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Synthesis of macromolecular mimics of small leucine-rich proteoglycans with a poly(ethylene glycol) core and chondroitin sulphate bristles

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ABSTRACT

Small leucine-rich proteoglycans (SLRPs) are a class of molecules prevalent in almost all tissues types and are thought to be responsible for collagen organization and macro-scale biological properties. However, when they are dysfunctional or degraded, severe pathological phenotypes are observed. Here we investigate macromolecular mimics to SLRPs using poly(ethylene glycol) (PEG) as a core (replacing the protein core of natural SLRPs) and chondroitin sulphate (CS) bristle(s) in an end-on attachment (via epoxide-amine reactions), mimicking the physical structure of the natural SLRPs. Poly(ethylene glycol)-diglycidyl ether (PEG-DEG) and ethylene glycol-diglycidyl ether (EG-DGE) monomers were used to incorporate CS bristles into a macromolecule that closely mimics the SLRP biglycan structure in a grafting-to strategy. The kinetics of these reactions was studied along with the specific viscosity and cytocompatibility of resulting CS macromolecules. Structures were found to incorporate two CS chains (similar to biglycan) on average and exhibited cytocompatibility equivalent to or better than CS-only controls.

1. Introduction

The extracellular matrix (ECM) constitutes the connective tissues of the body and is composed of two main kinds of molecules: (1) insoluble fibers (e.g. collagen) and (2) soluble polymers (e.g. proteoglycans) (Scott & Haigh, 1985, 1988). Mechanically, the insoluble collagen fibers provide tensile strength while the soluble polymers generally maintain the hydrostatic pressure that provides compressive properties. Proteolgycans (PGs) are also implicated in the regulation of collagen fiber structure and, as such, are altered in many disease states making them the focal point of much investigation in recent years (Gualeni et al., 2013, 2010; Parfitt et al., 2011; Stadlinger et al., 2008; Wang et al., 2012; Zhang, Zhang, Wang, & Li, 2014). These properties are thought to be a function of the many highly specialized structures observed in proteoglycans throughout the body (Hocking, Shinomura, & McQuillan, 1998; Iozzo, 1998).

Proteoglycans are composed of a protein core decorated with glycosaminoglycan (GAG) chains each attached by its terminal end (end-on) to the protein core through a glyosidic linkage (Hocking et al., 1998). However, the molecular weight of the protein core, the number of GAG chains and the type of GAG vary with the specific type of proteoglycan. Large proteoglycans such as aggrecan have 100–200 GAG chains attached to the protein core (approximately 300 kDa) in a dense, bottle brush configuration, (Chandran & Horkay, 2012; Roughley, 2006) while a subset of proteoglycans known as small leucine-rich proteoglycans (SLRPs), only generally have 1–2 GAG chains attached to a long, core protein (Hocking et al., 1998; Iozzo, 1998). The GAG chains can vary in composition among four types of GAGs: (1) keratan sulphate (KS), (2) dermatan sulphate (DS), (3) heparan sulphate (HS), and the most common (4) chondroitin sulphate (CS) and their variants (Iozzo, 1998). SLRPs in particular are known to regulate collagen fibrillogenesis and are highly implicated in the development of the crucial tissue-specific collagen ultrastructure organization (Hocking et al., 1998). Two class I SLRPs in particular, (1) biglycan and (2) decorin are found in significant concentrations throughout the extracellular matrix (ECM) in a number of types of tissue, including bone, cornea, tendon, muscle, cartilage, and heart and are found to bind to collagen in the 'd' and 'e' bands of the collagen fibrils (Chen, Shoujun, &

Abbreviations: SLRP, small leucine proteoglycan; PEG, poly(ethylene glycol); DGE, diglycidyl ether; EG, ethylene glycol; GAG, glycosaminoglycan; PG, proteoglycan; ECM, extracellular matrix; CS, chondroitin sulphate.

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David, 2013). When decorin or biglycan is introduced to the type I collagen fibrilogenesis process in vitro, it slows the rate of turbidity development and lateral aggregation, keeping the fibrils thinner and more regulated and leading to normal tissue function (mechanical or optical) (Rada, Cornuet, & Hassell, 1993; Zhang et al., 2009). Furthermore, when these SLRPs are dysfunctional or missing, negative phenotypic changes have been observed. In cornea, larger, more irregular collagen fibrils are observed with depleted decorin or biglycan, and when both are absent, the cornea is no longer transparent (Zhang et al., 2009).

Due to the key role of SLRPs in collagen organization and fibrillogenesis, it is of great interest to produce synthetic SLRP mimics. These mimics can be used to study the mechanics of the fibrillogenesis process as well as used as potential therapeutics in pathologies like Duchenne muscular dystrophy, rheumatoid arthritis, and Turner's syndrome, all of which are associated with SLRP dysfunction and/or degradation (Haslett et al., 2002; Iozzo, 2000). The present paper describes a method for synthetically mimicking SLRP structure by producing SLRP-like molecules consisting of a polymer core (PEG) and 1–2 bovine CS-4 GAG chains attached to the core in an end-on configuration (Sarkar, Lightfoot-Vidal, Schauer, Vresilovic, & Marcolongo, 2012). A PEG core was chosen in order to prevent enzymatic degradation, because the protein core has been identified as a target for degradation in vivo. (Lee, Han, Roughley, & Grodzinsky, 2013). Additionally, the use of a PEG core will allow for the study of the effects of GAG chains on the collagen fibrillogenesis and regulatory processes in addition to enabling the study of various GAG chain spacing without the confounding properties of the core protein, since these synthetic mimics preserve the molecular configuration and charge density of natural PGs without a core protein. Previous attempts have been able to study the effects of the core protein alone (Rada et al., 1993; Raspanti et al., 2008) or the natural SLRP (Garg, Berg, Silver, & Garg, 1989; Lewis et al., 2010; Rada et al., 1993). Enzymes that remove the GAG chain from the core protein produce freely floating GAG chains where any effects due to the molecular architecture of end-on GAG attachment are lost. Since it is unclear if GAG chains are able to interact with collagen fibers independent of their core protein (there is some evidence to suggest they can (Chen et al., 2013; Hocking et al., 1998; Redaelli et al., 2003; Scott, 1988)), it is important to evaluate how the structure and properties of the material contribute to the observed regulatory effects. Eventually this approach can also be used to study the effect of active cores or modifications on natural cores by synthesizing cores with different functionalities.

