Synthesis and Characterization of Chemically Modified Hyaluronan and Chondroitin Sulfate

Schiller J.[1,*], Becher J.[2], Möller S.[2], Nimptsch K.[1], Riemer T.[1] and Schnabelrauch M.[2]

[1]*University of Leipzig, Medical Faculty, Institute of Medical Physics and Biophysics, Härtelstr. 16-18, 04107 Leipzig, Germany*

[2]*INNOVENT e.V. Jena, Biomaterials Department, Prüssingstr. 27B, 07745 Jena, Germany*

Abstract: Glycosaminoglycans (GAGs) occur in many tissues. There is increasing evidence that the sulfation of GAGs does not occur at random, but a "sulfation code" exists that mediates the physiological functions of GAGs. Therefore, certain GAGs might also have interesting pharmacological properties. We will give here an overview on chemical functionalization strategies of hyaluronan (HA) and chondroitin/dermatan sulfate (CS/DS). This is a challenging task from the synthetic as well as analytical viewpoint because both, regioselective GAG modifications with defined molecular weights and methods to unequivocally characterize the structures of the modified GAGs must be adapted and/or developed. Consequently, in the last part of this review, methods of GAG characterization will be introduced and their capabilities and limitations discussed.

Keywords: Glycosaminoglycans, chondroitin sulfate, dermatan sulfate, hyaluronan, chemical functionalization, mass spectrometry, NMR spectroscopy, chromatography.

1. INTRODUCTION

1.1. Naturally Occurring GAGs

GAGs are natural, negatively charged, linear heteropolysaccharides classified into several groups on the basis of structure, namely hyaluronan (or hyaluronic acid, HA), keratan sulfate (KS), chondroitin sulfate (CS)/dermatan sulfate (DS), and heparin (Hep)/ heparan sulfate (HS) [1]. This review will focus exclusively on HA and CS/DS (Fig. (**1**)).

1.1.1. Chemical Structures of GAGs

HA, a non-sulfated GAG, is formed from repeating disaccharide units of β-D-*N*-acetylglucosamine (GlcNAc) and β-D-glucuronic acid (GlcA) linked by alternating β-1→3 and β-1→4 glycosidic bonds (Fig. (**1**)) [2]. In native HA the number of repeating disaccharide units can be larger than 10,000 resulting in molecular weights of about 4,000 kDa. In aqueous solution HA behaves as a randomly coiled stiffened chain molecule due to the disaccharide structure, internal hydrogen bonds, and interactions with the solvent [3]. A HA molecule occupies a large hydrated volume in solution and because of its polyelectrolyte nature the solution properties of HA are greatly affected by the ionic strength and/or the pH.

The galactosaminoglycans CS/DS are made up of β-1→4-linked repeating disaccharide units of GlcA β-1→3-linked to β-D-*N*-acetylgalactosamine (GalNAc). They form unbranched chains of variable lengths but possess lower molecular weights (that do normally not exceed 50 kDa) in comparison to HA. These repeating units are subject to considerable post-polymeric modification, i.e. epimerization of GlcA to α-L-iduronic acid (IdoA), and sulfation to varying degrees to yield a variety of disaccharide structures with sulfate residues at C4 or C6 (and rarely at C3) of GalNAc as well as C2 of IdoA [4]. Frequently occurring disaccharide units of CS, formerly named with capital letters (CS A, C, D, and E, resp.) are shown in Fig. (**1**). If in CS A or CS C GlcA is replaced to an higher extent by IdoA (normally more than 10 %), the GAG is named dermatan sulfate (DS), also known as chondroitin sulfate B (CS B). All members of the GAG family with the exception of HA occur as constituents of proteoglycans attached by a glycosidic linkage to a serine residue in the core protein *via* a tetrasaccharide spacer unit.

*Address correspondence to this author at the Institut für Medizinische Physik und, Biophysik, Medizinische Fakultät, Universität Leipzig, Härtelstr. 16-18, D-04107 Leipzig, Germany; Tel: +49-341-9715733; Fax: +49-341-9715709;
E-mail: juergen.schiller@medizin.uni-leipzig.de

Since sulfate is a strong electrolyte, the charge densities of the polysaccharides are strongly increased with each additional sulfate residue. This is very important for the water and ion binding capacity of the extracellular matrix (ECM) [5].

1.1.2. Biological Tissues and Body Fluids Rich in GAGs

Qualitative and quantitative variations give rise to complex GAGs that can differ in composition and sequence between cell types, tissues, and animal species [1].

CS (and DS) chains have - beside their structural meanings - important functions in central nervous system development, wound repair, infection, growth factor signaling, morphogenesis, cell division, differentiation and migration [1].

The most important source of GAGs is the ECM of connective tissues, where particularly CS occurs in a significant amount. The majority of commercially available GAGs are, thus, prepared from bovine nasal cartilage or trachea as well as from cartilaginous fish, such as shark. The synovial (joint) fluid is a particularly rich source of HA. However, HA is omnipresent in the human body and in other vertebrates, occurring in almost all biological fluids and tissues [6]. For instance, a human adult contains about 15 g HA. Nevertheless, the majority of "medical" HA is nowadays isolated from bacterial origin (grampositive *streptococci*).

1.2. Relevance and Medical Applications of GAGs

HA is involved in several key processes, including cell signaling, wound repair and regeneration, morphogenesis, matrix organization and pathobiology [7]. HA is used as a "lubricant" in the treatment of rheumatic diseases in order to improve the viscoelastic properties as well as the viscosity of synovial fluids that is decreased under inflammatory conditions [8]. For a more detailed survey of applications of HA see [8]. CS has become a widely used dietary supplement for the treatment of osteoarthritis [9]: CS is currently used and recommended by EULAR (The European League Against Rheumatism) as a SYSADOA (Symptomatic Slow Acting Drug for OA) drug for the treatment of knee and hand osteoarthritis [9].

1.3. The Sulfation Code - GAGs with Signaling Functions?

Recent studies suggest that GAGs may encode information in the form of a "sulfation code", whereby discrete modifications (for instance, the number and the positions of the sulfate residues) to the polysaccharide backbone may direct the location or activities of

1570-193X/10 $55.00+.00

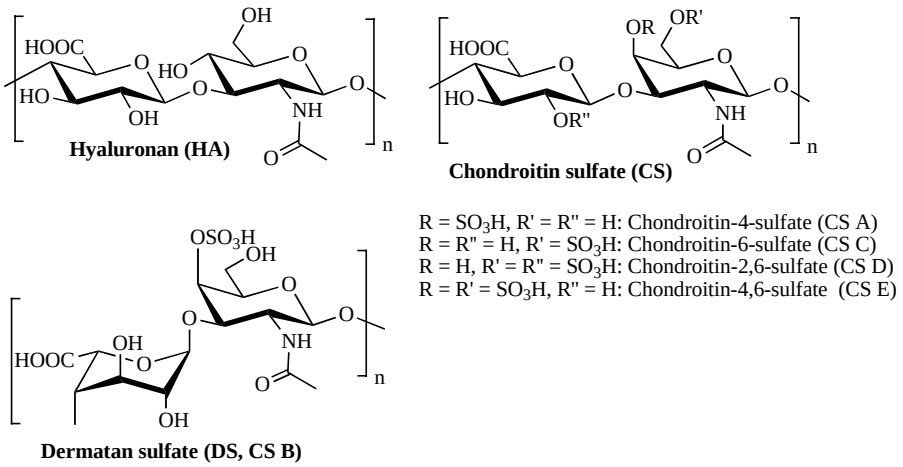

Fig. (1). Structures of the polymer repeating units of hyaluronan (HA), chondroitin sulfate (CS), and dermatan sulfate (DS). The carboxylic acid and sulfate groups are depicted as the free acids, not as salts.

proteins and particularly enzymes [10]. Very recently, even the term "heparanomics" has been introduced that intends to understand the systems involved in generating the developmental stage specific heparin or heparan sulfate sulfation patterns. Unlike other instances where biological information is encrypted as linear sequences in molecules such as DNA, sulfation patterns of GAGs are generated through a non-template driven process. Thus, deciphering the sulfation code and the dynamic nature of its generation has posed a new challenge - particularly to systems biologists [11].

2. SYNTHESIS OF MODIFIED GAGS

2.1. General Remarks

The systematic chemical derivatization of (native) GAGs represents a powerful approach to study structure-activity-relationships regarding the biomedical features of GAGs and to synthesize products with defined physical and biological properties for medical applications. Recent findings provided evidence that certain GAGs can attract or defeat selected cells under *in vivo* conditions and highly sulfated GAGs are able to interact with growth factors to stabilize them against proteolytic enzymes. This obviously opens up a new window to use GAGs as bioactive molecules in implant coatings and growth factor formulations. Unfortunately, highly sulfated GAGs such as HS are not easily available in large quantities and their short *in vivo* residence time as well as the poor mechanical properties of GAG-based hydrogel scaffolds represent further drawbacks regarding their clinical use. The defined chemical modification of GAG molecules is currently experiencing significant attention to qualify this unique class of biopolymers for extended biomedical applications.

