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Corresponding Author:	María Elisa Martínez-Barbosa Universidad de Sonora HERMOSILLO, Sonora MEXICO
Corresponding Author's Institution:	Universidad de Sonora
Corresponding Author E-Mail:	memartinez@polimeros.uson.mx
Order of Authors:	María Dolores Figueroa-Pizano
	Itziar Vélaz
	María Elisa Martínez-Barbosa
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TITLE:

A Freeze-Thawing Method to Prepare Chitosan-Poly(vinyl alcohol) Hydrogels Without Crosslinking Agents and Diflunisal Release Studies

AUTHORS AND AFFILIATIONS:
M. D. Figueroa-Pizano[1], I. Vélaz[2*], M. E. Martínez-Barbosa[1*]

[1]Department of Polymers and Materials, University of Sonora, Hermosillo, Sonora, Mexico
[2]Department of Chemistry, Faculty of Sciences, University of Navarra, C/Irunlarrea s/n, Pamplona, Navarra, Spain

Corresponding Author:
M. E. Martínez-Barbosa
elisa.martinez@unison.mx

I. Vélaz
itzvelaz@unav.es

Email Addresses of Co-authors:
mariad.figueroap@a2004.uson.mx

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SUMMARY:
The freezing-thawing method is used to produce chitosan-poly(vinyl alcohol) hydrogels without crosslinking agents. For this method, it is important to consider the freezing conditions (temperature, number of cycles) and polymer ratio, which can affect the properties and applications of the obtained hydrogels.

ABSTRACT:
Chitosan-poly(vinyl alcohol) hydrogels can be produced by the freeze-thawing method without using toxic crosslinking agents. The applications of these systems are limited by their characteristics (e.g., porosity, flexibility, swelling capacity, drug loading and drug release capacity), which depend on the freezing conditions and the kind and ratio of polymers. This protocol describes how to prepare hydrogels from chitosan and poly(vinyl alcohol) at 50/50 w/w % of polymer composition and varying the freezing temperature (-4 °C, -20 °C, -80 °C) and freeze-thawing cycles (4, 5, 6 freezing cycles). FT-IR spectra, SEM micrograph and porosimetry data of hydrogels were obtained. Also, the swelling capacity and drug loading and release of diflunisal were assessed. Results from SEM micrographs and porosimetry show that the pore size decreases, while the porosity increases at lower temperatures. The swelling percentage was higher at the minor freezing temperature. The release of diflunisal from the hydrogels has

been studied. All the networks maintain the drug release for 30 h and it has been observed that a simple diffusion mechanism regulates the diflunisal release according to Korsmeyer-Peppas and Higuchi models.

INTRODUCTION:

Recently, hydrogels have attracted great interest in the biomedical field because they are three-dimensional networks with high water content and are soft and flexible, so they can mimic natural tissues easily[1]. Also, they do not dissolve in aqueous medium at physiological temperature and pH but present a large swelling[2]. Hydrogels can act as tissue engineering scaffolds, hygiene products, contact lenses, and wound dressings; because they can trap and release active compounds and drugs, they are used as drug delivery systems[3]. Depending on their application, hydrogels can be made from natural or synthetic polymers, or a combination of both, in order to obtain the best characteristics[4].

The properties of hydrogels are a consequence of many physical and chemical factors. At the physical level, their structure and morphology depend on their porosity, pore size and pore distribution[5]. At the chemical and molecular level, the polymer type, the hydrophilic group content in the polymer chain, the crosslinking point type, and the cross-linking density are the factors that determine the swelling capacity and the mechanical properties[6,7].

According to the type of crosslinking agent used to form the network, the hydrogels are classified as chemical hydrogels or physical hydrogels. Chemical hydrogels are joined by covalent interactions between their chains, which are formed through UV and gamma irradiation or using a crosslinking agent[7,8]. Chemical hydrogels usually are strong and resistant but, generally, the crosslinking agent is toxic to the cells and its removal is difficult, so its application is limited. On the other hand, physical hydrogels form by the connection of the polymer chains through non-covalent interactions, avoiding the use of crosslinking agents[4,9]. The main non-covalent interactions in the network are hydrophobic interactions, electrostatic forces, complementary and hydrogen bounds[7].

Poly(vinyl alcohol) (PVA, **Figure 1a**) is a synthetic and water-soluble polymer with excellent mechanical performance and biocompatibility that can from crosslink agent-free hydrogels through the freeze-thawing method[10,11]. This polymer has the capacity to form concentrated zones of hydrogen bonds between -OH groups of their chains (crystalline zones) when they are freezing[12]. These crystalline zones act as crosslinking points in the network, and they are promoted by two events: the approaching of the polymer chains when the crystal water expands and the PVA conformational changes from isotactic to syndiotactic PVA during freeze[13]. Because of the freeze-drying, the water crystals are sublimated, leaving void spaces that are the pores in the hydrogel[14]. To obtain hydrogels with better properties, PVA can been easily combined with other polymers.

In that sense, chitosan constitutes an option as it is the only biopolymer from natural sources with positive charges. It is obtained by the deacetylation of chitin and it is composed of random combinations of β-1,4 linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine

(acetylated unit)[15,16] (**Figure 1b**). Chitosan is biodegradable by human enzymes and it is biocompatible. Also, by its cationic nature, it can interact with the negative charge of the cell surface, and this property has been associated with its antimicrobial activity[17]. This polymer is easy to process; however, their mechanical properties are not sufficient and some materials have been added to form complexes with better characteristics.