Previously, several PG mimics have been produced (Lee et al., 2010; Place, Kelly, & Kipper, 2014; Sharma, Lee, Choi, & Kim, 2013; Szente, Puskás, Csabai, & Fenyvesi, 2014; Wang et al., 2007). Panitch et al. present a variety of molecules consisting of CS chains conjugated to hyaluronic acid (HA)-binding peptides that can attach to hyaluronic acid (HA) within the cartilage structure and anchor charge density into the ECM in an attempt to increase osmotic pressure and draw moisture back into cartilage thereby improving its cushioning properties (Sharma, Lee et al., 2013; Sharma, Panitch, & Neu, 2013). Elisseeff et al. have utilized CS to mimic the aggregating properties of some proteoglycans by functionalizing CS with methacylate and aldehyde groups so that it can bond with both native tissue and biomateirals (Wang et al., 2007). These mimics were used as biomaterial adhesives in cartilage implants. Lee et al. used ring-opening metathesis polymerization (ROMP) to construct CS proteoglycan mimics with a synthetic norbornene backbone in a grafting-through process (Lee et al., 2010). These structures were used in microarray studies to probe protein-proteoglycan interactions. Place et al. produced variable GAG density grafted bottle-brush proteoglycan mimetics by attaching both HS and CS to hyaluronan backbones using reductive amination chemistry (Place et al., 2014). Finally, Szente et al., in an attempt to mimic the aggregating properties of aggrecan with HA, used a cyclodextrin based electrostatic crosslinking system to link HA chains together to form a hydrogel useful for drug delivery within cartilage and other connective tissues (Szente et al., 2014).

In this work, CS chains are attached by their terminal ends to a PEG-DGE monomers of varying molecular weight in an epoxide-amine ring-opening reaction. The result is PEG backbones with generally two CS chains attached, mimicking most closely the structure of biglycan which has two either CS or DS GAG chains attached to it, depending on the tissue type (Hocking et al., 1998). PEG has been demonstrated to be biocompatible at low concentrations and is generally considered a compatible biological material (Nishi, Nakajima, & Ikada, 1995; Petersen, Fechner, Fischer, & Kissel, 2002). The resulting molecules may be explored as biglycan mimics. In the present paper, reaction kinetics, molecular characterization, and solution physical behavior, as well as cytocompatibility, are reported.

2. Materials and methods

2.1. Materials

Chondroitin-4-sulphate (CS-4) from bovine cartilage was purchased from Sigma-Aldrich (CS-4, Sigma C6737) due to its primary amine content having an average of one amine (terminal-end) per CS chain (Sarkar et al., 2012). Fluorescamine and all other reagents were purchased from Sigma-Aldrich and used as received.

2.2. Reaction of CS to PEG-based di-epoxides

Epoxides are highly reactive due to the highly strained ring configuration, which facilitates the addition of nucleophilic species such as amines in the presence of protic solvents. This can occur with both primary and secondary amines; however, primary amines are the least sterically hindered thus the major product will be a single PEG-DGE monomer capped with two (2) CS chains on either end rather than long, alternating co-polymer aggregates in which CS chains are attached to more than one PEG-DGE monomer (Fig. 1).

CS was reconstituted in 0.10 M sodium borate buffer (SBB) (pH 9.4) to a concentration of 25 mg/mL. Next, either ethylene glycol-diglycidyl ether (EG-DGE) monomer (MW 174.2 g/mole, mean square end-to-end distance ~0.5 nm) or polyethylene glycol-diglycidyl ether (PEG-DGE) monomer (MW 526 g/mole, mean square end-to-end distance ~5 nm) was added to 10-mL aliquots of CS solution at various concentrations (10, 20, 40, 100, or 200 mM) and allowed to react at 25 °C, 37 °C, or 45 °C with continuous shaking for up to 96 h. During the reaction period (at 24 h, 48 h, 72 h, and 96 h), the reaction was monitored by means of the previous described fluorescamine assay (Sarkar et al., 2012) in which the amount of primary amine present in the sample was monitored. Briefly, 150 uL of sample was removed and assayed in triplicate with 50 mL of 3-mg/mL fluorescamine solution in DMSO. Absorbance was measured at 390 nm in a plate spectrophotometer and Eq. (1) was used to calculate the percentage of conjugation (Sarkar et al., 2012).

$$\%Conjugation = 100 \times \frac{([PA]\,in,\ CS\ without,\ monomer - [PA]\,in,\ CS,\ with,\ monomer)}{[PA]\,in,\ CS,\ without,\ monomer} \quad (1)$$

where [PA] denotes the concentration of primary amines as determined by a standard curve and linear regression fit (Sarkar et al., 2012). Reaction rates for the primary amine were determined by fitting a one-phase association curve (GraphPad Prism 5) to the per-