Progress in structural analysis of glycosaminoglycans and their applications in biomaterials

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The rising interest in the application of glycosaminoglycans (GAGs) is one of the main reasons for exploring different species and optimizing the extracting conditions of various GAGs in the last decade. Recent research data on GAGs have suggested that they have many new biological functions such as anti-atherogenesis, anticoagulation, prevention and cure of arthritis, morphogenesis and cell division. They are widely applied in functional food, clinical medicine, cosmetics and biomaterial. Especially, in the biomaterials industry, an increasing number of new applications have been found for GAGs such as tissue-engineering material, biodegradable film-forming materials and micro-encapsulating agents. However, these bioactivities and applications are dependent on their fine structure with different monosaccharide unit and substitute group patterns. This review provides recent information on GAGs preparation, determination and structural assay, as well as their potential novel or improved applications such as tissue engineering biomaterial.

Key words: Glycosaminoglycan, structure analysis, biomaterial.

INTRODUCTION

The glycosaminoglycan (GAG) containing hexosamine and uronic acid (or hexose) and other various substituent groups, are a group of structurally diverse polysaccharide polymers from animals and exist abundantly either natively or as part of proteolycans (PGs) in extracellular matrices and on cell surfaces. The GAGs chains consist of generally repeated disaccharide units that are negatively charged and assembled into long linear/acidic chains with variable lengths and composition (hexosamine, uronic acid, sulfate group). Thus conveying a high polyanionic charge to these macromolecules (Raman et al., 2003). There are four major classes namely hyaluronic acid (HA), chondroitin/dermatan sulfate (CS/DS), heparin/heparan sulphates (HS) and keratin sulfate (KS) according to different kinds and quantities of monosaccharide component and substituent group in GAG molecular chain. The bioactivities and their applications are quite distinct from each other. Some examples of GAGs uses in nature include heparin as an anti-coagulant, HA as a component in the synovial fluid lubricant in body joints and CS, which can be found in connective tissues, cartilage and tendons. Furthermore, the patterns and degree of sulfation, molecular mass, relative amounts of iduronic and glucuronic acid (GlcA) and hexosamine in the same kind of GAG considerably changes in quantity and position according to the species, age and/or tissues of origin and this plays an important role in the various bioactivities and applications (Volpi, 2004). For example, bovine tracheal cartilage is mainly sulfated in position four of GalNAc, which can protect high-dense lipoprotein against copper-induced oxidation (Albertini et al., 1999). CS from shark cartilage has a higher level of 2-sulfated GlcA residues as well as 6-sulfated N-acetylgalactosamine (GalNAc) which has anticancer function (Sim et al., 2007). GAGs reported to date have been used clinically for the treatment of chronic diseases such as degenerative arthritis, cirrhosis and chronic photo damage due to the emerging bioactivities such as antioxidant, anti-atherogenesis, anticoagulation, prevention and cure of arthritis, etc.
Table 1. Structural characteristics and main preparation methods of GAGs.

<table>
<thead>
<tr>
<th>GAG</th>
<th>Disaccharide unit</th>
<th>Modification state and rare moiety</th>
<th>Industrial preparation method and main source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chondroitin sulfate (CS)</td>
<td>β-D-glucuronic acid (1→3)-linked to a 2-acetamido-2-deoxy-β-D-galactopyranose.</td>
<td>2-O-(IdoA), 4O-, 6O-, on NAc-Gal and rare 3-O on GlcA.</td>
<td>Extraction from cartilage such as pig laryngeal cartilage, chick keel cartilage, and bovine nasal cartilage, etc.</td>
</tr>
<tr>
<td>Hyaluronic acid (HA)</td>
<td>β-D-glucuronic acid (1→3)-linked to 2-acetamido-2-deoxy-β-D-galactopyranose.</td>
<td>Unsulfated</td>
<td>Extraction from crista galli, umbilical cord and pig skin; fermentation method</td>
</tr>
<tr>
<td>Heparin</td>
<td>α-L-iduronic acid (1→4)-linked to glucosamine.</td>
<td>N-sulfation and acetylation as well as 2-O-, 4O-, 6O- as well as 3-O- sulfation coupled with C5 of -COOH epimerization on GlcA and IdoA.</td>
<td>Extraction from intestinal mucosa of pig or bovine.</td>
</tr>
<tr>
<td>Heparin sulfate (HS)</td>
<td>α-L-iduronic acid (1→4)-linked to glucosamine or β-D-glucuronic acid (1→4)-linked to glucosamine.</td>
<td>N-sulfation and acetylation as well as 2-O-, 4O-, 6O- as well as 3-O- sulfation coupled with C5 of -COOH epimerization on GlcA and IdoA.</td>
<td></td>
</tr>
<tr>
<td>Dermatan sulphates (DS)</td>
<td>α-L-iduronicacid (1→3)-linked to a 2-acetamido-β-deoxy-β-D-galactopyranose</td>
<td>2-O-(IdoA), 4O-, 6O- on NAc-Gal and rare 3-O on IdoA</td>
<td></td>
</tr>
<tr>
<td>Keratin sulfate (KS)</td>
<td>β-D-galactose (1→4)-linked to 2-acetamido-2-deoxy-β-D-glucopyranose</td>
<td>Only 6O-sulfation, KS-II may be fucosylated</td>
<td></td>
</tr>
</tbody>
</table>

