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CINNAMYL-IMINE-CHITOSAN HYDROGELS. MORPHOLOGY CONTROL

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Abstract: The study deals with the exploration of the possibilities to control the morphology of cinnamyl-imine-chitosan hydrogels in view of their bio-application. Three series of hydrogels were synthetized from chitosan of three different molecular weights and cinnamaldehyde, varying the molar ratio between the amine groups on chitosan and aldehyde functional groups. The hydrogel morphology has been monitored by scanning electron microscopy. The variation of the hydrogel morphology as a function of chitosan molecular weight, crosslinking degree, and incubation conditions has been monitored. It was concluded that there are multiple possibilities of tuning the morphology of these hydrogels in function of the targeted application.

Keywords: chitosan; cinnamaldehyde; hydrogel; morphology

Introduction

Hydrogels are a class of materials with a large area of applications in domains of contemporary interest, such as agriculture, environment protection and biomedicine. Among hydrogels, those based on biopolymers are especially interesting in biomedicine, because they usually promote good biocompatibility and biodegradability. A biopolymer largely used for

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the preparation of hydrogels is the chitosan. Chitosan is obtained by deacetylation of chitin, a waste material extracted from crustaceans and various insects.[1] Many studies dedicated to the chitosan, revealed that it is biocompatible, nontoxic (its degradation products are known natural metabolites), nonantigenic, haemostatic, antimicrobial, fungistatic, spermicidal, central nervous system depressant, immunoadjuvant and present antitumor activity, the ability to improve wound healing or clot blood, the ability to form protective films and coatings, selective binding of acidic liquids, thereby lowering serum cholesterol levels and hypertension prevention, property to accelerates bone formation or environmental protection.[1-5] All these features transformed the chitosan into a desired material for bio-applications. One direction which attracted the researchers' attention was the exploiting of chitosan for preparing hydrogels. To do this, chitosan is crosslinked, usually with glutaraldehyde, by forming imine bonds.[1,6] The main issue encountered for the as prepared hydrogels was the toxicity of the glutaraldehyde which strongly limits their use in biomedicine.[7] To surpass this problem, our group developed a new strategy to crosslink chitosan with monoaldehydes, based on the principles of dynamic covalent chemistry.[8-15] It was demonstrated that using this strategy, biocompatible hydrogels could be successfully obtained, because the method allows using a large variety of monoaldehyde crossslinkers.[9,10,15]

One important feature of the hydrogels is their ability to absorb large amounts of water or biologic fluids, which prompt them to mimic the tissues, or to encapsulate large amounts of drug to create drug delivery systems.[16] The ability of swelling is close related to the morphology of the hydrogels, so that, many efforts were dedicated to control the hydrogel morphology and consequently to control the swelling. In this context, our

paper is dealing with the synthesis of three series of chitosan based hydrogels using cinnamaldehyde as crosslinker in various conditions, in order to tune the hydrogel morphology. Thus, it was varied the crosslinking degree, the molecular weight of the chitosan and the incubation conditions. The results indicated many pathways of controlling the hydrogel morphology.

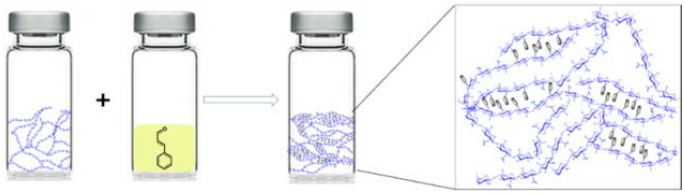
Experimental

Materials & Equipments

Cinnamaldehyde (≥93%) have been purchased from Aldrich and used without further purification. Chitosan of three different molecular weights: low molecular (125 kDa, DA=13.5%), medium molecular weight (1503 kDa, DA=20.5%) and high molecular weight (2259 kDa, DA=22.83%) has been purchased from Aldrich and used without any further purification steps.

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 comprised between 10 and 20 kV. The average pore size was estimated from four randomly chosen images. Since some images show pores with irregular shapes, the pore size was defined as the longest length that could be drawn in each pore.