Considering specific characteristics of chitosan and PVA, the successful manufacture of hydrogels has been reached by the freeze-thawing method[2,18] to avoid the use of toxic crosslinking agents. In chitosan-PVA hydrogels, the crystalline zones of PVA are also formed, and chitosan chains are interpenetrated and form simple hydrogen bonds with $–NH_2$ groups and –OH groups in PVA. The final chitosan-PVA hydrogel is mechanically stable, with high rates of swelling and low toxicity, and with antibacterial effect[18]. However, depending on the freezing conditions used in the preparation (temperature, time and number of cycles), the final characteristics may change. Some studies report that increasing the number of freezing cycles decreases the swelling degree and increases the tensile strength[19,20]. In order to strengthen the network, other agents such as gamma and UV radiation and chemical crosslinkers have been used additionally after the freeze-thawed preparation[21–23]. Hydrogels with a higher chitosan proportion have a more porous network and high swelling capacity but less strength and thermal stability. In this context, it is important to consider the preparation conditions to obtain suitable hydrogels for their target application.

The purpose of this work is to present in detail how the freezing conditions (temperature of freezing and number of cycles) affect the final characteristics of CS-PVA hydrogels. FT-IR spectra, morphological and porosity characteristics and swelling capacity were evaluated, as well as drug loading and release capacity. In the release studies, diflunisal (**Figure 1-c**) was used as model drug, due to its size suitable to the hydrogel structure.

PROTOCOL:

1. Preparation of chitosan-PVA hydrogels

1.1. Prepare 2% (w/w) chitosan and 10% (w/w) PVA solutions. Dissolve 0.2 g of chitosan in 10 mL of 0.1 M CH_3COOH solution (previously filtered) at room temperature and maintain continuous mechanical stirring overnight. Dissolve 1 g of PVA in 10 mL of distilled water and stir at 80 °C for 1 h.

1.2. Mix both solutions 1:1 using a magnetic stirrer until they are homogeneous at room temperature, and pour the mixtures on Petri dishes. Leave the samples for 2 h at atmospheric pressure to degas.

1.3. Freeze the hydrogels at -4 °C, -20 °C or -80 °C for 20 h and 4 cycles (samples CP4-4, CP4-20 and CP4-80, respectively). Freeze another hydrogel at -80 °C for 20 h using 5 or 6 freezing cycles (samples CP5-80 and CP6-80). After the third freezing cycle, wash the hydrogels with

deionized water. At the end, freeze-dry the hydrogels at -46 °C for 48 h and store for further characterization (methodology adapted from[2]).

2. FT-IR characterization

2.1 Place a little piece (1 mm x 2 mm) of hydrogel in the FT-IR spectrometer in ATR mode. Take the FT-IR spectra from 4000 to 600 cm^{-1} (2 cm^{-1} of resolution and average of 32 scans).

3. Swelling assays

3.1 Cut out discs (13 mm in diameter and 10 mm in height) from the hydrogel and weigh them. Incubate the discs in 50 mL of deionized water with shaking at 25 °C. Repeat three times.

3.2 Every 30 min remove the sample from the medium, blotter to eliminate the excess of water, and weigh. Calculate the swelling degree using the equation 1 and calculate the equilibrium state of swelling, q, at 24 h using the equation 2.

$$SD(\%) = \left[\left(\frac{Ws-Wd}{Wd}\right)\right] * 100 \ (Eq.1)$$

Where Wd is the weight of the dry hydrogel and Ws is the weight of the wet hydrogel.

$$q = \frac{Ws}{Wd} \ (Eq.2)$$

4. Electronic Microscopy

4.1 Cover a little piece of hydrogel with a thin gold layer (30 s and 10 mA) in a sputter coater.

4.2 Put the sample in a scanning electron microscope (SEM). Analyze the samples under vacuum at 20 kV and take the images with a 500x and 1500x magnification.

5. Porosimetry

5.1 Place discs 15 mm in diameter weighing around 0.26 g into the penetrometer (a solid penetrometer, having a bulk volume of 0.3660 mL and 5.7831 mL of stem volume). Analyze the porosity and pore size by Mercury Intrusion Porosimetry (MIP).

5.2 Conduct the experiment in the hysteresis mode (intrusion-extrusion). Measure the total intrusion volume (mL/g), total pore area (m^2/g), pore diameter (µm), porosity (%), permeability (mDarcy) and tortuosity. Repeat twice.

6. Drug loading and release

6.1 Before loading, prepare 4 L of 15 mg/L diflunisal solution and stir overnight. Confirm the concentration of the solution by UV-Vis spectroscopy (initial concentration). Indeed, swell 400 mg of freeze-dried samples of hydrogel in 6 mL of distilled water for 24 h.

6.2 For loading, fill a flask with 50 mL of diflunisal solution and maintain at 25 °C with constant stirring. Submerge each swelled hydrogel in the flask.

6.2.1. Take aliquots of remaining diflunisal solution (2 mL) at different times in order to determine the *plateau* region of the curve, for example: 3, 6, 24, 27, 30 and 48 h. After 24 h replace the solution with a fresh one.

6.3 Measure the absorbance at 252 nm of each aliquot, and determine the concentration of diflunisal present in the solution, using a calibration curve of diflunisal. Calculate the amount of diflunisal retained in the hydrogel at 24 and 48 h, as the difference of initial and final concentrations, taking into account the total volume (56 mL).

