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New methods to study the composition and structure of the extracellular matrix in natural and bioengineered tissues

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Abbreviations: 9-AA, 9-aminoacridine; amu, atomar mass unit; CP, cross polarization; CS, chondroitin sulfate; D, diffusion coefficient; Da, dalton; DHB, 2,5-Dihydroxybenzoic acid; DS, dermatan sulfate; ECM, extracellular matrix; ESI, electrospray ionization; FCD, fixed charge density; GAG, glycosaminoglycan; GalNAc, N-Acetylgalactosamine; GlcNAc, N-Acetylglucosamine; GlucUA, glucuronic acid; HA, hyaluronan; Hep, heparin; HPLC, high performance liquid chromatography; HR, high resolution; HS, heparan sulfate; Hyp, hydroxyproline; IdoUA, iduronic acid; IR, infrared; KS, keratansulfate; MALDI, matrix-assisted laser desorption and ionization; MAS, magic angle spinning; MRI, magnetic resonance imaging; MS, mass spectrometry; MW, molecular weight; *m/z*, mass over charge; NMR, nuclear magnetic resonance; Pa, pascal; PEG, poly-ethylene-glycol; PFG, pulsed-field gradient; PG, proteoglycan; pK, logarithm of the dissociation constant; PLGA, poly-D,L-lactide-co-glycolide; ppm, parts per million; ROS, reactive oxygen species; SDC, self-diffusion coefficient; T_1/T_2, relaxation time; TCP, tricalcium-phosphate; TLC, thin-layer chromatography; TOF, time-of-flight; U, (enzymatic) unit; U_D donnan potential; UV, ultraviolet

The extracellular matrix (ECM) comprises a gel of numerous biopolymers that occurs in a multitude of biological tissues. The ECM provides the basic support and mechanical strength of skeletal tissue and is responsible for shape retention. At the same time, the ECM is responsible for the viscoelastic properties and the elasticity of soft tissues. As expected, there are several important diseases that affect and degenerate the ECM with severe consequences for its properties. Bioengineering is a promising approach to support the regenerative capacity of the body. Unfortunately, the biomechanical properties of bioengineered ECM often only poorly meet the standards of their native counterparts. Many bioengineered tissues are characterized by an increased glycosaminoglycan (GAG) but decreased collagen content. This leads to an enhanced water content that strongly alters the viscoelastic and thus the biomechanical properties. Therefore, compositional analysis is important to estimate the tissue quality. We will show that nuclear magnetic resonance (NMR) spectroscopy and soft-ionization mass spectrometry (MS) represent useful techniques for ECM research both in natural and bioengineered tissues. Both methods are strongly complimentary: while MS techniques such as matrix-assisted laser desorption and ionization (MALDI) are excellent and very sensitive analytical tools to determine the collagen and the GAG contents of tissues, NMR spectroscopy provides insight into the molecular architecture of the ECM, its dynamics and other important parameters such as the water content of the tissue as well as the diffusion of molecules within the ECM.

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Introduction

The extracellular matrix (ECM) is a complex tissue component that is omnipresent in all vertebrates. The replacement or reconstruction of damaged ECM is the major subject of regenerative medicine and particularly important in industrialized countries where the population enjoys a high life expectation which, however, also leads to an over-aged population. Therefore, the interest in ECM is not stemming from the ECM itself but much more from its role in medicine.

Although ECM is found in almost all parts of the human body, we will focus here on bone, cartilage, and skin because these tissues are (1) in the focus of our current research interest and (2) of significant medical relevance.[1] Regenerative medicine[2] is a rapidly developing discipline of medical sciences and aims—in addition to the repair of more complex organs (such as liver)—particularly at the ECM. This field is particularly important as ECM-related diseases are of tremendous socioeconomic significance: for instance, about 46.4 million US citizens (approximately 21% of the overall population) are currently suffering from any form of arthritic diseases.[3] Logically, the related social consequences (e.g., early retirement and loss of life quality) and costs for the health care system are immense. Additionally, the number of people affected by the disease is increasing: By the year 2020, it is estimated that nearly 60 million Americans will be affected by arthritis.[4]

It is a common feature of ECM diseases that there are so far no convincing cures available and the majority of the medical interventions are symptomatic but not causal, i.e., the treatments are aimed exclusively to reduce the symptoms of the disease.

There are two major reasons for this surprising lack of knowledge: (1) the mechanisms of pathogenesis are so far largely unknown, i.e., it is not known which products of what cells contribute primarily to tissue degradation and (2) methods of early medical diagnosis are lacking so far.

The regenerative capacity of individual tissues also varies. While bone is known to provide a relatively good self-healing capacity provided the defect does not exceed a critical size, skin and in particular cartilage has a limited potential for regeneration. One particular problem of cartilage regeneration is the fact that this tissue represents a bradytroph tissue. This means that the ECM does not contain blood vessels and the transport of nutrients and metabolic waste products depends exclusively on diffusion.[5] As diffusion represents a very inefficient transport mechanism, the number of cells within the ECM is limited, which compromises the regeneration capacity of the cartilage ECM.

The transplantation of selected tissue cells or stem cells[6] that can be differentiated into the respective tissue cells represents a promising approach to treat ECM-related diseases. Larger defects are filled with scaffolding materials that can be loaded with the respective cells. Further, in alternative approaches autologous cells are extracted from the patients and cultured ex vivo prior to implantation. Unfortunately, the quality of the ECM that is generated under these conditions is normally inferior to the excellent properties of the native ECM. Since this is typically related to an insufficient concentration of the ECM biomolecules, an altered chemical composition, or insufficient cross-linking, a more detailed knowledge of the composition and of course methods to measure the composition are strongly required.

The Architecture of the Extracellular Matrix

Collagens. Before the early 1800s, the fibers of connective tissue were thought to be the basis of life. This opinion arose from the observation of the spontaneous generation of these aggregates. With the discovery of cells in connective tissue, however, the cellular theory became accepted as the basis of life in ≈1850.[7] Collagens are the by far most abundant proteins on earth and comprise about 25–30% of all proteins in vertebrate organisms.[8,9] The collagen superfamily is steadily growing, and at least 28 different collagen types encoded by more than 40 genes are currently known that function as highly essential structural tissue components:[10] collagens are located in the ECM of skin,[11] tendon,[12] bone,[13] cartilage[14] and many other tissues. With a tensile strength comparable with that of steel, fibrillar collagen represents an ideal material to impart stability to these various tissue types.[15] Of course, this is particularly important with respect to bone and cartilage that are continuously exposed to considerable forces: in articular cartilage, for instance, collagen forms an arcade-like structure that particularly helps to protect the cartilage cells from mechanical strain as the cells are surrounded by a basket-like web of collagen filaments.[10,16]

Independent of the specific type, collagens exhibit a highly specialized and complex organization. Collagen tripelhelices are formed from three individual polypeptide chains (α chains) that may be all of the same but also of different amino acid compositions.[17] These collagen tripelhelices with a molecular dimension of about 1.5 × 300 nm^2 are again aligned in the form of the so-called collagen fibrils that comprise a considerable part of the weight of the ECM. The structure of collagen is illustrated in **Figure 1**.

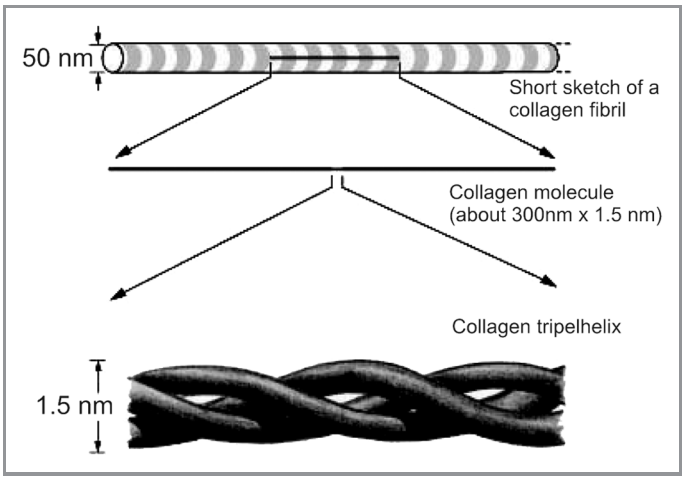

Figure 1. Structure of fibril-forming collagens. Collagen consists of tripelhelices of polypeptide chains that are aligned to fibrils. Rough molecular dimensions of the individual subunits are also given in this sketch. Reprinted with permission from reference 16.

It is conceivable that the unique collagen secondary and tertiary structure requires also a unique amino acid sequence. First, a considerable fraction of the residues should be small amino acids. Therefore, glycine (the smallest side chain with a hydrogen residue only) constitutes about one third of all amino acid residues in all collagen types[18] (**Fig. 2**).

