Carbohydrate Polymers 114 (2014) 344–351

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Synthesis of thiolated glycosaminoglycans and grafting to solid surfaces

Alexander Köwitsch[a], Mauricio Jurado Abreu[a], Ankur Chhalotre[a], Martin Hielscher[b], Steffen Fischer[b], Karsten Mäder[c], Thomas Groth[a,*]

[a] Biomedical Materials Group, Institute of Pharmacy, Martin Luther University Halle-Wittenberg, Heinrich-Damerow-Strasse 4, D-06099 Halle Saale, Germany
[b] Institute of Plant and Wood Chemistry, Dresden University of Technology, Pienner Strasse 19, D-01737 Tharandt, Germany
[c] Pharmaceutical Technology Group, Institute of Pharmacy, Martin Luther University Halle-Wittenberg, Wolfgang-Langenbeck-Strasse 4, D-06099 Halle Saale, Germany

ARTICLE INFO

Article history:
Received 11 March 2014
Received in revised form 4 August 2014
Accepted 5 August 2014
Available online 23 August 2014

Keywords:
Glycosaminoglycan
Thiol-ene chemistry
Surface grafting
Microcontact printing (μcp)
Ellipsometry
Water contact angle measurements (WCA)

ABSTRACT

Glycosaminoglycans (GAGs) with varying degree of sulfation were chemically modified to obtain thiolated analogues (tGAGs) for subsequent surface grafting on vinyl-terminated self-assembled monolayers. Thiolation was achieved by the use of the disulfide containing crosslinker 3,3'-dithiobis(propanoic hydrazide) and subsequent reduction of the disulfide with dithiothreitol. Two different molar ratios of the crosslinker were used for conjugation. The tGAGs were characterized by ^{1}H-NMR, Raman and flow-field-flow-fractionation (A4F) to determine the chemical composition, structure and molecular weight of the products. Ellman's reagent was used to quantify the thiol concentration of tGAGs. The tGAGs were immobilized onto vinyl-terminated glass and silicon via thiol-ene reaction. This was achieved by homogeneous immobilization from solution as well as with microcontact printing and exposure to UV light. The results of water contact angle measurement (WCA), ellipsometry and confocal laser scanning microscopy (CLSM) demonstrated that the resulting surface coverage was dependent on the degree of thiolation of GAGs.

1. Introduction

Glycosaminoglycans (GAGs) are charged polysaccharides that contribute to tissue structure and function in vertebrates by their partly large molecular weight and ability to bind huge quantities of water, but also because of their inherent bioactivity (Varki, 2009). Heparin, hyaluronic acid (HA) and chondroitin sulfate (CS) represent linear, unbranched GAGs that are widely used in different biomedical applications. Heparin is composed of different disaccharide units mainly of D–glucuronic acid or L–iduronic acid linked to a D–glucosamine by a α (1–4) bond. The hexuronic acid can be sulfated at 2–*O* position whereas the glucosamine unit primarily carries a *N*–sulfation and a sulfation at 6–*O* position (Murugesan, Xie, & Linhardt, 2008). Heparin is found in secretory granula of mast cells and released during tissue injury. Heparin is well–known for its anticoagulant properties due to amplification of activity of anti–thrombin III, which plays a major role in clinical use. On the other hand, heparin binds also to adhesive proteins, cytokines and growth factors, which may affect their activity (Capila & Linhardt, 2002). Chondroitin sulfate is composed of disaccharides consisting of β (1–4) D–glucuronic acid and β (1–3) *N*–acetyl–D–galactosamine with potential *O*–sulfation. CS is the most abundant GAG in human body as a component of many different proteoglycans like aggrecan, versican, etc. (Kjellen & Lindahl, 1991). Besides the important role of chondroitin sulfate for compressive resistance of cartilage and other tissues (Nishimura et al., 1998), it plays also a role during fibrilization of collagen (Kvist et al., 2006) and may also interact with regulatory proteins for development and homeostasis of the nerve system (Rogers et al., 2011). In addition, it was also found that CS is a negative regulator of apoptosis by binding of tumor necrosis factor TNF–α (Xu et al., 2008). Hyaluronic acid consists of two repeating saccharide units β (1–4) D–glucuronic acid and β (1–3) *N*–acetyl–D–glucosamine. HA represents the only non–sulfated glycosaminoglycan and is widely used in medical approaches and cosmetic applications (Beasley, Weiss, & Weiss, 2009; Falcone & Berg, 2008; Volpi, Schiller, Stern, & Soltes,

