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Chemical Modifications, Solid Phase, Radio-Chemical and Enzymatic Transformations of Hyaluronic Acid

From the chemical point of view, hyaluronan possesses four different types of functional groups: acetamide, carboxylic acid, hydroxyl and terminal aldehyde. After deacetylation, a free amine could be obtained from an acetamide group. All four functionalities permit characteristic chemical reactions. Such a wide variety of possible chemical modifications creates a sharp difference between hyaluronic acid and other polysaccharides whose reactivity depends mainly upon hydroxyl groups.

We realize that the description of the overall reactivity and chemical modifications of hyaluronan is a dense subject that could fill a separate monograph. However, this chapter will focus mainly on the chemical modifications of hyaluronan that lead to cross-linking. Such modifications play an important role for the creation of hyaluronic acid with valuable chemical and physical properties necessary for biological application of hyaluronan products.

Historically, hyaluronan was known for the chemical transformation of its hydroxyl groups. Only recently, several processes also described transformation of carboxyl groups and deacetylated amino groups. Generally, polysaccharides, including hyaluronan, possess all chemical reactions characteristic to hydroxyl-containing compounds including ethers and ester formation, substitution, elimination, and so on. The reactivity of hyaluronic acids depends mainly on the functionality of hydroxyl groups. Also, the contribution of the aldehyde group is relatively small. The presence of the free hydroxyl groups provides the possibility of structural modifications of the sugar base, which allows direct bio-specific modification to be carried out by means of, for example, bi-functional reagents that interact simultaneously with two functional groups of neighbouring macromolecules. We consider bi-functional reagents as chemical compounds that usually possess two of the same reactive groups separated by a spacer. Bi-functional reagents are widely used for a covalent

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linkage of sterically close fragments of polysaccharide macromolecules. One method of bio-specific modification is photo-initiating polysaccharide linkage with an initial introduction of photo-reaction fragments into its structure.

This chapter focuses on two subjects related to the field of mechanically stimulated reactions: (1) chemical and photochemical cross-linking of HA in aqueous solutions and (2) advanced methods of solid-phase polysaccharide modification. From these processes, the cross-linked hyaluronic hydrogels acquire a number of valuable properties that significantly extend the range of their medical applications.

5.1 Main Characteristics of Cross-Linked Hydrogels

In aqueous solution, hyaluronan forms a gel-like structure as a result of intermolecular interaction of linear macromolecules. Colloidal chemistry defines gels as structured systems with liquid dispersion media that exhibit mechanical properties more or less similar to those of solids. The particles of the dispersed phase are connected to each other in a three-dimensional web (which contains dispersion media in its cells and, in the case of hydrogels, water) that deprives the system of fluidity. It is obvious that the properties of hydrogels are mainly dependent on the strength of bonds and level of reticulation in the cross-linked structure. Cross-links in biopolymers can be categorized as either physical (formed by electrostatic interactions or hydrogen bonds) or chemical (formed by covalent bonds). The physical gels, upon heating, undergo web node decomposition that leads to a reduction in the shift modulus. Chemically cross-linked gels are significantly more resistant to heat but at high temperatures a complete and irreversible destruction of the gel's chemical structure takes place. The level of cross-linkage is determined by the average molecular weight of the polymer chain located between cross-links. A cross-link's density directly affects the fundamental properties of the hydrogels, such as degree of swelling, mechanical strength and elasticity, permeability and diffusion characteristics [1].

The hydrogels, formed by hyaluronan as a result of cross-linking, are amphiphilic polymeric substrates able to swell in water and form an insoluble bulky web. The polymeric network is in equilibrium with aqueous environment while there is the balance of elastic forces of cross-linked polymers with osmotic forces of solution. The chemical compositions and the molecular weight of the macromolecule fragment between two cross-links determine a density of the cross-links that, in turn, influences the swelling and size of gel pores [1, 2]. Besides that, the cross-linking characterizes hydrogel as a pseudo-solid compound, not the solution, thereby giving it viscoelastic properties [3]. Such properties are expressed through the physical characteristics of the hydrogels, including swelling level or amount of absorbed water. The swelling is directly related to the chemical structure of the polymer and is in inverse proportion to the density of the cross-links.

In 1943, Flory and Rehner were the first to find a connection between the level of cross-link density of the polymer and its swelling [4]. In the Flory–Rehner model, the swelling level is determined by equilibrium between elastic properties and the forces originated from mixing the polymer and solvent. In 1977, Peppas and Merrill modified the theory of Flory–Rehner to apply it to the behaviour of the hydrogels [5]. Due to elastic forces, the presence of water affects change in chemical potential inside the system [5] and the chemical structure affects the swelling. For example, hydrogels with hydrophilic groups, to which

HA is related, swell more than hydrogels with hydrophobic groups; the latter do not increase in volume in the presence of water [6]. The swelling hydrogel very often may depend on pH, temperature or other factors [7].

