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Synthesis, characterization, and evaluation of antifungal and antioxidant properties of cationic chitosan derivative via azide-alkyne click reaction

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ABSTRACT

In this work, quaternary ammonium group was introduced into chitosan backbone by cuprous-catalyzed azide-alkyne cycloaddition (CuAAC) to synthesize the cationic chitosan derivative bearing 1,2,3-triazole. The products were identified structurally by FTIR, ^{1}H NMR spectroscopy, XRD, and elemental analysis. The water solubility of chitosan derivatives at different pH values was determined by a turbidity measurement. The antifungal properties of cationic chitosan derivatives against *Botryis cinerea*, *Phomopsis asparagi*, *Fusarium oxysporum* f. sp. niveum, and *Fusarium oxysporum* f. sp. cucumerium were evaluated using the radial growth assay. Besides, the antioxidant activities of them were also tested by superoxide-radical scavenging and reducing power assays. Compared to chitosan, cationic chitosan derivative bearing 1,2,3-triazole showed the good water solubility especially at alkaline condition, excellent antifungal action with over 70% inhibitory indices against tested fungi at 1.0 mg/mL, and enhanced antioxidant activity with complete scavenging efficiency against superoxide-radical at 1.6 mg/mL because of the introduction of 1,2,3-triazole and quaternary ammonium moieties. These excellent biological properties present a promising prospect for this chitosan derivative in antifungal and antioxidant biomaterials.

1. Introduction

Chitosan, obtained from deacetylation of chitin, is one of the most abundant polyaminosaccharides [1]. Chitosan is a polycationic complex biopolymer that has an amino group at the 2-position of the glucosamine ring in a repeating glucosidic residue rather than a hydroxyl group compared with cellulose [2]. The concept of utilizing chitosan as an ideal biomaterial in the biomedical, pharmaceutical, agricultural, cosmetics, and food fields has received significant attention [3,4], due to its outstanding characteristics such as biocompatibility, biodegradability, and nontoxicity [5–7]. However, the main challenge in the wide utilization of chitosan is its insoluble nature in both organic and aqueous solvents at neutral pH [8,9]. To overcome this, tremendous efforts have been devoted to chemical modifications through the introduction of hydrophilic groups [4,10,11].

The modification of chitosan with cationic moieties has been performed by *N*,*N*,*N*-trialkylation of the amino group or by directly grafting cationic small molecules with covalent bond onto the primary amino and hydroxyl groups of chitosan backbone [12], because of cationic chitosan derivatives have particular characteristics such as improved water solubility over a broad pH range and excellent bioactivities such as antimicrobial and antioxidant activities [13–15]. Among the cationic chitosan derivatives, *N*,*N*,*N*-trimethyl chitosan and (2-hydroxy-3-trimethylammonium) propyl chitosan chloride have received particular attention [16]. The latter was usually prepared by etherification of chitosan and glycidyl trimethylammonium chloride in isopropyl alcohol or water [17]. Recently, the cuprous-catalyzed azide-alkyne cycloaddition (CuAAC) termed by Sharpless and coworkers [18] has been introduced into the chemical modification of chitosan due to its high specificity, modularity, tolerant to other functional groups [19,20]. Now our interest is in the preparation of cationic chitosan derivative by quaternization of amino group at C-2 and the introduction of *N*,*N*,*N*-trimethyl moiety into hydroxyl groups at C-3 or C-6 by CuAAC reaction simultaneously and its antifungal and antioxidant activities.

In the following, we aim to develop novel cationic chitosan derivative bearing 1,2,3-triazole via efficient CuAAC reaction. The chemical structure of cationic chitosan derivative was characterized in details by FTIR, ^{1}H NMR, and elemental analysis. The water solubility of the synthesized chitosan derivatives were also evaluated by a turbidity measurement. The antifungal activity of the derivatives against the four plant-threatening fungi, *Botryis cinerea* (*B. cinerea*), *Phomopsis asparagi* (*P. asparagi*), *Fusarium oxysporum* f. sp. niveum (*F. oxysporum* f. sp. niveum), and *Fusarium oxysporum* f. sp. cucumerium (*F. oxysporum* f. sp. cucumerium), was evaluated by hypha measurement in vitro.

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Meanwhile, the antioxidant activity was also investigated by the assessment of superoxide-radical scavenging activity and reducing power.

