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CHEMISTRY

CHITOSAN-BASED HYDROGELS. FROM CLASSIC TO DYNAMIC MATERIALS

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This review presents the current state-of-art in the obtaining of hydrogels by the use of a versatile polyaminosaccharide – chitosan. Presenting the main preparation strategies along with the properties of the resulting systems, the study focuses on the advantages conferred by chitosan's action, derived from its intrinsic properties, usually observable in the resulting hydrogels. Moreover, the literature survey reveals that the chemical structure of this polymer, by the presence of the amine groups on its backbone, renders it an excellent choice for obtaining of dynamic materials, especially hydrogels, through the reversible chemistry of imine linkage.

INTRODUCTION

Hydrogels are three-dimensional polymeric networks which are able to absorb and retain large amounts of water, up to thousand times higher compared to their dry weight [1]. Due to their properties, hydrogels are successfully applied in various fields, such as agriculture, health care or biomedicine. Hydrogels in either wet state or as xerogels (the form obtained after hydrogel freeze-drying) are versatile materials which can be used as controlled drug delivery systems or as scaffolds for cell growth and proliferations, as imaging agents or prosthesis, such as artificial cartilages or for contact lenses.

The main condition for hydrogels with applications in the biomedical field is their biocompatibility. This means that hydrogels must not cause an inflammatory reaction and should not present cytotoxicity, a condition which must be fulfilled by both hydrogels and by any component used for their synthesis or any other additives used in their preparation.

Another prerequisite is hydrogel biodegradability, with the exception of hydrogels, which are meant to be used in prosthesis or as contact lenses, when their resistance in the body as long as possible is desirable. This characteristic of a hydrogel simplifies the medical act by eliminating the need of posthealing surgery,

which would have been necessary to withdraw/ remove the implant after it has fulfilled its function. Their degradation products must be able to be metabolized or excreted by kidneys. Excretion of the degradation products requires them to have a certain size. In the particular case of hydrogels designed for tissue engineering, it is necessary for the material to gradually lose its strength, which stimulates healing of the tissue.

Numerous polymers have been used for hydrogels obtaining, both (i) natural: collagen, gelatin, fibrin, lysozyme, hyaluronic acid, agarose, dextran, chitosan, and (ii) synthetic: polyethylene glycol, poly (N-isopropylacrylamide), pluronic, poly (vinyl alcohol), poly (lactic acid), poly (ε-caprolactone), and many others. Among the natural polymers used in hydrogels preparation, chitosan holds a special place.

Chitosan is a linear polyaminosaccharide with polycationic structure, being the most widespread biopolymer after cellulose. It is biocompatible, biodegradable and it has low immunogenicity, reasons for which it has been investigated in many medical and pharmaceutical applications. Due to its moderate hydrophilicity, chitosan facilitates the adhesion, proliferation and differentiation of cells, while its antibacterial activity represents a supplementary gain compared with other natural polymers. Another advantage derives from the fact that the chitosan properties can be varied by controlling its molecular weight, its deacetylation degree and crystallinity – flexible parameters that can be modified to increase or decrease the solubility and biodegradability of chitosan-based systems. On the other hand, chitosan has some drawbacks, such as poor mechanical properties, inability to maintain a certain form and difficulty of purification – leading to the presence of impurities which worsen much more the mechanical properties [2, 3].

Chitosan's cross-linking and its transformation into a hydrogel improves considerably its mechanical properties, which eliminates these disadvantages. By controlling the crosslinking density and the hydrophilic/hydrophobic balance of the obtained systems, and by a targeted choice of the crosslinking agents, a wide range of hydrogels can be obtained, with suitable mechanical properties for specific medical applications, such as tissue engineering and controlled drug delivery.

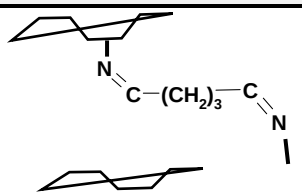
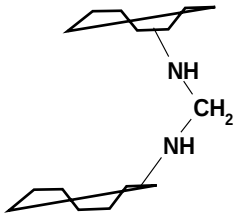
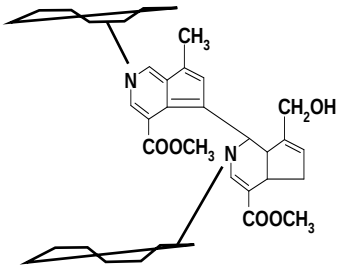
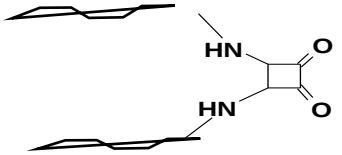
CHITOSAN-BASED HYDROGELS. OBTAINING AND RELATED PROPERTIES