The limit of swelling may be determined experimentally or calculated theoretically. The accurate measurement of swelling limit is useful in the calculations of cross-link density, mesh size and the diffusion coefficient. Many natural gels are formed by polyelectrolytes. The physical properties of these systems are greatly influenced by an osmotic pressure caused by counter-ions associated with the polymeric chains. Free counter-ions significantly increase the swelling of the charged gels and affect the elastic modulus. The mechanical characteristics of charged hydrogels determine the properties of many biological structures such as cartilage, synovial fluid, cornea and striated muscle. In order to measure the swelling of hydrogels, different experimental methods could be used. An important feature of hydrogels is the porosity or the size of the mesh. It is a structural property of the material, which is determined as a distance between adjacent cross-links. The study performed with polyethylene glycol diacrylate (PEGDA) experimentally established significant changes in the porosity upon the changes of the polymer molecular weight and small changes of cell sizes at the different concentration [8]. Direct measurements of porosity involve electron microscopy or quasi-elastic laser scattering. Indirect methods include mercury porosimetry and measurements of high elasticity and maximum swelling [7,9].

In designing cellular tissue, the diffusion rate of the solubilized compound is important in order to determine the rate of release of drugs or transport of nutrients and metabolites. The diffusion of nutrients, metabolites and other solubilized compounds depends on many factors, including the morphology of the network, the chemical composition of the hydrogel, the water content, the concentration of solubilized compounds and the level of the material swelling [7].

The nature of the cross-links affects the formation of the hydrogel, its shape, size and degradation. The formation of cross-links should be monitored for the biomedical applications of hydrogels. In this section, three different types of cross-linking in hydrogels are described: covalent, ionic and physical interactions [10].

The appearance of the chemical covalent cross-links can take place during the radical polymerization under exposure to high-energy radiation (gamma and electron) [10]. Before radical polymerization the polymers are usually modified by adding additional reactive groups. For example, acrylate is added to polyethylene glycol (PEG) in order to achieve covalent cross-linking [11]. The radical polymerization of acrylate groups can be initiated by light, high temperature and redox catalysis [12,13].

After the cross-linking process is started, it cannot be cancelled or stopped; it is controlled only by the initial process conditions. Photopolymerization is the conversion of a liquid polymer solution to gel under the action of photosensitizing additives and light [10] and is the most ideal method for synthesis of cross-linked hydrogels intended for use in medical practice since it allows for the reaction to be carried out with almost 100% efficiency.

The second type of chemical bonds in hydrogels represents the bonds based on ionic interactions. Several natural polysaccharides – for example alginate, a natural polysaccharide made from algae, hyaluronan and other charged polymers – form the hydrogel with ionic interaction in the presence of bivalent or multivalent cations. The reaction typically proceeds at ambient temperature and neutral pH [14]. The ionic interactions are weaker than covalent bonds, so such hydrogels undergo rapid degradation in physiological solution

(the media in which they are supposed to be used). One example of a synthetic polymer that forms hydrogel with ionic interactions is poly[di(carboxylatephenoxy)phosphazene] [15].

The weakest type of interaction realized by hydrogen bonding, hydrophobic interactions and Van der Waals forces also leads to gel structuring. Hydrogen bonds are usually stronger than hydrophobic and Van der Waals interactions – their energy values are in the range of 10–40 kJ/mol, but they are still an order of magnitude weaker than the ionic and covalent bonds. These weak interactions, however, play a central role in the process of molecular self-assembly, since the various combinations of these interactions in the macromolecules lead to a strong binding. R. Zhang et al. described the molecular self-assembly as a set of molecular building blocks that spontaneously form stable, physically connected network structures [16]. Despite the weakness of each act of physical binding, the multiplicity of such links makes the gel network structures quite stable. Thus, the various chemical and physical bonds can participate in the formation of stable cross-linked hydrogels.