The aim of this mini-review is

- to illustrate principal strategies of the chemical modification of HA and CS/DS in order to introduce different functional groups into these molecules and to deliver GAG derivatives with selective properties, and
- to summarize the most important methods of GAG analysis whereby both, the analysis of the native polymers as well as subsequent to specific degradation will be discussed.

Of course, several comprehensive reviews covering different aspects of GAGs have been already published [12,13], but to our best knowledge there is no survey available that deals exactly with the subjects indicated above.

In general, GAG chemistry is a challenging task considering the multi-functionality of GAG molecules, their limited solubility in organic solvents and their sensitivity against enzymatic or chemical degradations. Therefore, the careful selection of the applied modifying reagents and reaction conditions is essential in order to obtain products with the desired structure and structural uniformity.

2.2. HA and CS with Defined Sulfation Patterns

One of the most eminent challenges in the functionalization of GAGs is the synthesis of derivatives with a defined sulfation pattern. CS, the most abundant sulfated GAG, and HA, the only non-sulfated GAG, represent both ideal starting materials for this purpose. In early attempts to sulfate HA and CS, respectively, sulfuric acid or chlorosulfonic acid were used as reagents [14,15]. These relatively drastic reaction conditions did not result in a complete sulfation of all free OH-groups but caused a partial degradation of the polymer chain. Therefore, these reagents are usually used to synthesize low molecular weight sulfated GAGs

Since the 1980s complexes of SO_3 with organic amines or amides are the reagents of choice [16]. In the order SO_3/triethylamine < SO_3/pyridine < SO_3/DMF the reactivity of these sulfation reagents in aprotic solvents like DMF is increasing and sulfated derivatives of CS and HA with an adjusted degree of sulfation of $0 \leq DSS \leq 4$ can be obtained under mild reaction conditions with polymer yields up to 90 % [17-19]. Regarding the preparation of low-sulfated HA esters, SO_3/pyridine has been recently identified to be the reagent of choice whereas HA sulfates with high DSS values can be readily synthesized with SO_3/DMF (Fig. (**2**)) [20]. Examinations of the regioselectivity of this reaction evidenced that HA monosulfates completely sulfated at the primary C-6' position can be obtained even if only a slight excess of sulfation reagent is used and the resulting DSS values only slightly exceed 1.0 [19,20].

The modulation of the sulfation pattern does not only involve sulfation procedures, but also desulfation of sulfated CS and HA derivatives. The selective 6-*O*-desulfation of pyridinium salts of persulfated GAGs by means of silylating agents such as BTSA allows the synthesis of samples with the opposite regioselectivity, i.e. a free primary -OH group and sulfated secondary ones [21,22]. Based on the work of Toida *et al.* [22], the use of silyl acetamides as desulfating agents results in desulfated products with only slightly reduced molecular weights [22]. Applying reagents such as methanolic HCl [23] or a mixture of DMSO and water [23,24], the sulfated HA and CS derivatives are desulfated unselectively.

Since some GAGs (e.g. heparin) possess not only *O*-sulfate but also *N*-sulfate groups, methods have been established to *N*-deacetylate HA and CS, respectively, and to subsequently sulfate the free amino group with the above mentioned SO_3 complexes [25,26].

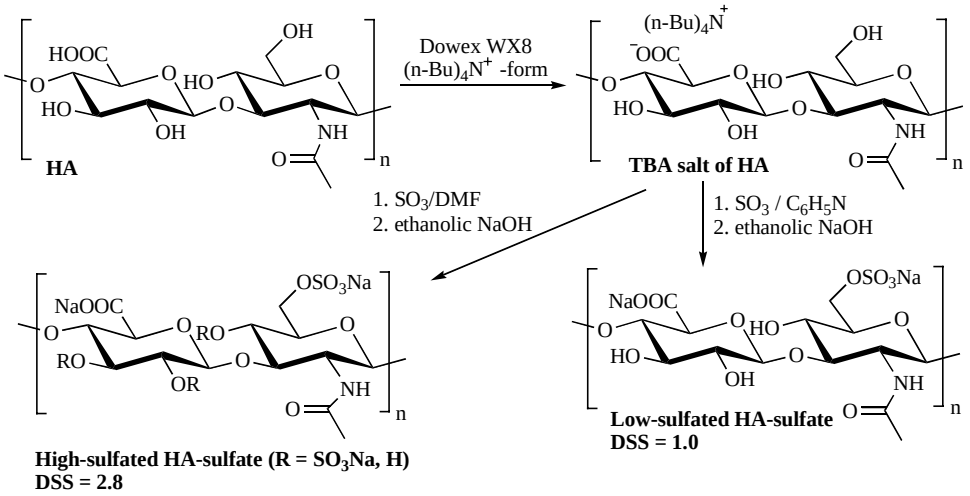

Fig. (2). Synthesis of HA sulfate esters with different degrees of sulfation.

2.3. Other Modifications than Sulfate

The multi-functionality of GAGs offers various options for further functionalization. Probably the most noted one is the esterification of the carboxylic acid group. This modification is already known since the 1950s and is normally performed with diazomethanes, alkyl halides or alcohols under acidic conditions [27,28]. In the case of HA, the esterification of the carboxylic acid group leads to products that are commercially available under the name "HYAFF®".

Another often described modification of GAGs is *N*-deacetylation which can be performed either with NaOH at elevated temperatures [29] or by means of hydrazine and catalytic amounts of hydrazine sulfate [14,25]. Applying the last mentioned method, the carboxylic acid groups are transformed into hydrazides, which have to be cleaved subsequently e.g. with HIO_3 or KI. Relatively high degrees of deacetylation of up to 80 % can be obtained with NaOH at about 100 °C and longer reaction times. Please note that the reaction conditions have to be carefully adjusted to avoid extensive reduction of the chain length.

The functionalization of the hydroxyl groups of GAGs with acid chlorides and anhydrides, respectively, to form the corresponding esters is also well known. Methacryl esters (Fig. (**4c**)) which represent important starting materials for cross-linked GAGs (see below) can be synthesized by reacting the free -OH groups of GAGs with methacrylic anhydride in water under slightly basic conditions [30] or upon reaction of HA or CS with glycidyl methacrylate by a reversible transesterification process [31]. The latter reaction competes with an irreversible epoxy ring-opening conjugation resulting in a structurally different methacryl ester. Epoxy-ring opening predominates at prolonged reaction times [32].

Recently, a series of melt-processable HA esters with long-chain alkyl residues soluble in organic solvents were synthesized by silylation of the cetyltrimethylammonium salt of HA followed by treatment of the formed trimethylsilyl ethers of HA (DS_{Silyl} ~ 4.0) with the corresponding acid chlorides (Fig. (**3**)) [33]. The acylation takes place at the oxygen of the trimethylsilyloxy group in the silylated HA resulting in the complete removal of trimethylsilyl groups from HA esters.

The EDC-assisted introduction of dihydrazides like adipic dihydrazide into GAGs provides derivatives with pendant hydrazide groups (Fig. (**4a**)) well-suited for the subsequent coupling of drugs [34], long alkyl chains or cyclodextrines [35] and for cross-linking of the GAG chains [36], respectively. The use of disulfide-linked dihydrazides (e.g. 3,3'-dithiobis-(propanoic hydrazide)) offers also a simple route to thiolated GAGs (Fig. (**4b**)) [37].

Besides esterification, several etherification procedures of GAG, using e.g. alkyl halides or epoxides [38,39] have been published. Additional carboxylic acid groups can be introduced into HA by carboxymethylation of -OH groups with chloroacetic acid under alkaline conditions (yield about 65 %) resulting in derivatives with $DS(CH_2COOH)$ values of about 0.5 and a preferential substitution of the primary -OH group [40]. An alternative approach to introduce carboxyl groups regioselectively represents the TEMPO-mediated oxidation of the primary -OH groups of HA [41]. High yields (between 60 and 80 %) of products oxidized at the pri-

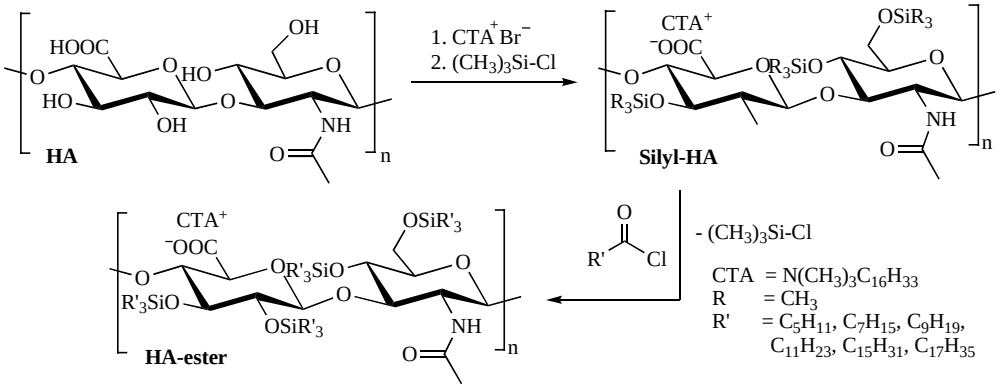

Fig. (3). Synthesis of long-chain carboxylic acid esters of HA *via* the trimethylsilyl ether.

