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Thiomers: Influence of molar mass on *in situ* gelling properties

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ABSTRACT

The aim of this study was to investigate the influence of molar mass of thiolated polymers (thiomers) on their *in situ* gelling properties. Chitosan–thioglycolic acid (chitosan–TGA) and pectin–cysteine (pectin–Cys) of increasing molar mass were chosen to produce *in situ* gels in combination with carbamide peroxide. Low molar mass chitosan (~2 kDa) was prepared by oxidative degradation with $NaNO_2$, whereas pectin was depolymerized by heat treatment. Thiomers, displaying 1271–1616 μmol (chitosan–TGA) and 305–403 μmol (pectin–Cys) free thiol groups per gram polymer, were synthesized via amide bond formation mediated by a carbodiimide. The results showed that a reduction of molar mass combined with increased concentrations of both cationic chitosan–TGA and anionic pectin–Cys leads to higher final viscosities and to a higher relative increase in viscosity within 60 min and 180 min, respectively. Using this method, the dynamic viscosity of a very low molar mass chitosan–TGA (~2 kDa) could be increased 100,000-fold within 60 min and 390,000-fold within 180 min. In view of these *in situ* gelling properties carbohydrate thiomers might be useful for various pharmaceutical applications such as vehicle for drug delivery or as wound dressing material.

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1. Introduction

The basis for *in situ* gelling systems is a biocompatible polymer that offers a phase transition from a solution to a gel within a very short period of time, by means of a conformational change. The process of phase transition, which implies an increase in viscosity, can be triggered by change in temperature, pH or electrolyte composition. Another method producing *in situ* gels is attributed to a crosslinking process initiated by oxidizing agents or UV-radiation. Several applications have been developed in this field, for instance nasal (Park et al., 2002) or oral (Miyazaki et al., 2001) drug delivery systems and wound dressing materials (Balakrishnan et al., 2006). So far, however, these systems are often not efficient, by reason of an insufficient increase in viscosity or too long time period needed for phase transformation.

In the last decade thiolated polymers, known as thiomers, have shown *in situ* gelling properties, which can be attributed to an oxidative crosslinking process (Hintzen et al., 2012; Hornof et al., 2003; Sakloetsakun et al., 2009). Thiomers are polymers that display thiol bearing ligands; therefore they can be crosslinked by the formation of intra- and/or intermolecular disulfide bonds. The resultant network causes a serious increase in viscosity, which can be raised up considerably by adding oxidizing agents. Furthermore, it could be demonstrated that both cationic and anionic carbohydrate thiomers are useful for *in situ* gelling systems, whereby hydrogen peroxide and carbamide peroxide were the most effective oxidizing agents (Hintzen et al., 2012; Sakloetsakun et al., 2009).

Up to now, however, the full potential of thiomers as novel type of *in situ* gelling polymers has not been reached. The molar mass of polymers determines many physical properties, for instance glass transition temperature and mechanical properties such as stiffness, strength, toughness and viscosity. As the molar mass of polymers is related to their viscoelastic properties (Fetters et al., 1994), it will likely also have an effect on the *in situ* gelling properties. The purpose of this study was therefore to investigate the influence of the molar mass of thiomers on their *in situ* gelling properties. Two thiomers with three molar masses each were chosen to identify the most suitable molar mass for fast increasing dynamic viscosity. In addition, one cationic and one anionic thiomer have been selected to ensure that the *in situ* gelling systems are suitable for a wide range of applications. As oxidizing agent carbamide peroxide was used and the tests have been carried out at physiological pH values (7.4 and 6.0) in order to render them useful for various pharmaceutical routes of administration.

2. Materials and methods

2.1. Materials

Two different commercially available chitosans (1. from shrimp shells, low-viscosity, degree of deacetylation ≥95%;

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2. from crab shells, middle-viscous, degree of deacetylation ≥95%) were purchased from Sigma–Aldrich (Austria). Two apple pectin samples with varying molar mass (1. Pektin Classic AU 202, degree of esterification: 68–76%, molar mass: ∼170 kDa; 2. Herbapekt SF 50 – LV, molar mass: ∼25 kDa) were graciously supplied by Herbstreith & Fox (Neuenbürg, Germany). *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), L-cysteine HCl, thioglycolic acid (TGA), sodium nitrite ($NaNO_2$), acetic acid (HAc), sodium acetate (NaAc) and carbamide peroxide ($CO(NH_2)_2{\cdot}H_2O_2$) were obtained from Sigma–Aldrich (Austria). All chemicals were of chemical grade.

2.2. Preparation of low molar mass polymers

2.2.1. Chitosan

The low molar mass chitosan was prepared by oxidative degradation with $NaNO_2$ at room temperature (Huang et al., 2004). First, 2 g of chitosan (middle-viscous) were dissolved in 150 ml of acetic acid (6%, v/v) under magnetic stirring and 80 mg of sodium nitrite, dissolved in 10 ml of distilled water, were added dropwise. After incubation for 1 h chitosan was precipitated by adding a 5 M solution of sodium hydroxide until pH 9 was reached. As next step, the precipitated chitosan was filtered and washed with cold acetone, followed by resolubilization of the residue in 15 ml 0.1 M acetic acid. The depolymerized product was purified by dialysis against water and lyophilized at −30 °C and 0.01 mbar (Christ Gamma 1-16 LSC Freeze dryer).