5.2 Methods of Hyaluronic Acid Cross-Linking

As was mentioned previously, hyaluronic acid possesses four functionalities: acetamide, carboxyl, hydroxyl and terminus aldehyde. All are suitable for cross-linkage reactions. Depending on the nature of the cross-linkage reagent, a large variety of hyaluronic acid materials, starting from the films with low water content up to hydrogels with high water content have been synthesized. The majority of the methods of the production of cross-linked hyaluronan is related to one of two schemes: (1) a one-stage process with a bi-functional reagent able to create cross-linked bridges or (2) a two-stage process in which highly reactive HA derivatives are synthesized then followed by second reaction that creates cross-links. Different reagents are typically used for hyaluronan cross-linking including diamines, aminoaldehydes (obtained from aminoacetals), dialdehydes, butadienesulfones, diepoxides, salts of divalent metals and others [17].

5.2.1 Cross-Linking with Carbodiimides

One of the most common reactions of the HA carboxylic group with amino acids and diamines is condensation in presence of HOBT in water/DMSO [18]. It is known that one of the best condensation methods of carboxylic and amino functions is the reaction in the presence of DCC (dicyclohexylcarbodiimide). One of the first studies of the condensation with DCC was conducted in 1991 [19]. A similar method was used for reaction with different amines [20]. Unfortunately, DCC required the reaction in non-aqueous conditions. However, there is a possibility to perform cross-linking with EDC – water soluble analogue of DCC [21]. A similar process, which mentioned condensation with EDC and resulted in cross-linked hydrogels is described in [22,23].

General methods for cross-linking of biopolymers such as hydroxyethylcellulose (HEC), carboxymethylcellulose sodium salt (CMC Na) and hyaluronic acid (HA) using water soluble carbodiimide are summarized in a review published in 2005 [24]. The interesting invention described the synthesis of water-insoluble derivative of hyaluronic acid cross-linked with biscarbodiimide [25].

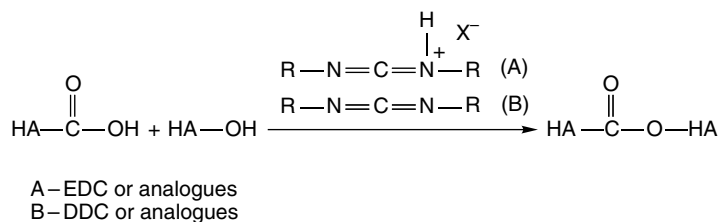


Figure 5.1 Hyaluronic acid cross-linking with carbodiimide

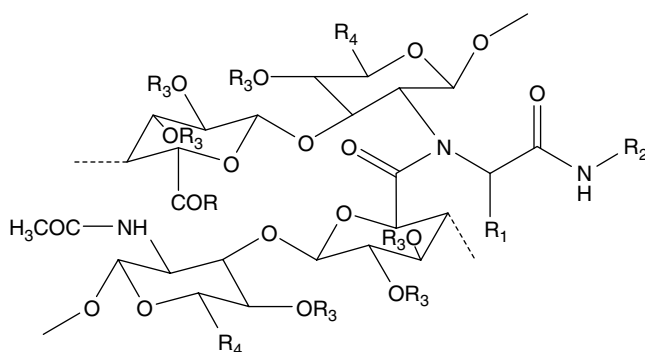


Figure 5.2 Cross-linked hyaluronic acid with amide bond after reaction with carbodiimide using the Ugi approach

One of the latest studies investigates the role of the solvent in carbodiimide cross-linking of hyaluronic acid. The cross-linked products were intended for use in ophthalmology. The conclusion was made that after the EDC treatment in the presence of an acetone/water mixture (85:15, v/v) the HA hydrogel membranes have the lowest equilibrium water content, the highest stress at break and the greatest resistance to hyaluronidase digestion. Irrespectively of the solvent composition (in the range of 70–95%), the cross-linked HA hydrogel membranes are compatible with human RPE cell lines without causing toxicity and inflammation [26].

An interesting method of connecting two hyaluronic acid fragments through carboxylic and primary amine functions using the Ugi approach is described in the patent [27]. In the method the primary amine was generated by deacylation, then two molecules were allowed to react in the presence of formaldehyde and cyclohexylisocyanide (Figure 5.2). The Ugi reaction allowed scientists to make cross-linked HA with an N-substituted amide bond, in which carboxylic and amino functions came from the different HA molecules.

Another possible way to synthesize cross-linked HA is by using bi-functional reagents. The cross-linked HA product is synthesized after reaction of HA with dihydrazide in the presence of HOBT and carbodiimide (Figure 5.3) [28]. In this reaction only the carboxylic functions from both HA molecules were used for cross-linking.

Another example of the synthesis of cross-linked HA with a dihydrazide bridge is described in [29]. For a formation of the bond between hyaluronan and primary amine, carbodiimide and N-hydroxysulfosuccinimide were used. The authors synthesized many

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