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Investigation of gelling behavior of thiolated chitosan in alkaline condition and its application in stent coating

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ABSTRACT

The gelling behaviors of thiolated chitosan (TCS) in alkaline condition were investigated. Thioglycolic acid was conjugated onto chitosan backbone through amide bond formation. The variations of thiol group content were monitored in presence of H_2O_2 or different pH values (pH 7.0, 8.0, 9.0) in dialysis mode. Different from the decreasing thiol group content upon time in acidic condition, increasing amount of thiol groups was detected in alkaline pH during 120 min dialysis attributed to alkaline hydrolysis of intra-molecular disulfide bonds. The extent of which was larger at higher pH values. Higher degree of thiolation, thiomer concentration or pH values promoted gelation of TCS. Entanglement and coagulation of chitosan molecule chains and re-arrangement of disulfide bonds acted closely and dynamically in the gelation process. Disulfide bonds, especially inter-molecular type, are formed by synergetic effects of thiol/disulfide interchange and thiol/thiol oxidation reactions. TCS coated vascular stent displayed wave-like microstructure of parallel ridges and grooves, which favored HUVECs adhesion and proliferation. The biocompatibility, peculiar morphology and thiol moieties of TCS as stent coating material appear application potential for vascular stent.

1. Introduction

Thiomers, termed as polymers bearing thiol groups on side chains, has been well investigated for their superior characteristics and biocompatibility especially in properties of enhanced mucoadhesion and therapeutic delivery (Kumar & Sinha, 2013; Albrecht & Bernkop-Schnürch, 2007). Such polymers include thiolated derivatives of chitosan, alginate, carboxymethycellulose, poly(acrylic acid), poly(methacrylic acid), etc (Bernkop-Schnürch, 2005). Wherein, thiolated chitosan is the unique cationic obtained via immobilization of thiol bearing ligands, such as cysteine (Atyabi, Talaie, & Dinarvand, 2009), thiobutylamidine (Bernkop-Schnürch, Hornof, & Zoidl, 2003), thioglycolic acid (Kast & Bernkop-Schnürch, 2001) or glutathione (Talaei, Azizi, Dinarvand, & Atyabi, 2011), on the primary amino groups of chitosan backbone.

Based on thiol/disulfide exchange reactions and/or a simple oxidation process, endogenous or exogenous disulfide bonds comparative to thiolated chitosan are formed, which endow chitosan with improved properties in regard to mucoadhesion, enhanced permeation, in situ gelation, efflux pump inhibition, tissue engineering, and controlled drug release (Sarti & Bernkop-Schnürch, 2011). With regard to the in situ gelling property, the sol–gel phase transition is mediated by the formation of inter- and/or intramolecular disulfide bonds at pH levels above 5 (Kast & Bernkop-Schnürch, 2001). Siganificant increase of elastic viscosity of chitosan–thioglycolic acid (chitosan–TGA) solution was measured in parallel with decreasing content of the thiol group within system (Hornof, Kast, & Bernkop-Schnürch, 2003). The variation of thiol group content was found to be environmental pH dependent. The thiol groups of the chitosan–TGA conjugate remained stable toward oxidation at pH 4, whereas, a significant decrease in the thiol group content could be observed at pH 5 and even lower at pH 6.5 (Kast & Bernkop-Schnürch, 2001). It is therefore postulated that chitosan–TGA is supposed to form gels of high elasticity at higher medium pH value, such as physiological pH. Although the postulation has been evidenced, existing concern is the phase transition occurs slowly in almost 6 h (Bernkop-Schnürch et al., 2003; Hornof et al., 2003; Krauland, Hoffer, & Bernkop-Schnürch, 2005). Velocity and extent of disulfide bond formation depends on the concentration of thiolate anions (S^-), representing the active form for oxidation. In order to shorten gelation time, on one hand, oxidizing agent was utilized along with chitosan–TGA which rapidly shifted to gel within 20 min successfully (Sakloetsakun, Hombach, & Bernkop-Schnürch, 2009). On the other hand, the S^- concentration depends on the p*K*a value of the thiol group and the pH

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of the surrounding medium (Bernkop-Schnürch, 2005). When the medium pH is higher than the p*K*a, comparatively higher amounts of S^- are supposed to lead to thiol/disulfide interchange and/or an oxidation process. So far, concerning literatures has been focused on the correlation between slightly acidic pH range within 4–6.5 and disulfide bond formation. Whether the gelling properties of thiolated chitosan can be improved by alkaline pH or not, however, has not yet been investigated.

In this research, chitosan–thioglycolic acid conjugate (TCS) was chosen as candidate to study its gelling behavior at pH 7–9. Effects of thiol group content, thiolmer concentration and polymer hydrophobicity on gelling process were systematically evaluated to elucidate the mode of action. Cytotoxicity and potential utilization as vascular stent coating material were evaluated.

2. Material and methods

2.1. Materials

Low-molecular weight CS (DD 85.31%, 150.9 kDa) was obtained from Qingdao Honghai, Biotechnology Co., Ltd. (Qingdao, China). Cobalt–chromium vascular stents were kindly gifted by medical college affiliated hospital of Qingdao University. 1-ethy-3-(3-dimethylaminopropylcarbodiimide) hydrochloride (EDC), *N*-hydroxysuccinimide (NHS) and Ellman's reagent, 5, 5′-dithiobis (2-nitro benzoic acid) were purchased from Sigma-Aldrich (St. Louis, USA). Calcein-AM was purchased from DojinDo laboratories (Japan). Thioglycolic acid (TGA) and other chemical reagents used in the study were all commercially acquired from Huasheng Chemical Reagent Company (Qingdao, China) with analytical reagent grade. Deionized water was used throughout experiments.