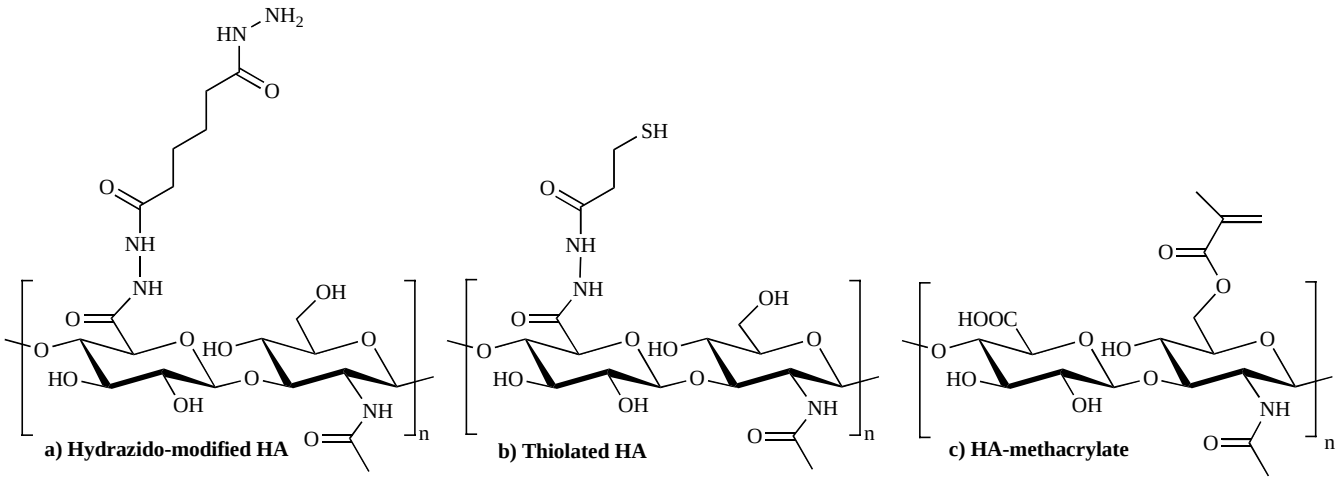

Fig. (4). Selected HA derivatives as useful starting compounds of cross-linking reactions and coupling of bioactive molecules.

mary -OH group can be obtained but the oxidation leads to a marked reduction of the HA molecular weight. Another common oxidation procedure, the Malaprade oxidation, is known to affect HS or CS much slower compared to other polysaccharides due to the rigid hydrogen bonding system of these GAGs. Nevertheless, aldehyde group containing HA with a degree of oxidation of up to 44 % has been prepared in reasonable yields (50-67 %) using this reaction [42]. However, in the presence of high amounts of periodate, a decrease in HA molecular weight has also been observed.

2.4. Cross-Linking of GAGs

The biomedical and pharmaceutical application of HA and other GAGs as fleeces, films, hydrogels, or micro- and nanoparticles normally requires their cross-linking to prevent fast dissolution or enzymatic degradation of water-soluble GAGs. Various low molecular weight bi-functional reagents have been used to cross-link HA leading to a variety of different hydrogel products with varying properties. A survey of these bi-functional cross-linkers is given in Table **1**.

Table 1. Survey of Bifunctional Cross-Linkers and Related Reaction Conditions for GAGs

Type of Cross-Linker	Examples	GAG	Reaction Conditions	Ref.
Dialdehydes	glutardialdehyde	HA (film)	0.01 N HCl/ acetone, RT, 24 h	[43,44]
	glutardialdehyde	HA-amine	PBS, RT, 5 min	[45]
	PEG-dialdehyde (MW = 3400 Da)	HA-hydrazide CS-hydrazide	H_2O, 4 °C, 24 h	[46]
Diepoxides	1,4-bisepoxybutane	HA	aq. NaOH (4 %), $NaBH_4$	[47]
	PEG-diglycidylether (MW = 8886 Da)	HA	0.1 N aq. NaOH, 60 °C, 45 min	[48]
	PEG-diglycidylether (MW = 512 Da)	CS	1 M aq. NaOH, RT, 24 H	[49]
Divinyl compounds	divinylsulfone	HA, HA, Hep	0.1 N aq. NaOH, RT, 75 min	[50]
Diamides	2,2'(ethylenedioxy) bis(ethylamine)	HA	H_2O (0.1 N NaOH, pH = 5.5), WSC, RT, 24 h	[51]
Di and polyhydrazides	adipic dihydrazide spermidine pentapropanoic pentahydrazide	HA	H_2O (0.1 N HCl, pH = 4.75), EDC, RT	[52]
Biscarbodiimides	ethyl[6[(benzyloxycarbonyl)amino]hexyl] carbodiimide	HA	H_2O (0.1 N HCl, pH = 4.75), RT, 2 h	[53]
Bis-NHS esters	PEG-bis(succinimidyl propionate 3,3'-dithiobis(sulfosuccinimidyl propionate)	HA-amine	PBS, RT, 5 min	[45]
	bis(sulfosuccinimidyl superate 3,3'-dithiobis(sulfosuccinimidyl propionate) ethyleneglycol bis(sulfosuccinimidyl succinate)	HA-hydrazide	0.1 M $NaHCO_3$, RT, 90 s	[54]
Di(meth)acrylates	PEG-di(meth)acrylate PEG-di(meth)acrylamide (MW = 3400 Da)	GAG-thiol	PBS, RT, 10 min – several days	[55,56]
Ditosylates	TEG-ditosylate	HA (TBA salt)	DMSO, RT, 24 h	[57]
Phosphates	trisodium trimetaphosphate	HA	$H_2O/C_2H_2Cl_2$, NaOH, 50 °C, 3 h	[58]

Using coupling agents such as WSCs (e.g. EDC) or 2-chloro-1-methyl pyridinium iodide, the self-cross-linking of HA can be performed by ester formation between activated carboxylic acid groups of HA and the hydroxyl groups of the same or an adjacent chain [59].

As already mentioned above, (meth)acrylate group-containing GAGs are often used as educts for generating biopolymer networks for tissue engineering purposes [60]. The cross-linking reaction can be initiated in aqueous medium either by the use of a water-soluble initiation system (e. g. potassium peroxodisulfate / triethanolamine) [31] or by irradiation with UV or visible light. In addition, UV light-initiated photodimerization of cinnamate-containing HA has also been used to prepare hydrogels able to prevent cell adhesion after surgery [61]. An interesting new approach to HA hydrogels comprises the "Click" reaction of a propargyl-modified HA with an azido group-containing HA in aqueous solution in the presence of Cu(I) leading to an *in situ* gel formation by a 1,3-dipolar cycloaddition [62].

In addition to the fabrication of GAG networks, the cross-linking procedures described above have also been used recently to synthesize composite networks containing GAGs and various other biopolymers including chitosan, collagen or gelatin. Using both thiolated GAGs (HA, CS) and thiolated gelatin as starting materials GAG-gelatin networks can be obtained by simple air oxidation under nearly physiological conditions [63]. Based on their structural and biochemical properties these biopolymer networks are able to mimic the ECM of living cells and serve as potent scaffolds in tissue engineering.

2.5. Chemical Methods to Decrease the Molecular Weight of GAG

Already in the 1970s it was observed that the reaction of GAGs with sulfuric or chlorosulfonic acid does not only result in a sulfation but also a depolymerization of the polysaccharides [64]. The reaction with other acids (e.g. HCl) causes chain scission of GAGs, too. Surprisingly, nitrogen oxides and products derived thereof are exclusively able to induce the cleavage of CS/DS but not of HA [25,65,66]. HA, however, can be easily depolymerized by means of heating an aqueous HA solution with diluted HCl [67].

Unfortunately, however, the degradation of GAGs under acidic conditions is not easy to control and side reactions such as elimination at the reducing end, *N*-deacetylation or desulfation may be also induced [25,65].

