ROMANIAN ACADEMY

School of Advanced Studies of the Romanian Academy

"Ilie Murgulescu" Institute of Physical Chemistry

DOCTORAL THESIS SUMMARY

SYSTEMS CONTAINING POLYSACCHARIDES STUDIED BY ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY

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KEYWORDS

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Polysaccharides

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Gels

Nanoparticles

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Note: Page numbering in the table of contents corresponds to the doctoral thesis.

Introduction

The doctoral thesis is structured in two parts, the first containing general literature data on the properties of polysaccharides and the physico-chemical methods of investigation, and the second comprising the original results. The polysaccharides described in the first part are those used during the doctoral research stage, namely alginic acid, hyaluronic acid, chitosan and cyclodextrins (as oligosaccharides). The general properties and areas of use of these polysaccharides were mentioned, emphasizing their ability to form hydrogels under certain conditions. The literature part further presents the physico-chemical methods used for characterizing the properties of hydrogels (rheology, electron microscopy, IR spectroscopy, fluorescence spectroscopy). The first part of the doctoral thesis ends with a chapter dedicated to electron paramagnetic resonance (EPR) spectroscopy, the main research method used in the study of polysaccharide hydrogels during the doctoral studies. This chapter focuses on the spectral characteristics of nitroxides used as spin probes or spin labels, which reflect their dynamics and interactions with the environment. Some applications of EPR spectroscopy in the study of gels/hydrogels are also presented.

In the second part of the thesis, representing the original contribution, we discuss the application of EPR spectroscopy as main spectroscopic method for investigating gel systems, complementary to classical methods of physico-chemical characterization (rheology, IR spectroscopy, SEM microscopy). In most cases, spin-labeling of polysaccharide chains was performed using the 4-amino-TEMPO or 4-carboxy-TEMPO free radicals in order to highlight gel formation and interactions in the gel network.

Research directions:

- highlighting inhomogeneities in gels at nanoscopic level using molecular probes of small dimensions;
- determination of gel encapsulation properties using spin-labeled derivatives with molecular masses covering two orders of magnitude (10^{-2}–10^{-4});
- evidencing the role of host–guest interactions by analyzing the features of EPR spectra and correlating the results with those provided by other physico-chemical methods; the results are of interest in the design of complex biomolecular systems containing alginate;
- formation of gold nanoparticles (AuNPs) by reduction of chloroauric acid ($HAuCl_4$) in polysaccharide solutions and gels;

- EPR study on the formation of interpenetrating (IPN) and semi-IPN networks of alginate and chitosan by the use of spin-labeled polysaccharides;
- the use of EPR spectroscopy in studying the gel accessibility of spin probes with variable molecular weights represents a novel approach;
- physico-chemical investigation on the riboflavin role in corneal collagen crosslinking (CLX).

Original contribution

In the third chapter of the thesis we studied the influence of host–guest supramolecular interactions occurring in alginate gels functionalized with host (cyclodextrin) and guest (adamantyl) units on the morphology, rheological behavior and encapsulation properties of these hydrogels.

This approach starts from several previous studies presented in the literature. Some of them, related to the synthesis of polymeric gels resulting from the coupling of polyethylene glycol chains with cyclodextrin units, were presented in detail in the introductory part of the thesis [2,3]. Another study refers to the spin-labeling of sodium alginate with TEMPO paramagnetic groups in order to highlight gel formation and ion exchange interactions through changes in the dynamic of the paramagnetic group [6].

The carboxyl groups present in the alginate structure allow the introduction of new functional groups, offering the possibility to modulate the properties of the gels and thus to use the new polymer in specific applications [4,5]. For example, spin-labeled alginate could be used to highlight the influence of complexing ions on chain dynamics and to highlight ion exchange processes between alginate and EDTA [6].

The influence of host–guest interactions on gel properties can be traced through TEMPO-type paramagnetic groups, using either the spin probe method or the spin-labeling method. The alginate (Alg) spin-labeling procedure, described in the literature [6], has been adapted (Figure 1) to introduce, in addition to the paramagnetic group TEMPO (T), also host-type structural units (β-CD functionalized with chains alkyl of different lengths) or guest units (adamantane, Ad).

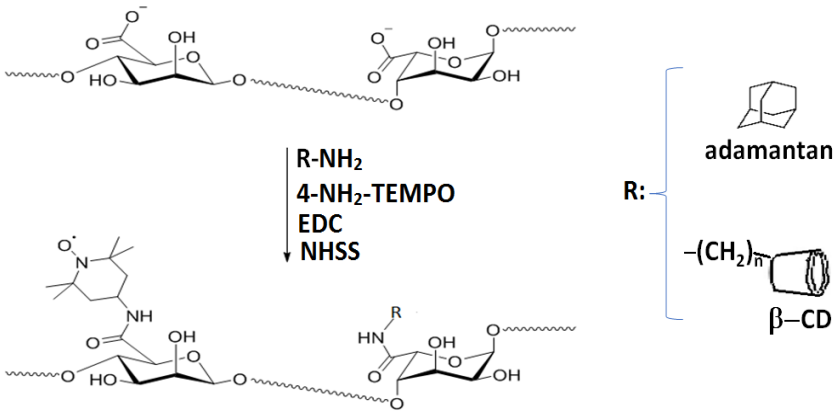

Figure 1. Functionalization of alginate with paramagnetic moieties and host (cyclodextrin) or guest (adamantane) units [6].

Functionalized alginate derivatives were denoted Alg-Ad, Alg-1,2-*β*-CD, Alg-1,3-*β*-CD and Alg-1,6-*β*-CD, and spin-labeled derivatives were denoted Alg -T, Alg-Ad-T, Alg-1,2-*β*-CD-T, Alg-1,3-*β*-CD-T and Alg-1,6-*β*-CD-T. The changes in EPR spectral parameters, as indirect proofs of non-covalent interactions, were correlated with the results provided by other physico-chemical methods.

The SEM images shown in Figure 2 evidence morphological changes induced by functionalization of alginate and host–guest interactions occurring in gel systems formed by alginates in the presence of calcium ions.

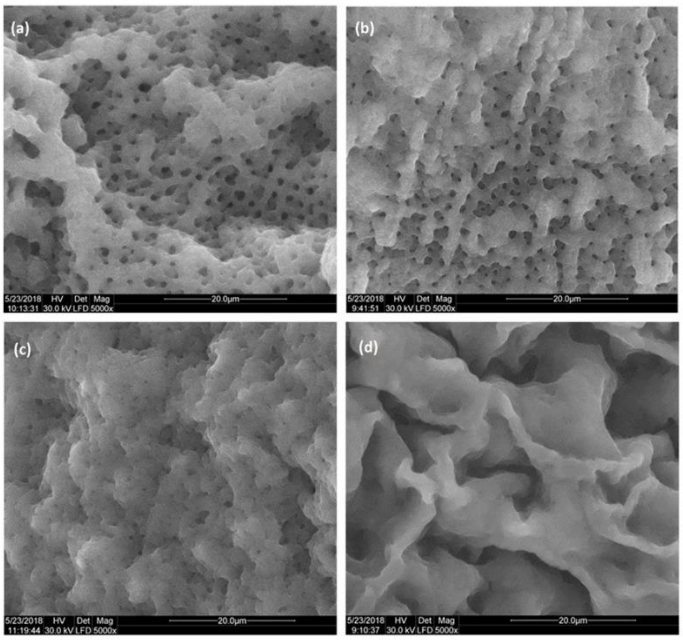

Figure 2. Morphology images of the surface of xerogels: Alg (**a**), Alg-Ad (**b**), Alg-1,3-*β*-CD (**c**) and Alg-1,3-*β*-CD / Alg-Ad (**d**).

Figure 2 shows the SEM images of the surfaces of the alginate xerogels investigated. In the case of non-functionalized alginate (Figure 2a) and adamantane-functionalized alginate (Figure 2b), the pores of the network are dense and characterized by an uniform distribution. Cyclodextrin functionalization results in larger sizes of both gel fibrils and network pores (Figure 2c). However, the SEM images of alginate, adamantane-functionalized alginate and cyclodextrin-functionalized alginate are very similar.

Interestingly, the SEM image of the alginate gel obtained by adding Ca^{2+} ions to a mixture of adamantane-functionalized alginate and cyclodextrin-functionalized alginate clearly shows a different morphology, characterized by considerably larger pores, deformed channels and a „wrinkled" network (Figure 2d). These differences in surface morphology indicate that host–guest interactions between alginate chains affect the overall properties of the gel. To confirm this, rheology and IR spectroscopy determinations were performed, which also provide information on the overall properties of the gels.

The rheological properties of alginate gels can be described in terms of two parameters, namely the storage modulus, G', and the loss modulus, G". Factors such as the characteristics of alginate (source, proportion and sequence of M and G blocks, viscosity), type, concentration of complexing cation and the presence in the system of other species such as monovalent cations or co-solvents, change the rheological properties of alginate gels. In our study, rheological measurements were performed immediately after gel preparation.

Figure 3 shows the evolution of the rheological parameters of alginate gel, functionalized alginate gel and gels prepared from their mixing, determined by the increase of shear stress, at a constant frequency of 1 Hz. In the case of all systems, it is observed that the storage modulus G' is greater than the loss modulus G" and that, from a certain value of the shear stress (σ*), characteristic to each system, the two rheological modules become equal. This phenomenon marks the appearance of plastic deformation. As can be seen from Figure 3a, the value of σ* increases significantly, by functionalization with either adamantyl fragments or with cyclodextrin. It should also be noted that the σ* value increases rapidly with the length of the alkyl chain. The highest value in this series is recorded for gels resulting from the complexation of Alg-1,6-β-CD alginate with calcium ions.