Synthesis: The hydrogels were prepared by acid condensation reaction of chitosan with cinnamaldehyde, by a protocol already reported.[8] Shortly, their synthesis was performed as follows.



Scheme 1. General representation of the hydrogels obtaining.

A 2% chitosan solution in acidic water (acetic acid in water of pH = 6.2) was warmed up to 50 ^{o}C. In parallel, a 3 % cinamaldehyde solution in ethylic alcohol has been prepared and also warmed up to 50 ^{o}C. Then, the warm cinnamaldehyde solution has been slowly dropped upon the chitosan solution, under vigorous stirring, till the viscous solution has been transformed into a soft, clear solid, which passed the test of inverted tube. The hydrogels were incubated 3 days at 25 ^{o}C in order to allow the ethanol to evaporate. Further, they were frozen in nitrogen liquid and lyophilised for 24 hours with a MartinChrist, Alpha 1-2LD freeze dryer system, obtaining xerogels for SEM analysis. They were stored into sealed vials and dried under vacuum before analysis.

Three different samples of chitosan with different molecular weight were used for hydrogel obtaining. The molecular weight of chitosan and cinnamaldehyde were calculated to correspond to 4 different molar ratios of the amine and aldehyde groups: 1/1; 2/1; 3/1 and 4/1. The obtained hydrogels were noted taking into consideration the molecular weight of chitosan and the molar ratio of amine/aldehyde groups (Table 1).

Table 1. The codes of the studied hydrogels.

Chitosan / **Codes**	**Molar ratio**[1]			
	1/1	2/1	3/1	4/1
Low molecular weight	**L1**	**L2**	**L3**	**L4**
Medium molecular weight	**M1**	**M2**	**M3**	**M4**
High molecular weight	**H1**	**H2**	**H3**	**H4**

[1]Calculated as molar ratio of glucosamine repeat units to cinnamaldehyde

The HP-MAS and FTIR spectra and X-ray diffraction which proved the hydrogelation mechanism has been reported elsewhere.[8]

Results and Discussions

Three series of hydrogels based on chitosan and cinnamaldehyde were synthetized by imination reaction followed by the self-ordering of the newly formed imine units into clusters playing the role of chitosan crosslinkers.[8-15] Scheme 1 represents the obtaining of the studied hydrogels, where the chitosan chains are depicted in blue, and the imine linkage with the aldehyde residue are represented by the black rectangles. Each series was prepared using chitosan of different molecular weight (low, medium and high, respectively) and different molar ratio of the amine and aldehyde groups (Table 1). Thus were prepared twelve hydrogels differing in chitosan molecular weight and crosslinking degree, features which allowed us to investigate the influence of these parameters on the hydrogel morphology.

Firstly, the influence of the molecular weight of chitosan on the hydrogels morphology was evaluated, by investigation of the SEM images on xerogels. As can be seen in figure 1, all the hydrogels presented a porous morphology, apparently with interconnected pores. In the case of chitosan of high molecular weight, the corresponding hydrogels showed an irregular morphology, with pores of various dimensions, which diameter spans from 10 to 50 μm.

The longitudinal cross-section revealed channels with thick walls, here and there decorated with holes. Decreasing the molecular weight of chitosan, the morphology became more regular. In longitudinal cross-section, a channelled morphology was observed too, but with a more holey structure of the walls. The medium molecular weight chitosan generated

pores with a medium diameter of 20 μm, while the low molecular weight chitosan provided pores with diameter around 40 μm. The different dimension of the pores was assigned to the variation in viscosity of the system, related to the molecular weight of chitosan.[17]

