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, 1H NMR spectroscopy, XRD, and elemental analysis. The water solubility of chitosan derivatives at different pHe 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 polyamino saccharides [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, cosmestics, 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, 1H 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.
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 (95%), 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 dimethylsulfide (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 (D₂O) 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° 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, hydrogen, and nitrogen (C/N) were converted into degrees of substitution (DS) values of carbon, hydrogen, and nitrogen (C/N) were converted into degrees of substitution (DS) and then expressed as the number of grafted functionalized groups of monomeric unit of chitosan.

2.3. Synthesis of chitosan derivatives

2.3.1. Synthesis of N,N-trimethyl-(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. Sodium azide 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 MgSO₄, 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-d₆) 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), 1.3 C NMR (125 MHz, DMSO-d₆) 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 CH₃I (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 flavescence precipitate (Elemental analysis: C: 31.43%, N: 4.52%, H: 5.49%, C/N: 6.95, DStrimethyl: 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: 6.2; DS.writhe: 92%. FTIR ν (cm⁻¹): 3428, 3274, 2927, 2121, 1473, 1076, 641. ¹H NMR (500 MHz, D₂O): δ (ppm) 5.60–2.00 (pyrazine rings), 4.32 (CH₃C=CH), 3.39 (N(CH₃)₃), 2.71 (CH₂C=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 dimethylsulfide (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, DSδ₂,₃-triazole: 28%. FTIR ν (cm⁻¹): 3421, 2931, 2884, 1650, 1477, 1376, 1060, 852. ¹H NMR (500 MHz, DMSO-d₆) δ (ppm): 8.19 (1.23-triazole-5-H), 5.03 (NCH₃CH₂N⁺), 4.65–2.06 (pyrazine rings), 4.00 (NCH₃CH₂N⁺), 3.43–3.15 (N(CH₃)₃).
2.4. Water solubility

The effect of pH on water solubility of chitosan and cationic chitosan derivatives was evaluated at room temperature [22]. The samples were dissolved in 1% acetic acid solution to reach 1.0 mg/mL concentration, followed by the dropwise addition of 1 M NaOH solution until reach a pH value of about 13. The transmittance of each solution was recorded on a TU-1810 UV spectrometer (General Instrument Co., Ltd., China) at 600 nm.

2.5. Antifungal assay

The effect of cationic chitosan derivatives with different concentrations on mycelial growth was tested by inoculating a mycelial disk (diameter: 5 mm) of 5-day-old culture of B. cinerea, P. asparagi, F. oxysporum f. sp. niveum, and F. oxysporum f. sp. cucumerium on a Potato dextrose agar (PDA) medium amended with chitosan derivative according to the literature [23]. The plates were incubated at 27 °C and the colony diameter was measured using decussating method when the control plate without samples was fully covered with mycelium. The inhibitory indices of cationic chitosan derivatives were calculated using the following equation:

\[
\text{Inhibitory index (\%)} = (1 - \frac{D_a}{D_b}) \times 100
\]

where \(D_a\) (mm) is the diameter of the growth zone in the test plates and \(D_b\) (mm) is the diameter of the growth zone in the control plate.

2.6. Antioxidant assay

2.6.1. Superoxide anion radical scavenging activity assay

The scavenging effect of samples against \(\text{O}_2^-\) was performed, using the method described by Tan with slight modification [24]. Briefly, in each tube, 1.5 mL of sample solution with different concentrations was mixed with 0.5 mL of nitroblue tetrazolium (NBT, 72 \(\mu\)M) solution and 0.3 mL of ferric chloride solution (0.1%, w/v). The absorbance values \(A_1\) was the absorbance of the sample solution with Tris–HCl buffer instead of NBT solution.

\[
\text{Inhibitory index (\%)} = (1 - \frac{A_1}{A_0}) \times 100
\]

2.6.2. Reducing power assay

The reducing power of chitosan and cationic chitosan derivatives was determined according to the method of Luan with some modifications [25]. In brief, the reaction mixture containing 1.0 mL of sample solution with different concentrations and 1.0 mL of potassium ferricyanide (1%, w/v) dissolved in sodium phosphate buffer (0.2 M, pH = 6.6) was shaken vigorously and incubated at 50 °C for 20 min. After cooling down, 1.0 mL of trichloroacetic acid (10%, w/v) was added to the mixtures and then centrifuged at 3500 rpm for 3 min. An aliquot of the mixture (1.5 mL) was retrieved and mixed with 1.2 mL of deionized water and 0.3 mL of ferric chloride solution (0.1%, w/v). The absorbance values of the reaction mixtures were determined at 700 nm. The reducing power could be assessed as follows:

\[
\text{Reducing power} = A_1 - A_0
\]

where \(A_0\) was the absorbance of the blank and \(A_1\) was the absorbance of the sample solution.

