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Research Paper

Novel bioadhesive polymers as intra-articular agents: Chondroitin sulfate-cysteine conjugates

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ABSTRACT

The aim of this study was to generate and characterize a chondroitin sulfate-cysteine conjugate (CS-cys) as a novel bioadhesive agent for intra-articular use. Mucoadhesive properties of synthesized CS-cys were investigated by rheological measurement of polymer-mucus mixture and rotating cylinder method, while bioadhesive features of CS-cys on porcine articular cartilage were evaluated via tensile studies. Thiolation was achieved by attachment of L-cysteine to CS via amide bond formation mediated by carbodiimide as a coupling reagent. The conjugate exhibited 421.17 ± 35.14 μmol free thiol groups per gram polymer. The reduced CS-cys displayed 675.09 ± 39.67 μmol free thiol groups per gram polymer after disulfide bonds reduction using tris(2-carboxyethyl)phosphine hydrochloride. The increase in dynamic viscosity of thiolated CS due to oxidative disulfide bond formation was demonstrated using capillary viscometer. The combination of CS-cys and mucus led to 4.57-fold increase in dynamic viscosity in comparison with mucus control. Furthermore, adhesion time to porcine mucosa of CS-cys-based test disk was enhanced by 2.48-fold compared to unmodified CS as measured by rotating cylinder method suggesting the interaction between thiomers and mucus gel layer via disulfide bonds formation. Tensile studies of thiolated CS on porcine articular cartilage showed 5.37- and 1.76-fold increase in the total work of adhesion and the maximum detachment force, respectively, in comparison with unmodified CS indicating bioadhesive features of CS-cys. Cytotoxicity of CS-cys was assessed in Caco-2 cells and rat primary articular chondrocytes using MTT and LDH release assay, thereby showing the safety of CS-cys at a concentration of 0.25% (w/v) in Caco-2 cells. Furthermore, 0.1% of CS-cys was found non-toxic to rat primary articular chondrocytes. According to these results, CS-cys provides improved bioadhesive properties that might be useful as an intra-articular agent for treatment of osteoarthritis.

1. Introduction

Chondroitin sulfate (CS) classified as a sulfated glycosaminoglycan (GAG) consisting of N-acetylgalactosamine and glucuronic acid has been commonly used as dietary supplement for osteoarthritis (OA) over several decades due to its anti-inflammatory, antioxidative and anti-apoptotic effects [1–3]. As a matter of fact, CS is the major component of extracellular matrix of numerous connective tissues, particularly skin, bone, synovial fluid and cartilage. Injection of CS into synovial fluid allows the direct contact to chondrocytes and provides a chondroprotective and anti-inflammatory effect as previously demonstrated in joint-defected rabbit models [4]. Furthermore, the combination of two GAGs, hyaluronic acid (HA) and CS, for intra-articular injection is effective and safe in treatment of patients with knee OA [5]. The efficacy of this treatment, however, might be radically further improved by a prolonged residence time of these polymeric compounds on the target tissue [6].

So far, thiolated polymers generated by the immobilization of thiol-bearing compounds on polymeric backbone have been widely investigated [7]. Owing to their capability to form disulfide bonds between sulfhydryl moieties of thiolated polymers and cysteine substructures of biological surfaces, enhanced adhesive properties of polymers can be achieved [8]. According to this, thiolation might effectively improve bioadhesive properties of CS in order to extend its residence time on articular cartilage, subsequently improving its chondroprotective and anti-inflammatory effects. In fact, lubricin (~227 kDa), a glycoprotein located on the superficial layer of articular cartilage, is herein paid attention. It plays an

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essential role as a joint lubricant, thereby decreasing weight-bearing joint friction and providing chondroprotective effects. Lubricin is composed of a central mucin domain flanked by globular N- and C-termini, somatomedin B-like domain and hemopexin-like domain [9]. Taken into account that cysteine residues are present at these regions, intra- and intermolecular disulfide bonds can be formed as demonstrated by Schmidt et al. [10]. This leads to mucin-like protein multimerization with ability to bind and lubricate cartilage surfaces. According to these contributions, thiolated CS might be a promising therapeutic polymer for treatment of OA.

It was therefore the aim of this study to generate thiolated CS and evaluate its bioadhesive properties as an intra-articular agent for OA treatment. Regarding bioadhesive effect evaluation, porcine intestinal mucus containing mucin glycoprotein was used in our experiments due to structural similarities of mucin to lubricin and for comparison reasons [9]. Bioadhesive properties of synthesized polymer were also evaluated on porcine articular cartilage to confirm the results. The cytotoxic effects of modified CS were additionally investigated using human colon carcinoma Caco-2 cells and rat primary articular chondrocytes.

2. Materials and methods

2.1. Materials

Chondroitin 4-sulfate sodium salt from bovine trachea, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimidehydrochloride (EDAC), amphotericin B, Alcian blue 8GX, calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$), collagenase D from *Clostridium histolyticum*, L-cysteine HCl monohydrate, 5,5′-dithiobis(2-nitroben zoic acid) (Ellman's reagent), dimethyl sulfoxide (DMSO), Dulbecco's Modified Eagle's Medium (DMEM), Hanks' balanced salt solution (HBSS), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tet razolium bromide (MTT), sodium borohydride ($NaBH_4$), sodium chloride (NaCl) and Triton-X®100 were purchased from Sigma–Aldrich (Vienna, Austria). Tris(2-carboxyethyl)phosphine hydrochloride (TCEP HCl) was received from Fluorochem (Hadfield, United Kingdom). 2,4,6-Trinitrobenzenesulfonic acid (TNBS) reagent was purchased from Thermo Fisher Scientific. Fetal bovine serum (FBS), phosphate-buffered saline (PBS) and gentamicin were obtained from Gibco (Invitrogen, Lofer, Austria). All chemicals were of analytical grade and other chemicals were obtained from commercial sources.

