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Ultrasonication - a Potential Method Toward Chitosan Hydrogels

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Abstract. *The main objective of the paper was to prepare eco-friendly chiral hydrogels from chitosan and betulinic aldehyde under ultrasonic radiation effect, targeting their use for enantiomeric separations. This strategy promoted the obtaining of the hydrogels by supramoleclar organization of the imine units bonding the betuline skeleton with chitosan into ordered clusteres, while not altering the physico-chemical properties of the reagents. FTIR, SEM, CD spectroscopy and POM techniques were used to prove the pelicularities of the hydrogelation mechanism under the ultrasonication. The stability of the hydrogels was investigated by monitoring the influence of the swelling in three media of diferent pH, by POM and CD. It was concluded that the chiral hydrogels prepared by ultrasonication are stable when the pH vary from acidic to basic, indicating the new synthetic approach as a valuable method to yield suitable materials for enatiomeric separations in medical field.*

Keywords*: chitosan hydrogels, ultrasonication, betulinic aldehyde, chiroptical, enantiomeric filtration*

1. Introduction

Chitosan is a polysaccharide obtained by deacetylation of chitin, one of the most naturally occurring biopolymers in nature and display several excellent properties such as biocompatibility, biodegradability, antifungal, antiviral, antibacterial as well as ability to form hydrogels, films or fibers. Due to these benefic properties, chitosan kept a great potential for many applications in fields such as biomedicine, agriculture, food, waste water treatment, cosmetics, textile industries and so on [1]. Moreover, chitosan is a cationic polysaccharide which contains hydrophilic and hydrophobic groups such as NH_2 or OH, which favor the preparation of different materials, one of them being the hydrogels. It is well-know that hydrogels are materials with great swelling ability, mimicking the natural tissues, which can be used for the preparation of drug carriers, matrix for tissue engineering, wound dressings and so on. Depending by the crosslinker nature, the chitosan hydrogelation can occur by physical or chemical bonding leading to physical or chemical hydrogels, respectively. They have distinct properties, e.g. physical hydrogels are less stable compared with chemical ones. Generally, the hydrogels are designed to present particular properties for the targeted applications. However this desideratum is sometimes difficult to attain due to the chitosan limitations, such as low solubility, (especially low hydrophilicity) and poor mechanical property. Over time, numerous methods have been developed to improve the properties of the chitosan hydrogels, such as copolymerization or blending with other polymers or monomers, adding hydrophilic materials or crosslinking with different monomers [2]. The use of different covalent crosslinkers to obtain chitosan hydrogels lead to different materials with improved properties, but over time they proved worse biocompatibility or biodegradability compared to the pristine chitosan. This is not acceptable for biomedical applications which require biomaterials without traces of organic solvent, acids or chemical reagents, which can inflict citotxicity and consequently biocompatibility loss [3-5]. Our previous work in the field of chitosan based hydrogels demonstrated that biocompatible and biodegradable chitosan based hydrogels, proper for biomedical applications can be achieved by an unusual crosslinking with different natural monoaldehydes, by simple stirring in homogeneous conditions. By varying the crosslinking ratio and the reaction conditions (temperature, gelation time and stirring velocity) it was possible to control the physico-chemical

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properties of the obtained hydrogels such as: swelling ability, porous morphology, thixotropic properties, biodegradability and capacity to encapsulate bioactive agents for controlled release applications [6-15]. However this new strategy was not appropriate in the case of the use of highly hydrophobic aldehydes which are not soluble in acidic water and system homogenicity can't be reached.

A new promising technology for the preparation of hydrogels is the use of ultrasonic radiation [16]. Prior used for the dissolution of solid polymers, preparation of nanomaterials, encapsulation of different fillers or various synthetic reactions, this technique also proved to be an excellent tool for the preparation of hydrogels, being able to provide strong physical or chemical interactions even at low frequencies [17,18]. Some studies demonstrated that ultrasonic radiation synthesis is a proper method which allows the tuning of the hydrogels properties, while not altering their chemical nature [19-21]. Thus, it was demonstrated that hydrogelation by ultrasonication allows the control of the molecular weight and improving the swelling ratio, reaction rate, solubility, mechanical property, adsorption capacity, water absorbency and so on [22-25].