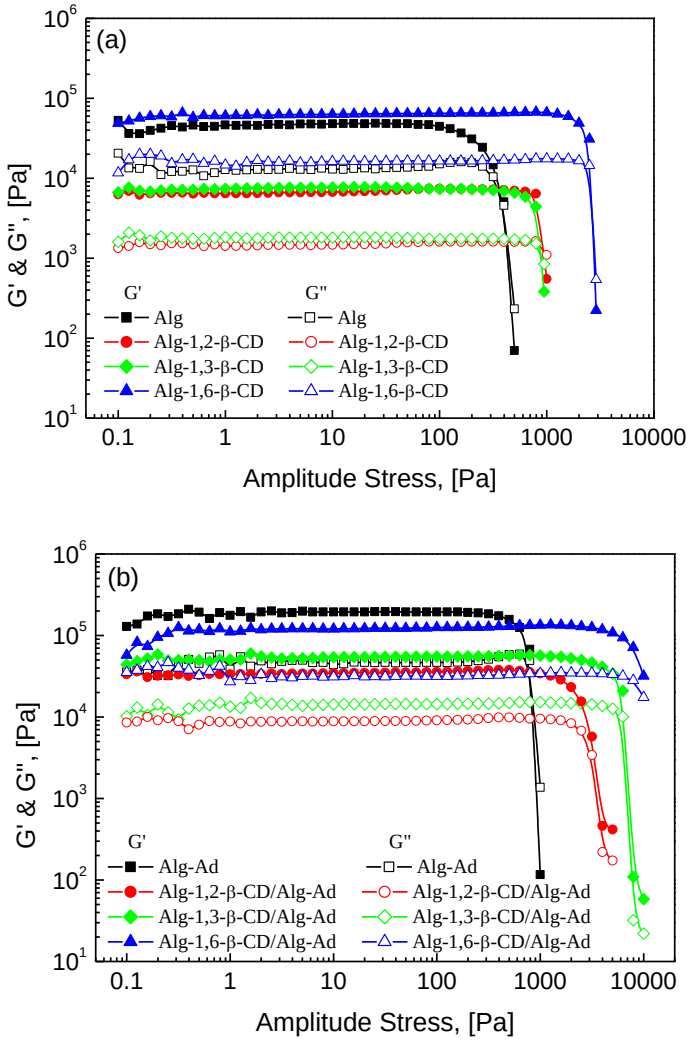

Figure 3. Dependence of modules G ' and G' on shear stress (σ*) for (**a**) alginate gel and cyclodextrin-functionalized alginate gels and (**b**) adamantane-functionalized alginate gel and alginate gels with host–guest interactions.

These data show that the functionalization of the alginate chain with β-CD units, and the increase of the length of the aminoalkyl chain that acts as a linker, determine an increase in the elasticity of the hydrogel network. Thus, the deformation resistance of the gel can be modulated by functionalization.

Host–guest interactions determine changes in the EPR spectral parameters of the paramagnetic groups attached to the alginate chains, namely the hyperfine splitting constant (a_N) and the rotational correlation time (τ). Thus, we can obtain additional information, at the nanoscopic level, on these interactions.

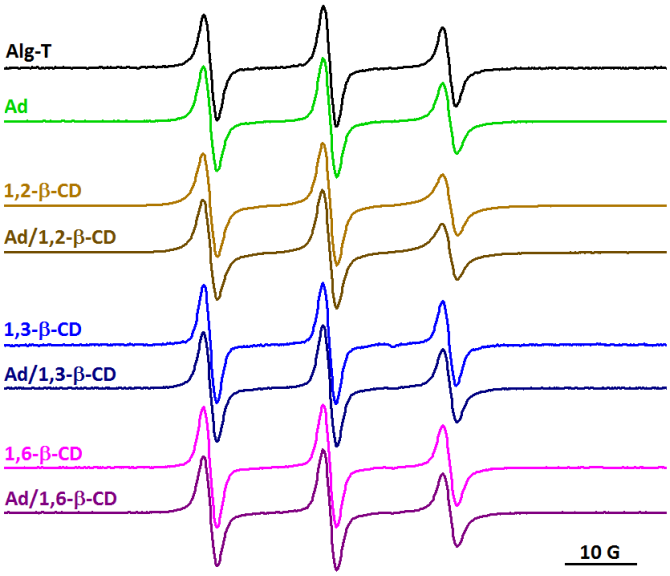

Figure 4. The EPR spectra of spin-labeled alginate (Alg-T) functionalized with host (1,n-*β*-CD) or guest (Ad) units and mixtures thereof (Ad/1, n-*β*-CD), recorded in solution at room temperature.

Figure 4 shows the EPR spectra of alginate labeled with the paramagnetic group TEMPO (T) and functionalized with adamantyl groups (Ad) or with cyclodextrin units (1, n-*β*-CD) and of the corresponding mixtures (Ad/1, n-*β*-CD), recorded in solution at room temperature.

The covalent attachment of the paramagnetic group to the alginate chain is accompanied by a slower dynamic, even if the EPR spectrum indicates the maintenance of a quasi-isotropic dynamic regime (the spectral lines are wider, the high field one having a lower height) [1]. We notice that, in solution, the changes of the EPR parameters are minimal in the case of mixtures of alginates functionalized with adamantyl groups and cyclodextrin units. This observation, correlated with small changes in EPR parameters, leads us to the conclusion that host–guest interactions between TEMPO groups and cyclodextrin cavities attached to alginate chains are less favored than the interaction of the free TEMPO radical with cyclodextrin in solution (Figure 5).

To confirm this hypothesis, we compared the EPR spectra of the free 4-amino-TEMPO spin probe in Alg-1,2-*β*-CD (solution or gel) with the spectra of the corresponding spin-labeled alginate (Alg-1,2-*β*- CD-T).

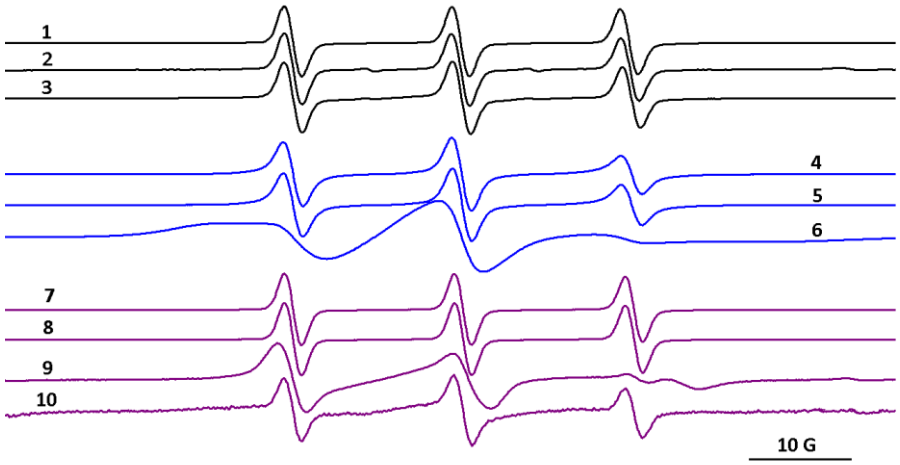

Figure 5. The EPR spectra of 1) 4-amino-TEMPO in water, 2) 4-amino-TEMPO in Alg-1,2-*β*-CD solution, 3) 4-amino-TEMPO in Alg-1,2 gel-*β*-CD, 4) Alg-1,2-*β*-CD-T solution, 5) Alg-1,2-*β*-CD-T solution, in the presence of 1-adamantanol, 6) Alg-1,2-*β*-CD-T gel, 7) 4-carboxy-TEMPO in Alg-1,2-*β*-CD solution, 8) 4-carboxy-TEMPO in Alg-1,2-*β*-CD solution, in presence of 1-adamantanol, 9) 4-carboxy-TEMPO in Alg-1,2-*β*-CD gel, 10) 4-carboxy-TEMPO in Alg-1,2-*β*-CD gel, in the presence of 1-adamantanol.

In the case of Alg-1,2-*β*-CD-T, host–guest interactions between TEMPO groups and cyclodextrin units covalently attached to the polysaccharide chain are, in theory, possible. The addition of 1-adamantanol to the Alg-1,2-*β*-CD-T solution did not lead to changes in the EPR spectrum, indicating that the attachment of TEMPO groups and cyclodextrin units to the alginate chain does not favor the host–guest interaction in solution. In the presence of Ca^{2+} ions, the restricted movement of the TEMPO group attached to the alginate chain is observed, without changes in the a_N value.

Another series of experiments aimed to analyze the behavior of the 4-carboxy-TEMPO spin probe in solution and in the Alg-1,2-*β*-CD gel. This spin probe can be involved in interactions with cyclodextrin as well as in the complexation of Ca^{2+} ions, thus competing with the carboxyl groups of the alginate chain. As with the 4-amino-TEMPO probe, the 4-carboxy-TEMPO probe does not form complexes with the cyclodextrin cavities attached to the alginate chain in solution. The EPR spectrum of 4-carboxy-TEMPO in the Alg-1,2-*β*-CD gel is characterized by the presence of two components with different dynamics. The slower component can be attributed to the probe complexation by the cyclodextrin units attached to the polysaccharide chain.

The analysis of the data presented in Figure 6 led to the conclusion that the structure of the spin probe significantly influences the interactions that are established with the gel matrix.

Surprisingly, the interaction between 4-carboxy-TEMPO and functionalized alginate chains with cyclodextrin units takes place in gel, but not in solution.

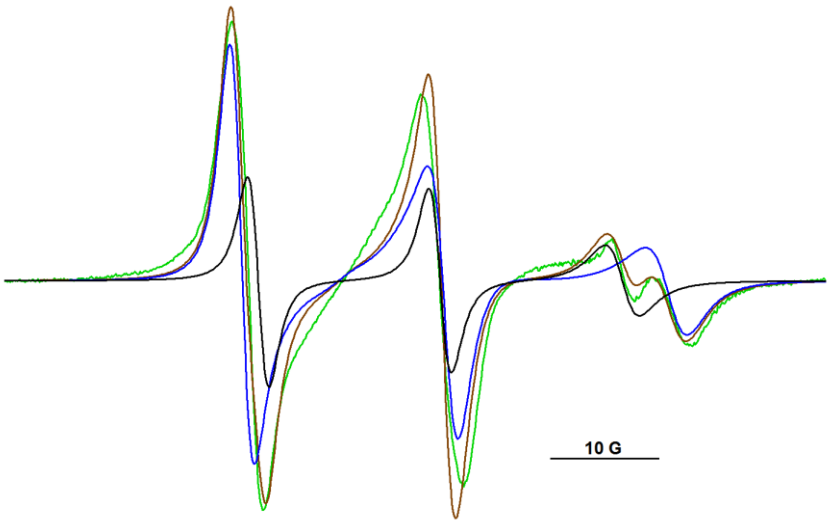

Figure 6. The EPR spectrum of 4-carboxy-TEMPO in the Alg-1,2-*β*-CD gel: green - experimental spectrum, brown - simulated spectrum, blue - slow dynamic component, black - fast dynamic component.