* Corresponding author. Tel.: +49 0345 55 28460; fax: +49 0345 55 27379.
E-mail addresses: Alexander.Koewitsch@pharmazie.uni-halle.de (A. Köwitsch), Jurado.Abreu@hotmail.com (M. Jurado Abreu), Ankurchhalotre@gmail.com (A. Chhalotre), Martin.hielscher@forst.tu-dresden.de (M. Hielscher), Sfischer@forst.tu-dresden.de (S. Fischer), Karsten.maeder@pharmazie.uni-halle.de (K. Mäder), Thomas.groth@pharmazie.uni-halle.de (T. Groth).

http://dx.doi.org/10.1016/j.carbpol.2014.08.027
0144-8617/

2009). While CS is always linked to a core protein, HA is synthesized as a polysaccharide chain, which can be composed by thousands of disaccharide units which are able to bind to multiple aggrecan units which can lead to the formation of huge proteoglycan aggregates (Fraser, Laurent, & Laurent, 1997; Prehm, 1983). Beside the mechanical function of high molecular HA, this GAG has also important regulatory functions during inflammation, tumor progression and metastasis (Toole, 2009). Recently, HA has been chemically sulfated to alter its biological functions because sulfation has shown to be an important precondition for the interaction with growth factors and proteins of the extracellular matrix (Cencetti, Bellini, Longinotti, Martinelli, & Matricardi, 2011; Hintze et al., 2009).

The bioactivity of GAGs is connected to the interaction with a plethora of proteins that possess specific binding regions to bind them (Kjellen & Lindahl, 1991). Particularly adhesion and growth of cells is also regulated by the presence of GAGs in the surrounding extracellular matrix, but also on the surface of cells (Guillame-Gentil et al., 2010; Kuschert et al., 1999). Hence, surface modification of biomaterials with GAGs can provide specific adhesive cues for cells, like HA or CS to cell surface receptors CD 44 (Knudson, Aguiar, Hua, & Knudson, 1996; Iida et al., 1998) or act indirectly by binding of adhesive proteins with heparin–binding domains and integrins (Haugen, Mccarthy, Roche, Furcht, & Letourneau, 1992). The alteration of surface composition is often applied to control interaction of biomaterials with proteins and to mediate adhesion, proliferation and differentiation (Guillame-Gentil et al., 2010; Lee et al., 2013; Murugesan et al., 2008).

Hence, immobilization of some GAGs has been introduced to control the bioactivity of surfaces to promote binding of growth factors, adhesive proteins and direct behaviour of cells in scaffolds (Baldwin & Kiick, 2010; Gribova, Auzely-Velty, & Picart, 2012). However, different methods have been applied for GAG immobilization. Some require rather harsh methods of surface modification and subsequent covalent grafting of GAGs to the surface (Huang, Guduru, Xu, Vienken, & Groth, 2010). Other approaches use physical forces to adsorb GAGs that may lead to more loosely or irreversible immobilization (Aggarwal et al., 2013). For most of the recent techniques for covalent immobilization of GAGs several steps for surface modification or GAG treatment are necessary (Edlund, Danmark, & Albertsson, 2008). Another disadvantage might be that some methods are limited to oligosaccharides of GAGs (Chuang & Masters, 2009; Seo et al., 2007). We have shown recently that covalent grafting of pre–activated hyaluronan leads to functional material surfaces that guide binding of protein ligands like aggrecan but also adhesion of cells (Köwitsch et al., 2011). Here we focus on the use of free thiols as active moieties that allow direct covalent linkage of GAGs via click chemistry to vinyl groups or gold and expand the range of GAGs from hyaluronan to chondroitin sulfate, heparin and one sulfated hyaluronan. For thiolation of GAGs a disulfide containing crosslinker which exposes reactive hydrazide groups is applied. The latter has the benefit that the thiol group can be released or reactivated with a thiol-reducing agent and also uses less material than direct application of unprotected thiolation agents, which have to be used in excess thus resulting in a less effective thiolation (Hermanson, 1996; Shu, Liu, Luo, Roberts, & Prestwich, 2002). Beside the details of synthesis and characterization of derivates that differ also in degree of thiolation, we show their grafting to self–assembled monolayers via thiol–ene reaction and the generation of 2–D–structured surfaces via microcontact printing (μCP).