2. Experimental section

2.1. Materials

Chitosan (molecular weight 200 kDa, the degree of deacetylation 83%) was supplied from Qingdao Baicheng Biochemical Corp. (Qingdao, China). 1,2-Dibromoethane (99%), iodomethane (98%), and propargyl bromide (80 wt% in toluene) were obtained from the Sigma-Aldrich Chemical Corp (Shanghai, China). Sodium azide (AR), magnesium sulfate (AR), trimethylamine (33 wt% in ethanol), sodium iodide (98%), sodium hydroxide (AR), triethylamine (AR), cuprous iodide (AR), *N*,*N*-dimethylformamide (DMF, AR), diethyl ether (AR), acetonitrile (AR), ethyl acetate (AR), *N*-methyl-2-pyrrolidone (NMP, AR), absolute ethanol (AR), and dimethylsulfoxide (DMSO, AR) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All these materials were used as received without further purification.

2.2. Structural characterization

Fourier transform infrared (FTIR) spectra of compounds were conducted on a Jasco-4100 Fourier Transform Infrared Spectrometer (Japan, provided by JASCO Co., Ltd. Shanghai, China) in transmission mode at a resolution of 4.0 cm^{-1} in the mid-infrared range (from 4000 to 400 cm^{-1}). The compounds were characterized by ^1H nuclear magnetic resonance (NMR) analysis run on a Bruker AVIII-500 MHz spectrometer (Switzerland, provided by Bruker Tech. and Serv. Co., Ltd. Beijing, China) using 99.9% Deuterium Oxide (D_2O) as the solvent and chemical shift was reported with the solvent residue as the reference. X-ray diffraction (XRD) measurements were performed using a Bruker D8 Advance X-ray diffractometer (Bruker AXS GmbH, Germany) with Cu Kα radiation (λ = 1.541874 Å) at a voltage of 40 kV and current of 30 mA. The 2θ diffraction diagrams were obtained between 5° and 50° at a scanning rate of $6°\cdot min^{-1}$. Elemental analyses of carbon, hydrogen, and nitrogen in the native and derived chitosan were determined on a Vario EL III (Elementar, Germany). The percentages of carbon and nitrogen (C/N) were converted into degrees of substitution (DS) values which were defined as the number of grafted functionalized groups of monomeric unit of chitosan. The DS values of functional groups in chitosan derivatives were calculated by the following equations [21]:

$$DS1 = \frac{n1 \times MC - MN \times W1}{n2 \times MC} \quad (1)$$

$$DS2 = \frac{MN \times (W2 - W1)}{n3 \times MC} \quad (2)$$

$$DS3 = \frac{MN \times (W3 - W2)}{n4 \times MC} \quad (3)$$

$$DS4 = \frac{MN \times (W4 - W3)}{n5 \times MC - n6 \times MN \times W4} \quad (4)$$

where *DS*1, *DS*2, *DS*3, and *DS*4 represent the deacetylation degree of chitosan, the degrees of substitution of *N*,*N*,*N*-trimethyl in chitosan derivatives, propargyl in chitosan derivative **2**, and 1,2,3-triazole groups in chitosan derivative **3**; *MC* and *MN* are the molar mass of carbon and nitrogen, *MC* = 12, *MN* = 14; *n*1, *n*2, *n*3, *n*4, *n*5, and *n*6 are the number of carbon of chitin, carbon of acetamido group, carbon of trimethyl, carbon of propargyl group, carbon and nitrogen of *N*,*N*,*N*-trimethyl-*N*-(2-azido)-ethyl ammonium bromide, *n*1 = 8, *n*2 = 2, *n*3 = 3, *n*4 = 3, *n*5 = 5, *n*6 = 4; *W*1, *W*2, *W*3, and *W*4 represent the mass ratios between carbon and nitrogen in chitosan derivatives).