2.7. Statistical analysis

All data from the three separate experiments were expressed as the mean ± the standard deviation (SD, \(n = 3\)). Statistical differences were performed by one-way analysis of variance followed by a Tukey’s test for multiple comparisons. A level of \(P < 0.05\) was considered statistically significant.

3. Results and discussion

3.1. Chemical synthesis and characterization

Cationic chitosan derivative bearing 1,2,3-triazole group was prepared via CuAAC reaction between cationic propargyl chitosan derivative 2 and \(N,N,N\)-trimethyl-N-(2-azido)-ethyl ammonium bromide (Scheme 1). Moreover, in order to improve the solubility and reaction efficiency of pristine chitosan in organic solvents, cationic propargyl chitosan derivative 2 was synthesized by \(N,N,N\)-trimethylation of amino group followed by propargylation of chitosan subsequently in the alkaline condition. And \(N,N,N\)-trimethyl-N-(2-azido)-ethyl ammonium bromide was obtained by a two-step nucleophilic substitution reaction of 1,2-dibromoethane with sodium azide and trimethylamine successively [26]. The successful synthesis of chitosan derivatives was confirmed by FTIR, \(^1\)H NMR, XRD, and elemental analysis.

Scheme 1. Synthetic routes for cationic chitosan derivatives.
3.1.1. FTIR analysis

FTIR spectra of pristine chitosan and its derivatives are illustrated in Fig. 1. The spectrum of chitosan shows basic characteristic peaks at 3417 cm$^{-1}$ (the N—H and O—H stretching vibrations), 2877 cm$^{-1}$ (the C—H stretching vibration of methylene), 1600 cm$^{-1}$ (the N—H bending vibration of free amino group), and 1072 cm$^{-1}$ (the C—O—C skeleton asymmetric stretching vibration of the glucosamine ring) [12,27]. For the cationic chitosan derivatives 1 and 2, inclusion of the –N+(CH$_3$)$_3$ is confirmed by the appearance of bands at the region 1473–1484 cm$^{-1}$ [28]. Effective propargylation of chitosan is supported by the development of FTIR features at 3274 cm$^{-1}$ (the C—H stretching vibration of terminal alkyne), 2121 cm$^{-1}$ (the C=C stretching vibration), and 644 cm$^{-1}$ (the C—H bending vibration of terminal alkyne) [29]. After CuAAC reaction, the synthesis of cationic chitosan derivative bearing 1,2,3-triazole 3 is confirmed by the reduction in FTIR bands at 3274, 2121, and 644 cm$^{-1}$ corresponding to the terminal alkyne, and the increasing intensity of bands at 1477 cm$^{-1}$ arising from the presence of the N,N,N-trimethyl moiety from the terminal alkyne molecule. However, the residual peaks of the terminal alkyne indicated the relatively low reaction efficiency, which agreed with the result of the substitution degrees 28% of 1,2,3-triazole group calculated by elemental analysis.

3.1.2. $^1$H NMR analysis

The $^1$H NMR spectra of chitosan and its derivatives are shown in Fig. 2 to further identify their chemical structures. The proton assignments in chitosan spectrum are assigned at 4.75 ppm (D$_2$O overlaps H-1), 3.44–3.93 ppm (H-3,4,5,6), 3.13 ppm (H-2), and 1.99 ppm (acetic acid overlaps NHCOC$_3$) [30]. In the spectrum of cationic chitosan derivative 2, the new signal at 3.39 ppm (a in the spectrum) corresponds to the methyl protons (–N+(CH$_3$)$_3$) of the quaternary ammonium group [31], and the new signals at 4.33 and 2.70 ppm (b and c in the

![Fig. 1. FTIR spectra of chitosan and cationic chitosan derivatives.](image1)

![Fig. 2. $^1$H NMR spectra of chitosan and cationic chitosan derivatives.](image2)
The intensity of signals at 3.21 ppm (a and g in the spectrum) indicates the amino group changing form the directional and intermolecular hydrogen bonds cause the poor solubility of ionic chitosan derivatives are depicted in Fig. 4. The strong intramolecular hydrogen bonding of the regularity of chitosan chain and the structure of hydrogen bonds, which is promising to expand the application of chitosan.

3.2. Water solubility

The results of water solubility and pH stability of chitosan and cationic chitosan derivatives are depicted in Fig. 4. The strong intramolecular and intermolecular hydrogen bonds cause the poor solubility of chitosan at neutral and alkaline condition [15]. The protonation of free amino group changing form –NH₂ to –NH₃⁺ at acidic pH could improve the water solubility of chitosan [34]. Therefore, the converse “S” type curve exists for the relationship between pH value and water solubility of chitosan. After quaternization of chitosan, chitosan derivative 2 shows higher water solubility with transmittance of above 93% at alkaline condition because of the hydrophilicity of quaternary ammonium groups. Finally, the introduction of more N,N,N-trimethyl moiety via CuAAC reaction could further improve water solubility of chitosan derivative 3 with 100% transmittance over the entire pH values range. This significantly improved water solubility might be due to the break of the regularity of chitosan chain and the structure of hydrogen bonds, which is promising to expand the application of chitosan.