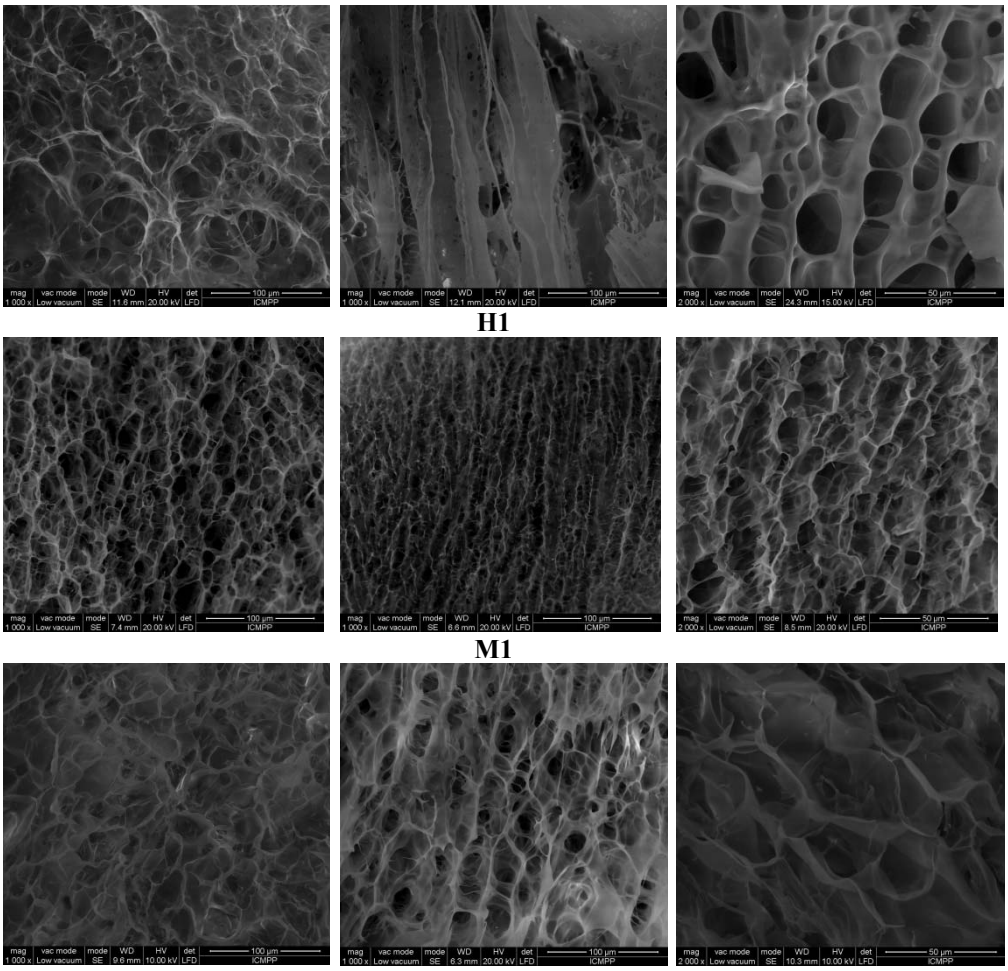


Figure 1. SEM microphotographs of the xerogels.

It is well documented in literature that the crosslinking degree strongly influences the morphology of the hydrogels.[1-14,18] To see how this parameter influenced the morphology in the particular case of cinnamyl-

imine-chitosan hydrogels, SEM images of the corresponding xerogels were acquired at high magnification. Representative images were given for the series **L1**-**L4** (Figure 2). As can be seen, the dimension of the pores was well correlated with the crosslinking degree. Except the xerogel with the highest crosslinking degree (**L1**), the others showed an increasing trend of the pore diameter along with the decreasing of the crosslinking degree. No evidence of a significant difference in the pore thickness was observed, indicating no influence of the crosslinking degree on this parameter.