In the present study chiral hydrogels based on chitosan and a natural originating monoaldehyde. i.e. betulinic aldehyde were succesfully prepared using the ultrasonication as an eco-friendly method. It was proved that ultrasonication is an appropriate tool for hydrogels synthesis, while not altering the chemical structure and properties of the reagents, i.e. chitosan molecular weight and chirality of the betulinic aldehyde. Moreover, this synthetic strategy promoted the supramolecular organization of the new imine compounds into clusters, leading to chiroptical hydrogels.

2. Materials and methods

2.1. Materials

Low molecular weight chitosan (217.74 kDa, 85% deacetylation degree (DA) was purchased from Sigma–Aldrich Co. (USA) and used as received. The betulinic aldehyde and acetate buffer solution of *p*H=4.2 were prepared in our laboratory, as already reported [9-15]. All the other solvents used in this paper were from Sigma–Aldrich Co. (USA) and used as received.

2.2. Hydrogel preparation by ultrasonication. Synthetic trials

To establish the procedure of preparation of chitosan/betulinic hydrogels, different reaction pathways were tried. The trials were performed varying the reaction conditions as will be described below:

The first synthetic protocol followed a procedure already reported for similar systems [9-15]. Briefly, in a 20 mL vial was prepared a 2% solution of chitosan by dissolving 0.1 g chitosan ($5.05x10^{-4}$ mmol glucosamine units) into 5 mL aqueous solution of glacial acetic acid (0.7%). Betulinic aldehyde was dissolved in ethanol to give a 1% solution. Two different molar ratios between amine and aldehyde functional groups were used: 3/1 and 5/1. The solution of betulinic aldehyde was heated at 55ºC, and then was slowly dropped into the chitosan solution, under vigorous magnetic stirring (1500 rot/min), at 55ºC. The reaction mixtures transformed into a suspension. After 5 hours, in both reaction systems (3/1 and 5/1) the hydrogelation didn't occur. By adding acetone, the system become macroscopically homogeneous, but also much diluted and the hydrogelation still didn't occur. It was concluded that the hydrophobicity of the betulinic aldehyde imped the obtaining of a homogeneous reaction system, and thus, the condensation reaction is hindered. Consequently, conditions to improve the solubility of the betulinic aldehyde in the system were created, by increasing the temperature or dissolving the aldehyde in solvents of higher polarity (Table 1). No hydrogelation was observed.

To further improve the solubility of betulinic aldehyde into the reaction system, *a second reaction* protocol has been developed, mainly consisting in dissolving chitosan into a 0.7% solution of acetic acid into a mixture of water/ethanol, 1/1 v/v, as follows. In a 20 mL vial was prepared a 2% solution of chitosan by dissolving 0.1 g chitosan ($5.05x10^{-4}$ mmol glucosamine units) into 5 mL solution of glacial acetic acid (0.7 %) obtained by mixing 2.5 mL water with 35 µl acetic acid and 2.5 mL ethanol. Betulinic aldehyde was dissolved in ethanol to give a 1% solution.For comparison reasons, the same two different molar ratios between amine and aldehyde functional groups were used: 5/1 and 3/1. The solution of

betulinic aldehyde was heated at 55ºC, and then was slowly dropped into the chitosan solution, under vigorous magnetic stirring (1500 rot/min), at 55ºC. The reaction mixtures transformed into a clear solution. After 5 h, in both reaction systems (3/1 and 5/1) the hydrogelation didn't occur. The reaction mixtures transformed into a suspension once the stirring and heating were stopped, and transformed again into a clear solution once they were re-heating.

