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*Professor Eugen Segal (1933-2013)*

# IMINO-CHITOSAN DERIVATIVES. SYNTHETIC PATHWAY AND PROPERTIES

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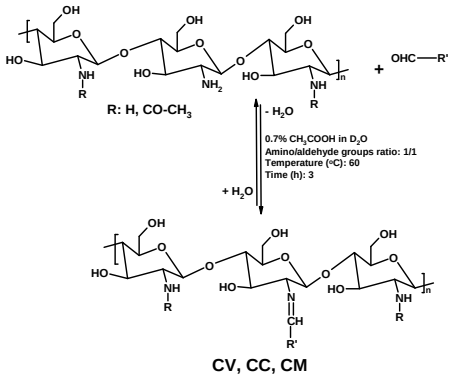
Three chitosan based biopolymers have been synthesized by condensation of three naturally occurring aldehydes with amine groups of chitosan yielding Schiff base modified chitosan. Since literature data lack or are poor in detailed experimental data on the chemistry involved in the preparation of chitosan Schiff bases, special attention was directed to enhance knowledge on this subject. Five different spectral methods, in solution, hydrogel and solid state have been employed with this aim. It was concluded that imine linkages are formed on chitosan chains while water leaves the reaction system.




## INTRODUCTION

Chitosan is a challenging biopolymer that attracts researchers' interest due to its outstanding biological properties: biocompatibility, nontoxicity, nonantigenicity, haemostatic, antimicrobial, fungistatic, spermicidal, central nervous system depressant, immunoadjuvant and antitumor activity, the ability to improve wound healing or clot blood, the ability to absorb liquids and to form protective films and coatings, selective binding of acidic liquids, thereby lowering serum cholesterol levels, accelerating bone formation and ability to act as matrix for obtaining advanced biocomposites.[1-4] Labeled by many natural product suppliers as "too good to be true", chitosan is commercialized for preventing plenty diseases, thus insuring a better quality of a longer life. However, specific scientific studies indicate that chitosan properties are not strong enough to allow its use as a drug, but mainly as a food supplement. Yet, its unique properties make chitosan an excellent candidate for the development of new biomaterials. To improve its characteristics or to

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use it as a drug carrier for various administration routes, many chitosan modification pathways have been explored. Among them, the reaction of chitosan amino groups with aldehydes bearing Schiff base derivatives received much attention in the recent decades, not only because Schiff base bonds emancipate amino groups from forming H-bonding networks and enhance chitosan solubility but also because it represents a pathway yielding new biopolymers with improved properties.[5-9] Although many papers report Schiff base derivatives (CSB) of chitosan, no data on the chemistry and yield of CSB derivatives have been published so far. This is a quite important aspect, taking into account the reversibility of imine formation in water, especially in acidic solutions, in which chitosan can be dissolved.

Taking all these into account, this paper is particularly addressing the yields of preparing three different imino-chitosan biopolymers with potential biomedical applications. To reach this target, three naturally occurring aldehydes extracted from natural oils, i.e. vanillin, cinnamaldehyde and menthone were reacted with chitosan in aqueous solution. The aldehydes are flavoring compounds and possess intrinsic antimicrobial, antifungic, antitumoral and so on[10-13] biological properties, creating the possibility of obtaining new chitosan based biopolymers with improved properties.

## RESULTS AND DISCUSSION

Three imino-chitosan biopolymers (**CV**, **CC**, **CM**) were obtained by reacting vanillin (**V**), cinnamaldehyde (**C**) and menthone (**M**) with chitosan in aqueous solution (Scheme 1), by an acid condensation reaction.[14,15] To reach a high imino yield, the solution was slowly evaporated in air to give free standing films. As cinnamaldehyde-imino-chitosan (**CC**) formed a hydrogel, it was lyophilized to obtain a xerogel.

Reaction evolution was followed by FTIR and NMR spectroscopy.