The susceptibility of GAGs to thermal stress is used for the solvolytic depolymerization at high temperatures [68] and for random scission of the polymer chain in autoclaves [69]. The molecular weight of *N*-acetylated GAG derivatives such as HA and CS can also be decreased using hypochlorite (NaOCl/HOCl), which initially attacks the *N*-acetyl group to form an intermediate *N*-chloramide and subsequently causes a site-specific chain scission [70].

Natural (non-sulfated) HA as well as CS/DS can be digested by hyaluronate lyase and chondroitin lyase, but these enzymes normally fail to digest chemically modified HA. However, such GAGs derivates can be oxidatively depolymerized by means of *in-situ* produced oxygen-derived free radicals. Such reactive oxygen species may be produced by reaction of a transition metal salt (e.g. $FeCl_2$, $FeSO_4$, $CuCl_2$), H_2O_2 and optional EDTA (Fenton type reaction) [71]. Finally, the degradation of GAGs by auto-oxidants such as Udenfriend's reagent (ascorbic acid, $FeCl_2$) or similar preparations [72,73] and with peroxynitrite presumably include intermediate oxygen-derived free radicals [69,74]. A survey of such reactions is available in [75].

3. ANALYSIS OF INTACT GAGS

There are two important properties of a macromolecule that must be carefully analyzed in order to obtain complete structural information: the molecular weight and the chemical structure (as well as inhomogeneities) of the polymer repeating unit. It will be outlined below that the latter parameter is best determined from polymer-derived oligomers, whereas the molecular mass must be obviously determined from the intact macromolecule.

3.1. NMR Spectroscopy

NMR is unequivocally a powerful tool to study the composition of a polymer as well as its tacticity [76]. However, the achievable line-shape is heavily influenced by the molecular weight and high mass polymers give only poorly resolved spectra [77]. This particularly holds for the most sensitive nucleus, ^1H, and is one reason why ^1H NMR is used to determine the extent of HA degradation in the synovial fluids [78] from patients suffering from arthritis and, thus, to determine the inflammation state: High mass HA (even in considerable concentrations) yields an extremely broad resonance that can be hardly differentiated from the noise. Subsequent to degradation, however, smaller fragments with reduced line-widths are obtained. Normally, the resonance of the *N*-acetyl side chain is observed because this resonance ($\delta \approx 2.04$ ppm) is much more sensitively detectable than the resonances of the more rigid groups of the carbohydrate backbone [78]. Thus, a high intense *N*-acetyl resonance indicates massive HA fragmentation. A similar approach can also be used to study the presence of small mass impurities such as detergents or solvents in a given HA preparation.

Despite its much lower sensitivity, ^{13}C NMR is the method of choice to investigate native GAGs and to monitor changes of the polymer repeat units. Selected ^{13}C NMR spectra of HA samples differing in the degree of sulfation are shown in Fig. (**5**).

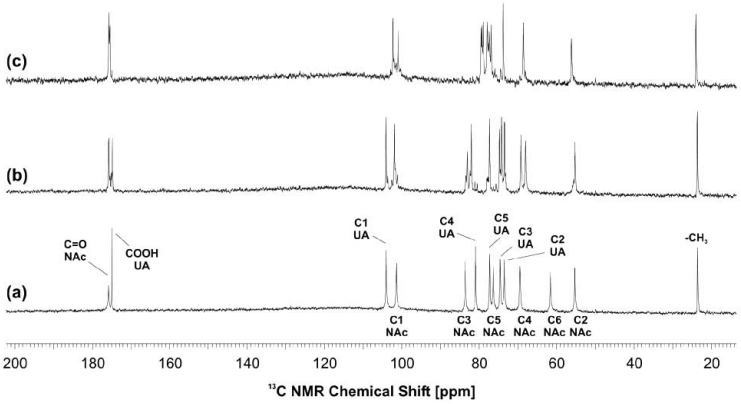

Fig. (5). ^{13}CNMR spectra of native HA (**a**), HA with 6.6% (**b**) and 13% sulfate content (**c**). All spectra were recorded in phosphate buffer, pH 7.4. Assignments of the individual resonances of the *N*-acetylglucosamine (NAc) and glucuronic acid (UA) repeating unit of HA are provided directly in the figure. So far unpublished data.

Please note that all C atoms of the native (non-sulfated) HA can be easily assigned due to their characteristic chemical shifts. Additionally, it is evident that the introduction of sulfate groups leads to characteristic changes. Since each carbon atom is characterized by a defined chemical shift, sulfate groups can be assigned to distinct positions. For instance, the C6 of the *N*-acetyl glucosamine residue is shifted to lower field upon sulfation. A more detailed description of the NMR spectra of intact GAG is available in [79].

3.2. Chromatography and Electrophoresis

HPLC is nowadays one of the most powerful tools to separate high molecular CS/DS as well as HA and this important topic has been previously reviewed [80]. The chromatographic separation of high-mass GAGs is normally based on differences of the molecular weights but not the functional groups. Separation is normally performed by common gel-permeation chromatography (GPC). As this is a relative method only, however, suitable GAG standards with defined MWs are necessary. Unfortunately, the availability of such compounds is still quite limited. Further reading is available in [81,82].

In addition to GPC, electrophoretic methods may also be used due to the charge of the native GAGs [83]. As the carboxylate and the sulfate residues of HA and CS provide very different charge densities, pH-dependent separation of both GAGs can be easily achieved. Nevertheless, it is evident that the separation of GAGs with comparable sulfate contents is rather difficult.

3.3. Viscosimetry

Viscosimetry is a quite old, but still well established method of GAG analysis and is based on the fact that the viscosity of GAG solutions increases with the molecular weight of the polymer if the concentration is fixed [84]. Thus, viscosimetry is a simple method to determine the extent of degradation processes because they are accompanied by a cleavage of the glycosidic linkages and, thus, by a reduction of the molecular weight. However, chemical changes of functional groups can be only indirectly assessed [85]. In contrast, structural changes (e.g. in dependence on the pH or the ionic strength) can be easily monitored by viscosimetry and modern rotational viscosimeters allow (in contrast to the previously widely used Ubbelohde devices) also the investigation of the polymer response to the applied shear stress, i.e. of conformational changes.

Standard rotational viscosimeters are typically made from steel and iron ions may be leached into the polymer solution and cause - particularly in the presence of atmospheric O_2 - partial depolymerization of the GAG [75]. Such effects should be carefully taken into consideration and - if possible - teflon-coated viscosimeters should be used. Nevertheless, the importance of viscosimetric measurements is continuously decreasing because only an overall parameter can be determined.

3.4. Quantitative Determination of GAGs

Although antibody-based [86] as well as radiochemical quantitative assays of GAGs are available, the assessment of the binding of certain dyes such as dimethylene blue [87] or alcian blue [88] (Fig. (**6**)) is most common in order to determine absolute amounts of native, high-mass GAGs.

Both assays are based on the binding between the cationic dyes and the acidic (negatively-charged) GAG. Therefore, the sensitivities of both assays depend on the charges of the analyte and, if present in the same concentration, CS is more sensitively detected than HA due to its higher charge density. Additionally, there is a certain minimum size of the polymer that is necessary for dye-GAG complex formation. This makes the quantitative determination of GAGs with strongly different MWs difficult.

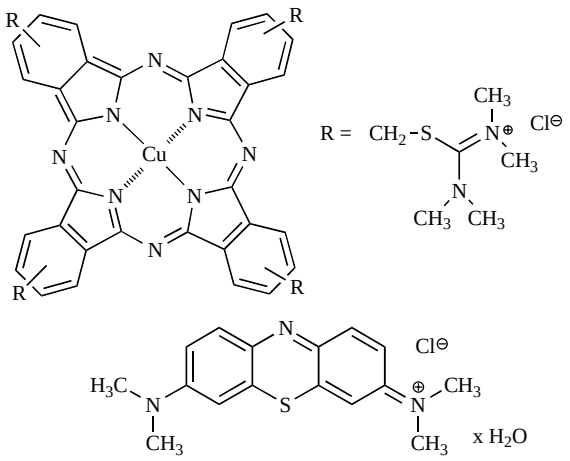

Fig. (6). Chemical structures of alcian blue (top) and dimethylene blue (bottom) that are normally used to determine GAG concentrations.

Another quite old but still very frequently used assay is the carbazole method according to Bitter and Muir [89]. Using this assay, the GAG of interest is fragmented by treatment with H_2SO_4 into free glucuronic acid that is subsequently determined by the carbazole color reaction. Thus, a differentiation between the individual GAG cannot be made and only the total amount of GAG is determined. It should also be noted that this assay is very sensitive to high salt concentrations and previous desalting is normally necessary. A more comprehensive review of quantitative GAG determinations is available in [90].

4. ANALYSIS OF DEPLETED GAGS

Although all information about the MW of the original GAG of interest is obviously lost by the degradation process, most GAG determinations of practical relevance are nowadays based on previous enzymatic (and to a lower extent chemical) degradation because a much higher extent of information is available from the analysis of the obtained defined oligosaccharides. A coarse survey of the involved steps is given in Fig. (**7**).

