Acta Biomaterialia 9 (2013) 8158–8166

Contents lists available at SciVerse ScienceDirect

Acta Biomaterialia

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

Probing the biofunctionality of biotinylated hyaluronan and chondroitin sulfate by hyaluronidase degradation and aggrecan interaction

Noomi Altgärde [a,1], Erik Nilebäck [a,b,1], Laura de Battice [a], Iva Pashkuleva [c,d], Rui L. Reis [c,d], Jana Becher [e], Stephanie Möller [e], Matthias Schnabelrauch [e], Sofia Svedhem [a,*]

[a] Department of Applied Physics, Chalmers University of Technology, 412 96 Göteborg, Sweden
[b] Biolin Scientific AB, Hängpilsgatan 7, 426 77 Västra Frölunda, Sweden
[c] 3B's Research Group – Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, AvePark, 4806-909 Taipas, Guimarães, Portugal
[d] ICVS/3B's – PT Government Associate Laboratory, Braga/Guimarães, Portugal
[e] Biomaterials Department, INNOVENT e.V., Pruessingstrasse 27 B, 07745 Jena, Germany

ARTICLE INFO

Article history:
Received 26 February 2013
Received in revised form 7 May 2013
Accepted 28 May 2013
Available online 5 June 2013

Keywords:
Glycosaminoglycan
Biotin
Surface-sensitive techniques
Hyaluronidase
Aggrecan

ABSTRACT

Molecular interactions involving glycosaminoglycans (GAGs) are important for biological processes in the extracellular matrix (ECM) and at cell surfaces, and also in biotechnological applications. Enzymes in the ECM constantly modulate the molecular structure and the amount of GAGs in our tissues. Specifically, the changeable sulfation patterns of many GAGs are expected to be important in interactions with proteins. Biotinylation is a convenient method for immobilizing molecules to surfaces. When studying interactions at the molecular, cell and tissue level, the native properties of the immobilized molecule, i.e. its biofunctionality, need to be retained upon immobilization. Here, the GAGs hyaluronan (HA) and chondroitin sulfate (CS), and synthetically sulfated derivatives of the two, were immobilized using biotin-streptavidin binding. The degree of biotinylation and the placement of biotin groups (end-on/side-on) were varied. The introduction of biotin groups could have unwanted effects on the studied molecule, but this aspect that is not always straightforward to evaluate. Hyaluronidase, an enzyme that degrades HA and CS in the ECM, was investigated as a probe to evaluate the biofunctionality of the immobilized GAGs, using both quartz crystal microbalance and high-performance liquid chromatography. Our results showed that end-on biotinylated HA was efficiently degraded by hyaluronidase, whereas already a low degree of side-on biotinylation destroyed the degrading ability of the enzyme. Synthetically introduced sulfate groups also had this effect. Hence hyaluronidase degradation is a cheap and easy way to investigate how molecular function is influenced by the introduced functional groups. Binding experiments with the proteoglycan aggrecan emphasized the influence of protein size and surface orientation of the GAGs for in-depth studies of GAG behavior.

1. Introduction

The interest in glycosaminoglycan (GAG)-related interactions is increasing due to their importance in various biological processes and the characteristics of their chemical structure. GAGs are involved in the mediation of cellular processes, the stabilization of growth factors and the development of various diseases [1,2]. Understanding interactions between GAGs and proteins in the extracellular matrix (ECM) (Fig. 1) and at cell surfaces is expected to bring important advances in drug development and tissue-contacting materials such as implants and cell culture substrates. For example, the GAG hyaluronan (also named hyaluronic acid, HA) is frequently used in research and in clinic as cell substrates and tissue scaffolds [3–5]. The naturally sulfated GAG chondroitin sulfate (CS) has gained increased attention because of its role in bone and cartilage tissue and its interactions with growth factors and other proteins [6–9]. The molecular structure of GAGs is heterogeneous and changeable over time, and remodeling occurs through the action of a range of different enzymes. More than 30 sulfotransferases that control the degree of sulfation of the GAGs have been identified [10,11], and are considered to be crucial in many protein interactions [1,12,13]. The amount of GAGs in the ECM is regulated by GAG-digestive enzymes like hyaluronidase, heparinase and chondroitinase [14,15]. Because of the changeable nature