2.2. Synthesis of CS-cysteine conjugate (CS-cys)

The CS-cysteine conjugate (CS-cys) was synthesized via amide bond formation between the primary amine of cysteine and the carboxylic acid group of CS [11]. As shown in Fig. 1, the coupling reaction was mediated by using EDAC as a cross-linking agent. Briefly, 1 g of CS was soaked in 50 mL of demineralized water for 30 min. Then, 100 mM EDAC and 500 mg of L-cysteine HCl were added into the polymer solution while stirring. The pH was subsequently adjusted to 5 and the reaction proceeded for 6 h at room temperature. The sample was then dialyzed against water acidified with HCl (pH < 3) at 4 °C in the dark. The dialysis water was changed 3 times a day for 3 days before lyophilization at −50 °C (Christ Gamma 1–16 LSC Freeze dryer, Germany). The dried product was stored at 4 °C until further used. Moreover, a control CS was prepared in exactly the same way as mentioned above, but EDAC was omitted during coupling reaction.

2.3. Disulfide bond reduction

In order to obtain a greater amount of free thiol groups, TCEP HCl was used to reduce disulfide bonds. In detail, 1 g of CS-cys

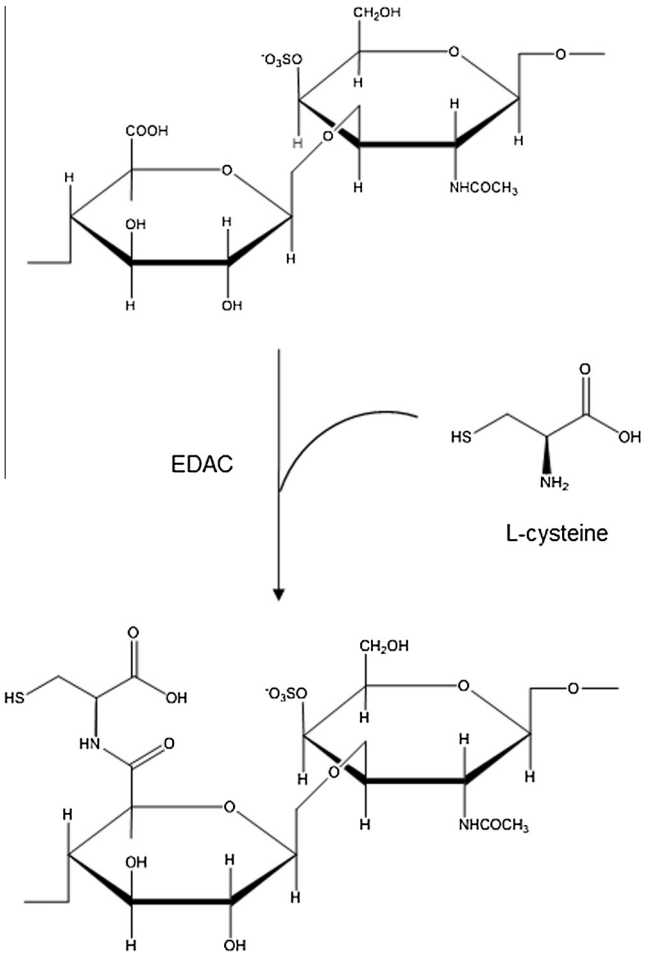

Fig. 1. Synthetic pathway of chondroitin-cysteine conjugates (CS-cys).

and 0.2 g of TCEP HCl were homogenized in 200 mL of demineralized water and kept stirring for 1 h. The reaction mixture was then dialyzed against water for 24 h and freeze-dried.

2.4. Determination of free thiol/total attached cysteine content

The degree of thiolation was determined using Ellman's reagent as previously described [11]. A calibration curve with increasing amounts of L-cysteine was established in order to calculate the amount of free thiol groups. Total amount of attached cysteine was also quantified after reduction with $NaBH_4$ followed by the addition of Ellman's reagent [12].

2.5. Determination of unbound cysteine content

To quantify the amount of remaining cysteine in the product, the free primary amine was determined using TNBS reagent [13]. In brief, polymer was soaked in 1 mL of 0.5% NaCl solution before 0.1 g of $CaCl_2{\cdot}2H_2O$ was added in order to precipitate CS. Afterward, sample was centrifuged at 13,400 rpm for 20 min and 500 μL of supernatant was incubated with 500 μL of 5% TNBS in 8% sodium bicarbonate for 90 min at 37 °C. Sample was thereafter centrifuged and supernatant (100 μL) was transferred to 96-well plate. TNBS reagent reacts with free primary amines of uncoupled cysteine which produces orange-colored derivatives. The absorbance was spectrophotometrically measured at 335 nm using microplate reader (Tecan infinite, M200 spectrometer, Grödig,