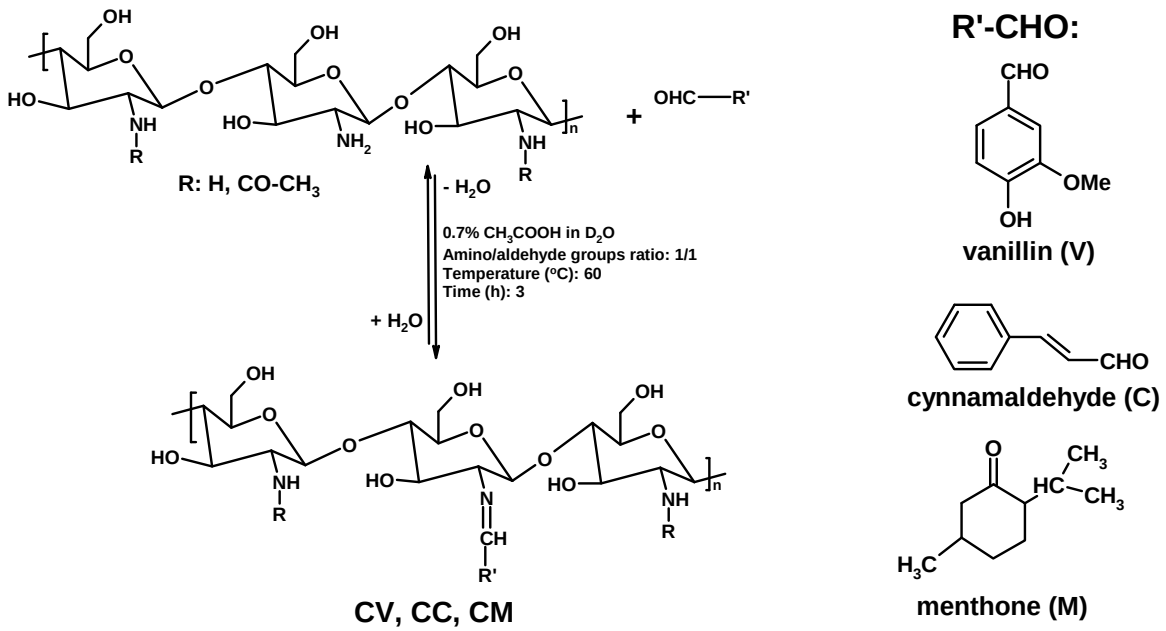
**$^1$H-NMR in solution** and **HRMAS in hydrogel** spectra are characterized by very intense peaks into the aliphatic region, belonging to chitosan protons,[16] while the peaks in the aromatic region – belonging to aromatic aldehyde residuum and to the newly formed imine proton – can be observed only by highly expanding the spectra. The vanillin/chitosan Schiff base derivative is given as an example in Fig. 1. Considering the integral of chemical shift of H2 as a measure of the amino groups of chitosan, the conversion of amino groups into imine units has been calculated, using the equation $\eta_{sol}=(A_{CH=N})/(A_{H2}*0.85)*100$, where $A_{CH=N}$ is the area of the imine proton peak, $A_{H2}$ corresponds to the H2 from the chitosan structure and 0.85 reflects the degree of deacetylation. While solutions of vanillin-imino-chitosan (**CV**) and menthone-imino-chitosan (**CM**) show small conversion degrees of amino groups into imine linkages, an amazing increase of the imine yield was observed for cinnamaldehyde-imino-chitosan (**CC**) hydrogel (Table 1).

The **FTIR spectra** of the obtained CSDs biopolymers very clearly indicate the formation of Schiff base linkages by the appearance of a peak in the 1630 – 1640 ppm spectral range, peak characteristic to imine unit vibration.[15,16] On the other hand, the deformation band characteristic to the N-H linkage into chitosan (1560 $cm^{-1}$) decreases in intensity and almost disappears in CSDs biopolymers spectra, indicating amino groups consumption during the condensation reaction. Moreover, the peaks characteristic to the aldehyde groups around 1760 $cm^{-1}$ are also missing (Fig. 2). The FTIR spectra of the CSDs containing double C=C bonds (aromatic or aliphatic ones) show their more or less well defined specific bands around 1602 and 1517 $cm^{-1}$. The peak attributed to the imino linkage is more intense for the vanillin and cinnamaldehyde based biopolymers and less intense for the menthone containing one, this suggesting a higher yield for the first two.