4.1. Enzymatic Methods to Obtain Defined Fragments of GAGs

The most widely used enzymes to fragment GAGs are bacterial or testicular hyaluronidases [91] as well as bacterial chondroitinases, in the majority of cases the ABC type from *proteus vulgaris* [13].

The latter enzyme digests chondroitin-4- (CS-A), chondroitin-6- (CS-C) as well as dermatan sulfate (CS-B) and has the additional advantage that it exhibits eliminase activity, leading to the introduction of a double bond into the uronic acid residue of the disaccharide (Fig. (**7**)). The corresponding UV absorption is helpful to quantitatively monitor the generation of the digestion products and, thus, the enzymatic activity. Unfortunately, these enzymes are inhibited by oversulfated GAGs and are, thus, often not capable of digesting chemically modified GAGs.

4.2. NMR Spectroscopy

Highly resolved NMR spectra can be obtained from the digestion products of GAGs that enable the simple differentiation as well as the determination of the sulfation site because the chemical shifts (e.g. of the *N*-acetyl residue) of the non-sulfated and the 4- and 6-sulfate are different [92]. This is a well-known method to determine the isomeric ratio of different CS preparations and to evaluate structural aspects.

4.3. Mass Spectrometry

One of the most frequently used methods for determining the composition of complex GAG mixtures is MS, whereby soft-

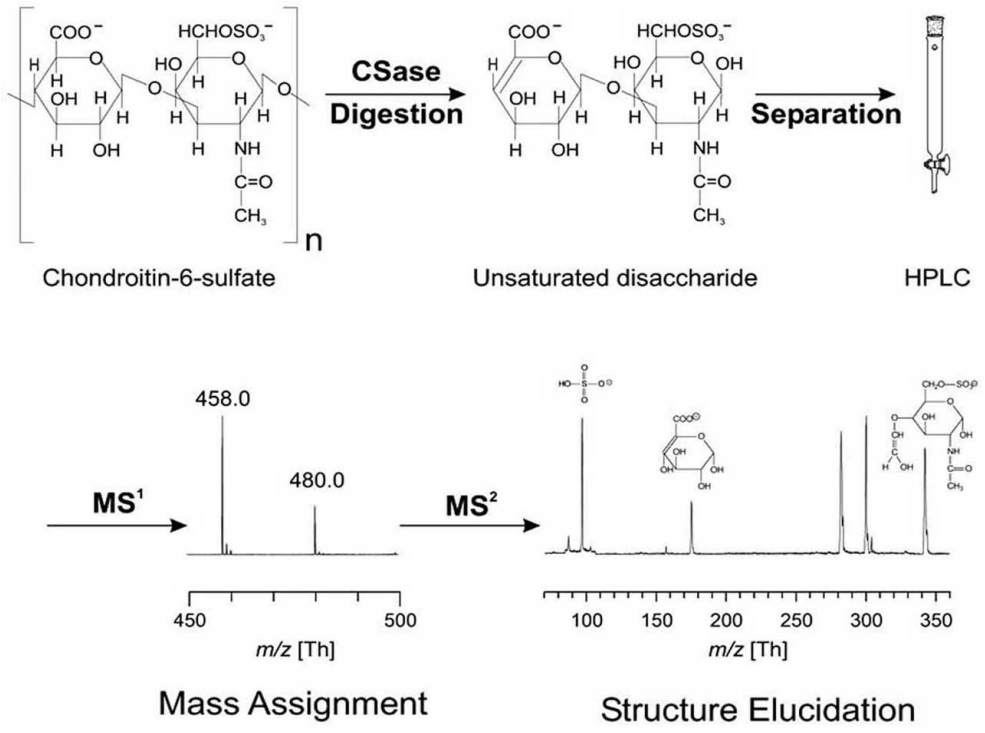

Fig. (7). Schema of the procedure normally used for GAG analysis. The GAG of interest is digested by the enzyme chondroitinase ABC that produces a mixture of unsaturated disaccharides depending on the structure of the GAG. The different disaccharides are subsequently separated - normally by means of HPLC or capillary electrophoresis - and analyzed by mass spectrometry that enables the mass assignment whereas subsequent MS/MS experiments allow detailed evaluation - particularly of the sulfate positions. Please note that only one isomer of CS is shown although this method can be also applied to more complex GAGs. A more detailed survey is available in [96].

ionization techniques such as electrospray (ESI) or matrix-assisted laser desorption and ionization (MALDI) are normally used that both enable (by MS/MS) even the localization of the sulfate residues. ESI MS is nowadays regarded as the method of choice [93], although it has been shown that MALDI-TOF MS also enables the quantitative determination of oligomers of HA [94] as well as CS [95]. Nevertheless, the most serious drawback of MS methods is the lability of the sulfate residue: Even if gentle ionization methods are applied, significant loss of sulfate residues will occur. This is a particular problem if highly sulfated compounds have to be analyzed. There is currently intense research activity to overcome this problem.

4.4. Chromatographic Methods

As already outlined above, bacterial lyases introduce a C4→C5 double bond into the non-reducing terminal hexuronate residue of the released disaccharide that is a characterized by a significant UV absorption (234 nm). These unsaturated disaccharides are often derivatized with fluorescent molecules to further increase detection sensitivity and specificity of HPLC [9] or other separation techniques. Furthermore, derivatization may also change the disaccharide properties to enable or improve resolution. 2-aminoacridone (AMAC) is a well-known fluorescent hydrophobic molecule that has been successfully used for the derivatization and separation of unsaturated (oligo)disaccharides [97].

Reverse-phase (RP)-HPLC offers a sensitive chromatographic separation of AMAC-(2-aminoacridone)-disaccharides sometimes providing better resolution than other analytical techniques [98]. Although not yet widely used, separation of enzymatic degradation products of GAG on a simple TLC plate is even also possible [99].

Using a combination of HPLC and MS subsequent to enzymatic degradation, the determination of even rather minor polymer repeating units (about 0.5%) of CS has recently been proven [100]: Isomeric nonsulfated HA and CS/DS disaccharides, isomeric monosulfated and isomeric disulfated CS/DS disaccharides, and the trisulfated species could be easily separated and unambiguously identified by their retention times and the corresponding mass spectra in negative ionization mode. However, the presence of desulfonated products from highly sulfated species due to the relative instability of the sulfo groups is still a problem regarding GAG analysis and makes the determination of the contribution of higher sulfated compounds less accurate.

SUMMARY AND CONCLUSIONS

This review was aimed to provide an overview about suitable chemical methods to modify natural GAGs that are of particular interest in the field of tissue regeneration and regenerative medicine. Although the chemical modification of GAGs is still a challenging task due to the need of regioselective reactions and retention of the original molecular weight, some useful synthesis routes were identified. Particular attention was paid to the introduction of sulfate groups because the elucidation of the sulfation code is nowadays a particular "hot" topic of research.

Hopefully, these authors were also able to show that significant attention has to be paid to the characterization of the modified GAGs: HPLC in combination with modern soft ionization MS techniques seem the methods of choice although the loss of sulfate residues is still a problem that complicates the analysis of highly sulfated species.

ACKNOWLEDGEMENTS

This work was supported by the German Research Council (DFG Schi 476/7-1 as well as TR 67, project A2).

LIST OF ABBREVIATIONS

AMAC	=	2-Aminoacridone
BTSA	=	N,O-bis(trimethylsilyl)acetamide
CS	=	Chondroitin sulfate
CTA	=	Cetyltrimethylammonium
Da	=	Dalton
DMF	=	Dimethyl formamide
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid
DS	=	Dermatan sulfate
DSS	=	Degree of sulfation
ECM	=	Extracellular matrix
EDC	=	1-Ethyl-3(3-dimethylaminopropyl)-carbodiimide
EDTA	=	Ethylenediaminetetraacetic acid
EULAR	=	The European League against Rheumatism
GAG	=	Glycosaminoglycan
GalNAc	=	N-acetylgalactosamine
GlcNAc	=	N-acetylglucosamine
GlcU	=	Glucuronic acid
GPC	=	Gel-permeation chromatography (Size Exclusion Chromatography)
HA	=	Hyaluronan
Hep	=	Heparin
HPLC	=	High-performance liquid chromatography
HS	=	Heparan sulfate
HYAFF	=	Benzyl ester of HA
IdoA	=	Iduronic acid
KS	=	Keratan sulfate
MALDI	=	Matrix-assisted laser desorption and ionization
MS	=	Mass spectrometry
MW	=	Molecular weight
NMR	=	Nuclear magnetic resonance
PEG	=	Polyethylene glycol
PBS	=	Phosphate-buffered salt solution
ppm	=	parts per million (unit of NMR chemical shift, δ)
RT	=	Room temperature (ca. 298 K)
SYSADOA	=	Symptomatic slow acting drug for OA
TBA	=	Tert.-butanol
TEG	=	Triethylene glycol
TEMPO	=	2,2,6,6-Tetramethylpiperidinyl-1-oxy
Th	=	Thompson (unit of *m/z* ratio)
TLC	=	Thin-layer chromatography
UV	=	Ultraviolet
WSC	=	Water-soluble carbodiimide

REFERENCES

[1] Sasisekharan, R.; Raman, R.; Prabhakar, V. Glycomics approach to structure-function relationships of glycosaminoglycans. *Annu. Rev. Biomed. Eng.*, **2006**, *8*, 181-231.