The EPR spectra shown in Figure 7 and the data in Table 1 are recorded for gels obtained from mixtures of spin-labeled alginates functionalized with cyclodextrin and adamantane (Alg-1,n-*β*-CD-T/Alg-Ad-T) has two components. The component characterized by a slow dynamic is attributed to the TEMPO groups located in the G region of the alginate chains, where Ca^{2+} ion complexation occurs predominantly, while the fast dynamic component is attributed to the TEMPO groups located in the M region of the alginate chain, as well as to the TEMPO groups located at a distance from the nodes of the gel network. The major change in the dynamics of the paramagnetic groups attached to the alginate chain is caused by the complexation of the carboxyl groups by Ca^{2+} ions and not by the host–guest interactions.

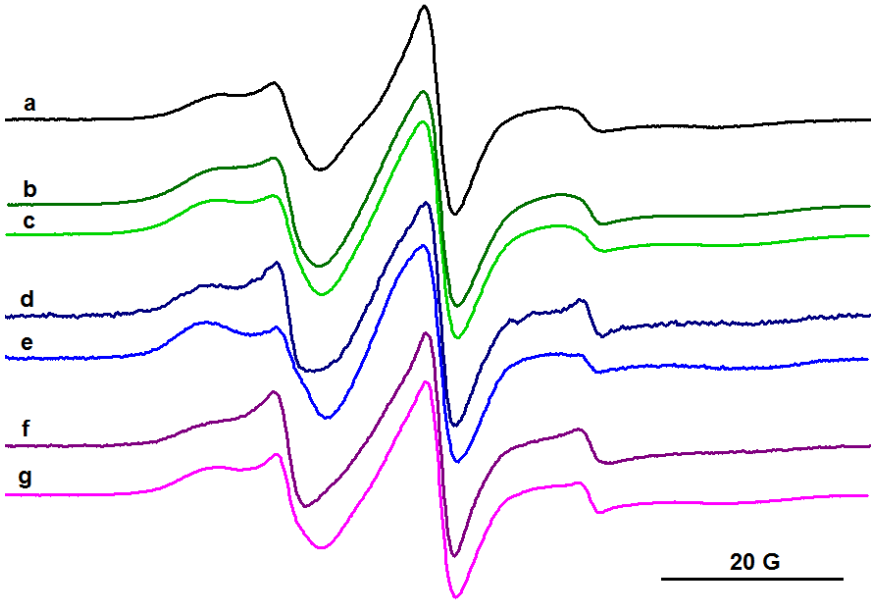

Figure 7. The EPR spectra of spin-labeled alginate gels: a) Alg-Ad-T, b) Alg-1,2-*β*-CD-T, c) Alg-1,2-*β*-CD-T / Alg-Ad-T, d) Alg-1,3-*β*-CD-T, e) Alg-1,3-*β*-CD-T / Alg-Ad-T, f) Alg-1,6-*β*-CD- T, g) Alg-1,6-*β*-CD-T / Alg-Ad-T, recorded at room temperature.

Table 1. The ratio of the contributions of the two components in the EPR spectrum and the distance between the extreme lines ($2A_{zz}$, in G) for spin-labeled alginate gels

System	**Individual alginate**		**Mixture of Alg-Ad-T and Alg-1,n-*β*-D-T**	
	Slow component / fast component	$2A_{zz}$	Slow component / fast component	$2A_{zz}$
Alg-Ad-T	-	57.4	-	-
Alg-1,2-*β*-CD-T	4.74	57.8	4.58	60.2
Alg-1,3-*β*-CD-T	3.66	62.1	4.64	63.5
Alg-1,6-*β*-CD-T	2.15	60.4	3.78	61.0

In conclusion, the complexation by Ca^{2+} ions of the guluronic blocks in the functionalized alginate is the main driving force in gel formation. Host–guest interactions, however, have a subtle contribution to the properties of gels, causing changes in rheological parameters and gel morphology. The EPR results demonstrate the local effect that host–guest interactions have on the dynamics of TEMPO groups covalently attached to alginate chains.

In chapter four are presented the results regarding the reduction of chloroauric acid in polysaccharide solutions (sodium alginate, dextran and chitosan), with formation of gold nanoparticles (AuNP) (Figure 8). Hydroxyl groups of polysaccharides have the ability to reduce Au^{3+} and to protect metallic nanoparticles in solution and in gel state [7-12, 13].

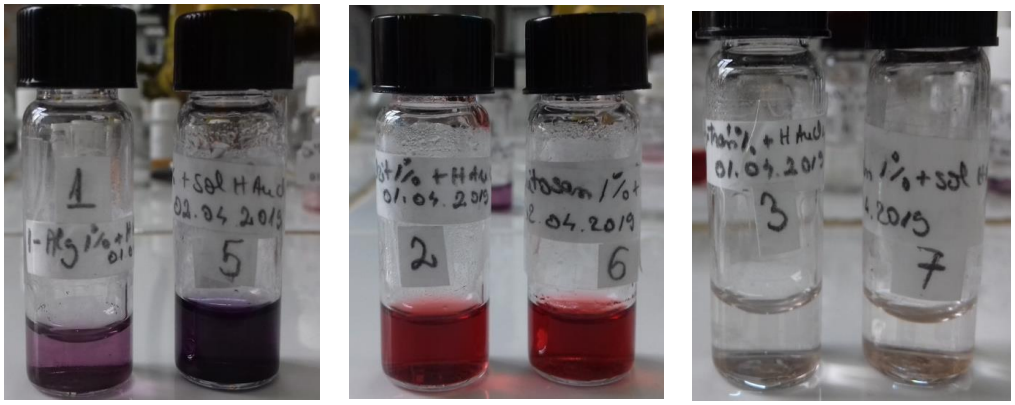

Figure 8. Solutions of 1) AuNP in alginate at room temperature, 2) AuNP in chitosan at room temperature, 3) AuNP in dextran at room temperature 5) AuNP in alginate at 90°C, 6) AuNP in chitosan at 90°C, 7) AuNP in dextran at 90°C.

Obtaining alginate gels decorated with gold nanoparticles was done either by adding Ca^{2+} ions to the alginate solution containing gold nanoparticles or by diffusion of Au^{3+} ions into the alginate gel, followed by *in situ* reduction. The first method of preparation results in a more uniform distribution of the gold nanoparticles in the gel. The processes of reduction and formation of gold nanoparticles take place much more slowly in gel than in solution (Figure 9). Alginate and chitosan have been found to promote the formation of nanoparticles smaller than 100 nm in size, while dextran causes the formation of larger nanoparticles.

The aim of this study was to obtain, from the analysis of EPR spectra, dynamic information that can be associated with the formation of metallic nanoparticles in solution and in alginate gel. For this, the EPR spectra of spin-labeled alginate were recorded in solution during the formation of gold nanoparticles. No change in the dynamics of the paramagnetic group attached to the alginate chain was observed (Figure 10).

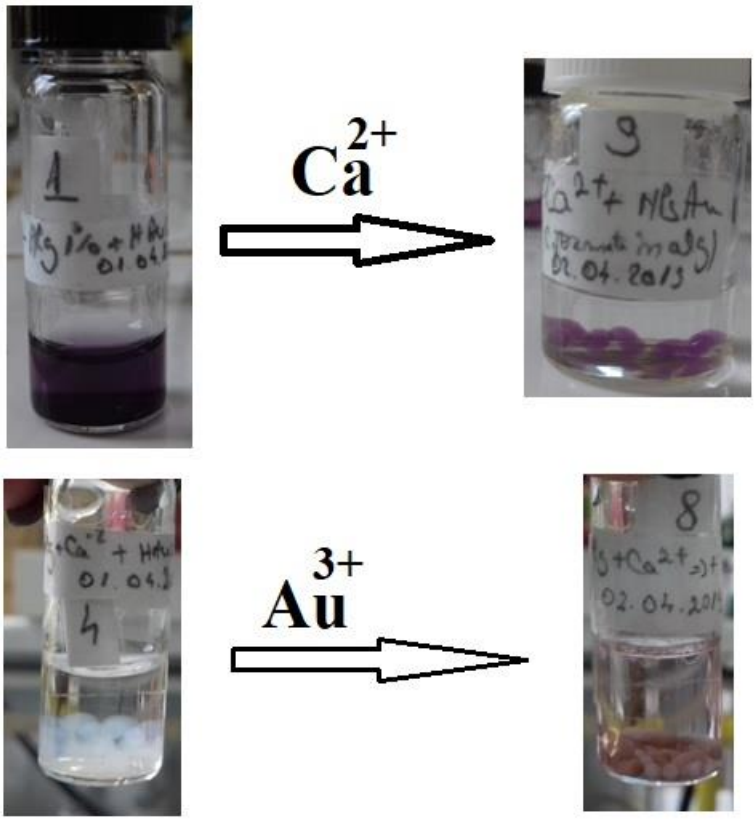

Figure 9. Formation of alginate gels by A) addition of Ca^{2+} ions to the AuNP solution in alginate and B) diffusion of Au^{3+} ions in alginate gel followed by reduction in gel.

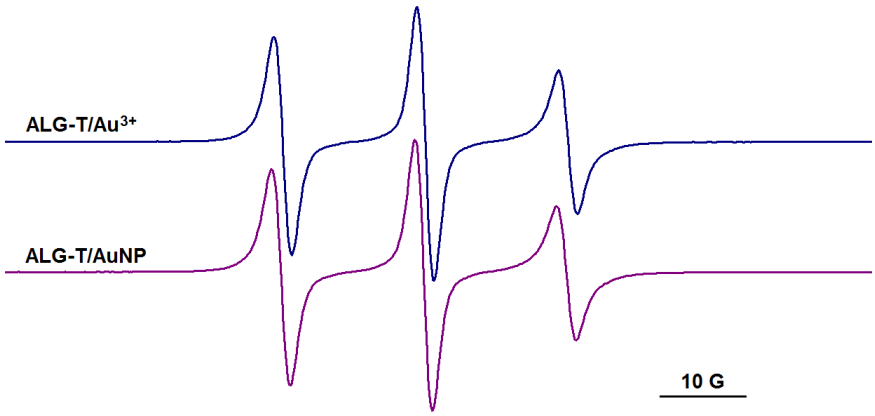

Figure 10. The EPR spectra of spin-labeled alginate in solution, in the presence of Au^{3+} ions, and in the presence of gold nanoparticles.