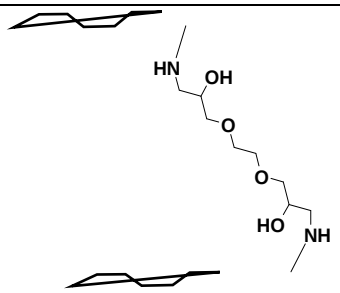
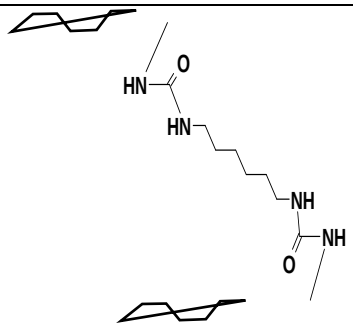
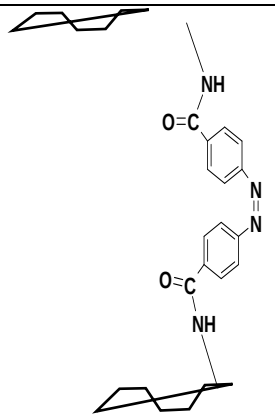
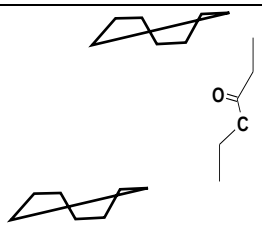
Generally speaking, chitosan-based hydrogels can be obtained by either *chemical* or *physical crosslinking*. Literature also reports the case of chitosan autocross-linking [4]. It is well known that the physically crosslinked hydrogels usually present weak mechanical properties and dissolution problems, that is why, for some applications, the chemically crosslinked hydrogels are preferred (applications in which the hydrogel stability is a main requirement).

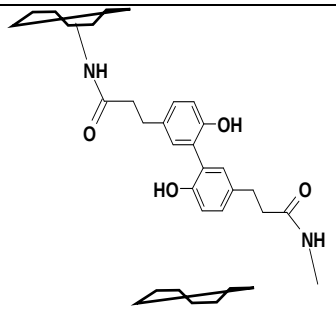
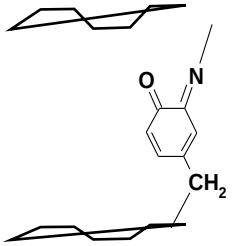
CHEMICAL CROSSLINKING

Chemical cross-linking of chitosan can be achieved by the ***use of crosslinking agents*** within a process known as secondary polymerization, or by ***irradiation-induced polymerization***. Table 1 presents the most common crosslinking agents used for chitosan-based hydrogel obtaining, the reactive functional groups which favor crosslinking and the possible chemical bonding.

Table 1
Crosslinking agents for chitosan and the resulting structures

	Crosslinking agents	**Crosslinking**
Small molecules	Glutaraldehyde	
	Formaldehyde	
	Genipin	
	Diethyl scuarate (DES)	

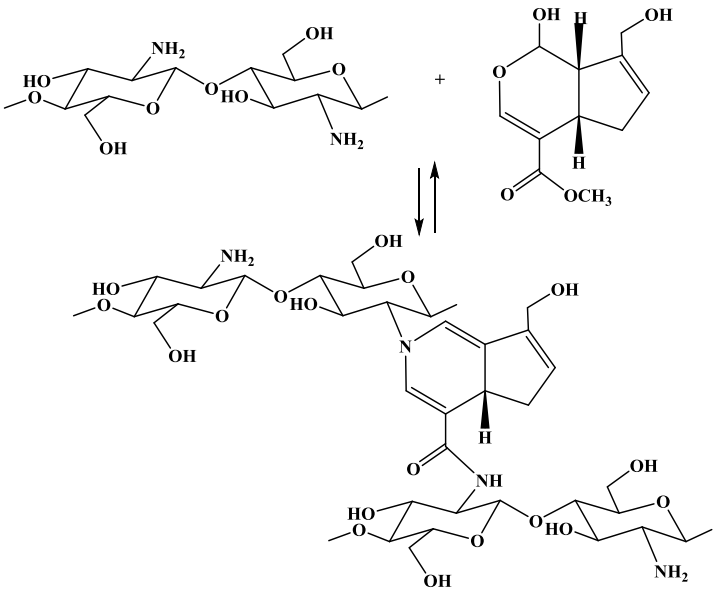
	Ethyleneglycol diglyci dilether (EGDE)	
	Diisocyanates	
Photosensitive molecules	Azides Functionalized azides	
	Acrylates	