6.3.1. Determine the encapsulation efficiency (EE) using the equation 3.

$$EE\% = \frac{Diflunisal\ concentration\ inside\ the\ hydrogel}{Diflunisal\ concentration\ in\ the\ solution} \times 100\ (Eq.3)$$

6.3.2. Freeze the loaded hydrogels at -80 °C and lyophilize them at -50 °C.

6.4 For drug release, submerge 30 mg of freeze-dried diflunisal loaded hydrogels in 50 mL of phosphate buffer (pH 7.4) at 25 °C. Maintain constant stirring. Withdraw aliquots of 2 mL at different times and replace with fresh medium to keep a constant volume.

6.4.1. Determinate the diflunisal released spectrophotometrically at 252 nm, according to a calibration curve.

6.5 Deduce the predominant drug release mechanism in the hydrogels adjusting the drug release data corresponding to the first 60%, to the Korsmeyer-Peppas model (Equation 4), to obtain the kinetic (*k*) and the diffusion (*n*) constants. The *n* values indicate the mechanism of drug release[24, 25]. Then, *n* values close to 0.5 are related to Fickian diffusion, meanwhile values of 0.5-1.0 for anomalous transport, where are involved diffusion and relaxation chains, and finally, values of 1.0 are related to case II transport.

$$Korsmeyer - Peppas\ model\ {}^{Mt}/_{M_\infty} = k_{KP}t^n\ (Eq.4)$$

6.5.1. To confirm the results, use the Higuchi, First order, and Zero order mathematical models (Equations 5 to 7) and select the better fit.

6.5.2.

$$Higuchi\ model\ {}^{Mt}/_{M_\infty} = k_H t^{0.5}\ (Eq.5)$$

$$First\ Order\ model\ {}^{Mt}/_{M\infty} = 1 - e^{-k_1 t}\ (Eq.6)$$

$$Zero\ Order\ model\ {}^{Mt}/_{M\infty} = k_0 t\ (Eq.7)$$

where *t* represents the release time, *Mt* the amount of drug delivered at a given time, and M_∞ the total amount of drug delivered at the end of the process.

REPRESENTATIVE RESULTS:

Hydrogels preparation

Chitosan-PVA hydrogels were obtained at -4 °C, -20 °C and -80 °C with 4 freezing cycles and at -80 °C with 5 and 6 freezing cycles by the previously reported freeze-thawing method[2]. All hydrogels were homogeneous, semi-transparent, flexible and resistant against manipulation.

FT-IR characterization

The FT-IR spectra are shown in **Figure 2**. Seven characteristics signals of chitosan and PVA polymers were detected: at 3286 cm^{-1} the stretching vibration mode of PVA hydroxyl group (-OH) and at 2918 cm^{-1} the stretching vibration mode of -CH group[26, 27]. The signals of amide groups, representative of chitosan structure, were found at 1652 cm^{-1} to the stretching vibration mode of C=O (amide I), at 1560 cm^{-1} to the flection vibration mode of N-H (amide II) and 1325 cm^{-1} to the vibration of amide III[28–30]. Other signals, at 1418 cm^{-1} to the flection vibration mode of C-H and at 1086 cm^{-1} to the stretching vibration mode of C-O groups, both of PVA, were detected[27, 31,32].

Electronic Microscopy

All CS-PVA hydrogels showed a highly porous surface (**Figure 3,** from left to right) and distinctive changes were observed according to the preparation conditions. Hydrogels prepared at -4 °C (CP4-4) presented larger pores than the hydrogels prepared at -80 °C (CP4-80). Moreover, the latter appears to have a more porous network. This effect may be due to the fact that, at lower temperature, the water crystal formation was faster and many small crystals emerged and were sublimated during the freeze-drying process, leaving void pores[14,33]. Meanwhile, the effect of the number of freezing cycles seems to promote more defined and circular pores in hydrogels CP6-80 (**Figure 3,** from top to bottom).

Porosimetry

Samples CP4-4, CP4-80 and CP6-80 presented more pronounced changes; in order to complement the information about morphology, they were analyzed by MIP (**Table 1**). The comparison between hydrogels CP4-4 and CP4-80 (**Figure 3-a**) showed that, at a lower temperature of freezing, hydrogels developed a more porous network, which presented a large total intrusion volume and higher total pore area. However, hydrogels CP6-80 showed less permeability than CP4-80 (**Figure 3-b**), probably due to their high tortuosity, which was also

reflected in a lower total intrusion volume. **Figure 3** presents the different pore sizes of these hydrogels. Two pore sizes were distinguished, one between 0.3-5.0 µm and other between 5.0-30 µm. In hydrogels CP4-80 and CP6-80, the porous network had a greater number of small pores than large ones, compared with CP4-4 hydrogel. These results were similar to those observed by SEM micrographs and suggested that, at lower temperature greater interactions between the PVA chains were favored and more crystalline zones were formed. In such a way, the formation of crystalline zones by PVA chains, was stimulated at low temperature.

Swelling assays

The swelling behavior of CS-PVA hydrogels can be seen in the **Figure 4**. They quickly absorbed large amounts of water; for the first 5 hours they retained 10x their weight, and after 20 hours they retain up to 15x their weight (equilibrium point). However, in relation to hydrogels prepared at the same number of freezing cycles, the hydrogel CP4-80 showed less swelling capacity in the first 5 hours as a consequence of the temperature that was used for its preparation (-80 °C). In the case of hydrogels prepared at different number of freezing cycles (CP4-80, CP5-80 and CP6-80) no differences were found at any time. Probably, the decreased swelling capacity observed in hydrogels prepared at -80 °C was caused by the small pore size of the hydrogel network.