As seen from **Figure 2** only four different amino acids make up for about two thirds of all residues in the collagen molecule.[18] This simplifies collagen analysis. Further, collagen contains some

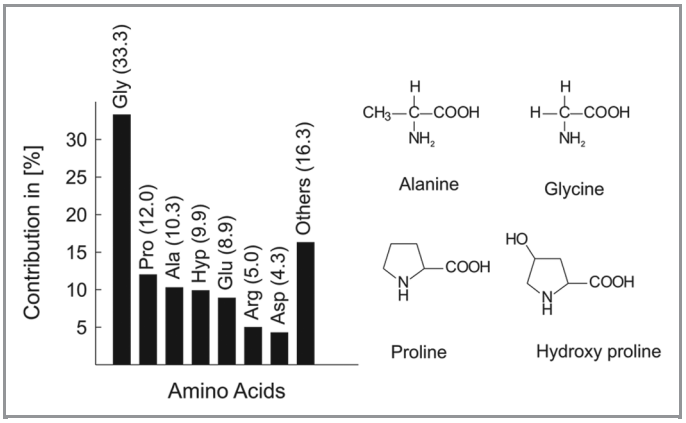

Figure 2. Approximate amino acid composition of collagen type II. Bars are labeled according to the three letter amino acid code. Hyp represents hydroxyproline. Although Hyp occurs in two isomeric forms, only the 3-hydroxy is given. Chemical structures of most relevant amino acids are also shown. Reprinted with permission and with modification from reference 16.

specific post-translationally modified amino acids such as hydroxyproline (Hyp) or hydroxylysine (Hyl) that are both very important: whereas the hydroxylysine residues are covalently attached to the carbohydrate moiety of the ECM (vide infra)[19] and mediate the supramolecular structure of the tissue, the Hyp residues are essential for the stability of the collagen.[20] Hyp is synthesized from proline exclusively within the polypeptide chain by the enzyme prolyl hydroxylase[21] that contains an essential ferrous ion within its catalytic center that is kept in its low valent oxidant state by the presence of reductants, particularly ascorbate. If ascorbate (vitamin C) is not supplied sufficiently via nutrition, collagen stability is not longer warranted, leading to a disease well-known as "scorbut."

Beside its important structural role, the collagen moiety of the ECM is also crucial to limit the swelling, i.e., the water uptake of tissues and it is well-known that the degradation of the collagen moiety of ECM with enzymes such as collagenase results in a strongly increased water content.[22] Therefore, an appropriate ratio between collagen and glycosaminoglycans (GAGs) is crucial in order to provide the specific (bio)mechanical properties of the ECM. It should be noted, that native tripelhelical collagen is not digestable by the majority of proteolytic enzymes with the exception of collagenase, whereas denaturated collagen (gelatin, which can be generated by heating of collagen) is highly susceptible to most proteases.[23] Finally, one major difference between cartilage and bone is the calcium content: mammalian bones contain huge amounts of calcium phosphate crystals in a protein matrix. The major form of the calcium phosphate is hydroxyapatite. Although type I collagen is uncharged, it plays a very important role for calcification and, thus, osteogenesis. These aspects have been recently reviewed.[24]

Glycosaminoglycans (GAGs). GAGs are natural, very complex, unbranched, polydisperse polysaccharides composed of disaccharide units normally of D-glucuronic acid (GlcUA) or L-iduronic acid (IdoUA) [only keratan sulfate (KS) has a galactose instead of an uronic acid moiety] linked to a D-glucosamine (GlcNAc) or D-galactosamine (GalNAc) residue (**Fig. 3**).

HA is the only GAG that is completely non-sulfated containing an unmodified N-acetyl-D-glucosamine (GlcNAc)-GlcUA repeating unit, while the other polysaccharides are generally modified through post-biosynthetic modifications, such as the addition of O-sulfo groups, C5-epimerization to form IdoUA residues, and de-N-acetylation to produce GlcN-sulfo residues.[25,26] These modifications often play a key role in a wide variety of biological and pharmacological processes but a more detailed discussion of these aspects is beyond the scope of this review. Although having a rather simple structure, HA is widely used in medicine and cosmetics.[27] HA is the by far largest GAG: For instance, the molecular weight of HA within articular cartilage and in healthy synovial fluid is about 10^7 Dalton.[28] At physiological pH, the majority of carboxyl groups is deprotonated and, therefore, HA (pK ≈3.21) negatively charged. To indicate all charge states, hyaluronan is the most commonly used term instead of hyaluronic acid and hyaluronate.

We will briefly deal with KS because this GAG is less abundant in the ECM than chondroitin sulfate (CS) or dermatan sulfate (DS). The repeating disaccharide unit of keratan sulfate (KS) $[Gal\ (\beta1\rightarrow4)\ GlcNAc\ (\beta1\rightarrow3)]_n$ contains a galactose residue instead of uronic acid and the glycosidic bonds are reversed in comparison to HA and CS/DS (**Fig. 3**). Sulfate esters are normally present at the C-6 of one or both monosaccharide units of KS.

CS A/C (i.e., the 4- and the 6-sulfate) and CS B, the latter also known as DS, comprise the disaccharide unit $[GlcUA\ (\beta1\rightarrow3)\ GalNAc\ (\beta1\rightarrow4)]_n$ variously sulfated in different positions of the hexosamine unit and/or the uronic acid. The regular disaccharide sequence of CS A, chondroitin-4-sulfate, is formed by the repeating unit sulfated in position 4 of the GalNAc unit, while CS C, chondroitin-6-sulfate, is sulfated in position 6. Disaccharides with varying numbers and positions of the sulfate groups can be located, in different moieties, along the polysaccharide chains, such as the disulfated disaccharides in which two sulfate groups are O-linked in position 2 of GlcUA and 6 of GalNAc (CS D) or in position 4 and 6 of GalNAc (CS E).[29]

In the case of DS, further enzymatic modifications in particular C-5 epimerization of GlcUA to IdoUA as well as O-sulfation at C-2 of IdoUA occur. Therefore, polysaccharide chains of DS feature a prevailing disaccharide unit $[IdoUA\ (\beta1\rightarrow3)\ GalNAc\ (\beta1\rightarrow4)]_n$ with a minor concentration of disulfated disaccharides, in particular sulfated in position 4 of GalNAc and 2 of the IdoUA unit.[29] These heterogeneous structures are responsible for the different and more specialized functions of these GAGs. One particular important point is the "heparin contamination crisis": this means that severe adverse events (even deaths) occurred when patients were treated with heparin that contained oversulfated CS. This has forced the Baxter Healthcare Corporation (as well as further companies) to recall many preparations of contaminated heparin[30] and significantly enhanced the interest in heparin analysis.[31]

Furthermore, it is known that the insertion of IdoUA residues imparts conformational flexibility to the DS chain altering the shape and spatial orientation of the sulfate residues, endowing the chain with a higher negative charge density than the GlcUA. Although the principles of the biosynthetic process have not yet been fully elucidated, it is well known that this process results in the generation of highly modified oligosaccharide domains separated by regions of relatively low-degree structural modifications within the polymer chain. Thus, the DS chain has a hybrid co-polymeric structure consisting of low modified (CS) and highly modified (DS) domains.[32] The IdoUA-containing units are often sulfated at C-4 of the GalNAc residue, while sulfation at C-6 is frequently associated with GlcUA-containing disaccharides. To our knowledge at least 23 different CS/DS disaccharide repeating units have been identified so far.[33]

Heparin (Hep) and heparan sulfate (HS) possess a distinctly different repeating disaccharide structure compared with the previous GAGs $[GlcA\ (\beta1\rightarrow4)\ GlcNAc\ (\alpha1\rightarrow4)]_n$ (**Fig. 3**). Hep is sometimes considered to be synonymous with HS, but this is incorrect: both compounds have been shown to differ in their degree of sulfation, with Hep being stronger negatively charged and displaying higher O- and particularly N-sulfation than HS. Most important, Hep has the highest charge density of any known biological macromolecule, while HS is generally less