Enzymatic systems	Fluoretic acid	
	Activated quinones	

Most of these crosslinkers react with the amine group of the chitosan structure, forming intermolecular bridges between the polymeric chains. The most widespread ***cross-linking agents*** are ***low molecular weight bifunctional compounds***, such as glutaraldehyde, diglycidyl ether, diisocyanate, and others [5–8]. The structural properties of the obtained hydrogels, related to the cross-linking agents and the targeted applications, were summarized by Berger [6]. Generally, it was found out that these hydrogels have many suitable properties for their application in the biomedical field, especially mechanical properties – which are superior to those of the physically crosslinked preparations. On the other hand, most of the above-mentioned bifunctional crosslinking agents (Table 1) have a certain degree of toxicity. To overcome the potential negative reactions produced by using this kind of crosslinking agents, a prior advanced purification of hydrogels is required, before their use *in vivo*. Even in this case, the toxicity of most of the bifunctional crosslinkers cannot be reduced, thus representing the main drawback of their use in obtaining hydrogels for biomedical applications.

The number of the non-toxic crosslinking agents, as proved up to now, is quite low. One of them is genipin, a natural compound extracted from gardenias [9]. This compound has been reported as capable of binding to the biological tissue [10] and to the biopolymers containing amino groups, such as chitosan (Scheme 1). Chitosan membranes obtained by chitosan crosslinking with genipin are characterized by a lower rate of degradation compared to their glutaraldehyde-based equivalent [11]. The use of genipin for the development of controlled drug delivery systems has led to a drug delivery capability superior to the previously existing ones [12, 13].

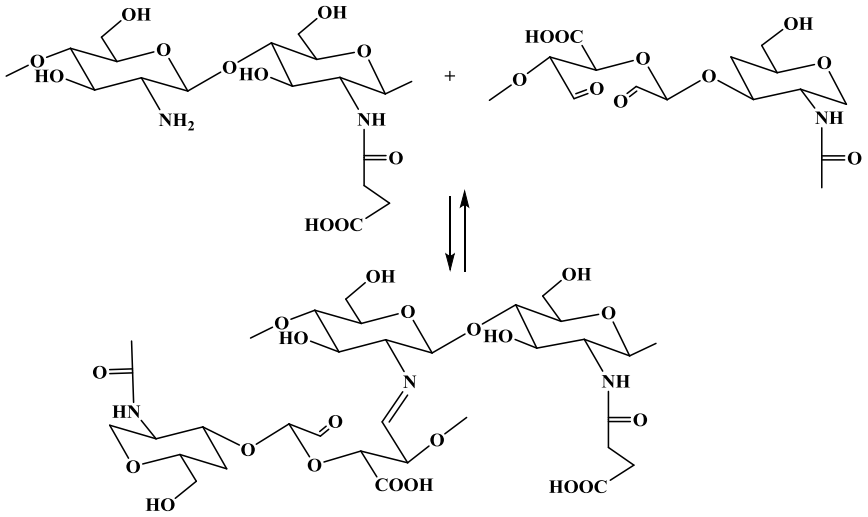
Although genipin shows good biocompatibility, a great disadvantage is the fact that it interacts often with the active principle, which is undesirable in the controlled release of drugs.



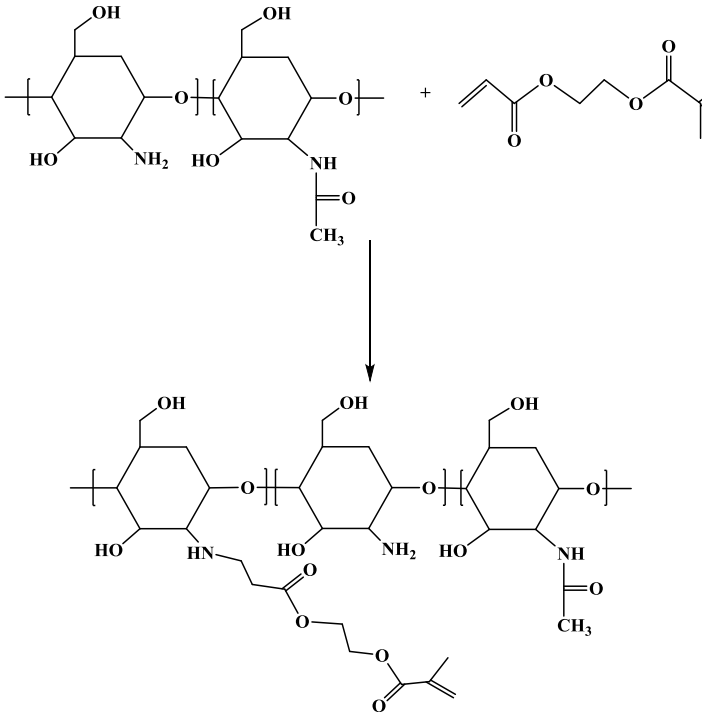
Scheme 1. Chitosan crosslinking with genipin [9]