The EPR spectra of Alg-T and Alg-1,2-*β*-CD-T gels in the presence of gold nanoparticles show a strongly restricted dynamics, having two spectral components. The dynamics of the paramagnetic groups attached to the chains is not significantly influenced by the formation of nanoparticles (Figure 11).

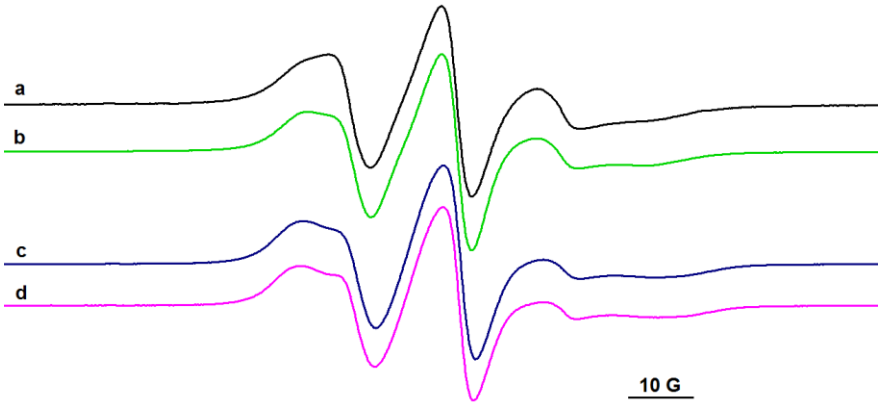

Figure 11. The EPR spectra of spin-labeled alginate gels in the absence or presence of AuNPs: a) Alg-T, b) Alg-T / AuNP, c) Alg-1,2-*β*-CD-T, d) Alg -1,2-*β*-CD-T / AuNP.

Chapter five presents the EPR study on the formation of interpenetrated polymer network (IPN) in polysaccharide systems. The IPN refers to polymeric materials that result from the interpenetration of two or more polymeric networks, without being covalently connected, but which can only be separated by breaking the chemical bonds [14,15,17]. When only the network of one of the polymers is crosslinked, a semi-IPN network results [16].

The polysaccharides pair was represented by chitosan and alginate, using as crosslinking agents glutaraldehyde (GA) and calcium chloride, respectively. In the case of the chitosan / alginate mixture, the addition of GA and Ca^{2+} was performed successively and alternately. The use of spin-labeled chitosan and spin-labeled alginate allowed to evidence the EPR spectral changes determined by the formation of semi-IPN and IPN networks. Figure 12 shows the images of the analyzed systems.

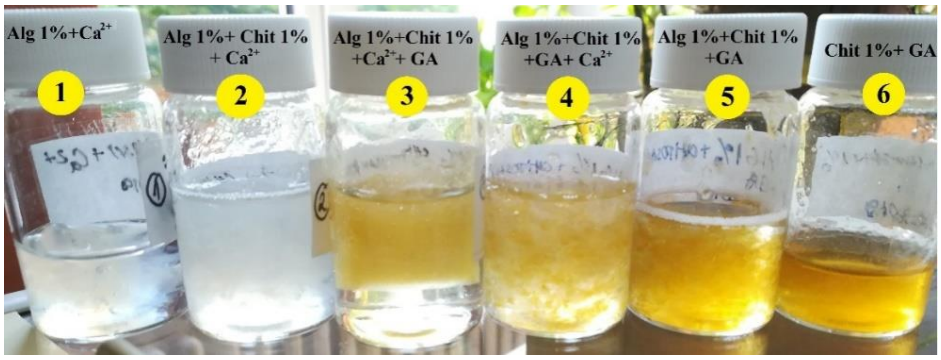

Figure 12. Gel samples 1) Alginate + Ca^{2+}, 2) Alginate + Chitosan + Ca^{2+}, 3) Alginate + Chitosan + Ca^{2+}+ glutaraldehyde, 4) Alginate + Chitosan + glutaraldehyde + Ca^{2+}, 5) Alginate + Chitosan + glutaraldehyde, 6) Chitosan + glutaraldehyde.

Figure 13 shows the EPR spectra of spin-labeled chitosan present in the prepared systems. For these systems, a similar fast dynamic of the paramagnetic groups covalently attached to the chitosan chain is observed (spectra a, b, c).

By adding Ca^{2+}, the alginate complex was made, the system turning into a gel (system 2, Figure 12). Compared to the alginate / chitosan solution, a slower dynamic of spin-labeled chitosan was observed in the corresponding gel. This indicates that the labeled chitosan chain is trapped in the alginate gel network. As can be seen, the gel has an EPR signal (pink spectrum), which indicates that alginate and chitosan form a semi-IPN network. The addition of glutaraldehyde to the chitosan and alginate solution leads to a more restricted movement.

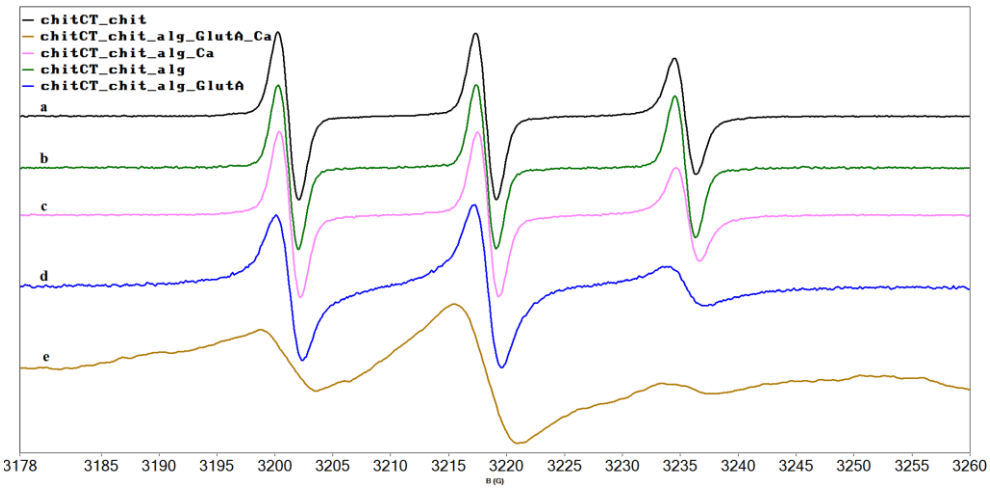

Figure 13. The EPR spectra of spin-labeled chitosan in alginate solution, in the presence or absence of crosslinking agents (Ca^{2+} ions and glutaraldehyde).

The addition of glutaraldehyde to the solution of spin-labeled chitosan and alginate leads to the formation of a gel. The spin-labeled chitosan has a slower dynamic (blue spectrum) revealed by the widening of the spectral lines corresponding to a semi-IPN network. The addition of Ca^{2+} to this solution leads to an even greater immobilization, this being the proof of the formation of an IPN network (brown spectrum). The EPR spectra of the solutions show no signal, which indicates that the spin probe is encapsulated in the gel networks.

Similar behaviors were observed in the systems containing spin-labeled alginate. The EPR spectra are shown in Figure 14. In alginate and chitosan solutions, spin-labeled alginate has a quasi-isotropic motion (spectra a and b in Figure 14). By adding Ca^{2+} to the alginate and chitosan solution, a semi-IPN gel is formed, and the EPR signal corresponds to that of a spin-labeled alginate gel (Figure 14, spectrum c). In the case of the alginate / chitosan / Ca^{2+} / glutaraldehyde

system, a more advanced immobilization of the paramagnetic group (spectrum d) corresponding to an IPN network was observed.

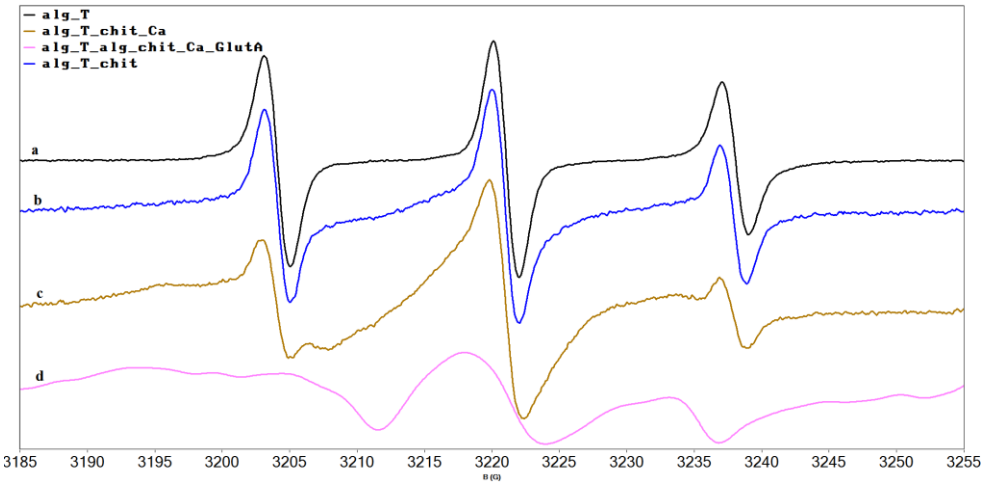

Figure 14. The EPR spectra of chitosan labeled in alginate solution, in the presence or absence of crosslinking agents (Ca^{2+} ions and glutaraldehyde).

The conclusion of this study is that EPR spectroscopy can evidence differences in IPN or semi-IPN networks determined by gel formation pathways. The study is to be completed by morphological investigations of the systems.

The sixth chapter presents the results on the use of EPR spectroscopy as a new method for estimating the encapsulation capacity of different molecular species in gel networks, for determining the mesh size of gel networks and as a method for highlighting gel inhomogeneities [16,20,21]. Thus, the diffusion of the probes indicated in Figure 15 and of spin-labeled bovine serum albumin (BSA) in the gels mentioned above was studied. The molecular weights of the probes used in the evaluation of the gel encapsulation capacity are presented in Table 2. Bovine serum albumin was labeled following the procedure described in the study of complexation / decomplexation of bovine serum albumin with sodium dodecyl sulfate [22].