The FTIR data are opposite to the solution $^1$H-NMR ones. While solution $^1$H-NMR shows unreacted aldehyde and low content of imine linkages, the FTIR spectra in solid state indicate the absence of aldehyde and the presence of imine linkages. To further clarify the evolution of the reaction, $^1$H-NMR and $^{13}$C-NMR spectra in solid state were registered for the CSD biopolymers obtained by reaction in solution and for the reagent mixture, too.

**$^1$H-NMR spectra in solid state** show a large peak ranged between 0 and 10 ppm with a maxim in the aliphatic region, at about 3.5 ppm, corresponding to chitosan protons (Fig. 3). The grinded mixture of reagents (Fig. 3a) shows a well defined peak at 9.00 ppm, corresponding to the chemical shifting of the C<u>H</u>O- proton in free aldehyde and a shoulder around 7.5 ppm, corresponding to the aromatic protons. On the other hand, in the biopolymer spectra (Fig. 3b) the peak assigned to aldehyde proton is missing,

while a new shoulder, attributed to the new formed imino-chitosan proton, appears around 8 ppm. This indicates the consumption of the aldehyde and the formation of the new imine linkage on chitosan when reagents are mixed together in aqueous solution.




Scheme 1 – Preparation of chitosan Schiff base biopolymers.

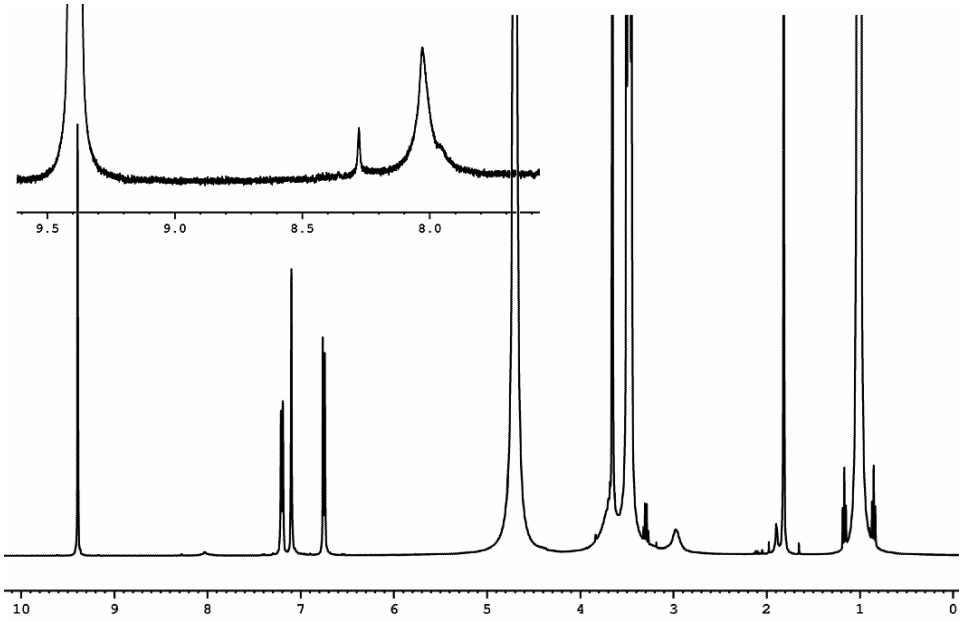


Fig. 1 – $^{1}$H-NMR spectrum of vanillin-imino-chitosan in acidic $D_2O/(CD_3)_2CO$.