To avoid the use of low molecular weight crosslinking agents, which are difficult to remove during the gelling process, a different approach is used, namely ***polymer-polymer chemical crosslinking***. In this way, the functionalized chitosan reacts *in situ* with a cross-linking agent, eventually a polymeric one. Following this technique, a biodegradable hydrogel based on chitosan and hyaluronic acid – able of *in situ* gelling – has been obtained. The reaction occurred between N-succinylated chitosan and the aldehyde groups of the hyaluronic acid, with the formation of a *Schiff base* (Scheme 2). The hydrogel was stable for four weeks and could be loaded with chondrocytes [14].

A similar approach was used to obtain hydrogels based on oxidized dextran and N-carboxyethyl-chitosan [15]. Hydrogels based on chitosan have been also obtained by *Michael addition reaction*, when the primary amine groups of chitosan acting as a nucleophile react with the vinyl group of another compound (Scheme 3). This reaction has the advantage that hydrogel formation is very fast and takes place through a bond different from the typically encountered imine one. By the reaction of acrylated chitosan with thiolated polyethylene oxide, a hydrogel with improved mucoadhesive properties was obtained, which has been proposed as a system appropriate for oral drug delivery [16].



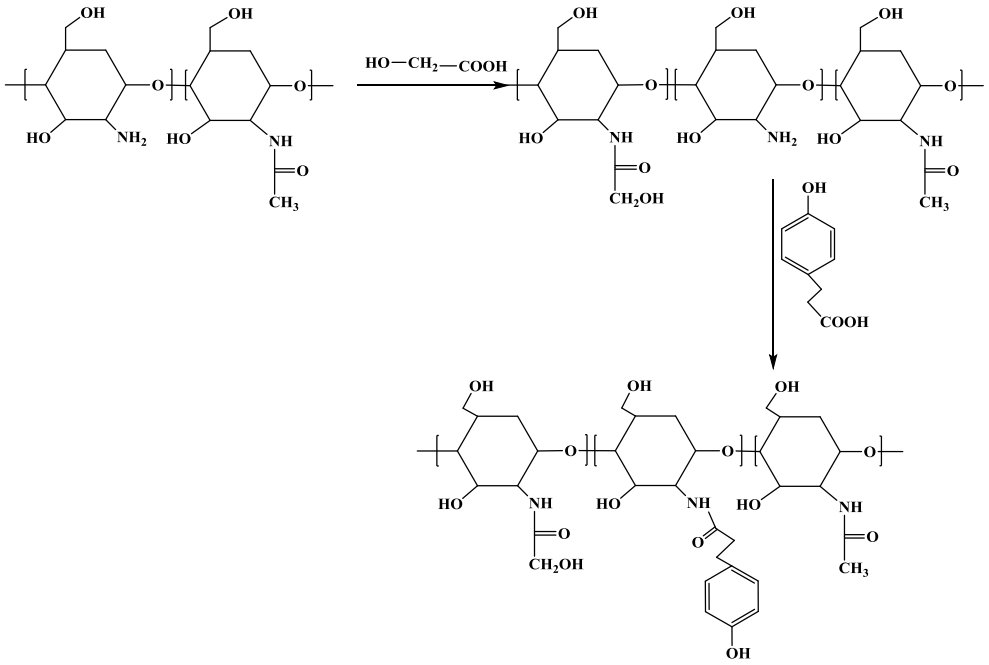
Scheme 2. N-succinylated chitosan crosslinking with the aldehyde groups of the hyaluronic acid [14]



Scheme 3. Synthesis of a photopolymerizable prepolymer from chitosan by Michael addition [16]

Even if preparation of hydrogels by using polymer-polymer systems has many advantages, this pathway also presents some drawbacks, especially the fact that it requires many steps, supplementary purification, as well as the risk of losing chitosan biocompatibility by its modification with different functional groups [16–18].