2.2.2. Pectin

Pectin was depolymerized by heat treatment at 100 °C under continuous stirring (Catoire et al., 1998). Therefore, 2 g of apple pectin (Classic AU 202) was dissolved in 100 ml distilled water and heated for 3 h. Vigorous boiling of the opaque mixture was observed for a certain period and then the solution became transparent with simultaneous formation of colored precipitate. Afterwards the solution was filtered and the filtrate was dialyzed against distilled water followed by lyophilization at −30 °C and 0.01 mbar (Christ Gamma 1-16 LSC Freeze dryer).

2.3. Determination of molar mass

The average molar mass can be determined by several methods. A common method is viscometry, because it is claimed to be the simplest, most rapid and probably the most precise determination method (Zhang and Neau, 2001). The viscosity average molar masses (M_v) of polymers were estimated by applying the Mark–Houwink–Sakurada (MHS) equation, relating $[\eta]$ with M_v

$$[\eta] = k[M_v]^a$$

where k and a are constants and $[\eta]$ is the intrinsic viscosity. The intrinsic viscosity is defined as

$$[\eta] = \lim_{C\to 0}\left(\frac{\eta_r - 1}{C}\right)$$

where η_r is the relative viscosity (solution to solvent) and C is the polymer concentration. Intrinsic viscosity determination has been carried out with an Ubbelohde capillary viscometer (SI Analytics GmbH, Mainz, Germany). Briefly, the relative viscosity η_r of polymer solutions was measured over a range of concentrations (0.1–2.5 g/l) in the capillary viscometer at 25 ± 0.05 °C. For this, chitosan was dissolved in 0.3 M HAc/0.2 M NaAc (Rinaudo et al., 1993) and pectin in 2.5 mM NaCl (Catoire et al., 1998). The intrinsic viscosity $[\eta]$ was deduced from the relative viscosity η_r measured for each polymer solution by extrapolation at zero concentration. In case of a chitosan solution the MHS-constants were k = 0.082 ml/g and a = 0.76 (Rinaudo et al., 1993) and the molar mass of pectin was calculated with k = 0.0436 ml/g and a = 0.78 (Catoire et al., 1998).

2.4. Preparation of thiolated polymers

Thiolated polymers were obtained by covalent attachment of thiol bearing ligands to the polymer backbone as previously described (Bernkop-Schnurch, 2005; Hornof et al., 2003; Majzoob et al., 2006). Chitosan–thioglycolic acid (chitosan–TGA) was obtained by treating chitosan with *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC) and thioglycolic acid. In this process the carboxylic acid groups of TGA and primary amino groups of chitosan formed amid bonds mediated by EDAC. In the same way, pectin–cysteine (pectin–Cys) was synthesized by activating the carboxylic acid groups of pectin with EDAC followed by a coupling reaction to primary amino groups of L-cysteine.

In brief, 1% (m/v) aqueous polymeric solutions were mixed with a thiol bearing ligand (L-cysteine, thioglycolic acid) in a weight ratio of 1:1. Afterwards, the carboxylic acid moieties were activated for conjugation by EDAC in a final concentration of 150 mM and reaction mixtures were incubated at pH 4.5 for 3 h at room temperature under stirring. The resulting conjugates were isolated in the dark by dialyzing two times at 10 °C against 1 mM HCl, two times against the same medium but containing 1% NaCl and then exhaustively against 1 mM HCl. All samples were lyophilized by drying frozen aqueous thiomer solutions at −30 °C and 0.01 mbar (Christ Gamma 1-16 LSC Freeze dryer). Thiomers were stored at 4 °C until further use.

Six thiomers with various molar masses, three chitosan–thioglycolic acid (chitosan–TGA C1–C3) and three pectin–cysteine (pectin–Cys P1–P3) conjugates, were produced. Chitosan–TGA samples C2 (low-viscous) and C3 (middle-viscous) were synthesized with commercially available chitosan from Sigma–Aldrich (Austria), and the chitosan specimen for C1 was achieved by depolymerization described in Section 2.2.1. In analogy, pectin for synthesizing thiomers P1 (Herbapekt SF 50 – LV) and P3 (Classic AU 202) were purchased by Herbstreith & Fox (Neuenbürg, Germany), whereas the pectin for sample P2 was obtained by preparation described in Section 2.2.2.

2.5. Determination of the thiol group content

The amount of immobilized thiol groups on the polymeric backbone was determined spectrophotometrically with Ellman's reagent (DTNB, 5,5′-dithiobis(2-nitrobenzoic acid)) as previously described (Bernkop-Schnurch et al., 1999). First, 500 μg of each thiomer was dissolved in 500 μl 0.5 M phosphate buffer at pH 8.0 and stirred for 30 min. Then 500 μl of a DTNB solution (0.3%, m/v) was added and incubated for 90 min protected from light at room temperature. Finally, the sample was centrifuged at 13,400 rpm for 5 min, an aliquot of 100 μl was transferred to a 96 wells microplate and the absorbance was measured at 450 nm (Tecan infinite M200). For quantifying immobilized thiol groups on the polymer, L-cysteine acted as standard for the calibration curve. All experiments were performed in triplicate.

2.6. Rheological measurements

Thiomers were hydrated in 1 ml distilled water under continuous stirring for 30 min. Achieving different concentrations 10 mg for 0.5% (m/v), 20 mg for 1% (m/v), 40 mg for 2% (m/v) and 200 mg for 10% (m/v) were weighed. Afterwards the pH of pectin–cysteine samples were adjusted to 7.4 with 500 μl 1 M phosphate buffer and the pH of chitosan–thioglycolic acid samples were adjusted to 6.0 with 500 μl 1 M acetate buffer. In case of chitosan–TGA a reaction pH of 6 was chosen to guarantee a complete solubility at

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