[2] Linhardt, R.J. Analysis of glycosaminoglycans with polysaccharide lyases. *Curr. Protoc. Mol. Biol.*, **2001**, Chp. 17: Unit17.13B.

[3] Hardingham, T. In: *Chemistry and Biology of Hyaluronan*; Garg, H.G.; Hales, C.A., Eds.; Elsevier; Amsterdam, **2004**; pp. 1-19.

[4] Fongmoon, D.; Shetty, A.K.; Basappa, Y.S.; Sugiura, M.; Kongtawelert, P.; Sugahara, K. Chondroitinase-mediated degradation of rare 3-O-sulfated glucuronic acid in functional oversulfated chondroitin sulfate K and E. *J. Biol. Chem.*, **2007**, *282*, 36895-36904.

[5] Sun, D.D.; Guo, X.E.; Likhitpanichkul, M.; Lai, W.M.; Mow, V.C. The influence of the fixed negative charges on mechanical and electrical behaviors of articular cartilage under unconfined compression. *J. Biomech. Eng.*, **2004**, *126*, 6-16.

[6] Fraser, J.R.; Laurent, T.C.; Laurent, U.B. Hyaluronan: its nature, distribution, functions and turnover. *J. Intern. Med.*, **1997**, *242*, 27-33.

[7] Volpi, N.; Schiller, J.; Stern, R.; Soltés, L. Role, metabolism, chemical modifications and applications of hyaluronan. *Curr. Med. Chem.*, **2009**, *16*, 1718-1745.

[8] Kogan G.; Šoltés L.; Stern R.; Schiller J.; Mendichi R. In: *Studies in Natural Products Chemistry*; Atta-ur-Rahman, Ed.; Elsevier: Amsterdam, **2008**; Vol. *34*, pp. 789-882.

[9] Volpi, N. Chondroitin sulfate: structure, role and pharmacological activity, Academic Press: Amsterdam, **2006**.

[10] Gama, C.I.; Hsieh-Wilson, L.C. Chemical approaches to deciphering the glycosaminoglycan code. *Curr. Opin. Chem. Biol.*, **2005**, *9*, 609-619.

[11] Lamanna, W.C.; Kalus, I.; Padva, M.; Baldwin, R.J.; Merry, C.L.; Dierks, T. The heparanome - the enigma of encoding and decoding heparan sulfate sulfation. *J. Biotechnol.*, **2007**, *129*, 290-307.

[12] Sugahara, K.; Mikami, T.; Uyama, T.; Mizuguchi, S.; Nomura, K.; Kitagawa, H. Recent advances in the structural biology of chondroitin sulfate and dermatan sulfate. *Curr. Opin. Struct. Biol.*, **2003**, *13*, 612-620.

[13] Linhardt, R.J.; Avci, F.Y.; Toida, T.; Kim, Y.S.; Cygler, M. CS lyases: structure, activity, and applications in analysis and the treatment of diseases. *Adv. Pharmacol.*, **2006**, *53*, 187-215.

[14] Wolfrom, M.L.; Juliano, B.O. Chondroitin sulfate modifications. II. 1 Sulfated and N-deacetylated preparations. *J. Am. Chem. Soc.*, **1960**, *82*, 2588-2592.

[15] Casu, B.; Naggi, A.; Torri, G. Chemical derivatization as a strategy to study structure-activity relationships of glycosaminoglycans. *Semin. Thromb. Hemost.*, **2002**, *28*, 335-342.

[16] Nagasawa, K.; Uchiyama, H.; Wajima, N. Chemical sulfation of preparations of chondroitin 4- and 6-sulfate, and dermatan sulfate. Preparation of chondroitin sulfate alike materials from chondroitin 4-sulfate. *Carbohydr. Res.*, **1986**, *158*, 183-190.

[17] Maruyama, T.; Toida, T.; Imanari, T.; Yu, G.; Linhardt, R.J. Conformational changes and anticoagulant activity of chondroitin sulfate following its O-sulfonation. *Carbohydr. Res.*, **1998**, *306*, 35-43.

[18] Barbucci, R.; Leone, G.; Chiumiento, A.; Di Cocco, M.E.; D'Orazio, G.; Gianferri, R.; Delfini, M. Low- and high-resolution nuclear magnetic resonance (NMR) characterisation of hyaluronan-based native and sulfated hydrogels. *Carbohydr. Res.*, **2006**, *341*, 1848-1858.

[19] Magnani, A.; Albanese, A.; Lamponi, S.; Barbucci, R. Blood-interaction performance of differently sulphated hyaluronic acids. *Thromb. Res.*, **1996**, *81*, 383-395.

[20] Hintze, V., Moeller, S., Schnabelrauch, M., Bierbaum, S. Viola, M., Worch, H., Scharnweber, D. Modifications of hyaluronan influence the interaction with human bone morphogenetic protein-4 (hBMP-4). *Biomacromolecules*, **2009**, *10*, 3290-3297.

[21] Matsuo, M.; Takano, R.; Kamei-Hayashi, K.; Hara, S. A novel regioselective desulfation of polysaccharide sulfates: Specific 6-O-desulfation with N,O-bis(trimethylsilyl)acetamide. *Carbohydr. Res.*, **1993**, *241*, 209-215.

[22] Toida, T.; Suzuki, A.; Nakajima, K.; Chaidedgumjorn, A.; Imanari, T. Effect of 6-O-sulfonate hexosamine residue on anticoagulant activity of fully O-sulfonated glycosaminoglycans. *Glycoconjug. J.*, **2000**, *17*, 393-399.

[23] McCarthy, M.M.U.; Baker, J.R. Isolation and desulphation of keratan sulphates. *Carbohydr. Res.*, **1979**, *69*, 151-164.

[24] Du, Y.; Taga, A.; Suzuki, S.; Liu, W.; Honda, S. Effect of structure modification of chondroitin sulfate C on its enantioselectivity to basic drugs in capillary electrophoresis. *J. Chromatogr. A*, **2002**, *947*, 287-299.

[25] Nadkarni, V.D.; Toida, T.; VanGorp, C.L.; Schubert, R.L.; Weiler, J.M.; Hansen, K.P.; Caldwell, E.E.O.; Linhardt, R.J. Preparation and biological activity of N-sulfonated chondroitin and dermatan sulfate derivatives. *Carbohydr. Res.*, **1996**, *290*, 87-96.

[26] Crescenzi, V.; Francescangeli, A.; Renier, D.; Bellini, D. New cross-linked and sulfated derivatives of partially deacetylated hyaluronan: synthesis and preliminary characterization. *Biopolymers*, **2002**, *64*, 86-94.

[27] Jeanloz, R.W., Forchielli, E. Studies on hyaluronic acid and related substances: I. Preparation of hyaluronic acid and derivatives from human umbilical cord. *J. Biol. Chem.* **1950**, *186*, 495-511.

[28] Bârzu, T.; Level, M.; Petitou, M.; Lormeau, J.C.; Choay, J.; Schols, D.; Baba, M.; Pauwels, R.; Witvrouw, M.; De Clercq, E. Preparation and anti-HIV activity of O-acylated heparin and dermatan sulfate derivatives with low anticoagulant effect. *J. Med. Chem.*, **1993**, *36*, 3546-3555.

[29] Wada, T.; Chirachanchai, S.; Izawa, N.; Inaki, Y.; Takemoto, K. Synthesis and properties of hyaluronic acid conjugated nucleic acid analogues - 1: Synthesis of deacetylhyaluronan and introduction of nucleic acid bases. *J. Bioact. Compat. Polym.*, **1994**, *9*, 429-447.

[30] Burdick, J.A.; Chung, C.; Jia, X.; Randolph, M.A.; Langer, R. Controlled degradation and mechanical behavior of photopolymerized hyaluronic acid networks. *Biomacromolecules*, **2005**, *6*, 386-3891.

[31] Möller, S.; Weisser, J.; Bischoff, S.; Schnabelrauch, M. Dextran and hyaluronan methacrylate based hydrogels as matrices for soft tissue reconstruction. *Biomol. Eng.*, **2007**, *24*, 496-504.