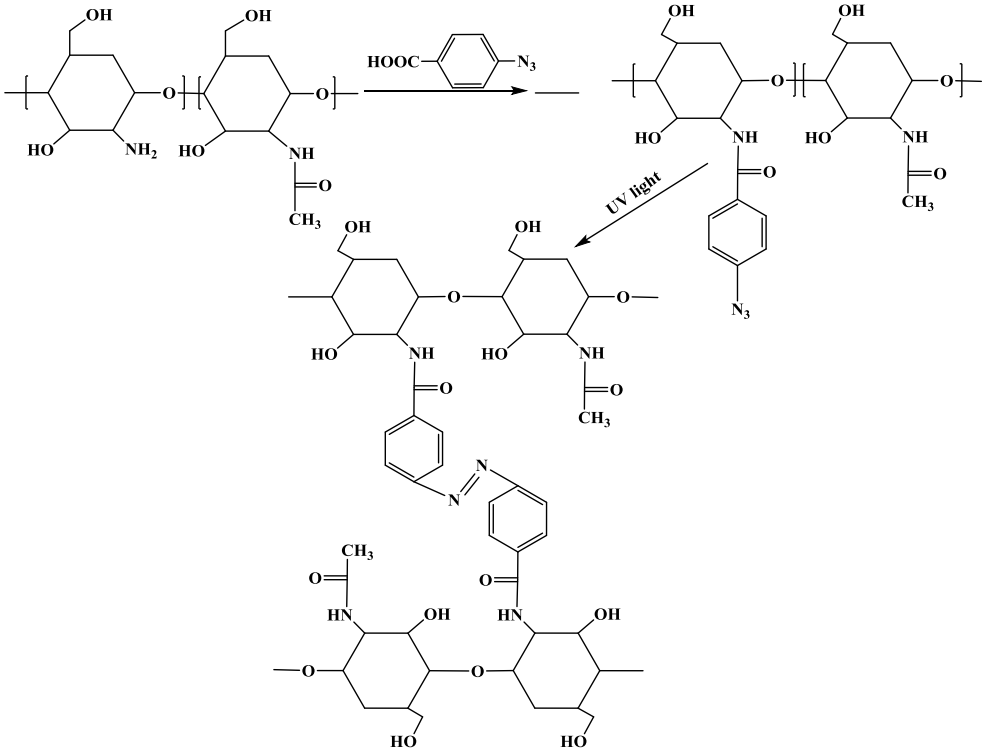
A quite new method aiming at obtaining hydrogels *in situ* is based on ***cross-linking by the use of enzyme-catalyzed reactions***. Thus, an injectable hydrogel was obtained through enzymatic crosslinking with peroxidase and hydrogen peroxide of a water soluble chitosan derivative (more precisely, chitosan grafted with glycolic and floretic acids) (Scheme 4). The gelling time of such a system varied from 4 min to 10 s, with increasing the polymer concentration from 1 to 3% [19]. Another hydrogel obtained *in situ* by enzymatic crosslinking was based on the use of tyrosinase enzyme as a catalyst for the reaction between chitosan and gelatin. Tyrosinase oxidized the tyrosyl residues of gelatin forming residues of quinone, which react with the amino groups of chitosan and lead to gelling after 30 min [20, 21].



Scheme 4. Chitosan grafting with floretic and glycolic acids [19]

Another method proposed for obtaining chitosan-based hydrogels is ***photo-chemically initiated crosslinking***. The method is based on the use of a polymer which contains photosensitive functional groups. Once irradiated with UV light, these groups act as active crosslinking centers. The technique offers considerable advantages, including the rate of response, safety, low cost, simplicity of the used

systems in comparison with conventional chemical methods, which require the presence of various reactive species, catalysts or initiators [22]. Following this procedure, a photocrosslinkable hydrogel has been obtained by functionalizing chitosan with azide groups. Thus, irradiation with UV light allows the conversion of the azide group into nitrene group which links the unreacted amino groups of chitosan's backbone, producing gelation (Scheme 5). Due to the presence of the azo group in the chemical structure, the resulted hydrogel is photo-responsive, showing its capacity to release various growth factors in a controlled manner and of being successfully used as a material that induces neovascularization *in vivo* [23–26].



Scheme 5. Chitosan grafting using UV light [23]

PHYSICAL CROSSLINKING

Chitosan-based hydrogels can be also obtained through physical crosslinking. To meet the requirements imposed to a hydrogel for its utilization in the bio-medical field, chitosan-based hydrogels should form a semi-permanent network able to swell but not to dissolve, which means that it must allow the access of water

molecules within, while also conferring resistance. Such semi-permanent networks require strong enough intermolecular interactions.

Typically, the systems obtained by noncovalent strategies are based on either the development of *electrostatic forces*, *hydrophobic-hydrophobic interactions* or *hydrogen bonds* between the polymer chains [27, 28]. Physically crosslinked hydrogels based on chitosan have inferior mechanical properties compared to the chemically crosslinked ones. For this reason, and also because of their pH sensitivity, these systems find applicability mainly in controlled drug delivery. Due to their porous structure and also to their structural peculiarities, the release of an active principle from such a system occurs mainly through a diffusion process [29].