(Xiong et al., 2007). More recently, there have been a number of reports on the biomedical activities including tissue engineering, drug delivery, regenerative medicine and surgery (Strehin et al., 2010; Fajardo et al., 2010; Eun et al., 2007).

GAGs have become well known owing to their biomedical bioactivities and material characterization, the market of nutraceuticals containing various GAGs have dramatically increased, they are being developed as functional food, biochemical pharmaceutical, cosmetics and biomaterial. In order to better utilize various GAGs, more research is being focused on the preparation, functional characteristics, bioactivities and their applications in different areas. In this review, we mainly focused on the latest preparation, separation, determination and identification methods and applications of GAGs.

### STRUCTURAL CHARACTERISTICS AND PREPARATION OF MAIN GAGS

As shown in Table 1, the disaccharide units of each GAGs are remarkably different, especially, the molecular components including kinds of hexosamine and uronic acid, position of sulfate group, which display a very large variety of structures. HA is the relatively simple polymeric species which has no sulfation pattern. CS can also be classified into some sub-groups such as CS-A (galactosamine can be sulfated on the C-4, mainly from pig laryngeal/ bovine nasal cartilage), CS-C (galactosamine can be sulfated on the C-6, mainly from pig laryngeal/ bovine nasal cartilage) and CS-E (galactosamine can be sulfated on the C-4 and C-6, originating from shark cartilage). Repeating disaccharide units in heparin and HS are either α-L-iduronic acid (1→4)-linked to glucosamine or β-D-glucuronic acid (1→4)-linked to glucosamine. The glucosamine is either N-sulfated or N-acetylated, and can be ester O-sulfated at the C-3 or C-6 position, while the uronic acid can be sulfated on C-2 position (Imberty et al., 2007). For example, HA is the sole GAG which is free from sulfate and protein. General boiling water extraction is suitable for it.
For the other GAGs, sodium salts such as NaCl and NaAc with different concentrations are the common extraction reagents. At present, GAGs are mainly extracted by enzymolysis of exogenous proteinase or sodium hydroxide for their attachment to a protein core and formation of GAG-protein/fat aggregates (Chen et al., 2011). Their usual and advanced separation and purification technologies are membrane separation, chromatographic separation (ion-exchange chromatography), hydrogen peroxide oxidation for decoloration and fractioned precipitation of alcohol. For promoting the bioactivities and applications, some of their chemical modifications are becoming hot topics in recent years. For example, polysulfated CS derivatives are prepared because of their higher antiviral and anticoagulant capacity, heparin with low molecular weight is generally popular for its stronger antithrombotic effect, longer half-time, weaker hemorrhage side effect and higher bioavailability. The industrial production methods mainly include degradation of nitrous acid/hydrogen peroxide, β-elimination and enzymolysis.

**COMPOSITION ASSAY OF GAG**

The difference of GAGs are attributed to the component including hexuronic acid, hexosamine and sulfate pattern, thus their determination and identification are becoming the basis of understanding the first structure which further illustrates the structure-effect relationship of various GAG.