*Table 1*

$^{1}$H-NMR data of Schiff base derivative samples

| Code | $\delta_{CH=N}$ | $\delta_{CH=N}$ | $\eta_{sol}$/% | $\eta_{solid}$/% |
|---|---|---|---|---|
| **CV** | 8.05 | 166 | 11.9 | 78.0 |
| **CC** | 8.10 | 168 | 49.1 | 90.1 |
| **CM** | 8.45 | 165 | 1.0 | 25.0 |

$\eta_{sol}$ – yield of imine in solution, as calculated from $^{1}$H-NMR or HRMAS spectra; $\eta_{solid}$ – yield of imine forming in solid state, calculated from $^{13}$C-NMR solid-state spectra

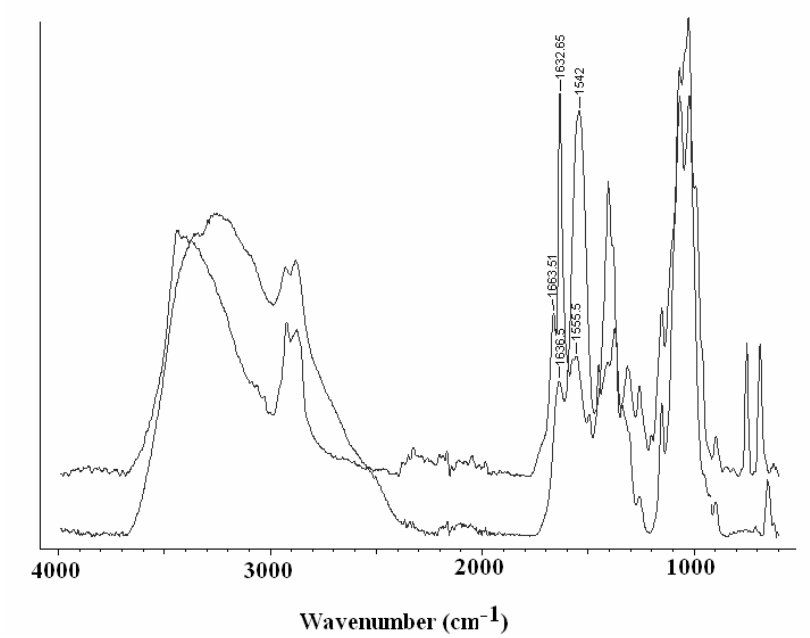



Fig. 2 – Chitosan and cinnamaldehyde-imino-chitosan biopolymer spectra, on dry films (ATR method).

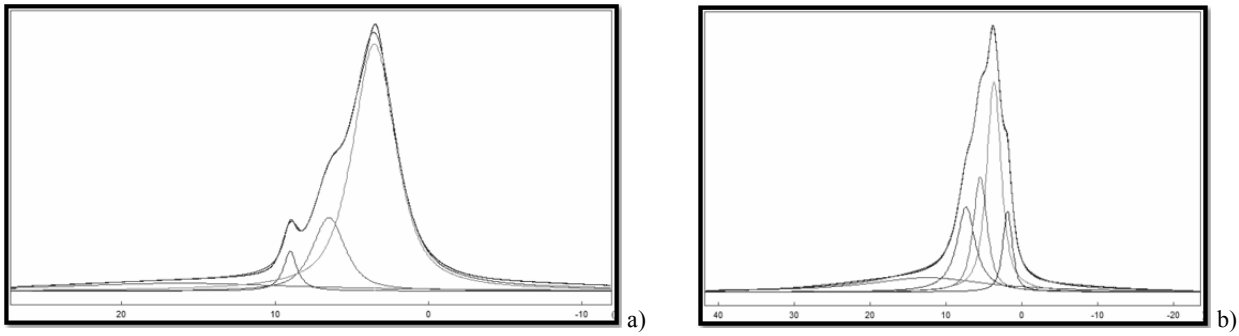



Fig. 3 – $^{1}$H-NMR spectra, in solid state, of a) chitosan/vanillin mixture obtained by grinding in an agate mortar and b) chitosan/vanillin Schiff base derivative obtained by reaction in water.