Drug loading and release

To evaluate the capacity of CS-PVA hydrogels as drug delivery systems, the anti-inflammatory drug diflunisal was loaded in the network and subsequently released. The encapsulation efficiency (EE) in all these systems was around 70%; however, the CP4-80 hydrogel presented more slightly EE at 73% (**Table 2**). Meanwhile, the releasing kinetics of diflunisal from the CS-PVA hydrogels were maintained for about 30 h in all cases. The CP4-80 hydrogel released the highest amount of diflunisal (**Figure 5**). This may be due to the fact this hydrogel showed a more porous structure in comparison with the other two types of hydrogel. This feature allowed the small molecule of drug to easily enter in the hydrogel network and, then, to be released. Between CP4-80 and CP6-80 hydrogels not differences were observed during release times (**Figure 6**). No *burst* effect was observed in any of the CS-PVA hydrogels, which is promising for pharmaceutical applications. Mathematical models were used to determine the main release mechanism in CS-PVA hydrogels. The results were adjusted to different mathematical models (**Table 3**) and according to the *n* values, it was found that the Fick diffusion dominates the drug release process.

FIGURE AND TABLE LEGENDS:

Figure 1 Chemical structure of PVA (**a**), chitosan (**b**) and diflunisal (**c**).

Figure 2 FT-IR spectra of pure chitosan and PVA and, chitosan-PVA hydrogels prepared at different conditions of freezing.

Figure 3 SEM micrographs of chitosan-PVA hydrogels at 1500x magnification. Pore size distributions of chitosan-PVA hydrogels: **a**) hydrogels prepared with 4 cycles of freezing and at -

4 °C and -80 °C. **b**) Hydrogels prepared at -80 °C and, 4 and 6 cycles.

Figure 4 Swelling kinetics of chitosan-PVA hydrogels: **a**) hydrogels with 4 cycles of freezing and **b**) hydrogels prepared at -80 °C.

Figure 5 Diflunisal release profiles in mg (**a**) and Mt/M_{∞} (**b**) for hydrogels CP4-4 and CP4-80.

Figure 6 Diflunisal release profiles in mg (**a**) and Mt/M_{∞} (**b**) for hydrogels CP4-80 and CP6-80.

Table 1 Porosimetry parameters of the porous structure of chitosan-PVA hydrogels.

Table 2 Encapsulation and release efficiencies for chitosan-PVA hydrogels.

Table 3 Kinetic parameters of diflunisal release from chitosan-PVA hydrogels.

DISCUSSION:

The freeze-thawing method is a suitable process to prepare biocompatible hydrogels focused in biomedical, pharmaceutical or cosmetical applications[34–36]. The most important advantage of this method, compared with other well-known methods to prepare hydrogels, is that crosslinking agent use is avoided, which could cause an inflammatory response or adverse effects in the human body[34]. This is a versatile method because it offers the possibility to prepare hydrogels from PVA or their mixtures with different polymers[11,37] in such way that new characteristics from the other polymers can be obtained in the new material (e.g., major capacity to absorb water, antimicrobial or antioxidant properties[2,18,35]). However, it is important to consider that the incorporation of other polymers could decrease the strength of the hydrogels[19,37].

The principal parameters to consider in the freeze-thawing method are the temperature of freezing, the time and the number of freezing cycles, and also, the polymer ratio (in case of polymer mixtures)[2,19,20]. A wide range of swelling, morphological and mechanical properties can be obtained with this method when the freeze conditions are controlled. These parameters affect directly the three-dimensional network configuration in chitosan-PVA hydrogels because the freezing conditions promotes the arrangements in PVA chains, which are joined by physical interactions, called crystalline zones[12,38]. These crystalline zones are concentrated regions of hydrogen bonds that act as crosslinking points in the hydrogels, which maintain and form the three-dimensional network and it is a retractor force when hydrogels are in the swelling state[2,39,40].

In this study, we evaluated the effect of a new range of freeze-thawing temperatures (-4 °C, -20 °C and -80 °C) combined with a different number of freezing cycles (4, 5 and 6) but the same time of freezing (20 h), to prepare 1:1 chitosan-PVA hydrogels. The lowest swelling capacity was observed at the lowest temperature (-80 °C). Indeed, hydrogels at this lowest temperature obtained the smaller pores and the more porous networks. These differences in chitosan-PVA hydrogels are useful for different applications such as drug delivery systems or scaffolds. In

general, chitosan-PVA hydrogels present high rates of swelling, due to chitosan hydrophilic groups ($–NH_2$)[41,42], and they are soft, flexible, easy to handle and resist the manipulation because of PVA characteristics. In that sense, the freeze-thawing method is easy, cheap and fast to produce chitosan-PVA hydrogels with different properties, avoiding toxic crosslinking.

Although the freeze-thawing is an easy and friendly method, it has some drawbacks. A complete homogenization of chitosan, in this case, and of the polymer mixtures is very important. Hydrogels can present more fragile zones and an irregular porous structure. Also, it is necessary to make a correct dissolution of PVA in water by heating at 70-80 °C[42, 43] for 1 h under magnetic stirring. The cooling of this PVA solution must be slow with constant stirring to prevent the formation of a solid layer of PVA.