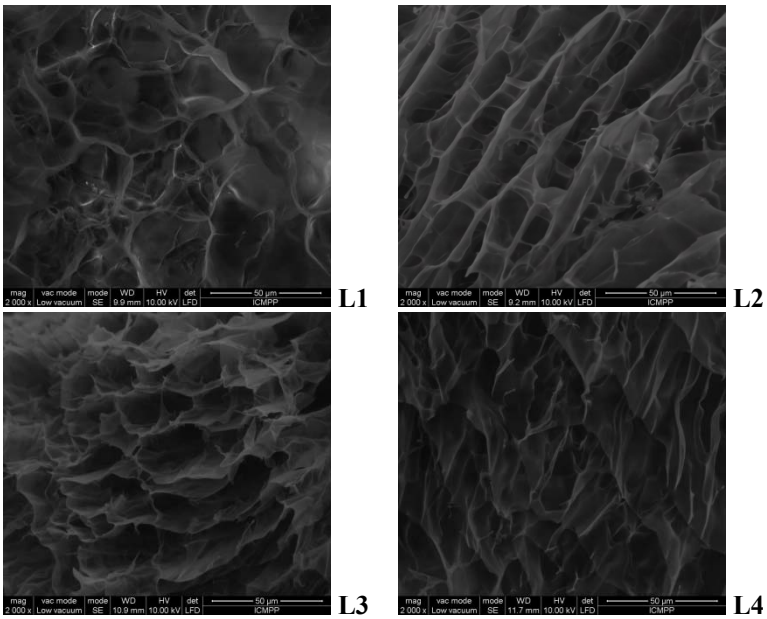


Figure 2. SEM images of the **L1**-**L4** xerogels.

Further, the morphological changes were monitored as a function of the incubation time. This investigation has been initiated, starting from the observation that hydrogelation of chitosan with cinnamaldehyde occurs slowly during the time, due to the reversibility of the imination reaction and self-ordering process.[8-14] It was demonstrated that in the particular case of cinnamyl-imine-chitosan system, the imination equilibrium was shifted to

the products during one-week.[8] Consequently, the morphology of the hydrogels incubated one-week before lyophilisation was tested, when drastic changes were noted. Representative SEM images of the xerogels after one-week incubation time (noted **L1*** – **L4***) were given in figure 3.

The drastic changes of the hydrogel morphology consisted in principal into an enhanced organization of the pores, correlated with an advanced structural organization at nanometric level. As can be seen, the morphology became more regular regarding the pore dimension and polydispersity of their diameter. In the case of the xerogel with the highest crosslinking degree, the pore walls appeared very cracked, fact assigned to the high rigidity of the stiffened chains grafted with rigid cinnamyl-imine units, which promoted cracks during freezing into liquid nitrogen. On the other hand, for a medium crosslinking degree (**L2***) the sample reveals a very straight morphology, reminiscent of honey-combs; the pores have almost hexagonal shapes. This structured morphology indicates a highly ordering at nanometric level, possible with a hexagonal structure too.[19] It should be highlighted that the highly organized morphology is beneficial for bio-applications, especially for tissue engineering.[20] The pore dimension is around 20 μm, size which fit well that of the ingrowth of hepatocytes and for the adult mammalian skin regeneration. Also, in the case of the hydrogels based on chitosan with high molecular weight, the pores dimensions up to 50 μm are appropriate for the osteoid ingrowth applications.[21]

An even more interesting morphology has been recorded for the xerogel sample with the lowest crosslinking degree, **L4***. In that case, there were observed pores with branches rising from the walls. The unexpected morphology has been explained by the presence of the large segments of hydrophilic, uncrosslinked, chitosan chains, which entangled together. The occurrence of these branches on the pore walls increases the active surface of the hydrogels, and can play the role of anchors for cells in tissue

engineering. It should be highlighted here that no such hydrogel morphology has been reported so far.