3.3. Antifungal activity

Growth inhibition of B. cinerea, P. asparagi, F. oxysporum f. sp. niveum, and F. oxysporum f. sp. cucumerium was studied to understand the antifungal effects of the prepared chitosan derivatives in vitro. The water-soluble low molecular chitosan was chosen in antifungal activity test instead of pristine chitosan with the poor water solubility in aqueous systems. The ability of the chitosan derivatives at 0.1, 0.5, and 1.0 mg/mL to inhibit the growth of the tested strains is shown in Fig. 5. It is demonstrated that all samples exhibit varying extent of antifungal activity towards four studied fungal pathogens in a dose-dependent manner. Decreases in the colony diameters were observed with an increase in the concentration of compounds. Although some extent of antifungal activity demonstrates for chitosan with inhibition percentages of 11.80–25.00% at 1.0 mg/mL, the inhibitory index of cationic chitosan derivatives towards different pathogens is obviously higher than that of chitosan. Meanwhile, the higher antifungal activity of cationic chitosan derivative 3 in comparison with chitosan and chitosan derivative 2 is observed. The inhibitory indices of cationic chitosan derivative 3 against B. cinerea, P. asparagi, F. oxysporum f. sp. niveum, and F. oxysporum f. sp. cucumerium are 94.31 ± 0.45, 81.40 ± 0.94, 74.85 ± 0.38, and 75.69 ± 0.56% at the maximum concentration of 1.0 mg/mL, respectively. Moreover, cationic chitosan derivative 3 still exhibits more effective activities against tested fungi even when the dosage is lowered to 0.5 mg/mL than that of chitosan and chitosan derivative 2 at the concentration of 1.0 mg/mL. The above results indicate that the enhanced inhibitory effect of compound 3 is most probably the result of the introduction of 1,2,3-triazole and N,N,N-trimethyl moieties. It is suggested that the strong antifungal efficiency of cationic chitosan derivative 3 results from electrostatic interactions between the quaternary ammonium moieties of chitosan derivative and negatively charged cell wall of microorganisms [35,36], which lead to the disruption in the cell membrane and the leakage of intracellular electrolytes and proteinaceous constituents [37,38].

3.4. Antioxidant activity

Antioxidant activities of chitosan derivatives are determined by the superoxide-radical scavenging activity and reducing power shown in Fig. 6 with a concentration-dependent manner, and the EC₅₀ value, which represents the antioxidant concentration required to obtain a
50% antioxidant effect, is also used to express the antioxidant capacity and to compare the activity of the compounds. A complete scavenging efficiency with an EC<sub>50</sub> value under 0.1 mg/mL against superoxide-radical for chitosan derivative 3, followed by 84.50 ± 3.62% scavenging rate with an EC<sub>50</sub> value of 0.68 mg/mL for chitosan derivative 2 and 34.72 ± 2.59% scavenging rate for chitosan at the maximum testing concentration of 1.6 mg/mL. Besides, the reducing power of chitosan and chitosan derivatives 2 and 3 at 1.6 mg/mL are measured as 0.40 ± 0.10, 0.62 ± 0.12, and 1.68 ± 0.13, respectively. The results indicate that the antioxidant activity of cationic chitosan derivative 3 bearing 1,2,3-triazole is significantly increased compared with cationic chitosan derivative 2 and chitosan. The enhanced antioxidant action is mainly ascribed to the function of 1,2,3-triazole group, which has the large dipole, conjugated double bonds, and lone pair electrons of nitrogen atoms and might act as electron donor to convert free radicals into more stable products to terminate the free radical chain reactions [14,24].

4. Conclusion

In present study, we successfully prepared cationic chitosan derivative bearing 1,2,3-triazole and N,N,N-trimethyl moieties using a facile cuprous-catalyzed azide-alkyne click chemistry approach. The synthesized cationic chitosan derivative showed the good water solubility over the entire pH values range at the concentration of 1.0 mg/mL. Moreover, the cationic chitosan derivative bearing 1,2,3-triazole exhibited enhanced antifungal activity against four kinds of plant threatening pathogens, as shown in Fig. 5. The antifungal activity of chitosan and cationic chitosan derivatives against B. cinerea (a), P. asparagi (b), F. oxysporum f. sp. niveum (c), and F. oxysporum f. sp. cucumerium (d).
fungal strains and strong scavenging effect against superoxide-radical as well as improved reducing power. These findings suggested that these excellent behaviors of the cationic chitosan derivative bearing 1,2,3-triazole were probably due to the introduction of quaternary ammonium and 1,2,3-triazole moieties. This simple and novel synthesis route described in this paper might provide a useful platform for design of chitosan-based antifungal and antioxidant agents, which could be able to find widespread use in biomedical and material fields.

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