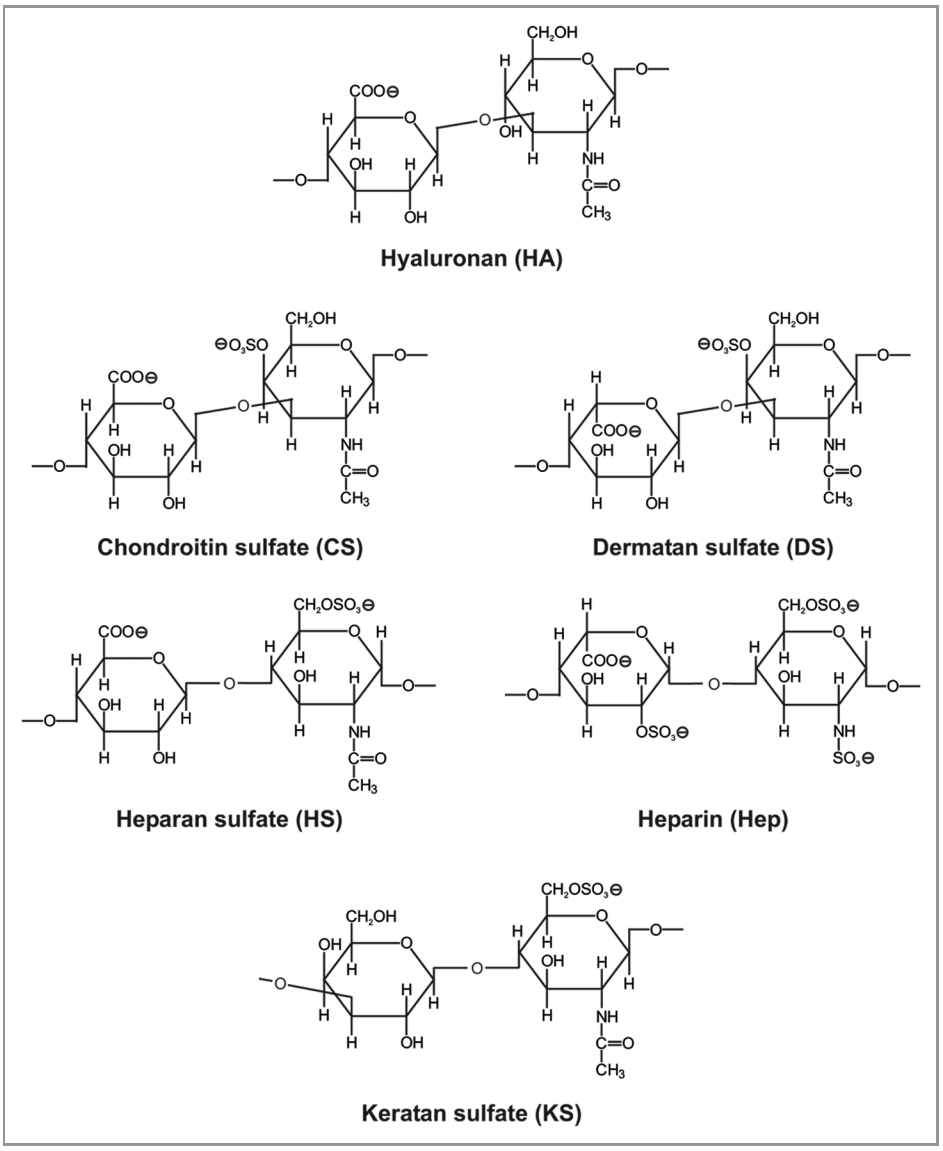

Figure 3. Structures of the disaccharide repeating units of the most important GAGs. In addition to the illustrated modifications, minor variations (e.g., differences of the sulfation patterns) are also common.

sulfated and possesses lower IdoUA contents. Both, Hep and HS exhibit diverse biological functions and participate in a large number of interactions with other effective extracellular and cellular membranes. However, as Hep and HS are less abundant in the ECM in comparison to DS/CS, we will not discuss Hep and HA here to a major extent.[34]

Biophysical Properties of the ECM and Tissues

As already outlined above, the chemical composition of the ECM is crucial for the function of the respective tissue since the GAG and collagen moieties determine the water contents that are crucial regarding the viscoelastic and the biomechanical properties.[35] Further, the motions of the biopolymers in the ECM themselves influence the elastic properties of the tissues.[36,37] Because of their high negative charge density (which depends of course on the pH and the ion concentration) the polysaccharides tend to bind large amounts of water and behave in principle like a sponge. In contrast, the collagen limits this high swelling tendency in the sense of a scaffold that ensures shape retention. Thus, both molecular moieties are very important. The biophysical mechanisms that help in establishing appropriate water contents will be shortly discussed in the forthcoming paragraphs.

Ion content and distribution within the ECM. The ion content of the ECM determines the water content and this is an extremely important parameter for different reasons: (1) As discussed above, the water content is important for the mechanical properties of the ECM. (2) If the composition of the ECM is determined, the collagen and GAG contents are normally related to either the wet or the dry weight of the tissue. Thus, knowledge about the native water content or the water content subsequent to drying (firmly bound water) is important. (3) Many biophysical

parameters such as diffusion processes or the nuclear magnetic resonance (NMR) relaxation times are directly affected by the water content.

Although a more detailed discussion of these aspects is surely beyond the scope of this short review, one should keep in mind some coarse guidelines: first, it is nearly impossible to obtain a completely dry ECM sample because some water is always tightly bound to the polysaccharides of the ECM. This type of water can hardly be removed. Second, high ion contents reduce the water content of the ECM because these ions screen the negative charges of the polysaccharides that mediate water binding. This particularly holds for bivalent cations such as Ca^{2+} or Mg^{2+} that interact massively with the sulfate residues of the polysaccharides. Third, many ECM samples are anisotropic with an uneven distribution of collagen and GAG.[38] This also leads to an uneven distribution of charges and ions. One should also note that these differences of charge densities are actually the reason why the ECM can be stained by cationic dyes such as Alcian blue that binds selectively to the negative charges of GAG. Finally, altered ion contents of tissues are typical of many diseases. Therefore, techniques for the determination of the ion content as well as the distribution within the tissues have been established.[39]

Donnan potential and counterion condensation. The charges of the sulfate and carboxylate groups of GAG in tissues are spaced by about 10–15 Å and give rise to the high charge density. This is termed fixed charged density (FCD) and is normally measured in mEq/ml (milliequivalents per milliliter) units, whereby in normal articular cartilage, for instance, the effective FCD ranges between 0.04 and 0.2 mEq/ml.[40]

These FCDs produce profound effects not only regarding tissue hydration and fluid movement as well as ion transport through the cartilage but also on a broad spectrum of other phenomena, e.g., streaming potentials. Because of the negative FCD of cartilage, Na^+ ions are attracted, whereas Cl^- ions experience repulsion and, therefore, a significant potential difference is established, the so-called Donnan potential U_D.[41] This theory may be used to explain the inhomogeneous ion distribution within tissues.

Osmotic activity of the ECM. As an example for the osmotic activity of the ECM, cartilage is briefly discussed here. The best way to understand the osmotic behavior of cartilage tissue is to consider a small part of cartilage and to image that this represents a very small osmotic chamber (Pfeffer cell). The required semi-permeable membrane of this osmotic chamber is realized by the collagen network that surrounds and traps the large negatively-charged GAG within the tissue. When the semi-permeable membrane of this micro-osmotic chamber is placed against an external electrolyte solution (e.g., NaCl of a certain concentration) water will flow into and ions will flow out of the chamber in order to maintain electro-neutrality and to achieve electrochemical equilibrium with the charged proteoglycan (PG). Already in 1924, F.G. Donnan[42] derived a mathematical expression for the equilibrium ion concentration (in this case primarily Na^+ ions) within such a semi-permeable chamber of charged macromolecules. This is important because the water content of tissue is of paramount interest regarding its biophysical properties: if tissue samples are incubated in de-ionized water, their water contents will increase while putting the tissue samples in concentrated salt solutions will decrease the water contents due to osmotic effects. Thus, when a higher osmotic pressure in comparison to the internal pressure is applied, this will result in a loss of water from the tissue and vice versa. This is of high relevance since cartilage for instance is steadily exposed to external pressures and compressive stresses as high as 20 MPa in the hip have been reported.[43] Experiments on the compression behavior of cartilage were often performed by mechanical compression,[44] as well as by osmotic compression.[45] However, it seems that both types of compression are rather difficult to compare and a comparison is only useful, when the tissue is mechanically loaded in an isotropic and uniform manner, with the applied load equal in magnitude to that of the osmotic pressure.[46] Although this topic would be worth an individual review, this emphasizes the importance of the water content.

Methods of In Vitro ECM Characterization

In the next chapters, methods allowing a comprehensive quantitative characterization of the ECM will be described. Although many further methods would also represent suitable analytical techniques, methods based on NMR and mass spectrometry (MS) (often in combination with methods of chromatography) will be emphasized in this review. In particular, we would like to emphasize the analytical power of these methods if they are used in combination because they yield different but complementary information.

Classical methods: histology and optical methods (characteristic dyes). Classical methods of histology are applied in the majority of clinical studies and a detailed discussion of all these techniques is beyond the scope of this review. Collagen is often monitored by either picrosirius red or Goldner's trichrome[47] but these approaches are normally not really quantitative.[48] Alcian blue (a cationic dye) is often used to monitor the distribution of the GAGs (that are strongly negatively charged) within the tissue sample.[49] Although this approach is widely used, one should keep in mind that the achievable color intensity is determined by (1) the molecular weight of the stained polymer (2) the charge state of the GAG and, thus, (3) the ion content of the sample.[50] Due to these problems, the composition of ECM samples is normally determined ex vivo: regarding GAG determination, the carbazole method according to Bitter and Muir is still widely used.[51] Using this assay, the GAGs of interest are fragmented by treatment with H_2SO_4 into free glucuronic acid (one carbohydrate repeat unit of HA and CS) that is subsequently determined by the carbazole color reaction. Unfortunately, a differentiation between the individual GAGs cannot be made and only the total amount of HA and CS is determined, while KS (due to the lack of the uronic acid) cannot be determined at all by this method. It should also be noted that this assay is very sensitive to high salt concentrations and previous desalting of the sample is normally necessary. A more comprehensive review of quantitative GAG determinations has been recently published.[52]