2.2. Synthesis of chitosan–TGA conjugation (TCS)

TGA was immobilized onto chitosan backbone followed literature with modification (Kast & Bernkop-Schnürch, 2001). In this study, TCS with low or high degree of substitution (TCS_L or TCS_H) was synthesized. Briefly, 0.5 g CS was dissolved in 0.1 M HCl under stirring to obtain a 1% (w/v) solution. TGA, EDC, and NHS (molar ratio of TGA:EDC:NHS = 2:5:5) were dissolved in 0.1 M MES buffer Saline (pH = 5.5) under continuous stirring to activate carboxyl groups (—COOH) of TGA. Then the activated TGA was added dropwise into the CS solution at molar ratio of 4:1 or 2:1 per sugar residues of CS. The pH of the mixture was adjusted to 5.0 with 1 M NaOH and stirred at room temperature for 6 h. The obtained solutions were dialyzed (molecular mass cut-off 12 kDa) for 3 days in darkness. In detail, they were dialyzed once against 5 mM HCl, and two times against 5 mM HCl medium but containing1% NaCl. Then, the conjugates were dialyzed exhaustively two times against 1 mM HCl. Finally, samples were lyophilized by drying frozen aqueous polymer solutions at −20 °C, 0.01 mbar (FD5-series freeze-dryer, Bartelt, Sim, America) and stored at 4 °C for further studies. The chemical structure of TCS was characterized by FTIR (NEXUS 470, Nicolet, America) and ^{1}H NMR (AV600 MHz, Bruker, Germany) in comparison with chitosan. DCl/D_2O and D_2O were utilized as solvents for chitosan and TCS in ^{1}H NMR analysis, respectively.

2.3. Determination of thiol group and disulphide content

The amount of thiol groups immobilized onto chitosan backbone was determined spectrophotometrically with Ellman's reagent (Hornof et al., 2003). Briefly, 5 mg TCS was hydrated in 0.1 M PBS (pH 8.0). Then 100 μL of this sample was mixed with 3 mL DTNB reagent solution and incubated for 15 min at room temperature, the absorbance of which was measured spectrophotometrically at 412 nm (UV-1200005, Meipuda, China). A calibration curve was established and used to determine the amount of reduced thiol groups.

For disulphide content determination, the disulphide bonds were firstly reduced by $NaBH_4$ and determined by Ellman's assay. The amount of disulfide bonds was obtained as difference value of the amount of total and reduced thiol groups (Bernkop-Schnürch, Hornof, Kast, & Langoth, 2002; Werle & Hoffer, 2006). In detail, TCS was hydrated in 3.5 mL H_2O and hydrated for 30 min, 6.5 mL 0.05 M Tris buffer (pH 6.8) and 10 mL of a freshly prepared 4% (w/v) $NaBH_4$ solution were added. The samples were incubated for 1 h in oscillating water bath at 37 °C. Thereafter, the remaining $NaBH_4$ was inactivated by addition of 5 M HCl. The pH of the mixture was adjusted to 8.0 with 1 M PBS (pH 8.0).

2.4. Redox or pH responsive behaviors of TCS

2.4.1. Redox responsive behavior of thiol group content

The Redox responsive behavior of thiol group content was studied by dialyzing TCS solution against H_2O_2 under different concentration (0.05%, 0.15% and 0.25%). The amount of thiol groups were quantified by Ellman's assay. Briefly, 3 mL TCS solution (TCS_L, 3 mg/mL) was added into dialysis tube (molecular mass cut-off 12 kDa) and immersed into H_2O_2 and stirred at 37 °C. The TCS solution was withdrawn every 20 min within 2 h for thiol group determination.

2.4.2. pH responsive behavior of thiol group content

The pH responsive behavior of thiol group content (TCS_L or TCS_H at concentration of 3 mg/mL or 5 mg/mL) was quantified at pH 7.0, 8.0 and 9.0 in PBS. The experiments were performed by dialysis method as mentioned in previous section. In the mean time, the light transmittance of TCS solutions was determined at 420 nm.

2.5. Evaluation of gelling property

In parallel to thiol group content detection, gelling properties of TCS solution were studied. The dialysis against H_2O_2 or PBS with different pH was ceased at designated time (20, 40, 60, 80, 100, 120 min), the TCS solutions were transferred into capped glass vial and stored at 37 °C overnight in still state to allow for gel formation. Gelling property of TCS solution was evaluated by whether gel formed or not in the glass vials, and the height ratio of gels were recorded if gel formed.

2.6. Hydrophobic characteristics study of TCS

Hydrophobic characteristics of TCS were studied based on previous literature (Kong & Park, 2011). Pyrene, used as a hydrophobic probe, was dissolved in ethanol at the concentration of 4.0 μg/mL. About 400 μL of this solution was pipetted into test tubes, and then the ethanol was driven off under reduced pressure. 4 mL TCS solution dialyzed in different pH medium for 1 h was added to the test tube, bringing the final concentration of pyrene to 2 μM. The mixture was incubated for 3 h under oscillating condition at room temperature. Pyrene emission spectra were obtained using a F-4500 fluorescence spectrophotometer (Hitachi). The probe was excited at 330 nm, and the emission spectrum was collected in the range of 350–500 nm at an integration time of 1.0 s. The excitation and emission slit opening were 10 and 2.5 nm, respectively.

2.7. Cytotoxicity assay

In vitro cytotoxicity was studied by MTT assay against human umbilical vein endothelial cells (HUVECs). HUVECs were seeded into a 96-well plate at a density of 2×10^4 cells per well and incubated for 24 h at 37 °C, CO_2 5% in culture medium (MEM