A limitation of this method, for cell culture assays, is the formation of whitish or semitransparent hydrogels. In this case, the application of glycerol or DMSO (toxic compound at room temperature) could be used to improve the appearance of hydrogel[23,44]. The freeze-drying step of the freeze-thawing method to prepare CS-PVA hydrogels is a critical step, because the hydrogels could present a constriction in the middle zone, which complicate the work and the characterization. To avoid this, the sample must be kept completely frozen before lyophilization. Concerning the drug loading and release studies, it is very important to ensure that there is no interference with the signals from the hydrogel components and the drug to be quantified.

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DISCLOSURES:

The authors have nothing to disclose.

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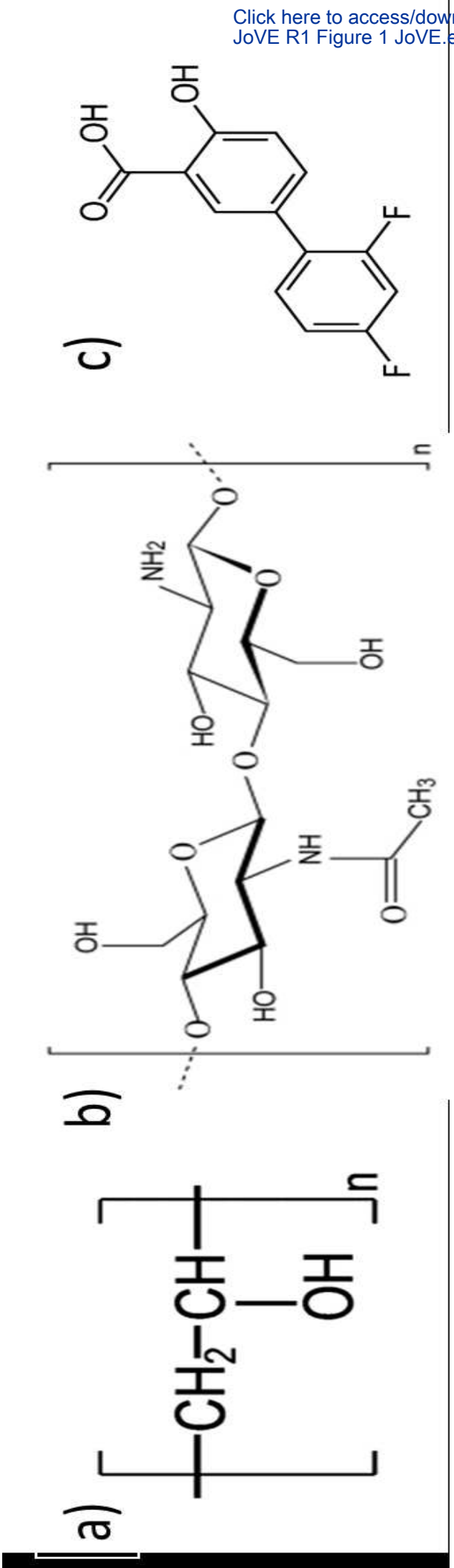