The TEMPO, 4-amino-TEMPO, 4-hydroxy-TEMPO and 4-carboxy-TEMPO spin probes were purchased from Aldrich, while the spin-labeled Pluronics (P123, L62, F127) were obtained following the method indicated in the literature [19].

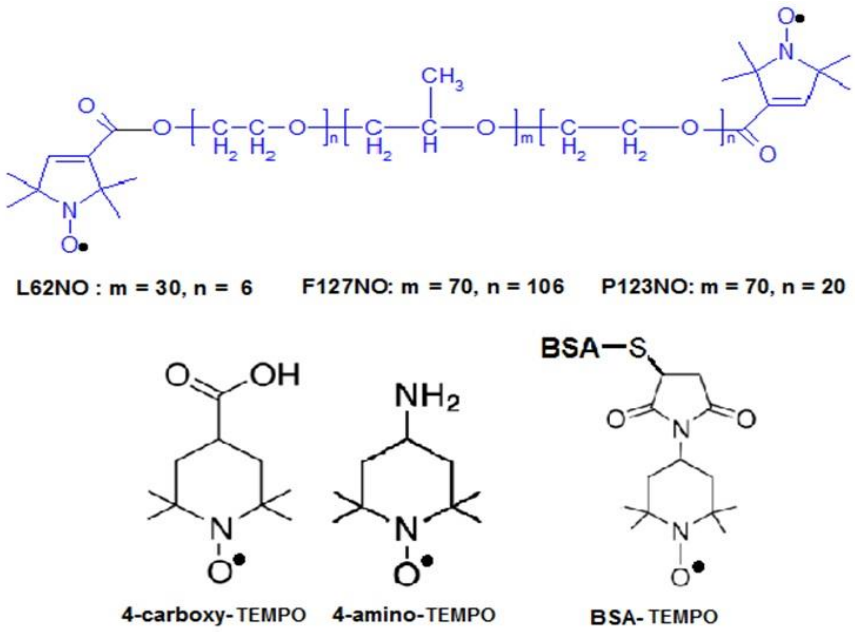

Figure 15. Spin probes used for diffusion in gels [19].

Table 2. Molecular weights of spin probes used for diffusion in gels [19].

Spin probe	Molecular weight (g/mol)
TEMPO (T)	156
4-hydroxy-TEMPO (T-OH)	172
4-carboxy-TEMPO (CT)	200
4-amino-TEMPO(TNH_2)	171
Pluronic L62NO	2500
Pluronic P123NO	5800
PEG8000-NO	8000
Pluronic F127NO	12600
Spin-labeled bovine serum albumin	≈ 66000

A number of chemical hydrogels resulting from the reaction between polyethylene glycol (PEG) or polypropylene glycol (PPG) functionalized with isocyanate groups that react with the hydroxyl groups of α- or β-cyclodextrin were analyzed. The aim was to highlight the encapsulation capacity according to several parameters: PEG chain length, the ratio between initial PEG / β-CD concentrations and the influence of chain polarity by comparing gels containing PPG and PEG (PEG900, PEG2000, PPG2000).

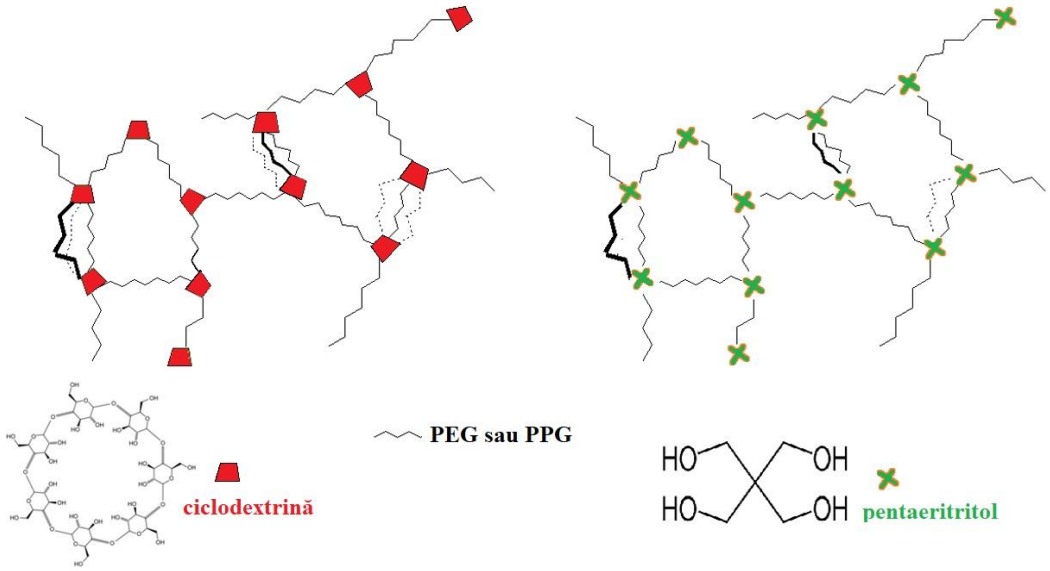

Figure 16. Schematic representation of polymer gel networks consisting of cyclodextrin or pentaerythritol with PEG or PPG, adapted after [18].

Depending on the initial ratio of reactants used in the synthesis of PEG / CD or PPG / CD polymer gels, it was observed by EPR spectral analysis that probes with molecular weight greater than 12000 g/mol do not diffuse into all polymer gels containing water as solvent. For instance, F127NO diffuses into the gel resulting from the reaction of PEG2000 with β-CD (Figure 17).

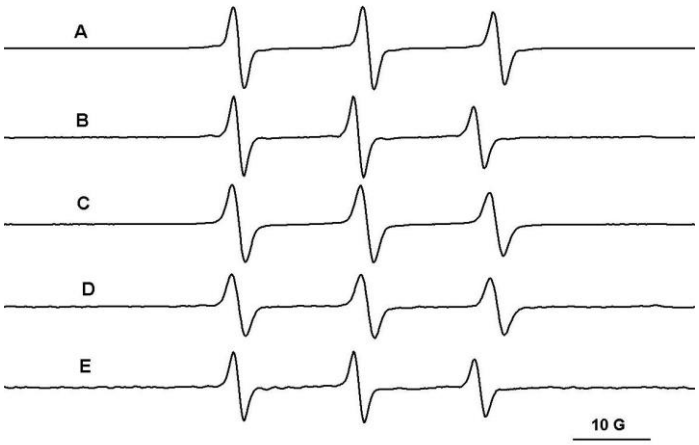

Figure 17. The EPR spectra of TEMPO (A), L62NO (B), P123NO (C), PEG8000-NO (D) and F127NO (E) spin probes encapsulated in PEG2000 / β-CD gel, in water.

Replacing the PEG chain with PPG (more hydrophobic - more compact conformations) changes the mesh size of the network. Replacing β-CD with α-CD changes the ability to encapsulate molecular probes, and thus the F127NO probe does not diffuse into these hydrogels. Replacing water with dichloromethane (DCM) / dimethylformamide (DMF) increases the mesh

size of the network allowing the encapsulation of higher molecular weight molecular probes (PEG8000-NO or F127NO). Spin-labeled serum bovine albumin does not diffuse into covalent polymeric gels (Figures 18, 19 and 20).

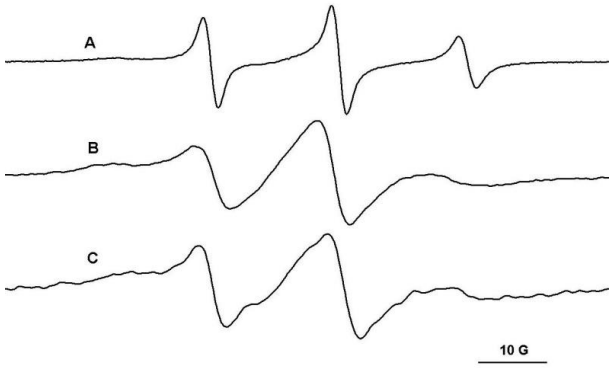

Figure 18. The EPR spectra of TEMPO (A), L62NO (B) and P123NO (C) spin probes encapsulated in PPG2000 / α-CD gel, in water.

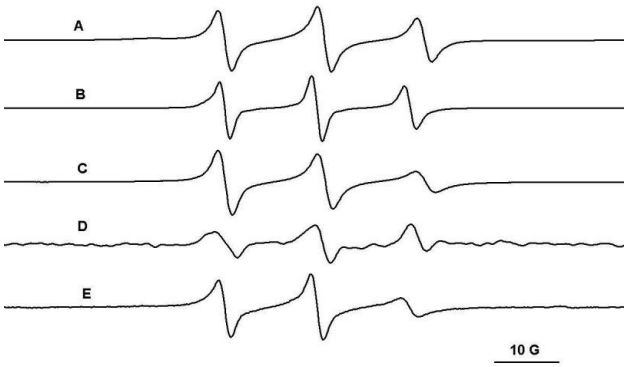

Figure 19. The EPR spectra of TEMPO (A), L62NO (B), P123NO (C), PEG8000-NO (D) and F127NO (E) spin probes encapsulated in PPG2000 / α-CD gel, in DCM.

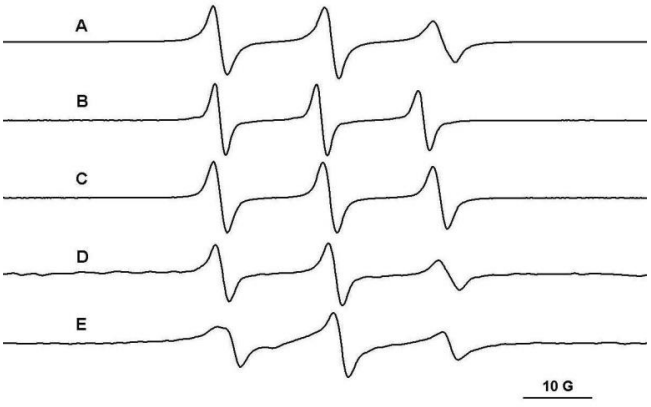

Figure 20. The EPR spectra of TEMPO (A), L62NO (B), P123NO (C), PEG8000-NO (D) and F127NO (E) spin probes encapsulated in PPG2000 / α-CD gel, in DMF.

Using a variety of spin probes, it was possible to obtain an overview of the permeability of covalent polymer gels. The EPR spectra of polymeric spin probes encapsulated in polymeric gels indicate in some cases a slow motion due either to the interaction with the gel network or to a constrained motion in the solvent pools delimited by the network.