The structure of the Schiff base derivatives was further investigated by **solid state $^{13}$C-NMR spectroscopy**. For accurate conclusions $^{13}$C-NMR spectra were recorded for both the studied biopolymers and their reagent mixtures. In the biopolymer spectra, a sharp peak, characteristic to the carbon involved into the imine bond, appears around 160 ppm.[15] The peaks belonging to the double linked carbon atoms into aromatic or unsaturated substituent are shown into the 110 – 150 ppm range while the peaks characteristic to the aliphatic carbons in chitosan and aldehyde residue lay between 10 and 110 ppm.[18] The weak peak around 175 ppm belongs to the carbon from the amide group. The peak characteristic to the aldehyde carbon (around 200 ppm) couldn't be observed in the biopolymer spectra but it was obvious in the reagent mixture, indicating the aldehyde consumption during water removing. The $^{13}$C-NMR in solid state of the studied Schiff base biopolymers and vanillin/chitosan mixture are presented in Fig. 4. The chemical shifts of the Schiff base carbon for all Schiff base derivatives are enclosed in Table 1.

The conversion degree of the amino groups on chitosan into imine bonds have been calculated from the $^{13}$C-NMR spectra in solid state, in a manner similar to that applied to the $^{1}$H-NMR spectra in solution.[7] The conversion degree was calculated as the ratio between the peak area of the imine C and the C1 chitosan carbon (110 ppm), this area being the least affected by superposing with other peaks. The obtained data are enclosed in Table 1.

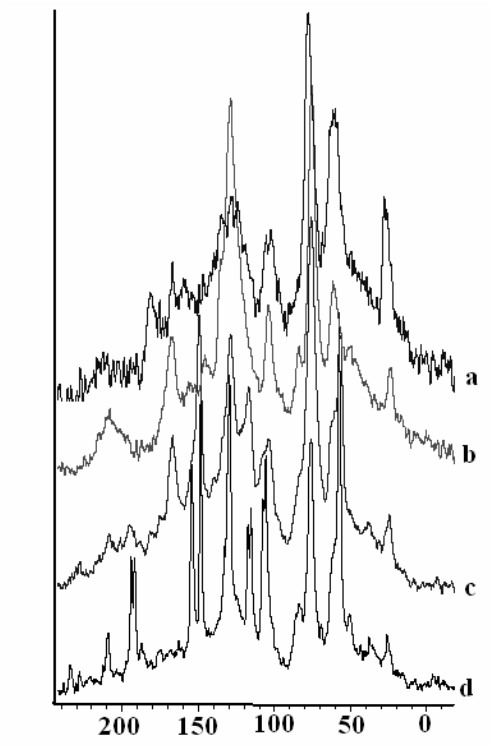



Fig. 4 – $^{13}$C-NMR spectra of the biopolymers (a: CM, b: CC, c: CV) and vanillin/chitosan mixture (d).

Comparing the imine forming yield in solution ($^{1}$H-NMR) with the one in solid state ($^{13}$C-NMR), a drastic increment can be observed. Moreover, the imine forming yield is significantly higher for the cinnamaldehyde-imino-chitosan hydrogel as compared to the vanillin-imino-chitosan and menthone-imino-chitosan solutions. This indicates that water plays an important role in imino-chitosan formation, the reaction equilibrium being moved to the products along with water removing from the reaction system, either by phase separation of the new biopolymer through hydrogel formation[19] or by take off.[20]

To see the **supramolecular arrangement of the imino-chitosan biopolymers**, **wide angle X-ray diffraction** on the biopolymer films has been performed. As can be seen in Fig. 5, the X-ray pattern of the menthone-imine-chitosan shows a broad halo which indicates an amorphous state, probably due to small conversion of the amine groups into imine linkages. The other two biopolymers exhibit X-ray patterns similar to the mesomorphic azomethines of low or high molecular weight, with layered structure.[21,22] They present a sharp peak in the small angle domain corresponding to a interlayer distance of 18.4 Å (CC) and 13.4 Å (CV), respectively, values which agree well with a interdigitated layering as simulated by HyperChem. In the wide angle domain, the samples present a broader reflection, which can be interpreted as a result of the polydispersity of the intermolecular distances around 4.5 Å. In the case of cinnamaldehyde, the intermolecular distance values range between 6.4 and 3.0 Å, while in the case of vanillin in the 5.1 – 3.2 Å range. This can be explained by the presence of aliphatic carbons in the cynamaldehyde structure which allows different conformations and as a consequence a larger intermolecular distance polydispersity. The medium angle peak around 11.2 $^{o}$ (7.8 Å) and 13.3 $^{o}$ (6.7 Å), respectively, corresponds to chitosan-chitosan interchain distances, their different values reflecting the influence of the different formed imines, too.