**Determination and identification of hexuronic acid**

During the past decades, high-performance liquid chromatography (HPLC), ion chromatography (IC) and gas chromatography (GC) were used to determine uronic acid (Kakit et al., 2006). These methods are not widespread because they easily give lower determination value due to the lability of uronic acids in aqueous acid which need complicate derivation process before column analysis. Thus, the most common assay method for the analysis of hexuronic acid is spectrophotometric method, which is based on the chromogenic formation between hexuronic acid produced by sulfuric acid hydrolysis of hexuronic acid and the color forming reagents including carbazole or meta-hydroxydiphenyl (Filisetti-Cozzi and Carpita, 1991). For determination and identification of hexuronic acid simultaneously, IC and GC methods are better than the above colourimetry. Owing to the fact that glucosidic bond which is consecutive to uronic acid is difficult to be hydrolyzed by acid and hydrolyzed uronic acid is easy to form lactone, uronic acid is determined and identified by GC analysis after reduction of carboxy group in uronic acid by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, aldononitrile acetates deviation (treated by hydroxylamine hydrochloride, pyridine and acetic anhydride, respectively). It makes uronic acid easier to be hydrolyzed by acid and avoids lactone production and use of uronic acid standard (Kakit et al., 2006).

**Determination and identification of hexosamine**

Elson-Morgan method is very easy for assay of hexosamine. However, the hydrolysis at 100°C and acetylation again usually demands intensive time and labor leads to incomplete hydrolysis and lower results. In recent years, more attentions have been paid to HPLC including amino-bound column, strong-anion exchange high-performance liquid chromatography (SAX-HPLC), reverse phase high-performance liquid chromatography (RP-HPLC) and so on. HPLC and pulsed amperometric detection (PAD) with a single isocratic analysis are adapted to isolate and assay simultaneously amino sugars as well as neutral saccharides (Cheng and Kaplan, 2003). In our laboratory, we used RP-HPLC procedures and ultraviolet and detection before automated precolumn derivation of ChS hydrolyzate with o-phthalaldehyde (OPA) to detect the content of GalNAc in CS from pig laryngeal cartilage. This method, similar to the above anion-exchange column chromatography, has higher resolution and reproducibility than Elson-Morgan method. Especially, it can also distinguish GalNAc from GlcNAc.

**Determination and identification of sulfate group**

Main methods used for the assay of sulfate group include gravimetric estimation as barium sulphate, turbidimetry and ion chromatography (Wang et al., 2004). In contrast to turbidimetry, the advantage of ion chromatography is simplicity, good reproducibility and sensitivity, and no other interference of impurities.

**COLORIMETRY ASSAY AND IDENTIFICATION OF GAG**

Considering the complexity and inaccuracy caused by hydrolysis or derivation procedure for determination of substituent groups, the direct determination methods such as colorimetry, chemical titrimetry and chromatography are rapidly developed. Owing to the polyanionic property of GAG, they can form soluble GAG-dye complex with cationic dye such as alcian blue, azure A, victoriapure blue BO or dimethymethyl blue, which alter the absorption spectra as compared to that of the dye alone (Liu and Qian, 2005). The merit of this method is rapid, simple and does not need strong acid or high temperature. However, it may be affected by the other
polyanionic macromolecules such as other carbohydrates, protein, DNA and RNA. At present, the emerging structure analysis or identification methods of GAG are spectrum analysis combined with enzymolysis technology (Ola et al., 2005). The GAGs are long chain molecules with higher molecular weight. Their complete analysis often needs hydrolysis before spectrum analysis. However, the occurrence of hexuronic acid, hexosamine and sulfate usually make them to inhibit partly the chemical hydrolysis such as hydrolysis, methylation analysis, smith degradation, periodate oxidation which are suitable for other general polysaccharide.