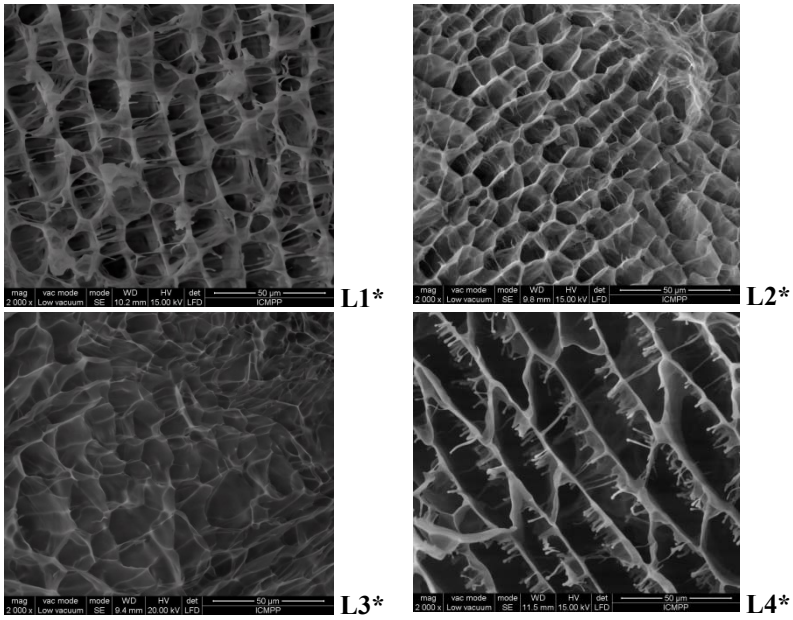
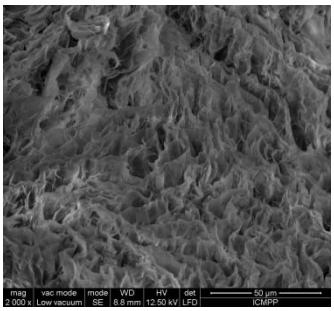
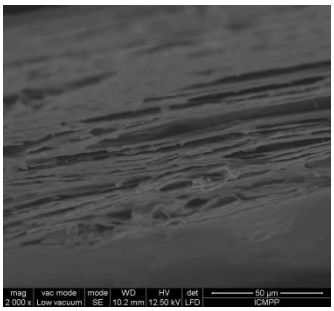


Figure 3. SEM images of **L1**-**L4** xerogels incubated one week

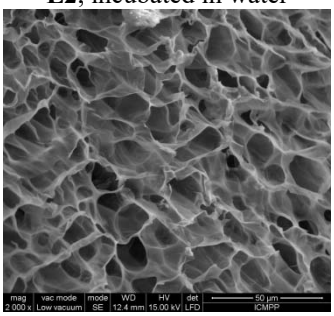
In order to create more parameters to control the hydrogels morphology, the morphological changes were monitored after the xerogels incubation into three different solvents: water, acetonitrile and ethanol, for one week, followed by free drying into atmosphere. Representative images were given in figure 4. As can be seen, drastic morphological changes took place in each case. When the xerogels were incubated in water, they shrunk reaching pores around 5 μm, the optimum pore size for neovascularization.[21] In the cross-section it could be observed a layered morphology at micrometric level, mainly consisting in the tightening of the walls of the channels. Less drastic changes were approached by incubation in organic solvents as ethanol and acetonitrile. The pore walls were obviously shrunk narrowing their dimension, but they kept the overall shape.[22, 23]



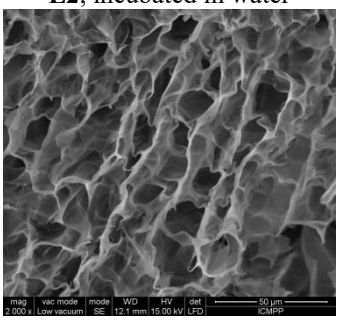
L2, incubated in water



L2, incubated in water



L2, incubated in acetonitrile



L2, incubated in ethanol

Figure 4. SEM images of the **L2** xerogel after incubation one week in different solvents.

Conclusions

Three series of hydrogels, based on chitosan of three different molecular weights and cinnamaldehyde were prepared by varying the crosslinking degree. Their morphology has been studied by scanning electron microscopy, either as prepared, or when applying different treatments, such as different time incubation in air or incubation in different solvent media. It was concluded that the morphology of the chitosan based hydrogels crosslinked with monoaldehydes can be strictly controlled function of the targeted application.

Acknowledgements

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