In contrast, the determination of the collagen content of a sample is normally based on the determination of characteristic

amino acids. Free amino acids can be easily obtained by acidic hydrolysis of the tissue of interest. This is an important advantage in comparison to the GAGs: amino acids "survive" the treatment with strong acids, while sugars are destroyed under these conditions under charring. Due to its high abundance in collagen, the determination of glycine seems the most straightforward approach.[53] However, one should keep in mind that glycine does of course also occur in the majority of proteins different from collagen. Accordingly, a value above the correct collagen concentration is normally determined if the glycine concentration is used. Therefore, the determination of the hydroxyproline (for instance by using chloramine T)[53,54] content is normally a better option because Hyp does nearly exclusively occur in collagen. Although beyond the scope of this review, antibodies against major proteins of the ECM are also available.[55]

NMR techniques. The great success of solution NMR spectroscopy is due to the fact that all NMR-active nuclei (i.e., those with a nuclear spin, such as ^{1}H, ^{2}H, ^{13}C, ^{15}N, ^{31}P, etc.) of a molecule with a MW smaller than about 40 kDa that tumbles freely in solution can be detected, assigned and structural and dynamical information can be derived.[56] As the polymers of the ECM are typically above the size limit for solution NMR the method is often considered less useful. However, solid-state NMR spectroscopy does not rely on molecular mobility and there is no intrinsic molecular mass dependent limitation. Therefore, solid-state NMR has been recognized as a very useful tool to study the immobile molecules of biological tissues, for instance in cartilage,[36,57,58] bone[59-61] or skin[62] for more than 30 years. In particular, collagen in its various types has been analyzed by solid-state NMR, as reviewed by several authors.[15,58,63-65] The particular use of solid-state NMR spectroscopy for tissue engineering has briefly been reviewed recently.[66]

The main difference between solution and solid-state NMR spectroscopy is the fact that the NMR interactions (such as chemical shift, dipolar coupling, and quadrupolar coupling) are orientation dependent in solids while the isotropic tumbling of the molecules in solution abolishes this effect. Therefore, solid-state NMR lines are typically broadened (up to many kHz) due to anisotropic spin interactions that are not fully averaged by molecular motion. However, these broad lines contain valuable structural information provided that the signal superposition can be reduced to a minimum (by isotopic labeling) or resonances can be separated by multidimensional NMR.[67]

Although quite successful, the single labeling approach is tedious, expensive, and time-consuming and particularly challenging for biological tissues. Therefore, research focused on improving the resolution of NMR spectra of solid materials. The innovation was magic-angle spinning (MAS) where the sample is oriented at an angle $\theta = 54.74°$ and spun around the length axis using MAS frequencies of ≈2–50 kHz.[68] Under MAS conditions the broad powder pattern collapses into a single small NMR signal with a line width comparable to that in solution NMR. Thus, isolated molecules of the ECM[69] as well as intact biological tissue such as cartilage,[36] bone[70] or skin[62] has been studied even at natural abundance of ^{13}C (only 1.1%) and other nuclei such as ^{31}P.[71]

In the past 10 years, high-resolution magic-angle spinning (HRMAS) NMR applications have also been demonstrated, where MAS is used to improve the resolution of NMR spectra of tissues but solution NMR type pulse sequences are applied.[72]

In the following, a few seminal contributions of ECM research by solid-state NMR methods are briefly reviewed.

Solid state NMR on bone, skin and cartilage. The first milestone in that field was the work by the Torchia group from the 1980s.[57] Using specific ^{2}H, ^{13}C, ^{15}N or ^{19}F labeling of individual amino acid residues in collagen, NMR spectra reflecting the presence of residual anisotropic nuclear spin interactions were detected.[73-75] These measurements provided amplitude and correlation time information of the molecular motions of all major amino acids in collagen. As expected, motional amplitudes were largest in non-cross-linked and non-biomineralized collagen while much more restricted motions were observed in biomineralized and cross-linked collagen.[76,77]

It should be mentioned that at the time MAS was not readily available. Therefore, these investigations had been performed under static NMR conditions and the structural information was extracted from the powder lineshapes. Likewise, the Torchia group studied the ECM of intact cartilage tissue under static NMR conditions.[57] While static spectra under high power decoupling provided broad spectra of collagen, scalar decoupled static ^{13}C NMR spectra showed surprisingly narrow lines that could be attributed to the GAGs of the tissue. The authors concluded that up to 80–100% of the GAGs in cartilage were highly mobile and could be detected under these conditions; an experimental finding that was also confirmed by other groups.[78,79]

Next, MAS applications on collagen without the need for isotopic labeling were conducted.[69,80-82] These studies reported the assignments of the collagen spectra as well as information on the hydration behavior of collagen and more importantly, provided the prerequisites for the solid-state NMR analysis of intact biological tissues.

The ^{13}C MAS NMR spectra that are recorded under typical solid-state NMR conditions (i.e., high power decoupling and cross polarization) of bone,[59,61,83-85] cartilage[36,45] and skin[62] show the typical fingerprint of the collagen molecule with some hints for immobile GAGs[36] in cartilage and citrate in bone.[84] Typical ^{13}C CP (cross polarization) MAS NMR spectra are given in **Figure 4**. Further, these biological tissues have been studied under dehydration[45,83,86] and the molecular dynamics has been characterized.[36,87,88]

In addition, ^{31}P MAS NMR is a convenient method to study the highly mineralized bone. A very thorough study has monitored the different stages of bone mineralization upon bone development.[60]

While the NMR characterization by CP MAS always detected the rigid collagen in these tissues, directly polarized ^{13}C NMR spectra of the respective samples showed much different molecules (**Fig. 5**). While the GAGs from the ECM dominate the NMR spectra of cartilage, lipids are the dominant contributors to the NMR spectra of bone and skin, whereas signals of the GAG are also present in NMR spectra of skin. The most comprehensive analysis has been performed for cartilage GAGs,

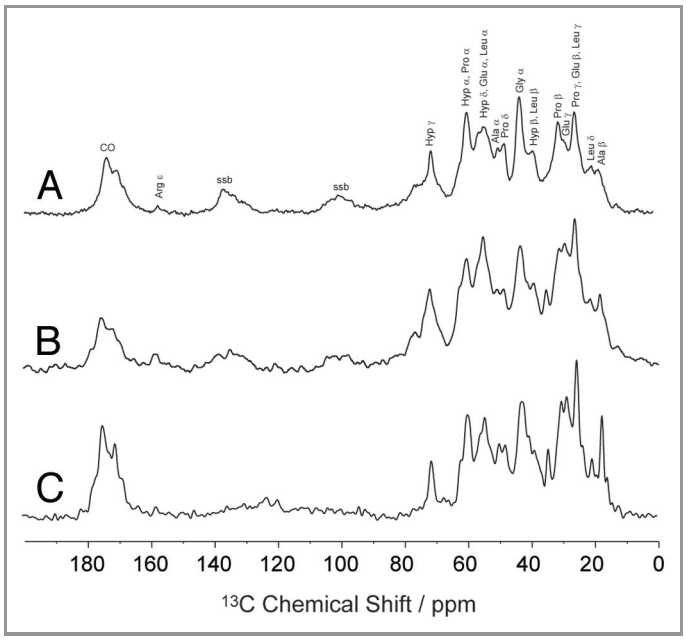

Figure 4. Cross-polarized proton-decoupled ^{13}C MAS NMR spectra of rat bone (A), porcine articular cartilage (B) and human skin (C). All spectra were acquired at a resonance frequency of 188.5 MHz and a MAS frequency of 7 kHz. Ala, alanine; Arg, arginine; Glu, glutamic acid; Gly, glycine; Hyp, hydroxyproline; Leu, leucine; Pro, proline.

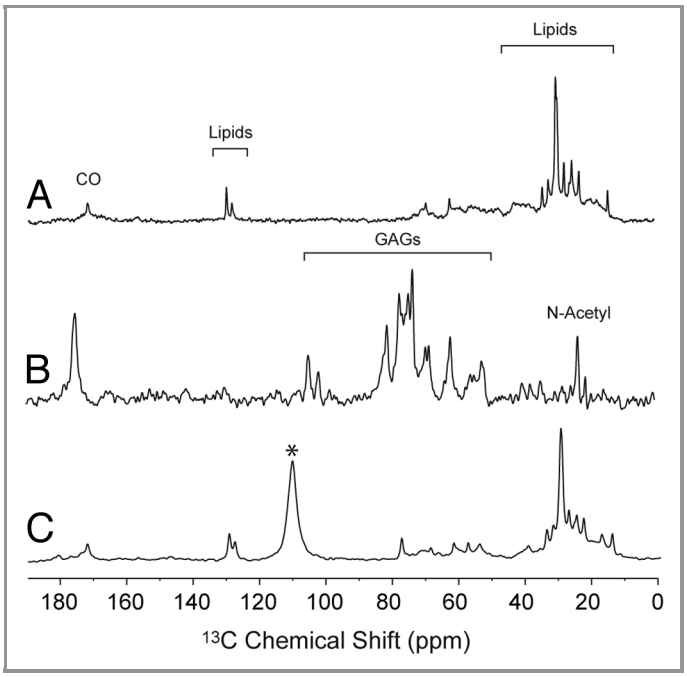

Figure 5. Proton-decoupled ^{13}C HR MAS NMR spectra of rat bone (A), porcine articular cartilage (B) and mouse skin (C). All spectra were recorded at a resonance frequency of 188.5 MHz. The main resonances are assigned in the figure. The peak marked with an asterisk in the skin spectrum refers to the signal of Teflon from the MAS rotor insert.

where mostly chondroitin sulfate has been analyzed. Here, a relaxation study characterized the molecular motions of the GAG component, which could be best described by a broad distribution function of more than 10 ns width.[37]

Solid state NMR of tissue engineered cartilage, bone implants and diseased cartilage. While solid-state NMR measurements on the intact tissues provide interesting insights into the tissue architecture and molecular dynamics, the method can also provide valuable results for tissue engineering and regenerative medicine. ^{13}C NMR spectra of agarose[89] and fibrin or whole blood based cartilage scaffolds[90] showed clear signatures of de novo synthesized GAGs of the ECM. Further, the molecular mobility of this new ECM was dynamically studied by ^{13}C relaxation analysis[37] showing that the molecular motions of the de novo formed ECM was still different from that of the native cartilage. The detection of collagen in these scaffolds by NMR techniques, however, has not been reported so far.