Ionically crosslinked chitosan based hydrogels

Given the polycationic nature of chitosan as a consequence of the presence of ionizable amine groups in its structure, anions or negatively charged molecules frequently can act as ionic crosslinkers. In such a way, hydrogels have been obtained by ionic complexation of chitosan with anionic small molecules, such as sulphates, citrates, phosphates [30, 31] or with metallic anions, such as Pt (II), Pd (II) and Mo (VI) [32, 33]. The properties of the ionically crosslinked hydrogels depend on the density and size of the anionically charged molecules, deacetylation degree of chitosan and on its concentration. Both the small molecules and the anions bind chitosan by means of protonated amino groups, while the metallic ions form covalent coordinative bonds with the polymer; these bonds are stronger than those formed between the anionic molecules and polycations. The chitosan solution has an acid dissociation constant of about 6.3, which limits its ability to form ionic complexes under physiological conditions (pH = 7).

An anionic molecule meant to be used in the formation of ionic complexes with chitosan must have a high charge density (to form strong ionic interactions) and low molecular weight (in order to easily diffuse through the polymeric matrix, resulting in the formation of electrostatic bonds).

Ionic complexation is also generated by other secondary interchains interactions, such as (i) the hydrogen bonds formed between the hydroxyl groups of chitosan, and (ii) the interactions between the deacetylated chitosan chains. These interactions improve the physical properties of hydrogels and can be adjusted for obtaining materials with suitable properties for application in various fields, from agriculture to biomedical ones [34].

Polyelectrolyte complexes

Even if these systems may be formed through electrostatic interactions, as well, they require the participation of a second electrically charged polymer, *e.g.* another polysaccharide, a protein or a synthetic polymer. The associations established between chitosan and other polyelectrolytes are stronger than other secondary

(hydrogen bonds or Van der Waals) interactions. The advantages of these types of associations are significant. First, due to the fact that their obtaining does not involve the use of organic precursors, catalysts or other reagents, these hydrogels are less toxic. On the other hand, as these systems contain only chitosan and another polyelectrolyte, their combination is simple and the process of their formation is a reversible one, providing the opportunity of controlling their morphology and properties.

Polyelectrolyte complexes based on chitosan have been obtained using anionic macromolecules soluble in water, such as DNA, anionic polysaccharide (alginate), glycosaminoglycans (chondroitin sulfate, hyaluronic acid, heparin), carboxymethyl cellulose, pectin, xanthan, dextran sulphate, proteins (gelatin, albumin, fibroin, keratin, collagen), and synthetic anionic polymers (polyacrylic acid). The stability of these polyelectrolyte complexes depends on many factors, including the charge density of the polymer, type of solvent, ionic strength, pH and temperature [35, 36]. The choice of an anionic macromolecule for the formation of a polyelectrolyte complex depends mainly on its charge at physiological pH, because the environment in which the hydrogel occurs modulates the ionic interactions and, obviously, the properties of the hydrogel.

Physical mixtures based on secondary interactions

Besides the already mentioned physical interactions, chitosan-based hydrogels can be formed using mixtures with other nonionic, water-soluble polymers, such as polyvinyl alcohol (PVA). These polymer mixtures form crystallite-type junctions and interpolymer complexations after freeze-drying or after several freeze-thaw cycles [37, 38]. The chain-chain interactions act as crosslinking points of the hydrogel. In the particular case of chitosan and PVA mixtures, increase of the used amount of chitosan decreases the ability of PVA to form crystallites, resulting in the formation of hydrogels with a less ordered morphology.

Recently, a hydrogel based on chitosan and polyethyleneimine (a polycation best known for its ability to act as a transfection agent) has been reported. By blending chitosan with this polymer, a hydrogel was obtained in less than five minutes. It was assumed that the formed hydrogel was the result of chitosan-chitosan interactions; when the polymer mixture is prepared at a pH of 7.5, chitosan is not soluble and can lead to the formation of crystallites between chains. This hydrogel was successfully used to support the growth of fetal skeletal human cells.

In an entirely unexpected manner, hydrogels have been obtained by chitosan self-crosslinking without using any crosslinker. The method is based mainly on the neutralization of the amine groups of chitosan with sodium hydroxide, which prevents the ionic repulsions between the polymeric chains, and allows the formation of hydrogen bonds, hydrophobic-hydrophobic interactions and also of crystallites [39]. An interesting phenomenon of diminishing the size of the hydrogel with neutralization has been observed. A hydrogel with a multilayered, “onion” type

structure, obtained by a gelling technique in several steps, has been reported. This type of hydrogel can be used to encapsulate drugs with the ultimate goal of co-transporting more therapeutic agents or of obtaining pulsatile drug delivery systems [40, 41].