* Corresponding author. Tel.: +46 0317723428.
E-mail addresses: noomi.altgarde@chalmers.se (N. Altgärde), nileback@chalmers.se (E. Nilebäck), battice@chalmers.se (L. de Battice), pashkuleva@dep.uminho.pt (I. Pashkuleva), rgreis@dep.uminho.pt (R.L. Reis), jb@innovent-jena.de (J. Becher), sm@innovent-jena.de (S. Möller), ms@innovent-jena.de (M. Schnabelrauch), sofia.svedhem@chalmers.se (S. Svedhem).
[1] These authors contributed equally to this work.

1742-7061/$ - see front matter © 2013 Published by Elsevier Ltd. on behalf of Acta Materialia Inc.
http://dx.doi.org/10.1016/j.actbio.2013.05.031

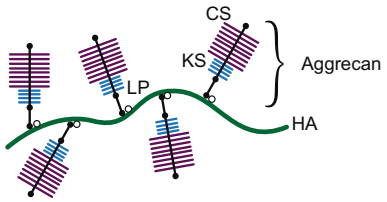

Fig. 1. In the ECM, the GAG hyaluronan (HA) interacts with the proteoglycan aggrecan, forming larger aggregates that are stabilized by a link protein (LP, not used in this study). Aggrecan consists of a core protein with attached GAG chains; chondroitin sulfate (CS) and keratan sulfate (KS).

of GAGs, samples with well-defined chemical and physical properties are required to unravel GAG-related interactions. An attractive approach to studying GAG-related interactions in real time is to employ surface-based analytical techniques, which require immobilization of the GAG derivatives to a sensor surface [16,17]. Often when immobilizing a biomolecule, specific chemical groups are introduced in order to have a directed coupling to the surface. Chemical modifications of GAGs (e.g. biotinylation) could not only influence the primary structure but also secondary arrangements of the GAGs, hence changing their biofunctionality. It has been shown that biotinylation may alter recognition in biomolecular interactions [18], and we have recently shown that the introduction of hydrazide groups along CS chains, to allow immobilization to model lipid membranes, altered subsequent interaction with the growth factor bone morphogenetic protein-2 (BMP-2) [19].

In this study, we have employed biotinylation as a method to immobilize HA and CS derivatives to sensor surfaces, via the tetravalent protein streptavidin. Because of its high binding affinity ($K_d \approx 10^{-15}$ M), the interaction between biotin and streptavidin is efficient in anchoring biomolecules to surfaces [20]. GAG derivatives were biotinylated either side-on, to a low and high degree, or end-on (Fig. 2). The main surface-sensitive technique used was quartz crystal microbalance with dissipation monitoring (QCM-D). The usage of this technique as a tool in carbohydrate research is increasing, since it is possible to probe biomolecular structures that are associated with a large amount of solvent [17]. GAG-layer build-up was followed using QCM-D and surface plasmon resonance (SPR) detection, and additional information was obtained by physico-chemical characterization with respect to surface hydrophilicity and surface charge. Hyaluronidase degradation was studied using QCM-D and high performance liquid chromatography (HPLC).

This study had several aims. The first aim was to characterize GAG layers obtained through immobilization of side-on and end-on biotinylated HA and CS chains. CS is a naturally sulfated GAG whereas HA is not. Since sulfation patterns of CS vary in vivo (see above), GAG derivatives where sulfate groups are systematically introduced could have great potential in molecular structure–function analysis [13]. Because of this, synthetically sulfated HA (sHA) and oversulfated CS (sCS) were also studied. The second aim was to investigate whether the biofunctionality of immobilized GAGs can be probed by the action of hyaluronidase. This enzymatic degradation is a well-known process where random cleavage of the β-1,4 glycosidic bond in HA and CS under acidic conditions yields tetra- or hexasaccharides as products [21–23]. The degradation of immobilized GAGs is hypothesized to yield a mass loss in surface bound material by QCM-D as long as the enzyme is able to recognize the GAG chains. The enzyme could thus be used to test how the biofunctionality of immobilized GAGs is affected by biotinylation and sulfation, prior to interaction studies using more complex and rare ligands. The third aim was to compare the degradation by hyaluronidase and the binding of the proteoglycan aggrecan to HA, as two measures of biofunctionality, using a subset of HA derivatives. Aggrecan is a common constituent in the ECM, where it interacts with HA (Fig. 1) [24,25].