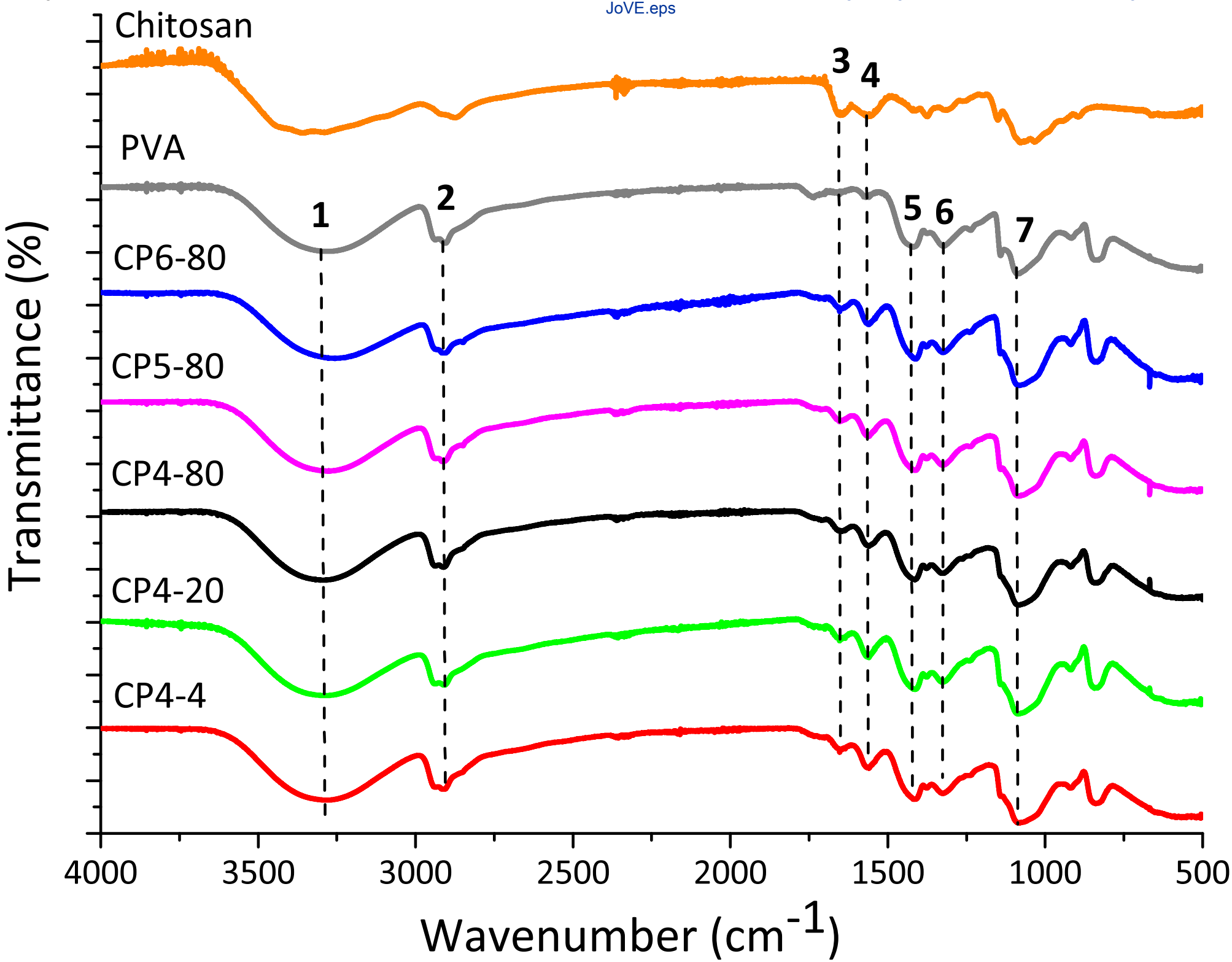

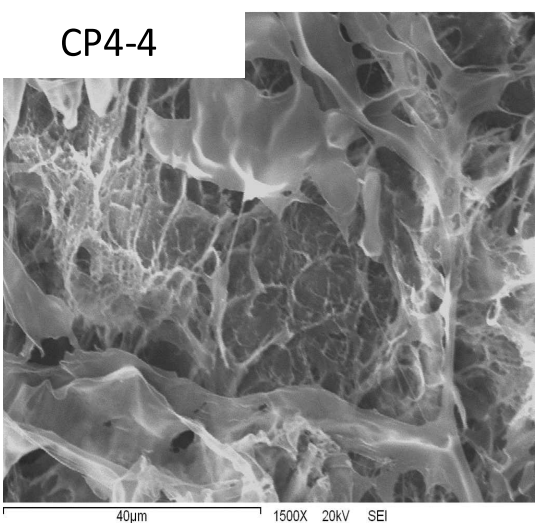

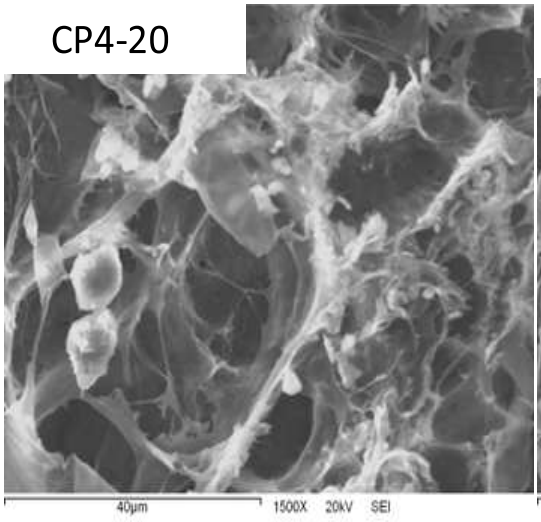