There are some studies on degenerated biological tissues by HR MAS NMR. In one contribution, the ^{1}H and ^{13}C HR MAS spectra of bovine nasal cartilage after digestion with different enzymes were analyzed.[91] Treatment with papain resulted only in small changes in the ^{1}H NMR spectrum, whereas a clear diminution of all resonances was detectable in the ^{13}C NMR spectra because the generated small mass fragmentation products are lost into the supernatant. On the other hand, treatment with collagenase caused the formation of peptides with an amino acid composition typical for collagen (glycine, proline, hydroxyproline and lysine).

Some interesting reports using ^{23}Na NMR have also been published on cartilage and diseased cartilage. Jerschow and coworkers[92] detected residual quadrupolar couplings in the ^{23}Na NMR signals of sodium associated with the proteoglycans of cartilage indicative of residual order of the GAG associated ions.[93] While the ^{23}Na signal of the spectra steadily decreased upon degradation with trypsin, the residual quadrupolar coupling actually increased, which suggested that higher GAG order was achieved upon depletion of collagen.[92] These experiments are particularly interesting with regard to ^{23}Na magnetic resonance imaging (MRI) for early detection of osteoarthritis.[94]

Solid-state NMR has also greatly contributed to study bone ECM development in implants. First papers studied tricalcium phosphate (TCP) implants by ^{31}P MAS NMR.[95,96] Typical ^{31}P MAS NMR spectra are shown in **Figure 6**. Clearly, bone apatite and TCP give rise to different NMR signals that can be deconvoluted to determine the relative contributions of "bioapatite" and TCP. Later, also an absolute quantification of the bioapatite content within the TCP implants was introduced using ATP as an internal standard to allow for a quantitative monitoring of the "bioapatite" formation in a rabbit model.[88]

The latter paper also reported ^{13}C CP MAS NMR spectra of collagen in bone. Interestingly, bone implants showed very well resolved collagen spectra in both a rabbit[88] and a rat model.[66] In the rabbit model the aforementioned TCP was used as a scaffold, while the latter study used a soft polymeric poly-D,L-lactide-co-glycolide (PLGA) scaffold. Absolute quantification was performed using isoleucine as an internal standard. In addition to the

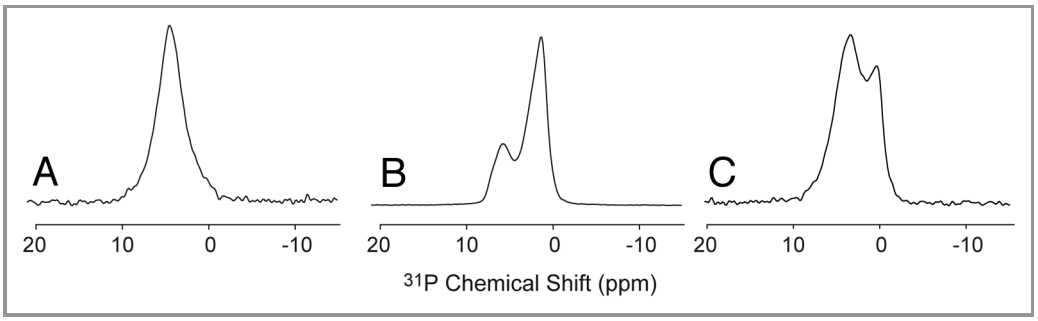

Figure 6. 161.9 MHz solid-state ^{31}P MAS NMR spectra of rabbit bone (A), pure β-TCP (B), and a rabbit implant removed after 3 mo. Spectrum (C) could be simulated from a superposition of spectra (A) and (B) at a 49:51 ratio. The NMR experiments were performed in a 4-mm MAS rotor using a Hahn echo pulse sequence at 37°C and a MAS frequency of 8 kHz. Spectra were acquired with a 1.9 μs 90° pulse and a relaxation delay of 400 sec.

high concentration of collagen formed in these implants, the molecular dynamics of the de novo formed collagen was also identical to that in native bone.

Water relaxometry. The majority of routine NMR investigations of the ECM are based on ^{1}H NMR because water is the most abundant compound in all tissues and the ^{1}H nucleus is most sensitively detectable. The determination of the water relaxation times is the basic principle of MR imaging (which will not be discussed here) since the contrast of MR images is primarily achieved by differences in the water relaxation times. Although a comprehensive discussion of the theory of MR relaxation would be outside the scope of this review, a very rough introduction is necessary. There are two different relevant relaxation times,[97] T_1 (spin-lattice relaxation) and T_2 (spin-spin relaxation), whereby T_2 is normally considered to be more important regarding the characterization of biological tissues. In a very simple fashion, one may state that relaxation times indicate the mobility of a certain nucleus. Free water that re-orients very quickly, for instance, possesses very long relaxation times, whereas water bound to the ECM polymers exhibits much shorter relaxation times.

There are so far only a few investigations on the relaxation behavior of tissue rich in ECM. In a rather pioneering work, Baker et al. determined T_1 relaxation times of 210 ms and T_2 relaxation times of 12 ms for canine articular cartilage.[98] These authors determined the overall relaxation times, whereas Xia et al. performed spatially-resolved measurements (in combination with self-diffusion studies) and explained the reduction of T_2 from 20 ms at the cartilage surface to 12 ms at the cartilage-bone interface by an increasing GAG content in the same direction.[99] Therefore, it is evident that also compositional parameters can be basically determined from NMR relaxation data. The explanation given[99] is, however, not absolutely convincing since also changes of the water content contribute to changes of the relaxation times. For instance, Lüsse et al. compared the relaxation data of two different types of cartilage,[100] porcine articular and bovine nasal cartilage that indicated different relaxation times under native conditions, i.e., at the normal water content (ca. 80%). However, when both cartilage types were compared at the same water content, differences were not observed.[100] These authors also investigated the influence of the ion content on the relaxation times and found only effects of Ca^{2+} ions, whereas there was no effect of increasing Na^{+} ion concentrations. Therefore, it was concluded that the water content of cartilage has the most important impact on relaxation processes and that the T_2 relaxation times can be used to estimate the water content of a given sample.

More recently, NMR relaxation studies on cartilage revealed that several components contribute to T_2 relaxation.[101,102] It is tempting to suggest that these numbers provide insights into water compartimentalization and arise from collagen-associated, GAG-associated, or free water. This also provides a tool for the quality control of artificially grown cartilage.[103] Nevertheless, this issue is discussed controversially and data on that subject, where the T_2 analysis only showed a single component have also been published.[104]

Water relaxometry measurements have also been performed for skin and it was claimed that the water content has to be carefully adjusted, for instance by using the osmotic stress technique in order to be able to perform reliable measurements.[105] It was also suggested that the relationship between relaxation times and water content can be used to assess the contribution of enzymes.[106] This is of course true because the removal of either collagen or GAG will significantly alter the water content of the tissue (vide supra). However, this only holds when the cartilage is completely isotropically-oriented with respect to the B_0 field since otherwise the so-called magic-angle effect has also to be considered, i.e., the appearance of individual cartilage layers depends on the angle between the sample and the static B_0 field.[107] Very similar effects are also observed when bone and tendon[108] samples are investigated in dependence on the orientation of the sample with respect to the magnetic field.

Diffusion NMR. Self-diffusion coefficients (SDC) of any given metabolite can be also determined by NMR measurements using the pulsed-field gradient (PGF) technique. The basis of this method is explained in more detail in NMR textbooks.[109] In brief, the principle of the technique is that the Larmor frequency along a given direction (typically the z-direction) is altered by the application of a gradient pulse. Thus, a molecule with a given z-coordinate is labeled by a spatially specific NMR frequency. A diffusion time Δ follows, during which the molecule is allowed to diffuse freely, before another gradient pulse abolishes the spatially dependent frequency encoding. When the molecules of interest did not diffuse, exactly the same echo amplitude is

detected. In contrast, a diminished echo amplitude indicates self diffusion and from the echo decay the diffusion coefficient can be calculated. For applications in tissue engineering, diffusion measurements can be combined with the MAS technique such that the diffusion of several molecules can be studied in one set of experiments.