Table 1. The reaction conditions for the protocol 1, for NH_2/CHO molar ratios of 3/1 and 5/1

No.	1	2	3	4	5	6
Solvent of betulinic aldehyde	ethanol	methanol	acetone	ethanol	Ethanol/DMSO 1/1	DMSO
Reaction temperature (ºC)	55	55	55	80	80	80
Stirring	magnetic	magnetic	magnetic	magnetic	magnetic	magnetic
Time (hours)	5	5	5	5	5	5
Observations	suspension	suspension	suspension	suspension	suspension	solution

Taking into consideration the ability of chitosan to self-order under the ultrasounds stimuli, *a third protocol* has been developed, replacing the magnetic stirring with sonication, as follow. In a 20 mL vial was prepared a 2% solution of chitosan by dissolving 0.1 g chitosan ($5.05x10^{-4}$ mmol glucosamine units) into a mixture of 2.5 mL water with 2.5 mL ethanol and 35 µL glacial acetic acid. Betulinic aldehyde was dissolved in ethanol to give a 1% solution, which was heated at 55ºC before use. Two different molar ratios between amine and aldehyde functional groups were used: 5/1 and 3/1. The vial containing the chitosan solution has been immersed in an ultrasonic bath, at 55ºC and ultrasonic frequency of 35 W. After 10 min, the solution of betulinic aldehyde was slowly dropped into the chitosan solution. While adding, a fine suspension started to appear, probably due to the different diffusion rate of the ethanol and aldehyde into the viscous chitosan solution. To avoid the aldehyde segregation before its interaction with chitosan, at each 10 min, the vial was taken off from the ultrasonic bath and vigorously stirred on a vortex (1500 rot/min). Moreover, in the case of the 5/1 molar ratio, 1 mL of acetone has been added as co-solvent to preserve the system homogeneity and thus to create proper conditions for interaction. The reaction mixture transformed into an opalescent semisolid material after 3 h, and became transparent after approx. 12 h (Table 2).

Table 2. The hydrogels codes and the reaction condition for their preparation

Code	**CB2**	**CB2.5**	**CB3**	**CB5**	**CB6**
NH_2-CHO	2:1	2.5:1	3:1	5:1	6:1
Solvent of betulinic aldehyde	Ethanol	Ethanol	Ethanol	Ethanol	Ethanol
Reaction temperature (ºC)	55ºC	55ºC	55ºC	55ºC	55ºC
Stirring	sonication	sonication	sonication	sonication	sonication
Ultrasonic frequency	35 W	35 W	35 W	35 W	35 W
Time (hours)	3	3	3	3	3
Observations	gel	gel	gel	gel	gel

2.3. Equipment

The corresponding xerogels were achieved by freezing the hydrogels in liquid nitrogen and further submitting to lyophilization using a Bench Top Pro with Omnitronics ™ equipment, for 10 h, at−55 °C and 152 Pa.

The hydrogels were obtained by using a BANDELIN SONOREX DIGITEC ultrasonic bath, with ultrasonic frequency of 35 W, at 55ºC.

Fourier-transform infrared (FTIR) spectroscopy has been made on the hydrogel lyophilized samples with a FT-IR Bruker Vertex 70 Spectrophotometer, by ATR technique. All spectra have been processed using OPUS 6.5 software.

The textures and their thermotropic behaviour were observed on thin slices of hydrogels or xerogels, by an Olympus BH-2 polarized light microscope equipped with a THMS 600 heating stage and a LINKAM TP92 temperature control system.

The xerogels microstructure was characterized by surface and cross-section viewing with a field emission Scanning Electron Microscope SEM EDAX – Quanta 200, run at 20 keV accelerating voltage.

The swelling behaviour was investigated applying a traditional procedure [26], and was assessed by calculating mass equilibrium swelling (MES) with equation MES = (Ms-Md)/Md, where Ms is the mass of the hydrogel in swollen state and Md is the mass of the hydrogel in dried state.

The Circular dichroism (CD) spectra were acquired via a Chirascan plus (Applied Photophysics) using 0.5mm path lamellar cells, at room temperature (22°C).

The molecular weight of the chitosan and the ultrasonicated chitosan was determined by viscometric analysis, using a Schott CT 52 (Schott AVS 350) viscometer with capillary No 0, at 25°C. Chitosan solutions of different concentrations in 0.3 M acetic acid and 0.2 M sodium acetate were prepared. The flow times of chitosan solutions and solvent were recorded for five times and the average value was calculated. The intrinsic viscosity [η] was graphically calculated by extrapolating the curve of reduced viscosity versus concentration to zero concentration. The molecular weight was then calculated by using Mark-Houwink equation (1) [27]:

$$[\eta] = k \cdot [Mw]^{\alpha} \quad (1)$$

where M_w is the viscosity average molecular weight of polymer, α and *k* are constants (α= 0.85 and k = 1.38×10^{-4} for 0.3 M acetic acid and 0.2 M sodium acetate solvent system), and [η] is intrinsic viscosity and can be determined from equation (2):