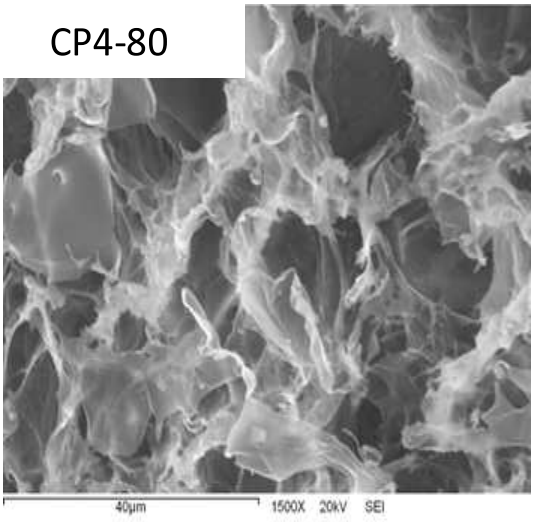

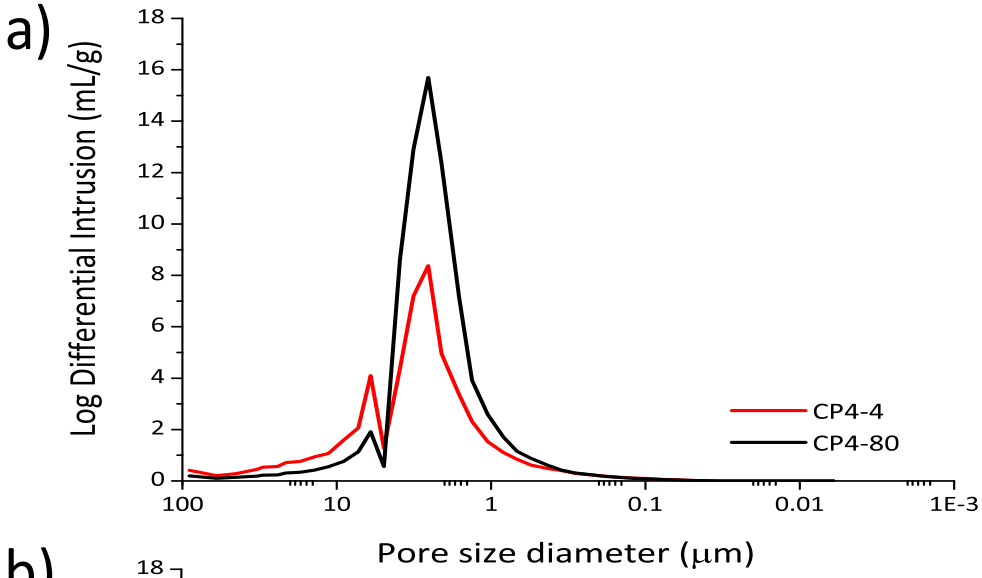

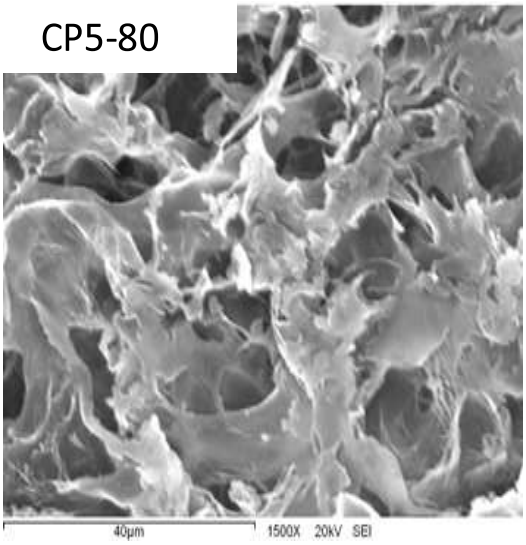

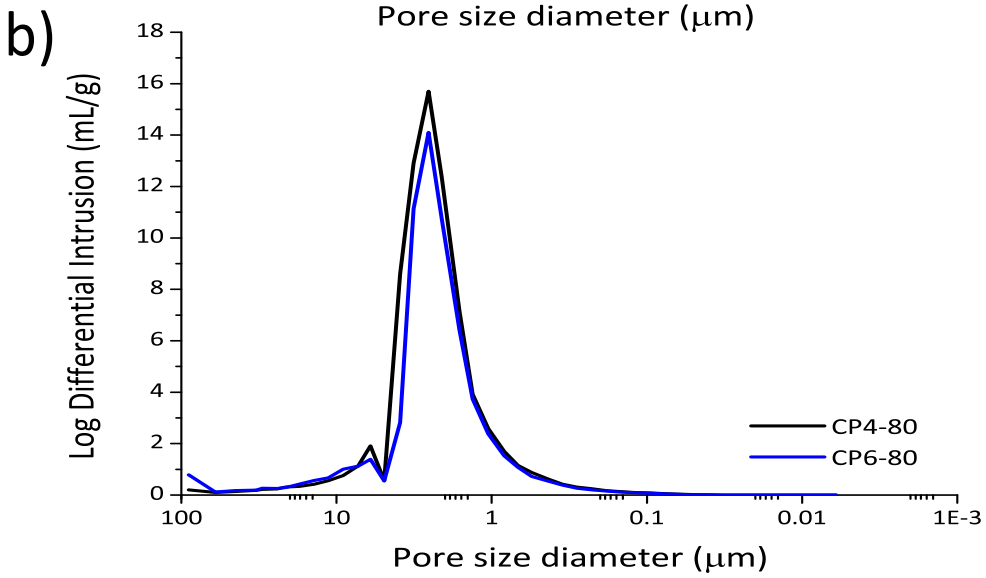