In the last decade, diffusion of molecules in polymer solutions or polymer gels as a first model for the extracellular matrix has been studied.[22,110] Next, water diffusion in intact cartilage tissue was also studied.[22] Surprisingly, water diffuses very fast in cartilage and decreasing self diffusion coefficients are only observed upon cartilage dehydration. In fully hydrated cartilage, diffusion coefficients only decrease at long observation times (Δ = 500 ms), which reflects the structural properties of the tissue, i.e., the collagen mesh size. In addition, the diffusion of cations[111] and polymers such as poly(ethylene glycol) (PEG)[112] or dextran[112,113] within the ECM of cartilage tissue have been studied. The diffusion behavior of water within the cartilage ECM was determined primarily by the water content, while water diffusion at long observation times clearly reflects the structural properties of the cartilage extracellular matrix.[22] Interestingly, a very similar trend was also found for small cations.[111] As known for polymer diffusion in concentrated polymer solutions,[114] in cartilage a scaling of the self diffusion coefficient (D) with the molecular weight (M) according to $D \propto M^{-m}$ with $0.9 \leq m \leq 1$ for PEG and $0.8 \leq m \leq 0.9$ for dextran was found.[112]

More recently, the interaction of more relevant biomolecules such as growth factors, hormones, cytokines or chemokines with the components of the ECM has become of interest in many applied fields such as tissue engineering and regenerative medicine.[115-117] These extracellular proteins can bind to the cells to stimulate the production of ECM; they can cause both pro- and anti-inflammatory reactions, along with other immune responses. While the biochemical understanding of the action of these mediators is progressing and their application in tissue engineering and regenerative medicine is developing, the basic physical questions of the interaction and diffusion of mediators with the complex architecture of the extracellular space is somewhat limping behind. Some studies have addressed the diffusion of growth factors in model fibrin gels[118] and brain tissue by optical methods.[119] It was found that diffusion in fibrin gels only negligibly influences diffusion coefficients.[118] Further, protein diffusion in brain tissue was shown to be much slower than free diffusion in dilute agarose gel, indicating significant receptor binding in the tissue.[119]

MS techniques. The advantages of NMR have been described in the previous chapter, which underlined the capabilities of the method. NMR is not only useful as an analytical tool to determine the chemical composition of a given tissue but also to study the structure and dynamics of selected compounds even in intact tissues. However, NMR does also exhibit significant drawbacks: the most serious disadvantage of NMR is its comparably low sensitivity. Therefore, relatively large sample amounts are necessary to acquire NMR spectra within an acceptable time. Isotopic labeling of course increases the sensitivity and represents a relatively straightforward approach in cell culture, but is usually not practical for animal experiments. Another problem is the analysis of crude mixtures: although there are sophisticated methods available that enable mixture analysis, these techniques are normally rather time-consuming.

In the next section we will demonstrate that MS provides means to overcome these problems and that the combination of NMR and MS is a promising and complementary approach in tissue characterization and the quality control of de novo formed tissues.

Soft-ionization MS techniques. From the chemical point of view, all biological tissues consist (beside water and small molecules such as dissolved glucose or lactate) of major amounts of proteins, DNA, lipids, and polysaccharides. Even 20 years ago, it would have been absolutely impossible to analyze such molecules by MS because the electron ionization (EI) method that was exclusively available at that time was not capable of ionizing such polar and/or high mass molecules. Even the analysis of amino acids and monosaccharides was difficult and derivatization of the sample was required in order to enhance the volatility of the compounds of interest because mass spectra are normally recorded under high vacuum conditions.

However, by the invention of soft ionization MS methods, the analysis of molecules refractive under EI conditions became possible and, thus, many "omics" approaches such as proteomics, lipidomics[120] and, more recently, glycomics have been launched. "Omics" can be defined as the complete characterization of a given sample regarding a certain class of molecules,[121] while soft ionization means that analysis is possible with only a minimum extent of fragmentation. As a consequence, intact molecules are detectable with their correct masses.[122]

Two of the most established soft ionization MS methods are electrospray ionization (ESI) MS and matrix-assisted laser desorption and ionization (MALDI) MS and the inventors of these techniques were honored by the award of the Nobel Prize.[123] Both methods enable the detection of large and polar molecules that are otherwise difficult to analyze by MS. Although ESI is an even gentler method of ion generation than MALDI,[124] we will focus here on MALDI MS due to its simplicity and the reader primarily interested in ESI MS is referred to one of the excellent reviews.[125] A simplified schema of the ion generation[126] within a MALDI mass spectrometer and the separation of the generated ions by a TOF mass analyzer are shown in **Figure 7**.

MALDI-TOF MS is based on the utilization of a matrix that is normally a small organic molecule, for instance, a cinnamic or benzoic acid derivate and some common matrix compounds are shown in **Figure 8**.

Most commercially available MALDI instruments are equipped with UV lasers (often with an N_2 laser emitting at 337 nm), while IR lasers are less often used.[127] The matrix (in a 100–1,000-fold excess over the analyte) has two important tasks: (1) to absorb the laser energy and (2) to prevent aggregation of the analyte molecules. A very homogenous co-crystallization between the matrix and the analyte is an important prerequisite for reproducible MALDI spectra. Unfortunately, despite of its profound importance, the process of ion generation in MALDI-TOF MS is so far only poorly understood.[128]

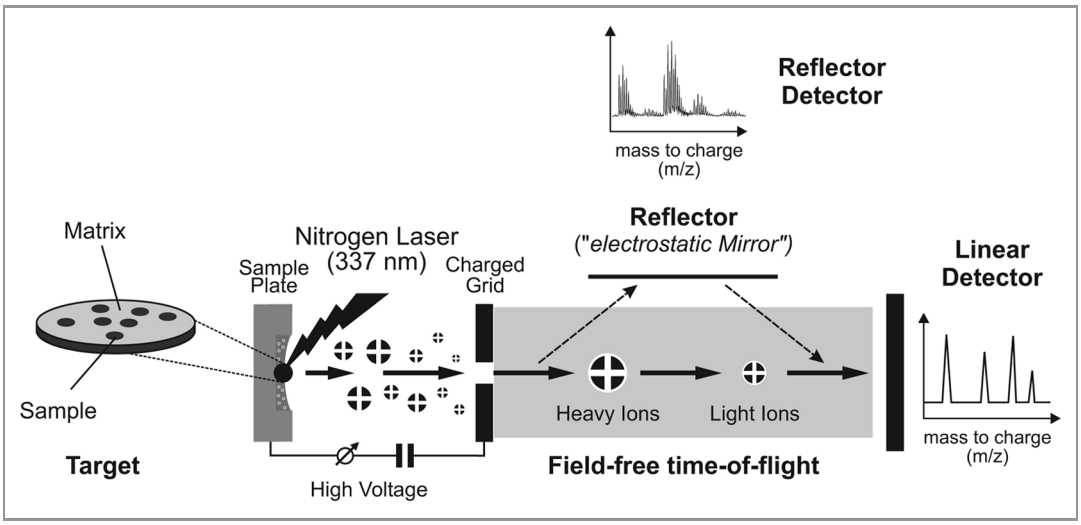

Figure 7. Schema of the processes occurring during the MALDI-TOF MS analysis of a sample (for details see text). Detection techniques using the "linear" and the "reflector" mode are emphasized in the figure. Reprinted with permission from reference 120.

When the pulsed laser beam hits the sample, the matrix is vaporized and carries intact analyte molecules into the vapor phase. During the expanding process of this gas cloud, H^+ and other ions (e.g., Na^+ or K^+) are exchanged between the analyte and the matrix molecules, leading to the formation of so-called "quasi molecular ions," e.g., $[M + H]^+$ or $[M + Na]^+$. Besides cation generation, anions can also be generated by abstracting H^+ or Na^+ from the analyte. The ratio between cations and anions is primarily determined by the (gas phase) acidities of the analyte and the matrix. As GAG, for instance, represent strong electrolytes, it is not very surprising that these ions are primarily detected in the negative ion mode.[129] After being formed, ions are accelerated in a strong electric field with typical accelerating voltages of the order of 20 kV. After passing a charged grid, the molecules are drifting freely over a field-free space where mass separation occurs: Low mass ions arrive at the so-called linear detector in a shorter time than high mass ions. Using a reflectron, the flight path can be enhanced and, thus, mass resolution can be improved. However, this is also accompanied by a loss of sensitivity. The particular advantages of MALDI MS in comparison to other MS techniques are the simple performance—often in combination with automation—and the high sensitivity down to a few attomoles of the analyte or even lower.[130] Typical positive and negative ion spectra of HA subsequent to digestion with testicular hyaluronidase are shown in **Figure 9**. Please note that the larger oligosaccharides such as the hexasaccharide (HA-6, *m/z* 1154) and the octasaccharide of HA (HA-8, *m/z* 1533) are more sensitively detectable in the negative ion mode due to the polyelectrolyte character of HA. Please also note the characteristic mass difference of 24 amu between peaks detectable in the