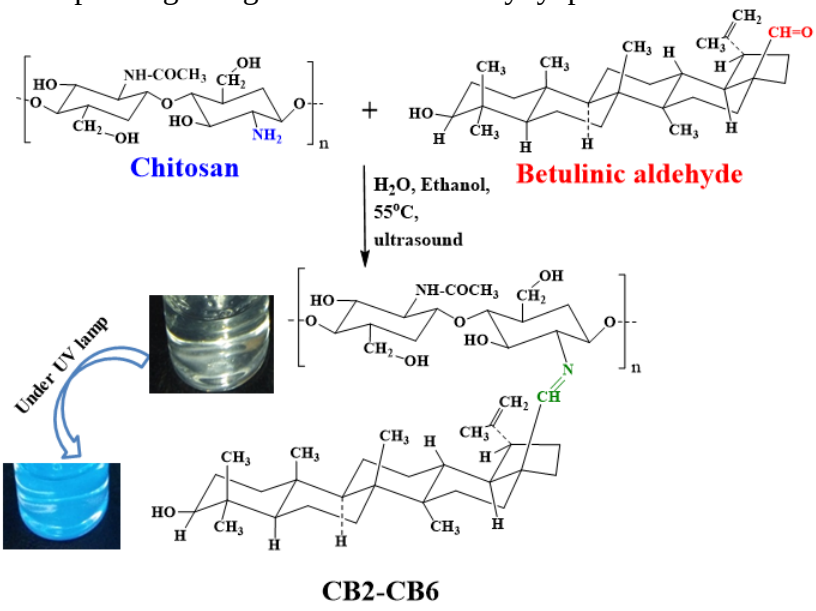
$$[\eta] = \lim_{c \to 0} \left(\frac{\eta - \eta sol)}{\eta sol \cdot C} \right) \quad (2)$$

where η is the solution viscosity and η_{sol} is the solvent viscosity and C is the solution concentration. As shown in equation (2), when $(\eta - \eta_{sol})/\eta_{sol}C$, that is, reduced viscosity (η_{red}) is plotted against concentration (C), the intercept corresponds to intrinsic viscosity [η].

3. Results and discussions

In the present study, chiral hydrogels were obtained by condensation reaction of chitosan with betulinic aldehyde using the ultrasonic radiation (Scheme 1).

As betulinic aldehyde is highly hydrophobic, hampering the attaining of a homogeneous system required for optimal condensation, its reaction with the water soluble chitosan was possible only under the effect of the ultrasound radiation. Moreover, the system homogenicity was maintained by solving chitosan in a mixture of water-ethanol. Hydrogels with different degree of crosslinking have been obtained by varying the molar ratio between the amine groups of chitosan and betulinic aldehyde (Scheme 1). In order to investigate the gelling mechanism and the structural characteristic of the hydrogels, the corresponding xerogels were obtained by lyophilization



Scheme 1. Synthesis of chiral chitosan based hydrogels under the effect of ultrasound

The effects of ultrasonication on the molecular weight and viscosity of the chitosan

It is know that ultrasonication led to an improvement of the polymers solubility and not only [22]. The effects of ultrasound radiation depend on several factors such as frequency, time and polymer used. In the case of chitosan, it was reported that by ultrasonication a slight decrease of the molecular weight occurs, along with an increase of its solubility and a higher ordering degree [25]. In this light, the molecular weight and intrinsic viscosity of the ultrasonicated chitosan were determined, in order to establish the role of ultrasound radiation upon these parameters, and indirectly upon the solubility. The viscosity of polymeric solutions is a direct measure of the hydrodynamic volume of polymeric molecules, which in turn are related to the molecular size or chain length, and therefore to the molecular mass. On a large scale, the viscosity of polymeric solutions is determined by the viscometric method and the molecular mass was determined using the Mark-Houwinq equation as was describe in Section 2.3. Such plots for chitosan and ultrasonicated chitosan are shown in Figure 1 and the corresponding intrinsic viscosities and calculated molecular weights are presented in Table 3.