Two other experiments aimed to highlight changes in the ability to encapsulate spin probes of gels in which cyclodextrin was replaced by pentaerythritol, both in the case of water and DCM as solvent. As with PEG900 / β-CD, it has been observed that only the P123NO probe can diffuse into the PEG900 / pentaerythritol hydrogel. In the case of DCM as solvent, the mesh size increases and all spin probes are encapsulated in the gel.

In the second part of this chapter we studied the ability of alginate gel and of IPN and semi-IPN networks formed by crosslinking sodium alginate and chitosan to encapsulate spin probes according to their molecular weight. It was observed that the spin-labeled block copolymers F127, L62 and P123 diffused into alginate gels and into semi-IPN alginate and chitosan gels (Figure 21).

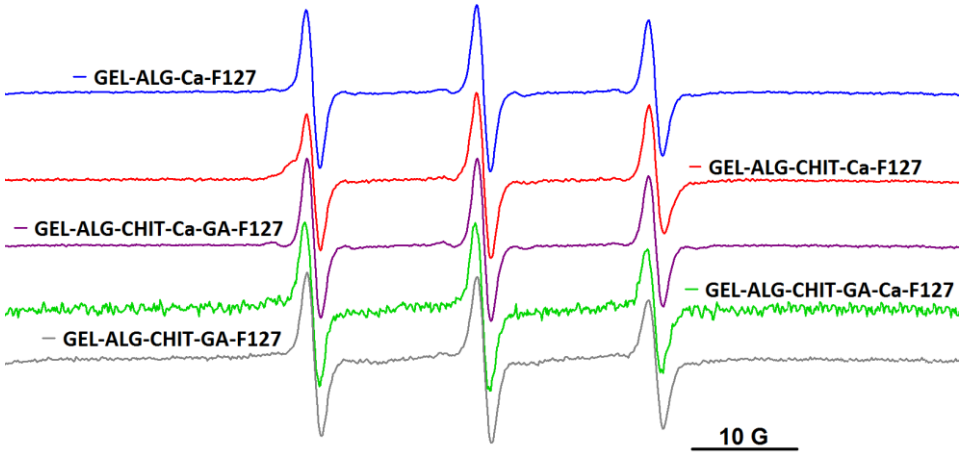

Figure 21. The EPR spectra of the F127NO spin probe in alginate, semi-IPN and IPN hydrogels.

Spin-labeled bovine serum albumin also diffuses into alginate gels and semi-IPN gels.

In the case of the IPN network, albumin diffusion is possible, but is dependent on the order in which crosslinking agents are added. The mesh size of IPN hydrogel networks resulting from the successive addition of glutaraldehyde and Ca^{2+} to the mixture of chitosan and alginate is smaller than the size of BSA, because spin-labeled albumin does not diffuse into the resulting IPN network.

Spin-labeled albumin has restricted motion in semi-IPN and IPN hydrogels (Figure 22). In the presence of glutaraldehyde, a very restricted motion is observed, which demonstrates a strong

interaction with the gel network (gray spectrum). In the presence of glutaraldehyde and Ca^{2+}, the EPR signal is very weak, indicating a gel network with small meshes (green spectrum).

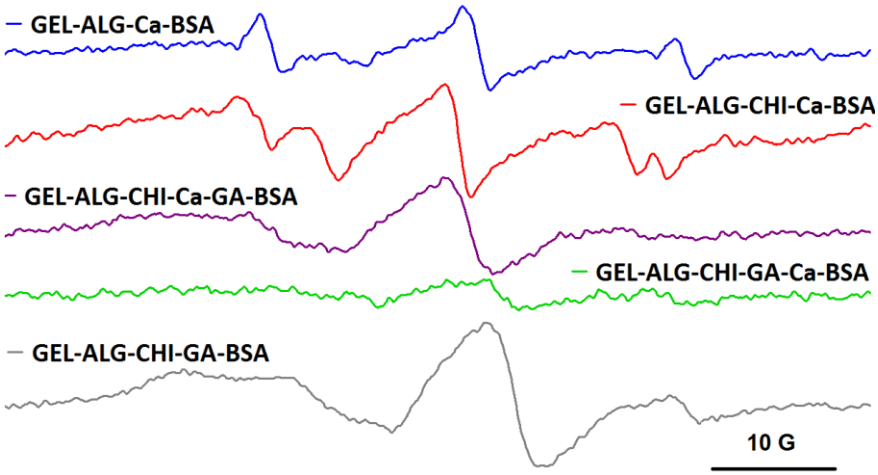

Figure 22. The EPR spectra of spin-labeled BSA in alginate, semi-IPN and IPN hydrogels.

Therefore, EPR spectroscopy can be used to monitor diffusion of molecular species with various molecular weights and thus to estimate the mesh size of gels. In the case of smaller mesh size compared to the size of the molecular probe, the latter cannot penetrate the gel network and thus the gel samples show no EPR signal. In some cases, the diffused probe in gel may have a change in dynamic. Experiments have shown that polymer gels have a mesh size of less than 10 nm (BSA size) compared to alginate and IPN hydrogels with chitosan. The mesh size of IPN hydrogels is larger than the BSA size.

The mesh size of the polymer gels depends on the length of the polymer linker and the order in which the crosslinking is done. The use of the EPR method to determine the mesh size by using spin probes with known sizes represents a novel approach.

The last chapter of the thesis refers to the modeling of the crosslinking process (CLX) of collagen under the action of riboflavin and UVA radiation, taking into account the involvement of the components of ophthalmic solutions (hyaluronic acid) and proteins in the tear film.

The studied systems were chosen as their components can be found in the eye structures and are involved in the chemical processes associated with the treatment of certain ocular diseases, such as keratoconus. This treatment involves the formation of intra- or interfibrillary bonds in the collagen network - a structural element of the cornea - mediated by radicals generated by the photodegradation of riboflavin under the influence of UVA radiation, as shown in Figure 23 [23]. This process can also have side effects because the free radicals formed can induce the death of endothelial cells or their destruction, so they can affect the retina or lens.

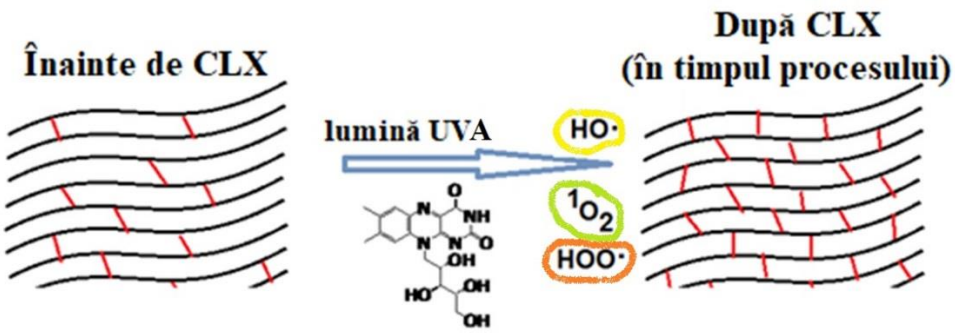

Figure 23. Schematic representation of the collagen crosslinking process mediated by riboflavin-generated radicals under the action of UVA [23].

Physico-chemical data on the processes involved in the CLX treatment are not reported in literature, although the procedure is described in several medical studies [24-28].

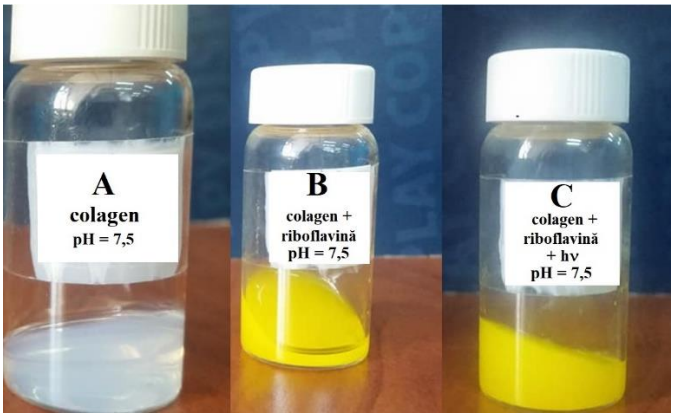

Figure 24. Collagen gels formed at pH = 7.5 in the absence of riboflavin (A), in the presence of riboflavin before exposure to UVA radiation (B) and after exposure to UVA radiation (C).

The research conducted in the study aimed to highlight by physico-chemical methods some transformations in systems containing hyaluronic acid, riboflavin, collagen. The physico-chemical methods used were: scanning transmission electron microscopy (STEM), rheology, microcalorimetry and EPR spectroscopy. In the presence of UVA radiation, riboflavin generates a series of oxygen-centered radicals (ROS) such as hydroxyl (HO•), superoxide ($O_2^{\bullet -}$), hydroperoxide (HO_2•) and singlet oxygen ($^1O_2$). In this study were used samples containing collagen extracted from bovine dermis. Collagen solutions of 0.25% in the absence and presence of riboflavin (0.1%) and / or hyaluronic acid (0.1%) at temperature ranges of 10-37°C and pH 3,5-7,5 were exposed to UVA radiation (Figure 24).

Collagen fibril formation was evidenced in STEM images at 37°C and pH 7.5, as a result of the transition from sol to gel. Samples were prepared using methods similar to those described in the literature [29-31].

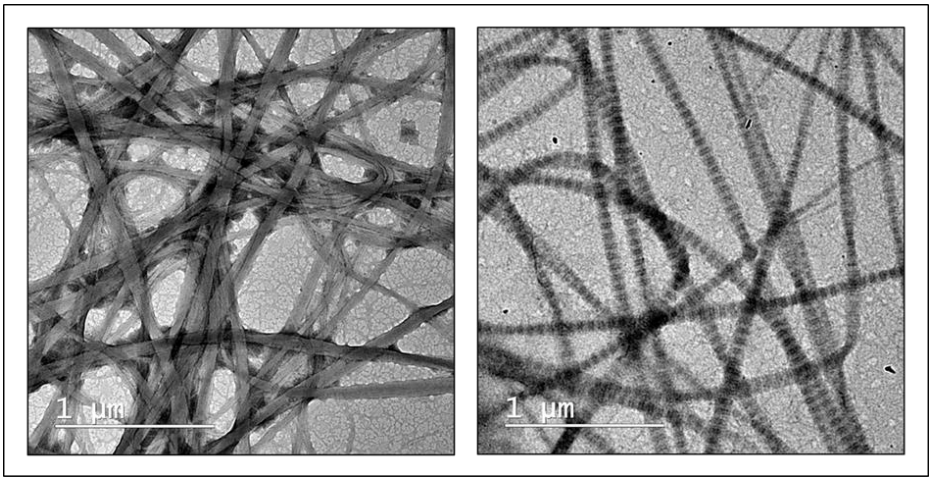

Figure 25. STEM images of collagen fibers in the absence (A) and presence (B) of riboflavin, pH 7.5.