2.3. Synthesis of chitosan derivatives

2.3.1. Synthesis of N,N,N-trimethyl-N-(2-azido)-ethyl ammonium bromide

To a solution of 1,2-dibromoethane (5 g, 27 mmol) in *N*,*N*-dimethylformamide (18 mL) was added sodium azide (1.04 g, 16 mmol) in batches at room temperature. The mixture was stirred at room temperature for 4 h. After the reaction was completed, the mixture was diluted with deionized water. Diethyl ether was then added and the mixture was partitioned between diethyl ether and water. The organic phase was washed with deionized water, then dried over anhydrous $MgSO_4$, filtered, and concentrated to give crude 2-azido bromoethane that was used in the next step without further purification. To a solution of trimethylamine (3.3 mL, 10 mmol) in acetonitrile (10 mL) was added the crude 2-azido bromoethane (1.49 g, 10 mmol) in acetonitrile (10 mL) dropwise at 0 °C. The mixture was stirred at room temperature for 4 h, vacuumed to remove all the solvent. The obtained dried residue was washed with ethyl acetate to give colorless solid. Yield: 1.16 g, 56%. FTIR ν (cm^{-1}): 3008, 2965, 2927, 2094, 1481, 1265, 944. ^1H NMR (500 MHz, DMSO d_6) δ (ppm): 3.98 (dd, J = 12.5, 6.9 Hz, 1H), 3.86 (m, 2H), 3.60 (m, 1H), 3.16 (d, J = 7.8 Hz, 9H). ^{13}C NMR (125 MHz, DMSO-d_6) δ (ppm): 65.04, 53.59, 52.90.

2.3.2. Synthesis of cationic propargyl chitosan derivative **2**

Into a 250 mL flask equipped with a reflux condenser and a stir bar were placed chitosan (1.61 g, 10 mmol of glucosamine) and *N*-methyl-2-pyrrolidone (75 mL). The mixture was stirred at room temperature for 1 h followed by addition of NaI (4.50 g, 30 mmol), 15% aqueous solution of NaOH (15 mL, 55 mmol), and CH_3I (15 mL, 240 mmol) in sequence. The suspension solution was refluxed under magnetic stirring at 60 °C for 1 h. After cooling the reaction solution, it was poured into absolute ethanol (750 mL) to afford flavescent precipitate (Elemental analysis: C: 31.43%, N: 4.52%, H: 5.49%, C/N: 6.95, $DS_{trimethyl}$: 59%). The resulting solid was collected by centrifugal separation and then dissolved directly in *N*-methyl-2-pyrrolidone (150 mL) in a 250 mL round bottom flask equipped with a condenser. This mixture was stirred at room temperature for 1 h after the dropwise addition of 5% aqueous solution of NaOH (36 mL, 45 mmol). To the homogeneous solution with constant stirring, propargyl bromide (3.50 mL, 45 mmol) was added dropwise to start the etherification at 60 °C for 48 h. Next, the resulting solution was cooled to room temperature and then poured into an excess amount of absolute ethanol (1000 mL) to produce yellowish precipitate. The resultant precipitate was washed with ethanol carefully and dried at −50 °C overnight in vacuum. Yield: 2.82 g, 76%. Elemental analysis: C: 38.94%, N: 4.18%, H: 6.69%, C/N: 9.32, $DS_{propargyl}$: 92%. FTIR ν (cm^{-1}): 3428, 3274, 2927, 2121, 1473, 1076, 644. ^1H NMR (500 MHz, D_2O): δ (ppm) 5.60–2.00 (pyranose rings), 4.32 ($\underline{CH_2}C{\equiv}CH$), 3.39 ($N^+(\underline{CH_3})_3$), 2.71 ($CH_2C{\equiv}\underline{CH}$).

2.3.3. Synthesis of cationic chitosan derivative **3**

A 100 mL three-necked round-bottom flask equipped with a magnetic stirring bar was charged with cationic propargyl chitosan derivative **2** (0.74 g, 2 mmol), *N*,*N*,*N*-trimethyl-*N*-(2-azido)-ethyl ammonium bromide (1.25 g, 6 mmol), triethylamine (0.28 mL, 2 mmol), cuprous iodide (38 mg, 0.2 mmol), and dimethylsulfoxide (40 mL). The mixture was stirred for 72 h at 75 °C under an argon atmosphere. After the reaction, the resulting product was precipitated in absolute ethanol (400 mL), then washed with absolute ethanol a few times and dialyzed against deionized water for 2 days before drying the product overnight in a vacuum oven at −50 °C. Yield: 0.88 g, 74%; Elemental analysis: C: 36.03%, N: 7.24%, H: 5.65%, C/N: 4.98, $DS_{1,2,3\text{-triazole}}$: 28%. FTIR ν (cm^{-1}): 3421, 2931, 2884, 1650, 1477, 1376, 1060, 852. ^1H NMR (500 MHz, DMSO-d_6) δ (ppm): 8.19 (1,2,3-triazole-5-$\underline{H}$), 5.03 ($NC\underline{H_2}CH_2N^+$), 4.65–2.06 (pyranose rings), 4.00 ($NCH_2C\underline{H_2}N^+$), 3.43–3.15 ($N^+(\underline{CH_3})_3$).