GAG lyases from bacterial sources but more recently of recombinant origin selectively degrade GAGs. For example, chondroitinase ABC will degrade virtually all chondroitin and DS, while leaving heparin (or HS, HA, KS) chains intact. Conversely, heparitinase enzymes will degrade nearly all forms of HS, but are unable to degrade CS, HA, DS and KS. Hyaluronidase will degrade HA but not others. More importantly, is that chondroitinase B only acts on the ChS-B, chondroitinase AC is for C4S and/or Ch6S and chondroitinase C for C6S. The generating oligosaccharides containing a Δ4,5-unsaturated uronic acid residue at the new non-reducing terminus exhibits an absorbance maximum at 232 nm, which permit the detection of the oligosaccharide products of the GAG lyases using ultraviolet spectroscopy. Thus, these specific GAG lyases, is followed by the diversified isolation techniques including size exclusion chromatography (SEC), SAX-HPLC, HPCE and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Ambrosius et al., 2008), which permit the identification of disaccharide characterization. Thus, analysis of unsaturated disaccharides is simple and rapid, avoiding any time-consuming derivatisation reactions. SAX-HPLC isolation and determination for unsaturated disaccharides after treatment with chondroitinase ABC is used to evaluate the content and biochemical properties (sulfation patterns) of ChS in shark cartilage powders and finished products containing shark cartilage powder. A linear gradient of 0 to 1.0 M NaCl (pH 3.5) for 41.55 min is used and the profile is monitored at 232 nm. The recovery ranges from 95.27 to 102.39% with precision from 2.27 to 3.95% (Xiong et al., 2007). Separation of milligram amounts of heparin oligosaccharides with sufficient purity ranging in degree of polymerization from four to 32 is achieved using continuous elution PAGE. The results of PAGE, strong anion exchange (SAX)–HPLC, electrospray ionization Fourier transform mass spectrometry (ESI-FTMS), and nuclear magnetic resonance (NMR) characterization of the gel-eluted oligosaccharides demonstrate that this method of separation does not introduce chemical artifacts and alter the oligosaccharide structure (Tatiana et al., 2010).

Mass spectrometry (MS) has also been applied to the analysis of oligosaccharides obtained from various GAGs. It is comparably sensitive and has the particular advantage that individual GAG can be easily differentiated by the characteristic mass differences of their polymer repeat units. Fast-atom bombardment MS (FAB-MS), matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) MS, ESI-MS, matrix-assisted laser desorption/ionization MS (MALDI-MS) and tandem MS are suitable for determining the characterization of oligosaccharides. ESI-MS/MS techniques provide high mass accuracy, structural information and ability to quantify the fragments. They have become more attractive and prominent for the analysis of oligosaccharides (Nimptsch et al., 2009; Connie and Leary, 2010; Seoa et al., 2011). A mixture of GAG chains from a plasma proteoglycan bikunin was isolated and identified using native, continuous-elution PAGE alone or in combination with partial enzymatic depolymerization, and the resulting fractions were analyzed by ESI-FTMS (Tatiana et al., 2011).

However, HPCE, SAX-HPLC or MS usually gives no information about linkage positions and anomic configurations. One- and two-dimensional NMR have been used to characterize their subtle structure.

**APPLICATION OF GAG IN BIOMATERIALS**

In many medicine system used in tissue engineering, surgery and drug delivery, there is need for materials which are both adhesive and in situ forming. These materials can be composed of synthetic, biological or a combination of such materials. Biological molecules such as GAGs have some advantages such as biodegradation, adhesiveness and bioactivities (stimulating cells to proliferate, migrate, differentiate and produce extracellular matrix) (Strehin et al., 2010). Recently, an increasing number of new applications of GAGs (mostly HA, CS and heparin) in biomaterial industry such as tissue-engineering material, biodegradable film-forming materials and micro-encapsulating agents were presented due to their physical-chemical (polaronic, biodegradable and biologically compatible) and biological properties.

**APPLICATION OF GAGS IN TISSUE ENGINEERING**

Tissue engineering is a new and exciting technique which has the potential to create tissues and organs de novo (Chen et al., 2002). The scaffold acts as a physical support structure and insoluble regulator of cell activity. It has emerged as a promising alternative approach in the treatment of malfunctioning or lost organs. For facilitating cell adhesion, promoting cell growth and allowing the retention of differentiated cell functions and regeneration of tissue, the scaffold should be biocompatible, biodegradable, highly porous with a large surface/volume ratio, mechanically strong and malleable. Thus, various
CONCLUSION

The remarkable physical-chemical, therapeutic and biological importance of GAGs ensures a continued interest in advancing new technologies for their extraction, separation and purification, identification and applications in functional food, biochemical pharmaceutical, cosmetics and biomaterial.

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