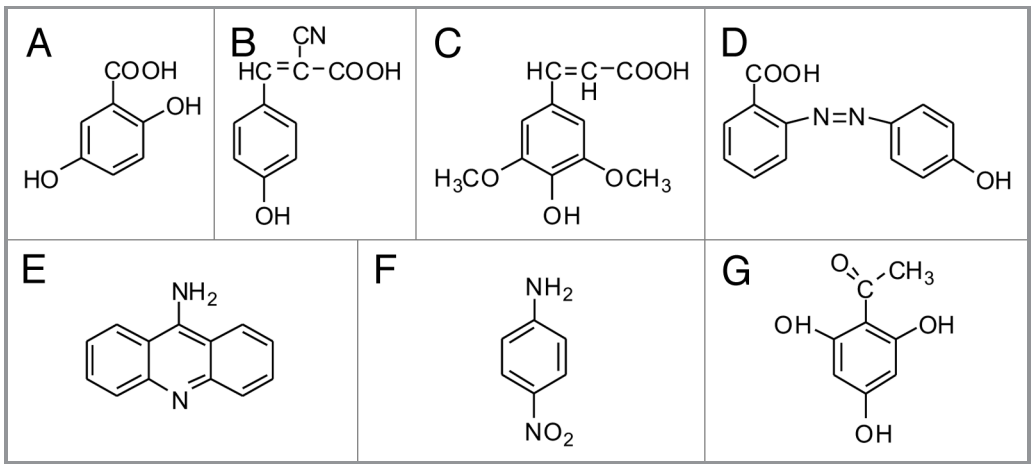

Figure 8. Chemical structures of important, frequently used UV MALDI matrices: (A) 2,5-dihydroxybenzoic acid (DHB); (B) α-cyano-4-hydroxycinnamic acid (CHA); (C) sinapinic acid (SA); (D) 2-(4-hydroxyphenylazo)benzoic acid (HABA); (E) 9-aminoacridine (9-AA); (F) p-nitroaniline (PNA); (G) 2,4,6-trihydroxyacetophenone (THA). Note that 9-AA, THA, and PNA are particularly useful for recording negative ion mass spectra.

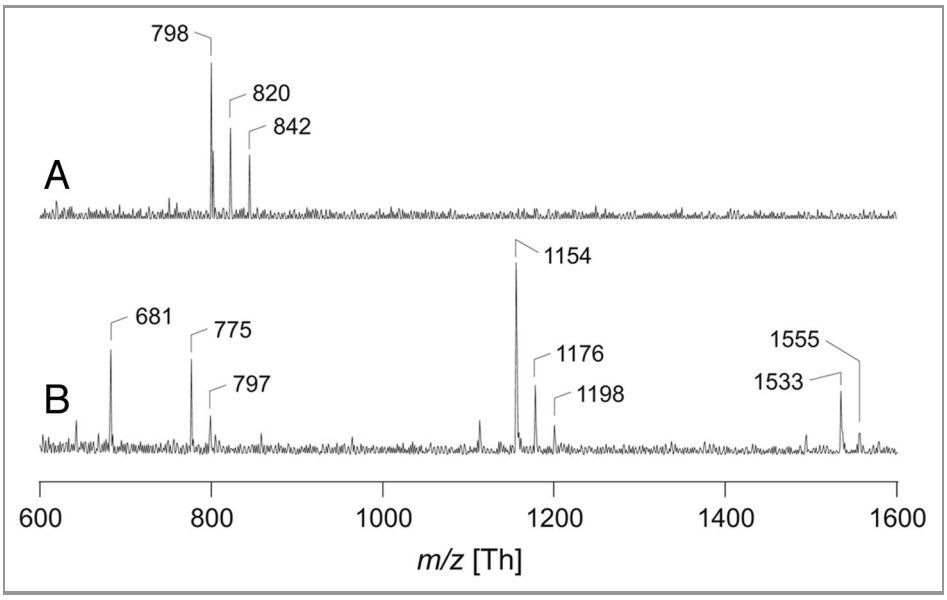

Figure 9. Positive (A) and negative ion (B) MALDI-TOF mass spectra of a 10 mg/ml HA solution digested with 2 U hyaluronidase for 9 h. HA-4, HA-6 and HA-8 are detectable in the negative ion mode, while only HA-4 is detectable in the positive ion spectrum. Both spectra were recorded using DHB as matrix after a 1:10 dilution of the carbohydrate solution. Reprinted with modification and permission (from Elsevier) from reference 129.

positive and negative ion mode. This corresponds to one Na^+ (23) and H^+ (1) that provide two charges which are required to come from a negatively charged to a positively charged analyte ion.

Applications of MALDI MS to collagen. MALDI-TOF MS is particularly suitable for the direct (without the need of major purification of the sample prior to MS characterization) detection of proteins and peptides in biological samples, because it is (1) highly sensitive and (2) robust with regard to sample impurities such as salts and detergents. Accordingly, MALDI-TOF MS has been already successfully applied to the qualitative analysis of collagens: fibril-forming collagens are composed of linear polypeptide chains of about 100 kDa, which form closely coiled triple-stranded helical macromolecules. The detection of type III collagen from calf skin by employing UV MALDI MS and sinapinic acid as the matrix has been attempted,[131] but this approach confers many limitations: the intact collagen triple helix is not detected, and the individual chains were only detectable after thermal denaturation of the sample.[132] Thus, UV MALDI is no suitable tool to characterize native collagen.

In contrast, the analysis of native collagen, even that of the non-covalently bound triple helices, becomes possible if infrared (IR) instead of UV MALDI MS is used. For instance, chicken collagen of types I, II, and XI have been identified by IR MALDI MS by using succinic acid as the IR matrix (personal communication), whereas Dreisewerd and coworkers have identified whole fibril-forming collagen proteins of types I, III, and V in fetal calf skin by using glycerol as IR matrix.[133] Using this approach even the high-mass collagen type III of several hundred kDa was detectable.

Although the detection of intact collagen is thus basically possible, the reproducibility of protein mass spectra ($>$ 100 kDa) is unequivocally poor and not suitable for the estimation of the collagen contents of biological samples. Consequently, Henkel and Dreisewerd[134] performed a CNBr degradation of the fibrillar collagens I, III and V prior to MS analysis. This enabled the identification of distinct peptides that may serve as reference signals of the individual α-chains. Additionally, peptide glycosylation and the cross-linking of peptides can also be monitored. However, no quantitative data regarding the collagen contents of tissues could be obtained.[134]

Collagen type II (a main constituent of articular cartilage) has been investigated by Zhang and coworkers.[135] These authors demonstrated that characteristic fragments enable the differentiation between the collagen types I and II by using isolated bovine collagen and cartilage subsequent to thermal denaturation and tryptic digestion. Denaturation is necessary because native collagen cannot be digested by trypsin. Unfortunately, this work did not provide any quantitative data. We have recently shown that the cleavage of native collagen by the readily available enzyme collagenase A prior to MS enables the determination of the collagen content of an unknown sample:[136] using collagenase A digestion a variety of tripeptides is generated with Gly-Pro-Hyp being the most abundant one. Using a defined tripeptide as internal standard in a known concentration, the intensities of Gly-Pro-Hyp can be directly used to determine the collagen content of a tissue sample. Unfortunately, however, this approach does not enable the differentiation of the different collagen types but exclusively the estimation of the overall collagen content. Therefore, further attempts are necessary to overcome this problem. According to the so far available data, trypsin-digestion of thermally denaturated collagen seems a promising approach.

Applications of MALDI MS to glycosaminoglycans. The analysis of intact GAGs is even more challenging than that of collagen: in addition to the high molecular weight of native GAGs, the high

charge density (primarily due to the sulfate groups) renders these molecules extremely refractive to MS. Therefore, although all information about the MW of the original GAG of interest is obviously lost by the degradation process, all mass spectrometric GAG determinations are 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 **Figure 10**.