[32] Li, Q.; Wang, D.-A.; Elisseeff; H.-J. Heterogeneous-phase reaction of glycidyl methacrylate and chondroitin sulfate: Mechanism of ring-opening-transesterification competition. *Macromolecules* **2003**, *36*, 2556-2562.

[33] Zhang, M.; James, S.P. Synthesis and properties of melt-processable hyaluronan esters. *J. Mater. Sci. Mater. Med.*, **2005**, *16*, 587-593.

[34] Pouyani, T.; Prestwich, G.D. Functionalized derivatives of hyaluronic acid oligosaccharides: drug carriers and novel biomaterials. *Bioconjug. Chem.*, **1994**, *5*, 339-347.

[35] Charlot, A.; Heyraud, A.; Guenot, P.; Rinaudo, M.; Auzely-Velty, R. Controlled synthesis and inclusion ability of a hyaluronic acid derivative bearing beta-cyclodextrin molecules. *Biomacromolecules*, **2006**, *7*, 907-913.

[36] Creuzet, C.; Kadi, S.; Rinaudo, M.; Auzély-Velty, R. New associative systems based on alkylated hyaluronic acid. Synthesis and aqueous solution properties. *Polymer*, **2006**, *47*, 2706-2713.

[37] Shu, X.Z.; Liu, Y.; Luo, Y.; Roberts, M.C.; Prestwich, G.D. Disulfide cross-linked hyaluronan hydrogels. *Biomacromolecules*, **2002**, *3*, 1304-1311.

[38] Mlcochova, P.; Hajkova, V.; Steiner, B.; Bystricky, S.; Koos, M.; Medova, M.; Velebny, V. Preparation and characterization of biodegradable alkylether derivatives of hyaluronan. *Carbohydr. Polym.*, **2007**, *69*, 344-352.

[39] Serban, M.A.; Yang, G.; Prestwich, G.D. Synthesis, characterization and chondroprotective properties of a hyaluronan thioethyl ether derivative. *Biomaterials*, **2008**, *29*, 1388-1399.

[40] Möller, S.; Schmidtke, M.; Schnabelrauch, M.; Wutzler, P. Antivirale Kombinationen sowie ihre Verwendung. *Patent* 2005, DE 10 2005 2004 2643.

[41] Jiang, B.; Drouet, E.; Milas, M.; Rinaudo, M. Study on TEMPO-mediated selective oxidation of hyaluronan and the effects of salt on the reaction kinetics. *Carbohydr. Res.*, **2000**, *327*, 455-461.

[42] Weng, L.; Pan, H.; Chen, W. Self-crosslinkable hydrogels composed of partially oxidized hyaluronan and gelatin: *in vitro* and *in vivo* responses. *J. Biomed. Mater. Res. A*, **2008**, *85*, 352-365.

[43] Tomihata, K.; Ikada, Y. Crosslinking of hyaluronic acid with glutaraldehyde. *J. Polym. Sci. Pol. Chem.*, **1997**, *35*, 3553-3559.

[44] Lu, P.L.; Lai, J.Y.; Ma, D.H.K.; Hsiue, G.H. Carbodiimide cross-linked hyaluronic acid hydrogels as cell sheet delivery vehicles: characterization and interaction with corneal endothelial cells. *J. Biomater. Sci. Polym. E.*, **2008**, *19*, 1-18.

[45] Bulpitt, P.; Aeschlimann, D. New strategy for chemical modification of hyaluronic acid: Preparation of functionalized derivatives and their use in the formation of novel biocompatible hydrogels. *J. Biomed. Mater. Res. A*, **1999**, *47*, 152-169.

[46] Kirker, K.R.; Prestwich, G.D. Physical properties of glycosaminoglycan hydrogels. J. *Polym. Sci. Pol. Phys.*, **2004**, *42*, 4344-4356.

[47] Laurent, T.C.; Hellsing, K.; Gelotte, B. Cross-linked gels of hyaluronic acid. *Acta Chem. Scand.*, **1964**, *18*, 274–275.

[48] Segura, T.; Anderson, B.C.; Chung, P.H.; Webber, R.E.; Shull, K.R.; Shea, L.D. Crosslinked hyaluronic acid hydrogels: a strategy to functionalize and pattern. *Biomaterials*, **2005**, *26*, 359-371.

[49] Wang, S.C.; Chen, B.H.; Wang, L.F.; Chen, J.S. Characterization of chondroitin sulfate and its interpenetrating polymer network hydrogels for sustained-drug release. *Int. J. Pharm.*, **2007**, *329*, 103-109.

[50] Balazs, E.A.; Leshchiner, A. Cross-linked gels of hyaluronic acid and products containing such gels. Patent 1986, US 4582865.

[51] Bodnar, M.; Daroczi, L.; Batta, G.; Bako, J.; Hartmann, J.F.; Borbely, J. Preparation and characterization of cross-linked hyaluronan nanoparticles. *Colloid Polym. Sci.*, **2009**, *287*, 991-1000.

[52] Vercruysse, K.P.; Marecak, D.M.; Marecek, J.F.; Prestwich, G.D. Synthesis and *in vitro* degradation of new polyvalent hydrazide cross-linked hydrogels of hyaluronic acid. *Bioconjug. Chem.*, **1997**, *8*, 686-694.

[53] Kuo, J.W.; Swann, D.A.; Prestwich, G.D. Chemical modification of hyaluronic acid by carbodiimides. *Bioconjug. Chem.*, **1991**, *2*, 232-241.

[54] Pouyani, T.; Harbison, G.S.; Prestwich, G.D. Novel hydrogels of hyaluronic acid: Synthesis, surface morphology, and solid-state NMR. *J. Am. Chem. Soc.*, **1994**, *116*, 7515-7522.

[55] Shu, X.Z.; Liu, Y.C.; Palumbo, F.S.; Lu, Y.; Prestwich, G.D. *In situ* crosslinkable hyaluronan hydrogels for tissue engineering. *Biomaterials*, **2004**, *25*, 1339-1348.

[56] Cai, S.S.; Liu, Y.C.; Shu, X.Z.; Prestwich, G.D. Injectable glycosaminoglycan hydrogels for controlled release of human basic fibroblast growth factor. *Biomaterials*, **2005**, *26*, 6054-6067.

[57] Huin-Amargier, C.; Marchal, P.; Payan, E.; Netter, P.; Dellacherie, E. New physically and chemically crosslinked hyaluronate (HA)-based hydrogels for cartilage repair. *J. Biomed. Mater. Res. A*, **2006**, *76A*, 416-424.

[58] Dulong, V.; Lack, S.; Le Cerf, D.; Picton, L.; Vannier, J. P.; Muller, G. Hyaluronan-based hydrogels particles prepared by crosslinking with trisodium trimetaphosphate. Synthesis and characterization. *Carbohydr. Polym.*, **2004**, *57*, 1-6.

[59] Young, J.J.; Cheng, K.M.; Tsou, T.L.; Liu, H.W.; Wang, H.J. Preparation of cross-linked hyaluronic acid film using 2-chloro-1-methylpyridinium iodide or water-soluble 1-ethyl-(3,3-dimethylaminopropyl)carbodiimide. *J. Biomater. Sci. Polym. Ed.*, **2004**, *15*, 767-780.

[60] Ifkovits, J.L.; Burdick, J.A. Review: photopolymerizable and degradable biomaterials for tissue engineering applications. *Tissue Eng.*, **2007**, *13*, 2369-2385.

[61] Miyamoto, K., Sasaki, M., Minamisawa, Y., Kurahashi, Y., Kano, H., Ishikawa, S. Evaluation of *in vitro* biocompatibility and biodegradation of photocrosslinked hyaluronate hydrogels (HADgels). *J. Biomed. Mater. Res. A.*, **2004**, *70A*, 550-559

[62] Crescenzi, V.; Cornelio, L.; Di Meo, C.; Nardecchia, S.; Lamanna, R. Novel hydrogels *via* click chemistry: synthesis and potential biomedical applications. *Biomacromolecules*, **2007**, *8*, 1844-1850.

[63] Shu, X.Z.; Ahmad, S.; Liu, Y.; Prestwich, G.D. Synthesis and evaluation of injectable, in situ crosslinkable synthetic extracellular matrices for tissue engineering. *J. Biomed. Mater. Res. A*., **2006**, *79*, 902-912.

[64] Nagasawa, K.; Inoue, Y. Reaction between carbohydrates and sulfuric acid. 2. Depolymerization and sulfation of chondroitin sulfate by sulfuric acid. *Chem. Pharm. Bull.*, **1971**, *19*, 2617-2621.

[65] Bienkowski, M.J.; Conrad, H.E. Structural characterization of the oligosaccharides formed by depolymerization of heparin with nitrous acid. *J. Biol. Chem.*, **1985**, *260*, 356-365.