2. Materials and methods

2.1. Materials

Heparin (Hep) from porcine intestinal mucosa was purchased from Serva Electrophoresis GmbH (Heidelberg, Germany). Hyaluronic acid (HA) sodium salt (Mw 1.3 MDa) was provided by Kraeber & Co GmbH (Ellerbek, Germany). Low molecular weight HA (Mw 15 kDa) was obtained by acidic hydrolysis as reported previously (Köwitsch et al., 2011). Sulfated HA with a sulfation degree (D_{Sulf}) of 1.3 was provided by Innovent e.V. (Jena, Germany) (Hintze et al., 2009). Chondroitin sulfate A (from bovine trachea), N–Hydroxysuccinimide (NHS) and 6–aminofluorescein were obtained from Sigma–Aldrich (Schnelldorf, Germany). The dialysis bag (Spectra/Por membrane, Mw cutoff = 3500), D_2O and organic solvents were provided by Carl Roth GmbH (Germany). 3,3‘–dithiobis(propanoic hydrazide) (DTPH) was synthesized and analyzed as described before (Vercruysse, Marecak, Marecek, & Prestwich, 1997). 7–octenyldimethychlorosilane was purchased from ABCR GmbH & Co. KG (Karlsruhe, Germany), dithiothreitol (DTT) and 2–(N–Morpholino)ethanesulfonic acid monohydrate (MES) from VWR International GmbH (Dresden, Germany), tris(hydroxymethyl)aminomethane (TRIS) from Merck KGaA (Karlsruhe, Germany), 5,5′–dithiobis–(2–nitrobenzoic acid) (DTNB, Ellman‘s reagent) and 1–ethyl–3–(3–dimethylaminopropyl) carbodiimide hydrochloride (EDC) from Alfa Aesar (Karlsruhe, Germany).

2.2. Synthesis of thiolated glycosaminoglycans (tGAGs)

The synthesis of thiolated GAGs was performed with some modification according to a recent paper (Shu et al., 2002). All reactions were conducted at room temperature (RT). Briefly, 0.8 mmol of GAG (Hep, CS, HA, sHA) was dissolved in micropure water (80 mL). Then, 3,3‘–dithiobis(propanoic hydrazide) (DTPH, 0.2 mmol (representing 0.25 equivalents of the available —COOH groups) or 0.04 mmol (0.05 eq.)) was added to the stirring solution and the pH was adjusted to 4.75. Thereafter, EDC (0.5 mmol (for 0.25 eq.)/0.1 mmol (for 0.05 eq.)) was added while the pH was maintained at 4.75. After additional 3 h incubation 1 M NaOH was used to increase the pH to 7.0. Then, DTT (2.0 mmol) was added and the pH was raised to 8.5. The solution was stirred over night. Then, the pH of the reaction mixture was lowered to 3.5 before the solution was dialyzed against diluted HCl (pH 3.5) containing 100 mM NaCl for 2 days and against diluted HCl (pH 3.5) for 2 days. Finally, the solution was freeze–dried and a white product was obtained.

For the visualization via fluorescence microscopy the thiolated CS (tCS1.16) was labelled with 6–aminofluorescein. Therefore, 0.35 mmol of tCS1.16 was dissolved in 50 mL MES buffer (50 mM, pH 4.7). Then EDC (0.7 mmol) and NHS (0.7 mmol) were added and the solution was stirred for 1 h. The pH was adjusted to 7.0 and 9 mg of 6–aminofluorescein in 4 mL of DMSO was added. The container was wrapped in aluminium foil and stirred over night. Afterwards, the solution was acidified and dialyzed against diluted HCl (pH 3.5) for 5 days. Finally, the solution was freeze–dried and a pale yellow product was obtained.

2.3. Characterization of thiolated glycosaminoglycans (tGAGs)

The chemical structure of the thiolated GAGs was analyzed by ^{1}H–NMR (Varian Gemini 2000, Palo Alto, CA, USA). ^{1}H–NMR samples were dissolved in D_2O and measured with 256 repetitive scans at a frequency of 400 MHz to obtain the corresponding spectra.

Raman spectroscopy was performed with a Bruker MultiRam spectrometer (Ettlingen, Germany) equipped with a germanium diode as detector that is cooled with liquid nitrogen. A cw–Nd:YAG–laser with an exciting line of 1024 nm was applied as light source for the excitation of Raman scattering. The spectra were recorded over a range of 3500–0 cm^{-1} using an operating spectral resolution of 3 cm^{-1} and a laser power output of 125 mW. The thiol content of tGAGs was determined according to the results of the