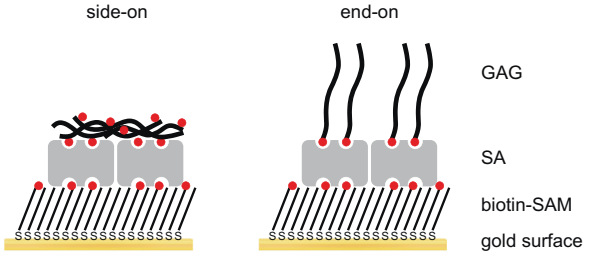

Fig. 2. Side-on and end-on immobilization of biotinylated GAG chains via the strong binding of biotin to streptavidin (SA). Biotinylated gold surfaces were obtained through the formation of self-assembled monolayers (SAMs).

2. Materials and methods

2.1. Chemicals

Oligo-ethylene glycol (OEG) disulfides with terminal hydroxyl (dS-OEG, structure: $-[S-CH_2-(CH_2-O-CH_2)_7-CH_2-OH]_2$, MW: 771.0 Da) and biotin groups (dS-OEG-biotin, structure: $-[S-C_2H_4-CO-NH-(CH_2-O-CH_2)_9-NH-CO-C_4H_8-\text{Biotin}]_2$, MW: 1539.9 Da) were purchased from Polypure, Norway. Phosphate buffered saline (PBS) buffer was made from tablets (NaCl 137 mM, KCl 2.7 mM, phosphate buffer 10 mM, pH 7.4) from Sigma. Streptavidin (Sigma), hyaluronidase type I-S and type IV-S from bovine testes (Sigma), aggrecan (Sigma), HA for preparation of synthetically modified derivatives (Aqua Biochem, Germany), HA for reference sample in HPLC measurements (Sigma), CS (degree of sulfation ($DS_{sulfate}$) = 0.9, Kraeber, Germany) and modified GAG derivatives (see Table 1 and below) were aliquoted in water and stored at −20 °C. Water was purified and deionized to a resistivity of >18.2 MΩ cm with a MilliQ system (Millipore, France). Biotin-LC-hydrazide (6-(biotinamido)hexanehydrazide) was purchased from Pierce (Thermo Fisher, Germany).

Table 1
GAG derivatives used in the study.

GAG	Name	M_W (kDa)	DS_{biotin}[a]	$DS_{sulfate}$[b]
Hyaluronan (HA)	HA[c]	1000[c]	–	–
	b-HA end-on	23	end-on (4.2 μg mg^{-1})	–
	b-HA 2.6%	1000	2.6% (15.7 μg mg^{-1})	–
	b-HA 4.3%	90	4.3% (25.3 μg mg^{-1})	–
	b-sHA end-on	30	end-on (4.7 μg mg^{-1})	3.1
Chondroitin sulfate (CS)	CS	20	–	0.9
	b-CS end-on	20	end-on (6.3 μg mg)	0.9
	b-CS 0.4%	20	0.43% (2.6 μg mg^{-1})	0.9
	sCS	20	–	3.4
	b-sCS 0.5%	20	0.47% (2.9 μg mg^{-1})	3.4

[a] The degree of biotinylation is given both in percentage as well as mass of biotin per mass of GAG. DS_{biotin} = 100% would correspond to one biotin group per repeating unit.
[b] $DS_{sulfate}$ indicates the average number of sulfate groups per repeating unit.
[c] In HPLC experiments, HA from Sigma with a wider MW distribution was used.