The most widely used enzymes to depolymerize native GAGs are bacterial or testicular hyaluronidases[137] as well as bacterial chondroitinases, in the majority of cases the ABC type from *Proteus vulgaris*.[138] 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. 10**). The arising UV absorption (normally determined at 232 nm) is helpful to quantitatively monitor the generation of the digestion products and, thus, the endpoint of the enzymatic digestion. Although bacterial hyaluronidases do also generate unsaturated products, the application of hyaluronidases is less favorable because they lead to oligosaccharide mixtures. Unfortunately, all these enzymes are inhibited by oversulfated GAGs and are, thus, not capable of digesting chemically modified GAGs.[139] This is a serious problem because chemical degradation methods do normally also generate a variety of site reactions that massively complicate the MS characterization of the released oligosaccharides. Another problem is the sulfate loss that occurs upon the ionization process. Many efforts were already made to avoid this unwanted fragmentation, but so far this process can only be minimized, for instance, by using ionic liquid crystalline matrices[140] or the cesium salts of the GAG,[141] but never completely avoided. The sulfate loss is unwanted regarding quantitative data, but extremely welcome regarding the differentiation of isomers: of course, the 4- and 6-isomers of CS have exactly the same mass. Therefore, differentiation is possible only by MS/MS spectra: it has been shown by using MALDI[89] as well ESI[142] that there are peaks in the MS/MS spectra that enable a clear differentiation of the isomers, i.e., some fragment ions are exclusively arising from a certain isomer. We have recently used a combination between MS and MS/MS to investigate the de novo generation of dermatan sulfate by skin fibroblasts.[143]

As the quantitative evaluation of MS/MS spectra is more difficult, separation of the individual isomers prior to MS analysis is normally the method of choice. This separation can be performed by a variety of methods including HPLC[144] and thin-layer chromatography:[145] in particular, the direct combination between MALDI and thin-layer chromatography (TLC) is currently a hot research topic because this combination can be easily established and gives convincing data with high resolution. This topic has been recently reviewed.[146]

Some selected negative ion MALDI-TOF mass spectra of selected GAGs subsequent to enzymatic digestion and investigated directly on a TLC plate are shown in **Figure 11**.

Nevertheless, there are two problems that complicate the situation: The first problem is coming from the use of high amounts of formic acid that are required to be able to separate GAGs on a normal phase TLC plate.[147] Under these conditions, esterification occurs (detectable by the mass shift of 28) and, thus, a certain part of the GAG is not detectable as such but only as the corresponding formyl ester. This is, however, not a major problem because the extent of formylation is only moderate. A second much more serious problem is the loss of the sulfate residue. This does, however, occur under all so far tested MS conditions[148] and is only slightly enhanced if measurements are performed directly on the TLC plate. From our point of view, there are two prime issues that have to be improved in order to make the TLC/MALDI approach routinely applicable: first, normal phase TLC should be replaced by reversed phase TLC because this would prevent the need to use highly polar solvents such as formic acid. Second, it has to be evaluated whether fragmentation of the sulfate residues can be further minimized. For instance, it has been shown recently that recording positive ion spectra results in reduced fragmentation in comparison to the negative ion spectra.[149]

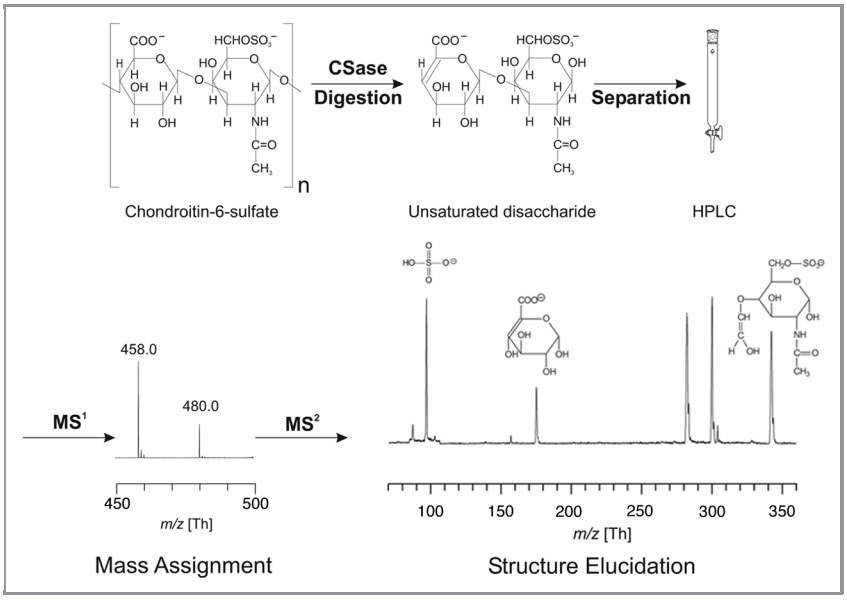

Figure 10. Schema of the procedure normally used for GAG analysis. The GAG of interest is digested by the enzyme chondroitinase ABC that produces a mixture of unsaturated disaccharides depending on the structure of the GAG. The different disaccharides are subsequently separated, normally by means of HPLC or capillary electrophoresis, and analyzed by MS 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. Reprinted with permission and with modification from reference 139.

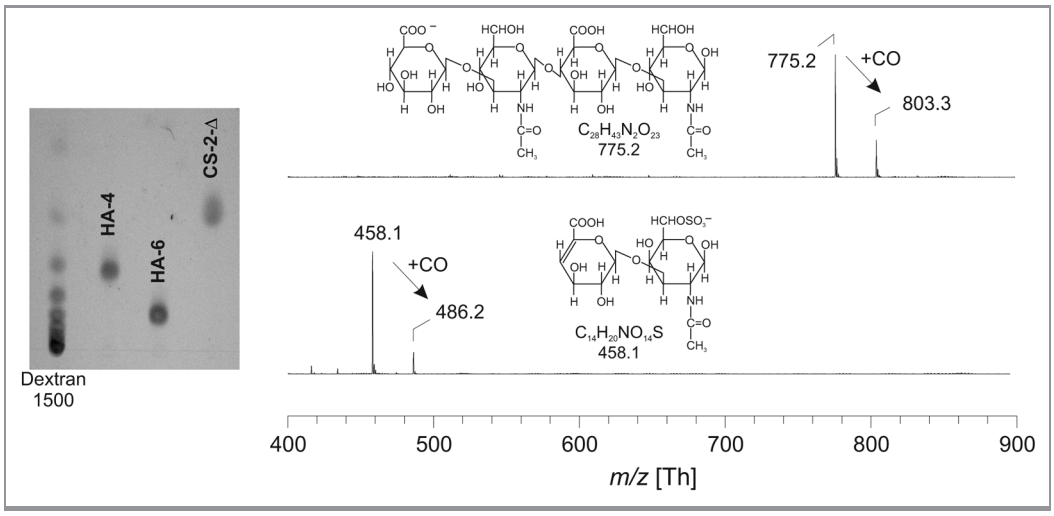

Figure 11. Negative ion MALDI-TOF mass spectra of selected oligosaccharides obtained by enzymatic digestion of HA-4 (upper spectrum) and the unsaturated disaccharide of chondroitin sulfate (lower spectrum). Mass spectra were recorded directly from the TLC plate (shown at the left). The molecular structures and the *m/z* ratios are provided along with the mass spectra. Please note that "+CO" indicates the formation of the formyl ester because the separation of the carbohydrates was performed in the presence of formic acid. Dextran 1500 as well as HA-6 are only shown for comparative purposes on the TLC plate (left) but no mass spectra of these compounds are provided. Reprinted with modification and with permission from reference 145.

Summary and Outlook

Since diseases of the ECM are extremely widespread, thousands of papers appear annually on this topic. The majority of these studies, however, deals with biochemical or immunological aspects of the corresponding diseases or is dedicated to the characteristics of a certain cell type that is involved in ECM degeneration.

The aim of this review was to show that the knowledge about the composition of the ECM of a given tissue is extremely important because the composition determines the structure and the water content of a tissue that are both crucial parameters regarding the tissue function. Compositional analyses are particularly important as bioengineered tissues do so far not exhibit the excellent biomechanical properties of the native tissues and are normally characterized by an elevated GAG and reduced collagen content. Two methods, NMR spectroscopy and MS were in the focus of this review. Both are not typical techniques of tissue research but feature some important advantages in comparison to established immunological or histological methods. For instance, NMR and MS do not make use of specific dyes or antibodies and are capable of detecting basically all types of molecules without any alteration of the tissue of interest. The combined use of NMR and MS provides a lot of advantages. NMR is capable of providing information about all major tissue constituents as well as structural and dynamical parameters such as order parameters or the interactions with other molecules. Additionally, NMR is a method that provides direct quantitative information as the intensities of the individual resonances correlate directly with the concentration of a given metabolite. Several parameters detectable by NMR are well understood with regard to their meaning for the tissue quality and properties. Since these parameters can be measured with reasonable precision, the detection of those values in tissue engineered samples is just on the verge of representing tools for the quality control/quality assurance analysis of de novo formed tissues. Due to the quantitative character and the direct relation to important parameters of tissue quality, we expect that tissue characterization by NMR will develop into a more or less routine technique for the ECM characterization in tissue engineering approaches. It should be emphasized that intact tissue samples can be investigated by solid-state NMR without the need for any preparative procedures. The most serious argument against the use of NMR is its relatively low sensitivity requiring sample amounts in the low mg range. Nevertheless, higher magnetic field strengths, improved probe technology and polarization methods become more frequently available suggesting that NMR techniques will definitely contribute to the development of the tissue engineering field.

Sensitivity, in contrast, is the most important strength of MS: using this method, sample amounts in the μg range are always sufficient. Additionally, MS enables the accurate differentiation of compounds with a similar structure but with different molecular weights. For instance, MS is the method of choice to differentiate compounds with a different extent of polymerization. Thus, NMR and MS should not be regarded as alternative but as complementary methods and combining both techniques will result in excellent information about the sample composition and the biophysical properties of the respective tissues.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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