[66] Hassan, M.S.; Mileva, M.M.; Dweck, H.S.; Rosenfeld, L. Nitric oxide products degrade chondroitin sulfates. *Nitric Oxide*, **1998**, *2*, 360-365.

[67] Tommeraas, K.; Melander, C. Kinetics of hyaluronan hydrolysis in acidic solution at various pH values. *Biomacromolecules*, **2008**, *9*, 1535-1540.

[68] Toida, T.; Sato, K.; Sakamoto, N.; Sakai, S.; Hosoyama, S.; Linhardt, R.J. Solvolytic depolymerization of chondroitin and dermatan sulfates. *Carbohydr. Res.*, **2009**, *344*, 888-893.

[69] Al-Assaf, S.; Navaratnam, S.; Parsons, B.J.; Phillips, G.O. Chain scission of hyaluronan by peroxynitrite. *Arch. Biochem. Biophys.*, **2003**, *411*, 73-82.

[70] Rees, M.D.; Hawkins, C.L.; Davies, M.J. Hypochlorite-mediated fragmentation of hyaluronan, chondroitin sulfates, and related N-acetyl glycosamines: evidence for chloramide intermediates, free radical transfer reactions, and site-specific fragmentation. *J. Am. Chem. Soc.*, **2003**, *125*, 13719-13733.

[71] Ofman, D.; Slim, G.C.; Watt, D.K.; Yorke, S.C. Free radical induced oxidative depolymerisation of chondroitin sulphate and dermatan sulphate. *Carbohydr. Polym.*, **1997**, *33*, 47-56.

[72] Uchiyama, H.; Dobashi, Y.; Ohkouchi, K.; Nagasawa, K. Chemical change involved in the oxidative reductive depolymerization of hyaluronic acid. *J. Biol. Chem.*, **1990**, *265*, 7753-7759.

[73] Matsumura, G.; Pigman, W. Catalytic role of copper and iron ions in the depolymerization of hyaluronic acid by ascorbic acid. *Arch. Biochem. Biophys.*, **1965**, *110*, 526-533.

[74] Corsaro, M.M.; Pietraforte, D.; Di Lorenzo, A.S.; Minetti, M.; Marino, G. Reaction of peroxynitrite with hyaluronan and related saccharides. *Free Radic. Res.*, **2004**, *38*, 343-353.

[75] Schiller, J.; Fuchs, B.; Arnhold, J.; Arnold, K. Contribution of reactive oxygen species to cartilage degradation in rheumatic diseases: molecular pathways, diagnosis and potential therapeutic strategies. *Curr. Med. Chem.*, **2003**, *10*, 2123-2145.

[76] Mathur, A.M.; Scranton, A.B. Characterization of hydrogels using nuclear magnetic resonance spectroscopy. *Biomaterials*, **1996**, *17*, 547-557.

[77] Schiller, J.; Fuchs, B.; Arnold, K. The molecular organization of polymers of cartilage: an overview of health and disease. *Curr. Org. Chem.*, **2006**, *10*, 1771-1789.

[78] Schiller, J.; Arnhold, J.; Sonntag, K.; Arnold, K. NMR studies on human, pathologically changed synovial fluids: role of hypochlorous acid. *Magn. Reson. Med.*, **1996**, *35*, 848-853.

[79] Bociek, S.M.; Darke, A.H.; Welti, D.; Rees, D.A. The ^{13}C NMR spectra of hyaluronate and chondroitin sulphates. Further evidence on an alkali-induced conformation change. *Eur. J. Biochem.*, **1980**, *109*, 447-456.

[80] Stylianou, M.; Triantaphyllidou, I.E.; Vynios, D.H. Advances in the analysis of chondroitin/dermatan sulfate. *Adv. Pharmacol.*, **2006**, *53*, 141-166.

[81] Capila, I.; Sasisekharan, R. In: *Chemistry and Biology of Hyaluronan*; Garg, H.G.; Hales, C.A., Eds.; Elsevier: Amsterdam, **2004**; pp. 21-40.

[82] Cowman, M.K.; Mendichi, R. In: *Chemistry and Biology of Hyaluronan*; Garg, H.G.; Hales, C.A., Eds.; Elsevier: Amsterdam: **2004**; pp. 41-69.

[83] Volpi, N.; Maccari, F.; Linhardt, R.J. Capillary electrophoresis of complex natural polysaccharides. *Electrophoresis*, **2008**, *29*, 3095-3106.

[84] Mendichi, R.; Soltés, L.; Giacometti Schieroni, A. Evaluation of radius of gyration and intrinsic viscosity molar mass dependence and stiffness of hyaluronan. *Biomacromolecules*, **2003**, *4*, 1805-1810.

[85] Soltés, L.; Valachová, K.; Mendichi, R.; Kogan, G.; Arnhold, J.; Gemeiner, P. Solution properties of high-molar-mass hyaluronans: the biopolymer degradation by ascorbate. *Carbohydr. Res.*, **2007**, *342*, 1071-1077.

[86] Hascall, V.C.; Midura, R.J.; Sorrell, J.M.; Plaas, A.H. Immunology of chondroitin/dermatan sulfate. *Adv. Exp. Med. Biol.*, **1995**, *376*, 205-216.

[87] Sabiston, P.; Adams, M.E.; Ho, Y.A. Automation of 1,9-dimethylmethylene blue dye-binding assay for sulfated glycosaminoglycans with application to cartilage microcultures. *Anal. Biochem.*, **1985**, *149*, 543-548.

[88] Gold, E.W. The quantitative spectrophotometric estimation of total sulfated glycosaminoglycan levels. Formation of soluble alcian blue complexes. *Biochim. Biophys. Acta*, **1981**, *673*, 408-415.

[89] Bitter, T.; Muir, H.M. A modified uronic acid carbazole reaction. *Anal. Biochem.*, **1962**, *4*, 330-334.

[90] Frazier, S.B.; Roodhouse, K.A.; Hourcade, D.E.; Zhang, L. The quantification of glycosaminoglycans: A comparison of HPLC, carbazole, and alcian blue methods. *Open Glycosci.*, **2008**, *1*, 31-39.

[91] Prabhakar, V.; Capila, I.; Sasisekharan, R. The structural elucidation of glycosaminoglycans. *Methods Mol. Biol.*, **2009**, *534*, 147-156.

[92] Kreil, G. Hyaluronidases - a group of neglected enzymes. *Protein Sci.*, **1995**, *4*, 1666-1669.

[93] Volpi, N.; Maccari, F. Structural characterization and antithrombin activity of dermatan sulfate purified from marine clam Scapharca inaequivalvis. *Glycobiology*, **2009**, *19*, 356-367.

[94] Zaia, J.; Li, X.Q.; Chan, S.Y.; Costello, C.E. Tandem mass spectrometric strategies for determination of sulfation positions and uronic acid epimerization in chondroitin sulfate oligosaccharides. *J. Am. Soc. Mass Spectrom.*, **2003**, *14*, 1270-1281.

[95] Busse, K.; Averbeck, M.; Anderegg, U.; Arnold, K.; Simon, J.C.; Schiller, J. The signal-to-noise ratio as a measure of HA oligomer concentration: a MALDI-TOF MS study. *Carbohydr. Res.*, **2006**, *341*, 1065-1070.

[96] Nimptsch, A.; Schibur, S.; Schnabelrauch, M.; Fuchs, B.; Huster, D.; Schiller, J. Characterization of the quantitative relationship between signal-to-noise (*S/N*) ratio and sample amount on-target by MALDI-TOF MS: Determination of chondroitin sulfate subsequent to enzymatic digestion. *Anal. Chim. Acta*, **2009**, *635*, 175-182.

[97] Ambrosius, M.; Kleesiek, K.; Götting, C. Quantitative determination of the glycosaminoglycan delta-disaccharide composition of serum, platelets and granulocytes by reversed-phase high-performance liquid chromatography. *J. Chromatogr. A*, **2008**, *1201*, 54-60.

[98] Deakin. J.A.; Lyon, M. A simplified and sensitive fluorescent method for disaccharide analysis of both heparan sulfate and chondroitin/dermatan sulfates from biological samples. *Glycobiology*, **2008**, *18*, 483-491.

[99] Zhang, Z.; Xie, J.; Zhang, F.; Linhardt, R.J. Thin-layer chromatography for the analysis of glycosaminoglycan oligosaccharides. *Anal. Biochem.*, **2007**, *371*, 118-20.

[100] Volpi, N. High-performance liquid chromatography and on-line mass spectrometry detection for the analysis of chondroitin sulfates/hyaluronan disaccharides derivatized with 2-aminoacridone. *Anal. Biochem.*, **2010**, *397*, 12-23.

Received: March 11, 2010 Revised: April 12, 2010 Accepted: April 12, 2010