Thermoreversible hydrogels and hydrophobic-hydrophobic associations

There exist systems, such as the thermoreversible gels, which suffer a sol-gel transition by changing the temperature of the environment in which they occur. Hydrophobic-hydrophobic interactions, or secondary interactions, are established between the chains of the polymers able to form such hydrogels, leading to the possible obtaining of a semi-rigid gel from a liquid. Thus, when the system temperature exceeds a certain value, known as the lower critical solution temperature (LCST), the material undergoes a hydrophilic-hydrophobic transition, due to the formation of aggregates/crystals.

A polymeric solution with low viscosity at room temperature, yet able to form a gel upon LCST, brings essential advantages for the use of such systems in the biomedical field. Therefore, the material can be injected as a liquid into the body, and, when the body temperature exceeds the value of LCST, it gels, and it can release the previously encapsulated active principle [42, 43]. The use of this method simplifies very much things, eliminating invasive surgery and offering the possibility to release the drug at the desired site without secondary effects, such as local heating, use of organic solvents, formation of toxic byproducts, and so on. In clinical applications, injectable hydrogels are especially recommended to treat irregularly shaped areas, eliminating the need to use conventional matrices with special design.

Based on this principle, thermoreversible hydrogels have been obtained by the aggregation of chitosan copolymers or by neutralization with polyol salts [44, 45]. Such a system has been obtained by mixing disodium glycerol phosphate and chitosan. The phosphate groups serve to neutralize the positive charges on chitosan, allowing the increase of chitosan hydrophobicity at elevated temperature. The mixture is a clear liquid at room temperature, which gels at 37°C [46]. This system has been successfully used in the transport of bone bioactive protein and as a support for chondrocytes cultures, which were implanted *in vivo*, leading to the formation of new cartilages in about three weeks.

Another thermoreversible gel was obtained through gelation of a chitosan- and polyethylene glycol -based (PEG) copolymer, based on the interactions between the chitosan chains. The copolymer was synthesized by grafting PEG units with a hydroxyl end group on the chitosan chains and formation of an imine bond, followed by its reduction with sodium cyanoborohydride [47]. By optimizing the content of PEG (45–55% by weight) and of its molecular weight (which actually influence the hydrophobic/hydrophilic ratio in the final copolymer), the resulting

molecule shows a sol-gel transition when shifted from room temperature to the human body temperature. The solution could be injected through a needle at a temperature lower than the LCST, and subsequently turned into a transparent gel, once the temperature exceeded the LCST. This phenomenon has been attributed to the fact that the hydrogen bonds established between PEG and water are governing at low temperatures, while at higher temperatures hydrophobic-hydrophobic interactions dominate the polymer chains [48, 49].

Based on the same principle, hydrogels of chitosan and poly (N-isopropylacrylamide) (PNIPAM) or chitosan and poloxamer have been obtained [50]. PNIPAM is a thermosensitive polymer known for its LCST, which is very close to the human body temperature, of 32°C. To change this value, more precisely to increase it around 36.5–37°C, PNIPAM was modified with many polymers, among which, chitosan. Such a system could be used for the transport and release of drugs, proteins or peptides. A copolymer obtained using chitosan and poloxamer has a sol-gel transition temperature of –25° C, and it has been successfully used to support cartilage regeneration [51].

A comparison between the chemically crosslinked hydrogels and the physically crosslinked ones reveals that the crosslinking type governs hydrogels' stability and their properties. Therefore, the covalently crosslinked hydrogels, regardless of the type of the new formed bonds, are stable, resistant to environmental factors, and have superior mechanical properties, compared to those of the physically crosslinked ones. A disadvantage is that these systems require additional purification processes to remove the unreacted cross-linking agents, which most often are toxic.

On the contrary, the physically crosslinked hydrogels show higher biocompatibility and are better tolerated by the body. Moreover, the interactions generating these systems are reversible. Thus, the physically crosslinked systems do not show high stability and may be sensitive to environmental changes: pH, temperature and ionic strength. This last characteristic can be easily speculated and used to obtain systems capable to respond in a controlled and desired manner to the environmental conditions [52].