To conclude, the X-ray diffraction data indicate a self-organizing of the imino-chitosan biopolymers in lamellar structures with well-defined layering, with less order inside the layers, somehow similar to the smectic mesophase arrangement.[23,24]

As cinnamaldehyde-imino-chitosan has been obtained as hydrogel and xerogel, respectively, its **morphology** has been further monitored by **scanning electron microscopy**. To see the influence of the reticulation degree on xerogel morphology, different ratios between the amino groups onto chitosan and aldehydes were used – 4/1, 3/1, 2/1, 1/1 –, giving CC41, CC31, CC21 and CC11 biopolymer hydrogels, respectively. As one can see in Fig. 6, the xerogel morphology drastically depends on the amine/aldehyde ratio. Thus, the pore diameter is bigger as the cinnamaldehyde content is smaller, and so is the reticulation degree. In the case of 1/1 amine-aldehyde molar ratio (CC11), the broken walls of the hydrogel can be seen, probably due to the high wall rigidity determined by the high content of the rigid cinnamaldenhyde units.

## Cinnamaldehyde-imino-chitosan as scaffold for bone regeneration

It is well known that chitosan hydrogels are promising materials as scaffolds for bone regeneration. Since chitosan-cinnamaldehyde biopolymer was obtained as a hydrogel, its ability to act as a matrix for osteoblasts growth has been checked according to a published procedure.[25] The biopolymer scaffolds were obtained by freeze-drying technique, which resulted in the already given (Fig. 6) microporous morphological structure. Preliminary tests showed the best osteoblasts cell viability in the case of CC31 xerogel, this indicating a close dependence on xerogel porosity (Fig. 7). The cell viability seems to be lower for the xerogels with too small pores –

unable to allow cell penetration in the bulk xerogel, or too large pores – unable to insure their good cell filling. Further studies on using these xerogels as matrices for bone regeneration are in progress.

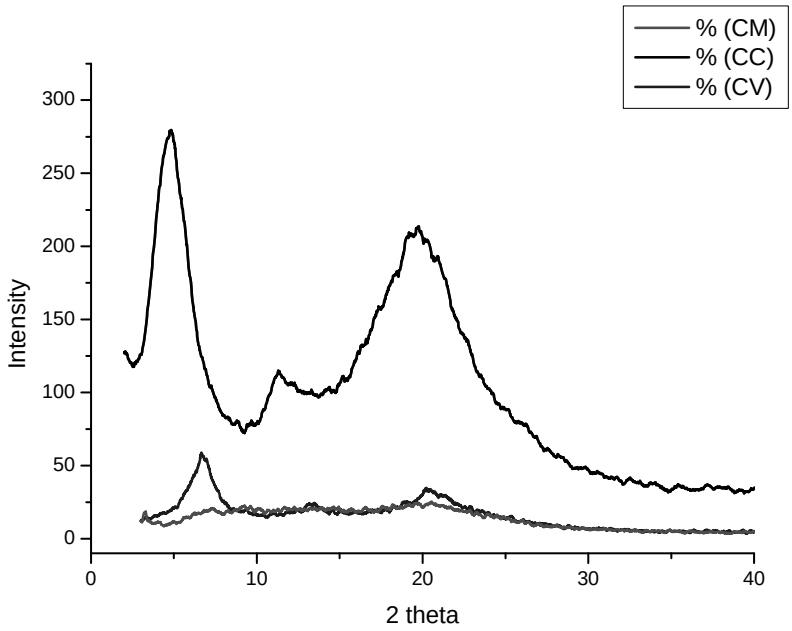



Fig. 5 – XRD pattern of the imino-chitosan derivatives.