Table 3. Intrinsic viscosity and viscosity average molecular weight of chitosan.

Samples	Intrinsic viscosity [η], dL/g	Molecular weight, M_w
Chitosan	4.7548	Mw=217,743 kDa
Ultrasonicated chitosan	4.7209	Mw=215.918 kDa

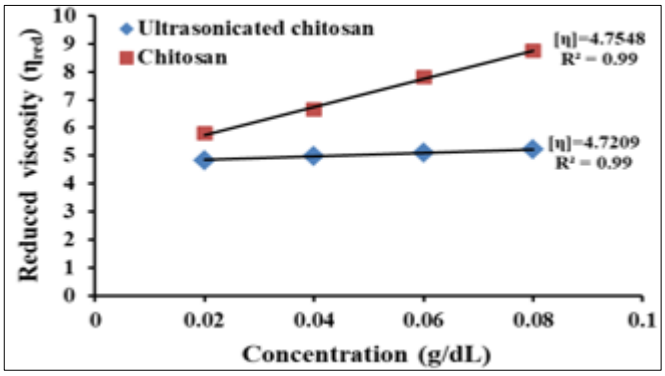


Figure 1. The reduced viscosity of the ultrasonicated chitosan and chitosan

From the Figure 1, it can be observed that the chitosan viscosity and implicitly the molecular weight was not affected by ultrasonication. On the other hand, ultrasonication leads to the improvement of the gelling mechanism of the chiral hydrogels, through the better disolution of the betulinic aldehyde and chitosan, which favored the formation of supramolecular ordering imine clusters. The reaction conditions, time and the power of ultrasound, favoured the condensation reaction of chitosan with betulinic aldehyde, especially for the 3:1 molar ratio between amine groups of chitosan and betulinic aldehyde. This fact was also demonstrated using FTIR spectroscopy, when the presence of the absorption band characteristic to the imine linkage was clearly observed at 1647 cm^{-1}. The absorption band characteristic of the imine bond was of medium intensity and overlaped with the characteristic vibration bands of the C=C groups, from 1642 cm^{-1}. In the case of the other samples, the characteristic absorption bands of the aldehyde groups still could be observed at 1725 cm^{-1}, indicating the incomplete condensation. The clear evidence of the characteristic imine band was gained by curve fitting in the region 1800-1600 cm^{-1} of the FTIR spectra, as shown in Figure 2. Spectral changes observed in the region 3700-2700 cm^{-1}, (associated with the stretching vibrations of the amine and hydroxyl groups, involved in intra- and inter-molecular hydrogen bonds), confirmed the supramolecular rearrangements expected as result of the ultrasound effect. It could be concluded that for an optimal balance of the functional units, the ultrasound favoured the condensation reaction concomitant with the supramolecular arrangements, emerging to the hydrogelation process.