In the case of the sample prepared at pH 7.5, following the increase of the temperature at 37°C, it was possible to observe macroscopically the sol-gel transformation as evidence of fibrillogenesis, but microscopically there was no uniform process (Figure 25 A). In contrast, in the presence of riboflavin, even in the absence of irradiation with UVA light, the formation of fibrils was observed microscopically in a much more orderly manner (Figure 25 B). Fibrils are also formed in the presence of hyaluronic acid.

The aim of the EPR measurements was to highlight the presence of short-lived radicals (especially those centered on oxygen) in various systems exposed to riboflavin and UVA radiation. Therefore, spin trapping experiments were performed for solutions of lactoferrin (LF), lysozyme (LYZ), human serum albumin (HSA) and mixtures thereof in the presence of UVA-exposed riboflavin. For this, the three spin traps presented in Figure 26 were used: DMPO - used to identify hydroxyl radicals, CPH - used to highlight superoxide and hydroxyl radicals and TEMP - used to identify singlet oxygen [32]. For most of the analyzed systems, the dominant radical species was the hydroxyl radical.

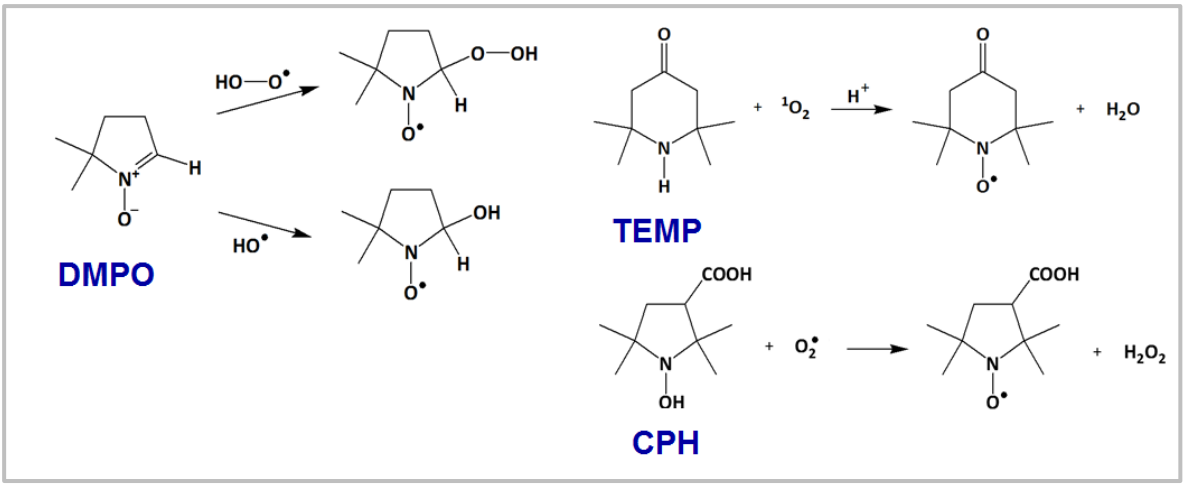

Figure 26. The structures of the spin traps used [32].

The CLX method of stopping the evolution of keratoconus involves irradiating riboflavin with UVA light, so to demonstrate the role of light, we recorded the EPR spectrum of the solution in the dark, noting that there is no signal (black spectrum); in the presence of light (cyan spectrum), the 4-line EPR signal characteristic of the DMPO-HO• adduct is observed (Figure 27).

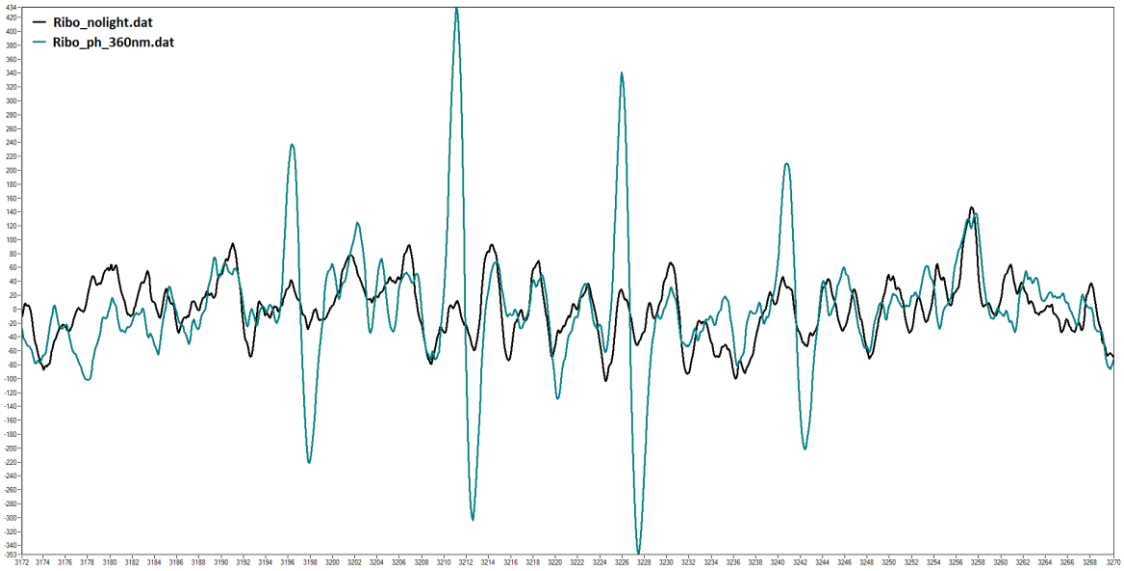

Figure 27. The EPR spectrum of riboflavin / DMPO solution in the absence (black) and in the presence of light (cyan).

The EPR spectra of spin adducts formed in the protein systems mentioned above contain two components. One component corresponds to the DMPO-HO• adduct, and the other corresponds to a DMPO adduct with a carbon-centered radical that is formed by the interaction of ROS radicals generated by riboflavin with proteins. The lines corresponding to each adduct are marked with a red dot for DMPO-HO• and a blue dot for the carbon-centered radical adduct

(Figure 28). In the presence of hyaluronic acid or dextran it was found that the formation of carbon-centered radicals is inhibited. Thus, we can say that hyaluronic acid has not only a role in restoring the integrity of the tear film, but also an antioxidant role against highly reactive radicals.

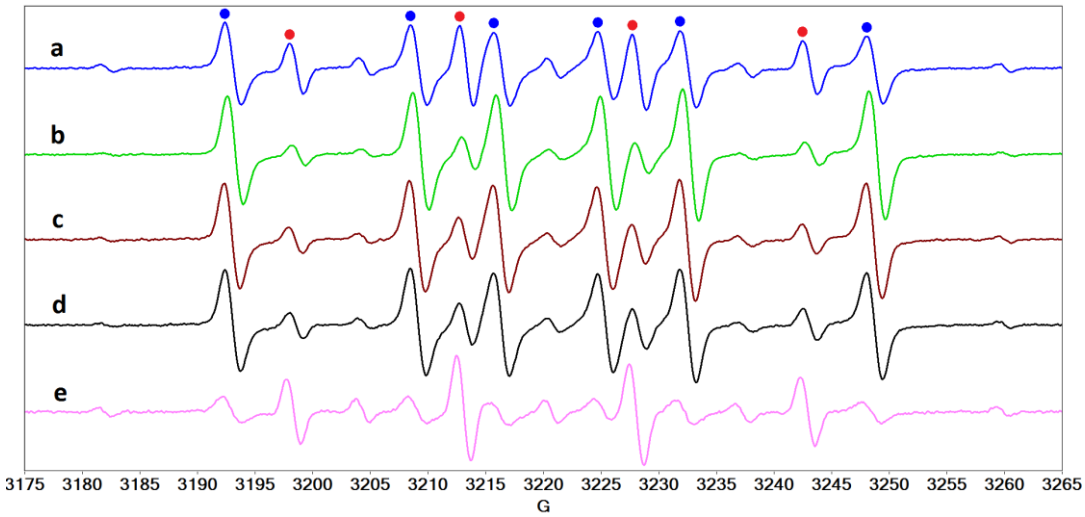

Figure 28. The EPR spectra of DMPO adducts formed after UVA exposure in the presence of riboflavin in solution of a) HSA, b) LF, c) LYZ and in the mixture of the three proteins in the absence (d) and in the presence of HA (e).

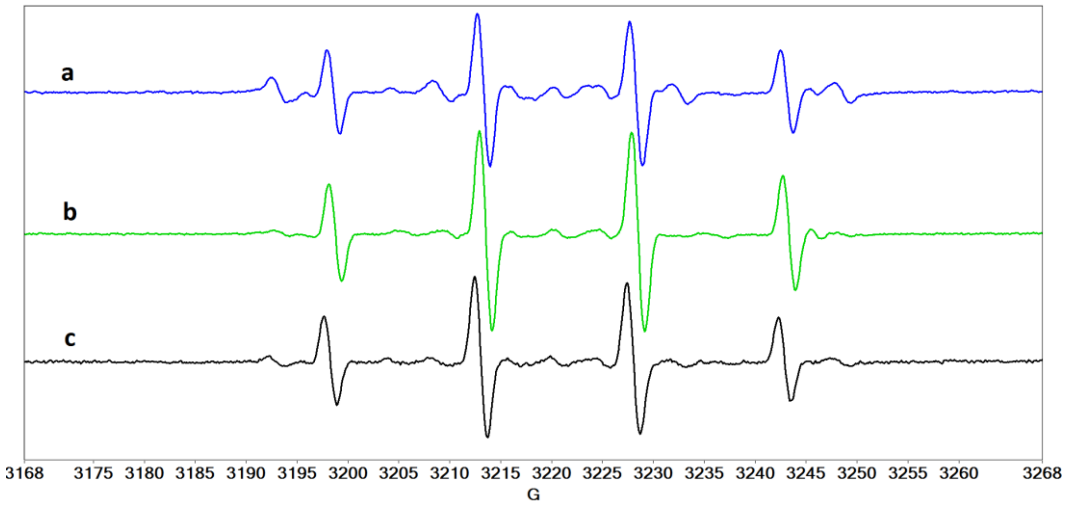

Figure 29. The EPR spectra of DMPO adducts formed after UVA irradiation of the collagen solution in the presence of riboflavin and in the absence of HA (a), in the presence of HA (b) and in the presence of the mixture HA + HSA + LF + LYZ (c).