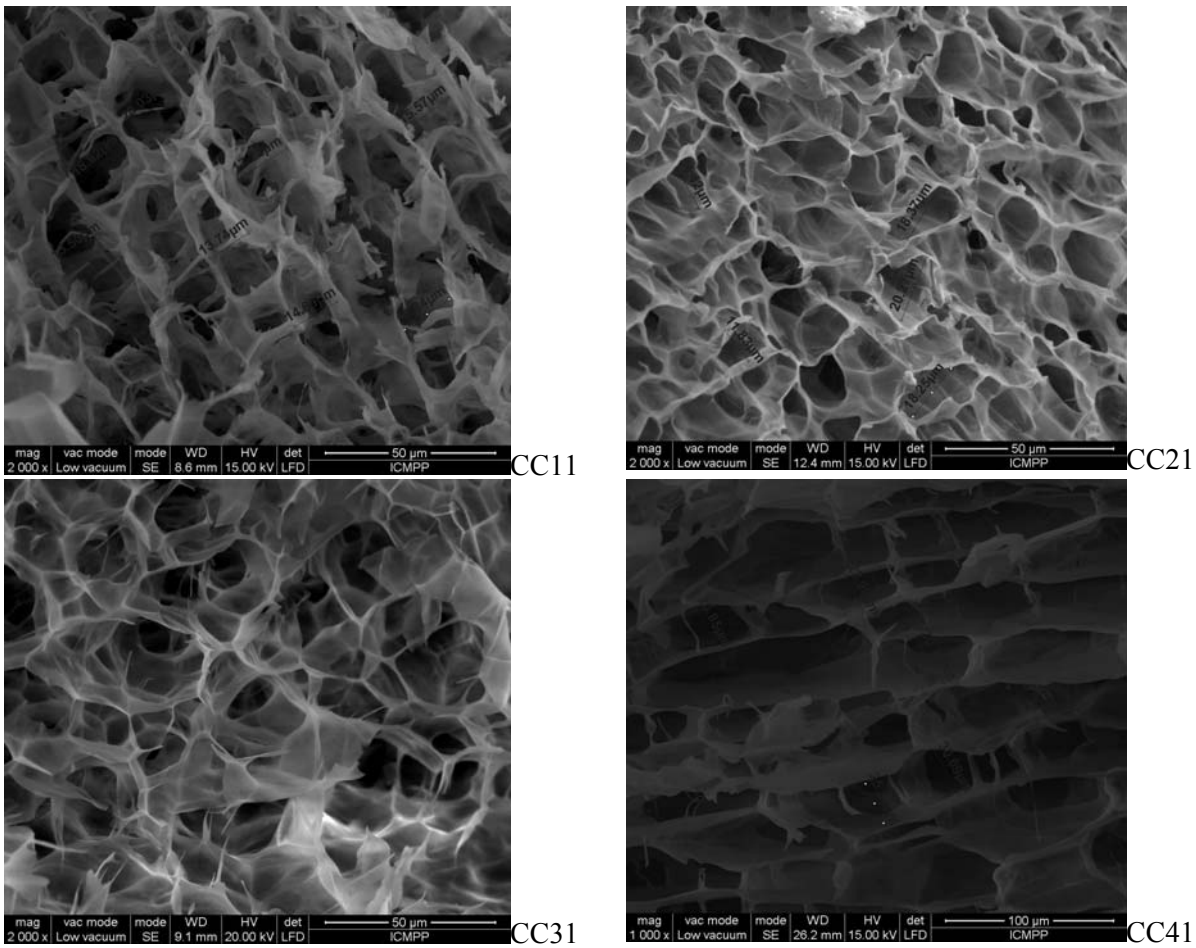



Fig. 6 – SEM microphotographs of the cinnamaldehyde-chitosan hydrogels.

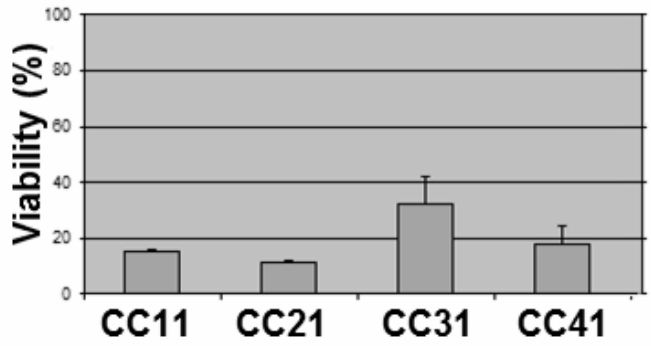



Fig. 7 – Cell viability of the cinnamaldehyde-imino-chitosan xerogels.