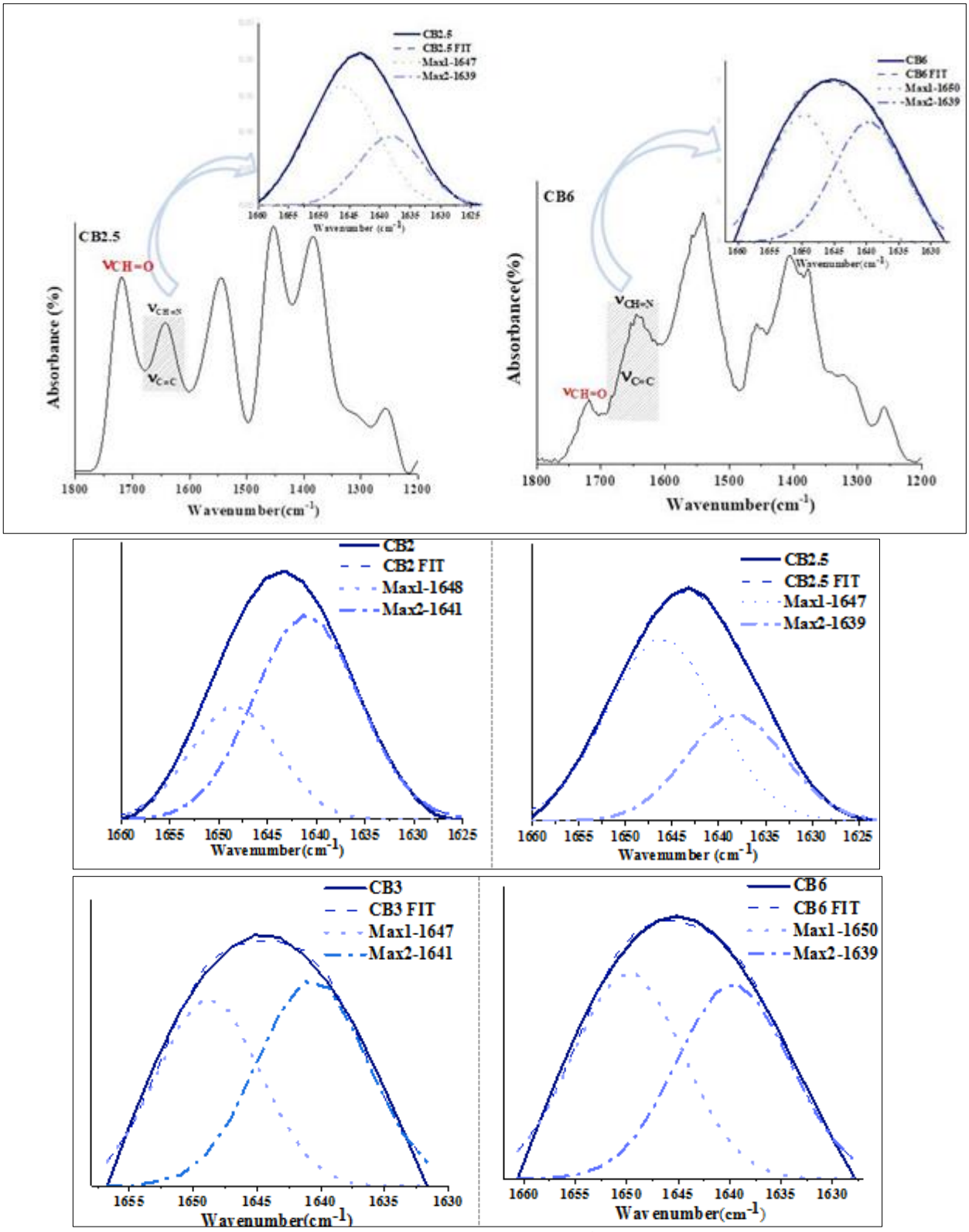
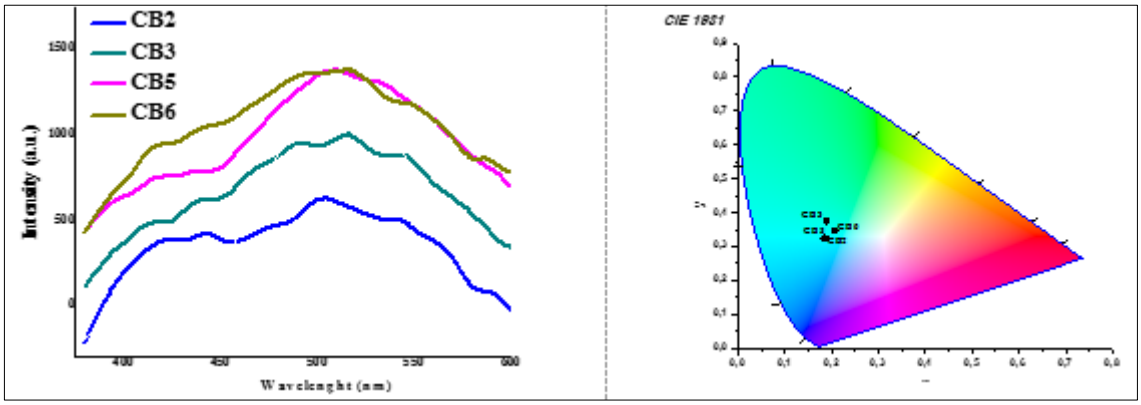


Figure 2. Curve fitting in the region 1800–1600 cm^{-1} of the xerogels FTIR spectra

These hydrogels prepared under the ultrasound effect showed bright blue luminescence when illuminated with an UV lamp, in agreement with the extensive conjugation of the rigid imine units, and also with the supramolecular architecturing of the chitosan-betulin units (Scheme 1) [28, 29].