DYNAMIC CHEMISTRY IN THE OBTAINING OF SUPRAMOLECULAR CHITOSAN-BASED STRUCTURES

Supramolecular chemistry represents a new field with different transdisciplinary branches, developed on many research directions, one of them concerning the building of complex dynamic systems and of advanced materials by designing components assembled through intermolecular forces. The spontaneous, yet still controlled obtaining of supramolecular architectures of nanometric size through selforganization represents a pathway of programmed engineering and processing of functionalized

nanostructures, offering a simple alternative to nanofabrication and nanomanipulation. The supramolecular chemistry is basically a dynamic chemistry, if considering the labile nature of the noncovalent or covalent interactions which assure the integrity of the supramolecular architecture, a dynamic material thus resulting.

The concept of dynamic material, introduced by Jean Marie Lehn, is based mainly on the idea that molecular entities bound together through reversible chemical linkages allow, under the influence of external stimuli, a continuous modification of their constitution through changes and reorganizations of the molecular blocks. External stimuli can be light [53], chemical effectors, such as ionic metals (metalo-selection) [54] or factors which influence the state of equilibrium – *e.g.* temperature or pH [55]. These materials, able to respond at external stimuli by constitutional changes, gain adaptative characteristics, thus opening a new perspective to the adaptative, evolutive chemistry, which offers many opportunities in materials technology. Due to their special, both theoretical and applicative importance, the principles of dynamicity were also extended on polymers, leading to the generation of dynamic constitutional polymers known as "Dynamers". By virtue of the reversible chemical connections between the monomeric units, dynamers may show dynamic functions as a response to physical stimuli or chemical effectors, by creating adaptative networks. They represent a distinct class of adaptative polymers: the "Adaptamers".

In relation with dynamic materials, the chemistry of imine linkage formation from simple precursors: aldehydes and amines, proved to be one of the most important instruments of dynamic covalent chemistry involved in assembling dynamic materials and molecular discrete objects with exotic topologies, such as: molecular containers [56], porous, crystalline or amorphous molecules [57] or extended molecular systems: epitaxial crystals [58], microtubes [59], self-healing films [60]. The dynamicity of imine formation is affected by both intrinsic structural characteristics (steric and electronic factors), and external factors, such as solvent, concentration, temperature and pH.

Taking advantage of the thermodynamic characteristics of the C=N linkages, formation of a imine bond is associated with the ability of "errors checking" and "model reading" in the process of selfassembling, thus orienting the synthesis towards complex topologies. The functional associated aspects, alongwith their nanometric characteristics place these nanostructured materials at the border of materials science, and bring new opportunities for the exploitation of new functional materials.

CHITOSAN – A GENEROUS PLATFORM FOR SUPRAMOLECULAR CHEMISTRY

As a polymer containing amine groups, chitosan can be used in this context for the obtaining of biodynamic materials, more precisely it can be transformed into imino-chitosan derivatives, generically called biodynamers.

The advantages of chitosan as a biopolymer refer to its numerous biological properties, such as biocompatibility, lack of toxicity against the human body and the environment, and also to its therapeutical properties: hemostatic, antimicrobial, fungistatic, antitumoral activity, ability to enhance wound healing and to accelerate bond formation, the capacity to bind the fat acids and therefore to reduce the cholesterol level, etc. [61, 62]. Due to all these, chitosan is used as an alimentary supplement, its properties being insufficient for its utilization as a drug. To increase chitosan's properties, the literature mentions many chemical modifications with different benefic compounds.

From a chemical point of view, chitosan is a polyamine – therefore, the main modification is the condensation reaction of the amine groups with different aldehydes, which constitutes an important workbench for the development of biodynamic materials through the reversible chemistry of the imine linkage. Moreover, the ability of chitosan to be processed as films, nanoparticles, micro and nanofibers or hydrogels, increases even more its applicative value and therefore the interest in obtaining biodynamers.

Therefore, the literature of the field reports hydrogels based on iminochitosan derivatives with dynamic properties conferred by the presence of the reversible imine linkage. In this manner, hydrogels with strong antifungal or antitumoral activity were obtained, which, in the presence of water and in a biomometic medium, were able to relese the aldehyde with the corresponding biological activity.

CONCLUSIONS

Chitosan, a biopolymer derived from chitin, presents interesting biological properties, which make it an important candidate for biomedical applications, and not only. On the other hand, chitosan has an important drawback: its weak mechanical properties, which can be highly improved by its crosslinking, thus converting the linear polymer into a tridimensional network - hydrogels. Obtained by chemical or physical crosslinking, the hydrogels based on chitosan represent an important class of materials with different properties, which render them suitable for a large range of applications. Moreover, the presence of amine groups on the chitosan backbone makes this polymer an important tool for designing dynamic materials with unique properties.

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