## EXPERIMENTAL

Low molecular weight chitosan, menthone, vanillin and cinnamaldehyde were purchased from Sigma-Aldrich and were dried at 50 $^{o}$C, overnight, before use. The molecular weight of chitosan was 125 kDa and its deacetylation degree (DA) was15%.[5]

### Synthesis

To a 2% solution of chitosan (0.05 g, 0.29 mmol glucosamine repeat units) in 0.7% acetic acid a 3% solution of aldehyde in acetone has been slowly added, under vigorous stirring at 50 $^{o}$C, during 3 hours. The reaction was performed into a two-necked flask equipped with a condenser and a magnetic stir bar, for a 1/1 amino/aldehyde groups molar ratio. To check reaction progress by H-NMR or HRMAS spectroscopy, the synthesis was performed using deuterated solvents.

While the color of the reaction mixture remained almost unchanged in the case of menthone, in the other two cases the color changed in beige for cinnamaldehyde and in deep yellow for vanillin, clearly indicating the obtaining of Schiff base. Cynnamaldehyde yielded a gel-like product.

After 3 hours reaction time, 3 mL of viscous solution was casted into a Petri dish, with a diameter of 3 cm, and allowed to dry. The obtained crude films were further dried in vacuum, at 70 $^{o}$C. By grinding with a pestle into an agate mortar, powders of different colors were obtained.

### Equipment

**ATR-FTIR spectra** were recorded on a FTIR Bruker Vertex 70 Spectrophotometer, by ATR technique. The **liquid state NMR spectra** were performed on a BRUKER Avance DRX 400 MHz spectrometer, at room temperature. The **HRMAS spectra** have been recorded on a Bruker Avance III 400 MHz spectrometer equipped with a 4 mm dual direct detection HRMAS probe with z-gradients. The samples were introduced in zirconium rotors and spun at 5 kHz. $D_2O$ was used for the lock and all spectra were recorded with presaturation of the water signal**.** **$^{13}$C-NMR solid-state** spectroscopy was conducted by single-contact 50.32 MHz $^{13}$C CP-MAS on a Bruker MSL CXP-200 spectrometer fitted with a Bruker-z32DR-MAS-DB probe. Powder samples were contained in a ceramic cylindrical rotor and spun at 4.5 KHz. Contact time for cross polarization was 2.5 ms and 1400 – 4000 scans were accumulated. Spectra were referenced indirectly to a zero value for tetramethylsilane (TMS). **Wide Angle X-Ray Diffraction** (WAXD) was performed on a Bruker D8 Avance diffractometer, using the Ni-filtered Cu-K$\alpha$ radiation ($\lambda$ = 0.1541 nm). Specimen cross-section of studied xerogels were viewed with a **field emission scanning electron microscope**, Scanning Electron Microscope SEM EDAX – Quanta 200, at an accelerated electron energy of 15 KeV. The obtained hydrogels were **flash frozen** in liquid nitrogen and lyophilized for 24 hours with a MartinChrist, Alpha 1-2LD freeze dryer system.

## CONCLUSIONS

Schiff base chitosan derivatives were synthesized by acid condensation reaction. The spectral analysis – $^{1}$H-NMR in solution, HRMAS in hydrogel, $^{1}$H-NMR in solid state, $^{13}$C-NMR in solid state and FTIR – proved that imine obtaining takes place during the water removing process and is facilitated by hydrogel formation. Cinnamaldehyde and vanillin gave biopolymers with high conversion degree of the amino groups into imine linkages, self-structured into a lamellar supramolecular structure, while menthone yielded a low conversion rate, giving amorphous biopolymers. The cinnamaldehyde based chitosan hydrogel shows promising potential as scaffold for bone regeneration.

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