($2\theta = 10.8^\circ$).

CONCLUSIONS

NO can be reacted with the primary amine group of chitosans and form $[NONO]^-$ groups. The effect of molecular weight and degree of acetylation on the NO release properties was investigated. The total NO release of the chitosan-NO adducts is closely related with the molecular weights and DAs: a bell-shaped distribution in the range of degree of acetylation from 6 to 95% with the total NO release at DA $\approx$ 50 %. The total NO release was directly proportional to the molecular weights for chitosans with the same degree of acetylation. From XRD pattern, it can be seen that heterogeneous reaction of chitosan and NO was influenced greatly by the crystalline form of chitosans, and the variety of total NO release of chitosan-NO adducts was in generally agreement with the intensity of reflections at low Bragg angles $2\theta = 8.6^\circ$–11.1°, which denoted that a relaxed hydration crystalline form and NO molecules could penetrate this crystal and react with the chitosan molecules. This study will contribute to the synthesis of an effective NO donor using chitosan derivatives as the matrix, which can regulate the loading and release rate of NO according to the demand of biomedical applications.

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