The presence of HO• radicals and of radicals centered on carbon atoms was noticed in solution containing collagen and riboflavin after exposure to UVA radiation (Figure 28 a). The presence of hyaluronic acid, but also of the three proteins (LF, LYZ, HSA) reduces the contribution of the carbon-centered radical (Figure 29 b and c).

Irradiation of riboflavin-containing solutions can form, in addition to the hydroxyl radical, the superoxide radical which has a higher reactivity and, in theory, could have a greater negative side effect in the treatment of CLX. The CPH spin trapping agent can highlight the formation of this radical by the use of superoxide dismutase (SOD). Figure 30 shows the spectra of CPH adducts formed in the collagen solution in the absence (Figure 30, blue spectrum) and in the presence of SOD (Figure 30, green spectrum).

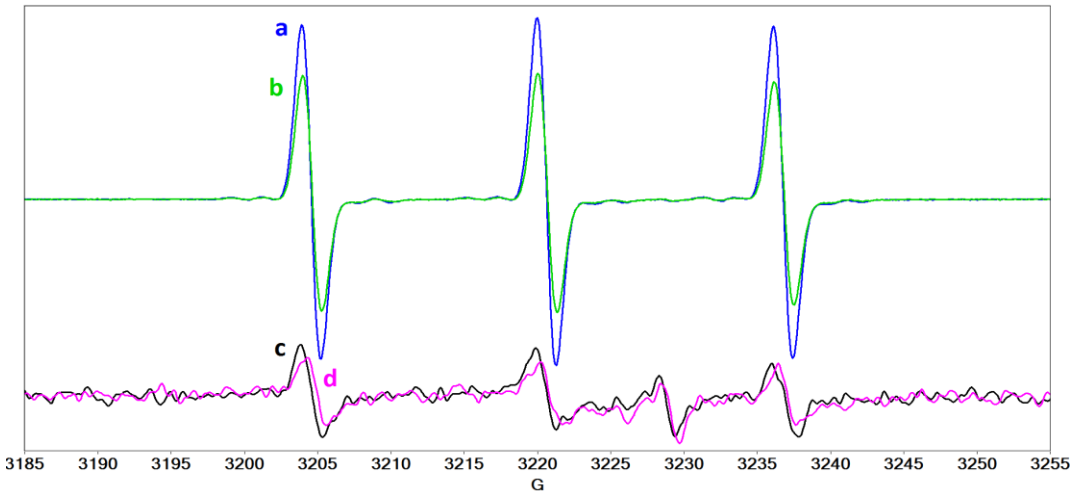

Figure 30. The EPR spectra of CPH adducts formed by UVA irradiation of a solution containing riboflavin and a) collagen, b) collagen + SOD, c) collagen + HA and d) collagen + HA + SOD.

It is observed that the intensity of the green spectrum is slightly lower than that of the blue spectrum, which could lead to the conclusion that the superoxide radical can be generated in the collagen solution. In the case of the solution containing collagen and hyaluronic acid, it is observed that the intensities of the two spectra are similar and lower (Figure 30 black and magenta spectrum). This result highlights the protective role that hyaluronic acid may play during CLX treatment.

Among the possible oxygen-centered reactive radicals (ROS) generated in the presence of UVA and riboflavin, singlet oxygen is the one that would have a higher potential for destruction of eye tissues following CLX treatment (Figure 31). The generation of TEMPONE (4-oxo-TEMPO) made it possible to detect singlet oxygen in riboflavin solution in water, in riboflavin and collagen solution, in collagen solution, but also in Peschke solution (pharmaceutical formula containing riboflavin and dextran).

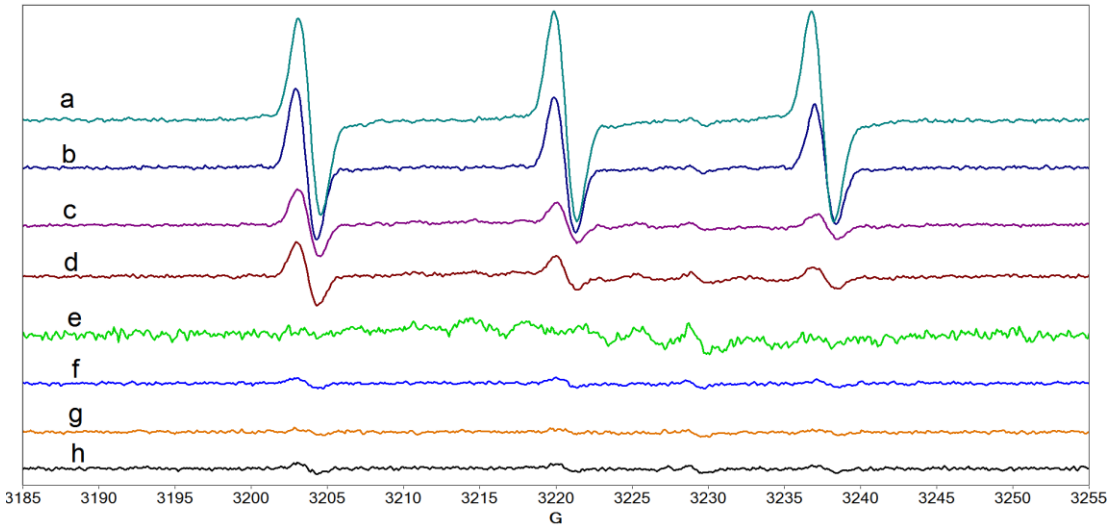

Figure 31. The EPR spectra of TEMPONE formed after UVA irradiation of systems: a) H_2O, b) collagen + riboflavin, c) collagen, d) PESCHKE solution, e) tears, f) collagen + HA, g) collagen + HA + HSA + LF, h) HSA + LF + LYZ.

In contrast, singlet oxygen formation was not observed in tears, in a mixture of collagen and hyaluronic acid or in a mixture of collagen, hyaluronic acid and protein. Experiments have shown that hyaluronic acid has the role of neutralizing the excess of reactive species formed. In the presence of hyaluronic acid or dextran, it was found that the formation of carbon-centered radicals is inhibited, which proves that hyaluronic acid has an antioxidant role against highly reactive radicals.

The results of physico-chemical determinations on systems containing collagen and hyaluronic acid exposed to the action of riboflavin and UVA support the roles that riboflavin and hyaluronic acid play in the treatment of CLX. Riboflavin, by generating free radicals, ensures the formation of interfibrillary bonds that lead to increased mechanical strength of the cornea, while hyaluronic acid has the role of regulating or neutralizing some of the free radicals formed. The dextran that is part of the PESCHKE solution has the same role.

The thesis focuses on the use of EPR spectroscopy as a method of investigating systems containing polysaccharides. The EPR data were supplemented by those obtained by rheology, calorimetry, IR spectroscopy and electron microscopy.

The data presented in this thesis offer the prospect of further studies on the functionalization of polysaccharides in order to modulate their properties according to the desired applications. In particular, studies on the formation of metal nanoparticles in polysaccharide gels will be detailed.

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List of scientific articles published in the field of the doctoral thesis:

1. S. Mocanu, I. Matei, A. Leonties, V. Tecuceanu, A.M. Hanganu, Z. Minea, A. Stancu, E.I. Popescu, G. Ionita, "New flexible molecular probes bearing dansyl and TEMPO moieties for host–guest interactions in solution and gels", New J. Chem., 2019,43, 11233-11240(F.I.=3.288/2019). doi:10.1039/C9NJ01554J
2. E. I. Popescu, L. Aricov, S. Mocanu, I. Matei, E. Hristea, R. Baratoiu, A. Leonties, C. Petcu, E. Alexandrescu, G. Ionita, "Subtle influence on alginate gel properties through host–guest interactions between covalently appended cyclodextrin and adamantane units", *New J. Chem.*, **2021**, 45, 8083-8091 (F.I.=3.591/2020). doi:10.1039/D1NJ01278A
3. I. Matei, E. I. Popescu, S. Mocanu, E. N. Hristea, F. Savonea, R. Baratoiu, G. Ionita, "Non-covalent interactions evidenced by EPR spectroscopy in cyclodextrin complexes", *Rev. Roum. Chim.*, **2021**, 66 (1), 9-23. doi:10.33224/rrch.2021.66.1.01
4. I. Matei, M. Bem, A. R. Leonties, C. Radutiu, E. I. Popescu, S. Mocanu, F. Savonea, R. Baratoiu, G. Ionita, "Processes mediated by gold nanoparticles encapsulated in polymeric gels evidenced by EPR spectroscopy", *Rev. Roum. Chim.*, **2021**, 66 (3), 295-301. doi:10.33224/rrch.2021.66.3.10

Participation in scientific events:

1. G. Ioniţă, I. Matei, E. I. Popescu, Z. Mînea, S. Mocanu, Prezentare poster: Host–guest interactions in polysaccharide hydrogels evidenced by spin probes, 2019, The 52nd International Meeting of theESR Spectroscopy Group of the Royal Society of Chemistry, Glasgow
2. G. Ioniţă, E. I. Popescu, E. Hristea, R. Bărăţoiu, S. Mocanu, I. Matei, Prezentare poster: Host-guest interactions in polymeric gels evidenced by EPR spectroscopy, 2019, XIth EFEPR Conference, Bratislava
3. E. I. Popescu, Z. Mînea, S. Mocanu, I. Matei, G. Ioniţă, Prezentare poster: Spin probes - Tools to investigate loading capacity and inhomogeneity of hydrogels, 2019, 21st Romanian International Conference on Chemistry and Chemical Engineering (RICCCE), Constanța

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