The blue luminescence was confirmed by photoluminescence spectra and the chromaticity diagrams, when the hydrogels were excited with the wavelength of 395 nm. The photoluminescence spectra

displayed a large emission band, from 380 to 600 nm, with a maximum around 500 nm (Figure 3a), giving a blue-greenish color of the emited light (Figure 3b).



a b

Figure 3. a) Emission spectra and b) chromaticity diagram of the hydrogels

Scanning electron microscopy (SEM) have shown a porous morphology with pore dimeter around 10 micrometers, appropriate as fillers for chromatographic separations (Figure 4) [30].

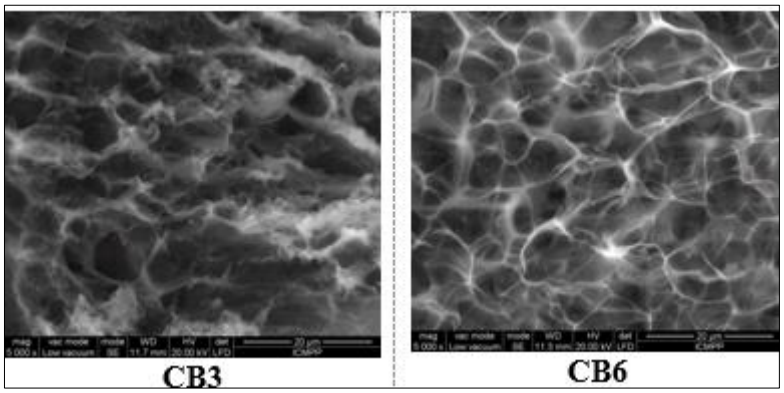
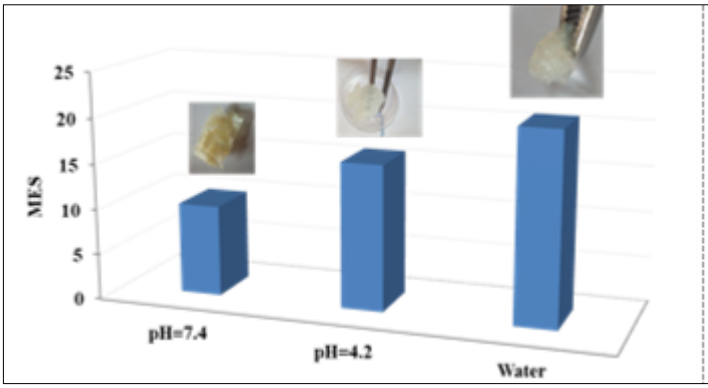


Figura 4. SEM microphotographs of **CB3** and **CB6** xerogels

As the chiral hydrogels based on chitosan and betulinic aldehyde are potential suitable materials for enatiomeric separations, which require the use of different pHs, it is necessary to know the stability of these systems in environments with different *p*H. Thus, by hydration the corresponding xerogels in three media of different pH (water, phosphate buffer solution, buffer solution with *p*H=4.2) the swelling capacity and also their stability was investigated. The mass equilibrium swelling (MES) was achieved relativ quickly, in 10 min in water and acid buffer solution, and slower, in 3 h, in basic buffer solution. An excellent stability was observed for the samples with higher betulinic aldehyde content, which retained their integrity in water and basic buffer solution, even for three months. Figure 5a displays the MES values of the sample **CB3,** calculated for the three swelling environments. Figure 5b gave MES values for all samples.



Code	Water	pH=7.4	pH=4.2
CB2	25	9	28
CB2.5	18	8	14
CB3	21	10	16
CB5	53	14	29
CB6	69	18	49

a b

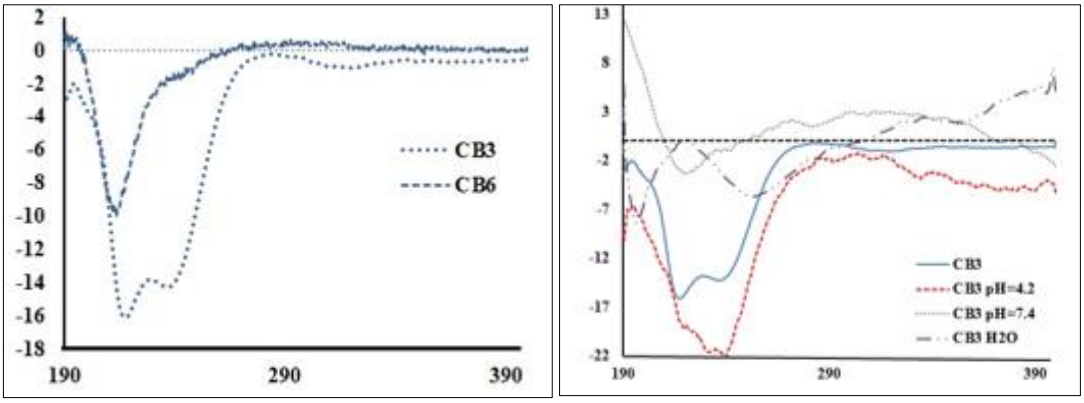
Figure 5. a)Mass equilibrium swelling of **CB3** after swelling in different;
b) MES values for all samples

The supramolecular ordering of chiral chitosan-betulin hydrogels was evidenced by polarized light optical microscopy (POM), which revealed fingerprint textures characteristic of cholesterol liotropic liquid crystals, atributed to the ordering potential of chitosan and the new betulinic based clusters [31 33]. Moreover, in the case of the amine/aldehyde molar ratio equal to 3 (**CB3**), in the thin layer, clearly aligned domains with visible steps were observed, confirming the helical ordering of the betulin-substituted chitosan (Figure 6).

In this context, from the presented data, it can be considered that in the case of the **CB3** hydrogel, the ultrasound radiations has led to a good correlation between the structure and properties, which recommends it as a matrix for the enantiomer separations. Further, in view of its application as column filling for enantiomer separation, the chiroptical stability of **CB3** was monitored by POM on samples swollen in environments of different *p*H. As can be seen in figure 6, after swelling, the xerogels displayed a texture similar to that of the pristine hydrogel, with no significant modifications, indicating a good stability of the helical organization under *p*H stimuli. This points for the long term use of the **CB3** hydrogel for enantiomeric separation [34].

Figure 6. POM microphotograps of **CB3** hydrogel and xerogel swollen in media of various *p*H for 3 h, aquired at room temperature (200x magnitude)

Moreover, circular dichroism spectroscopy was used as a complementary method to verify the stability of the helical structure of **CB3**. The **CB3** hydrogel showed a negative absorption in the far-UV spectra, divided into well-defined bands, while in the near UV field a new negative absorption band appeared around 319 nm, which was assigned to the R-enantiomer of the chiral imine chromophore [35]. Remarkably, the absorptions occurred entirely in the negative region, indicating the dominance of the right configuration (Figure 7).



a b

Figure 7. Circular dichroism spectra of the a) **CB3, CB6**, and b) **CB3** in different *p*H media

When the **CB3** swelled in water or acidic medium, no changes of the right configuration were noted; the adsorption bands shifted to left or right and modified their shape, but were mentained in the negative spectral region. Even if in the physiological *p*H, a broad haloo seems to occur in the positive region, the main absorption peak persisted in the negative domain, indicating the dominance of the right configuration. All these are in accordance with a good chiroptical stability, as already indicated by POM, indicating their safe use as column filling for enantiomeric separation.

4. Conclusions

Chiral hydrogels were successfully obtained from chitosan and betulinic aldehyde, under the effect of ultrasonic radiation. By FTIR, CD spectroscopy and POM it was demonstrated that the hydrogelation mechanism based on the imination reaction followed by the supramolecular chiral organization of the betulinic skeleton, either bonded to chitosan or unreacted, under ultrasonication effect. The hydrogels presented chiral supramolecular organization and porous morphology, features proper for filling materials of the chromatography columns for enantiomeric separations. Moreover, by swelling in media from acidic to basic pH they completely rehydrated and preserved the chiroptical properties, while being stable, over 3 months. All these characteristics recommend these hydrogels for use in medical domain as filling materials in enantiometric separations.

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