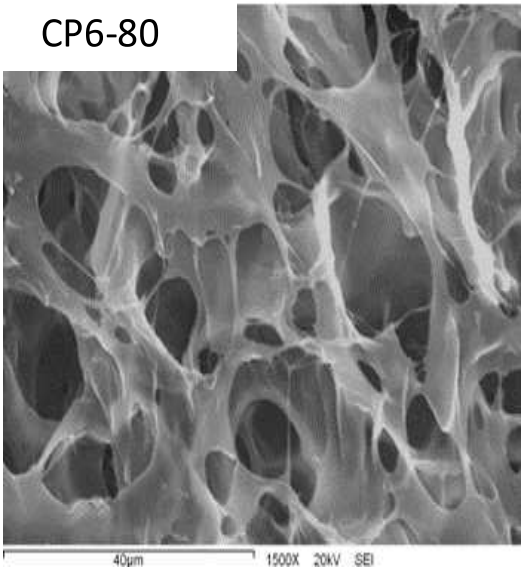

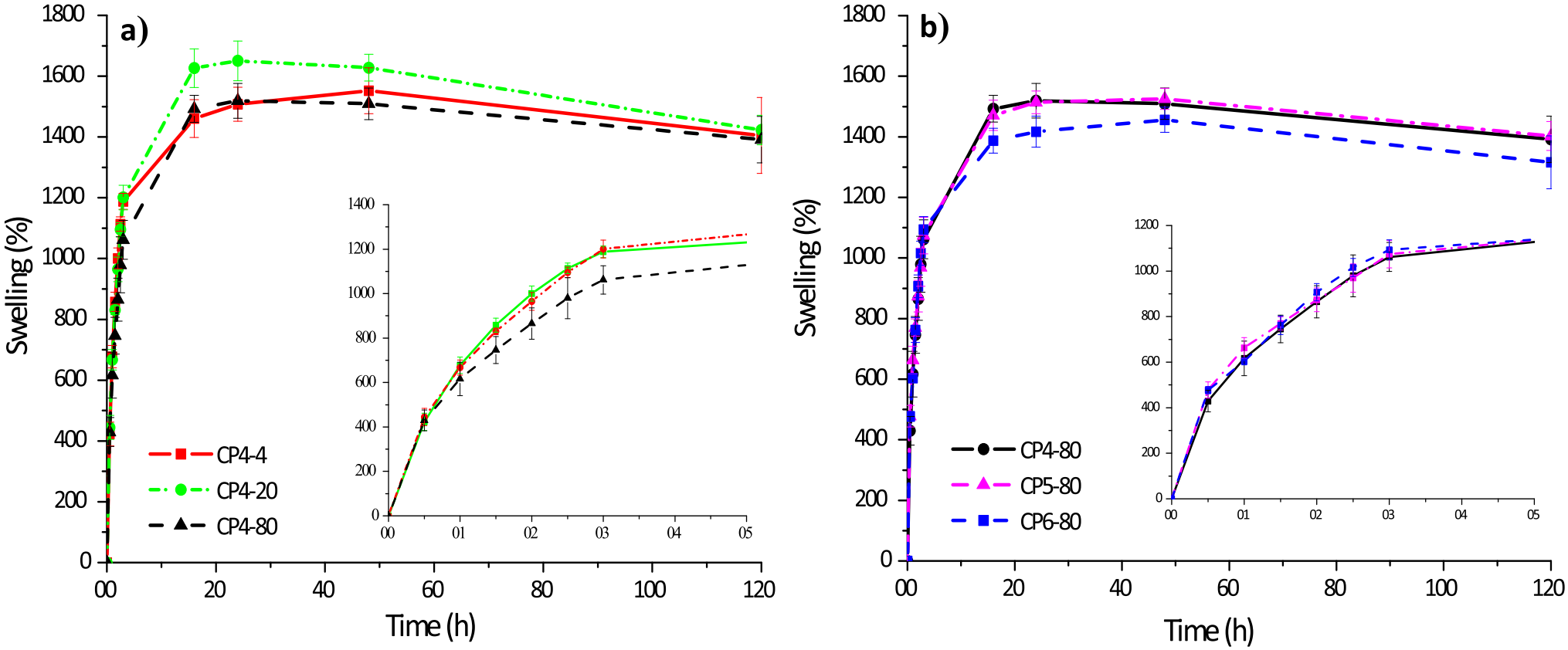

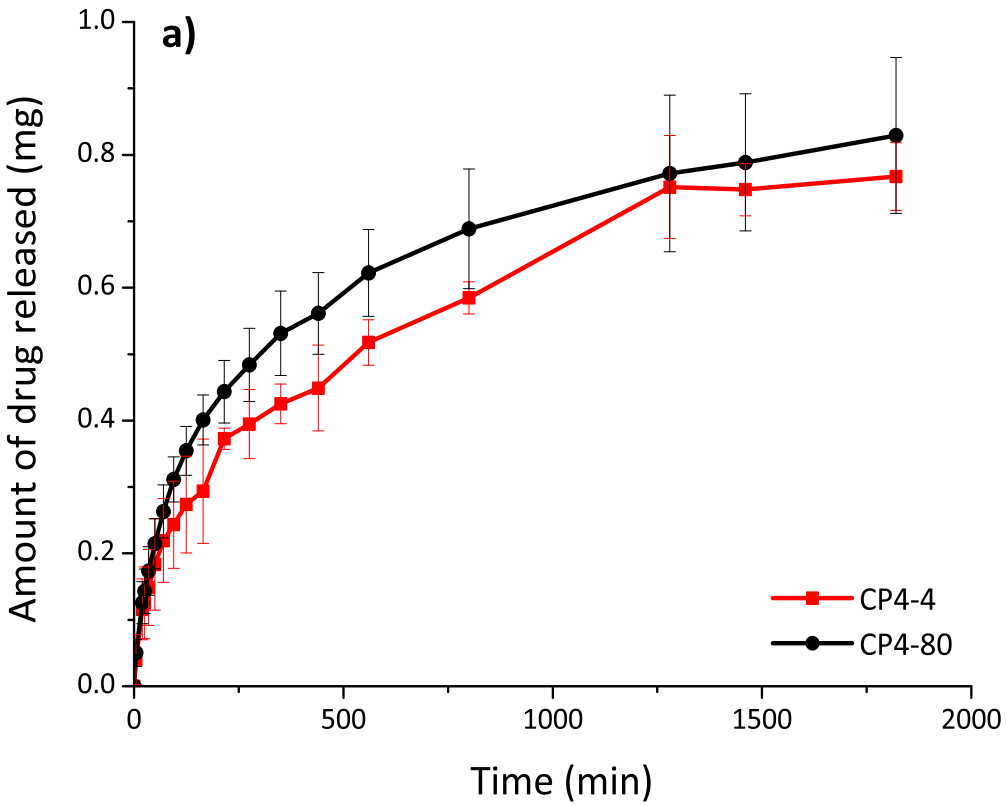

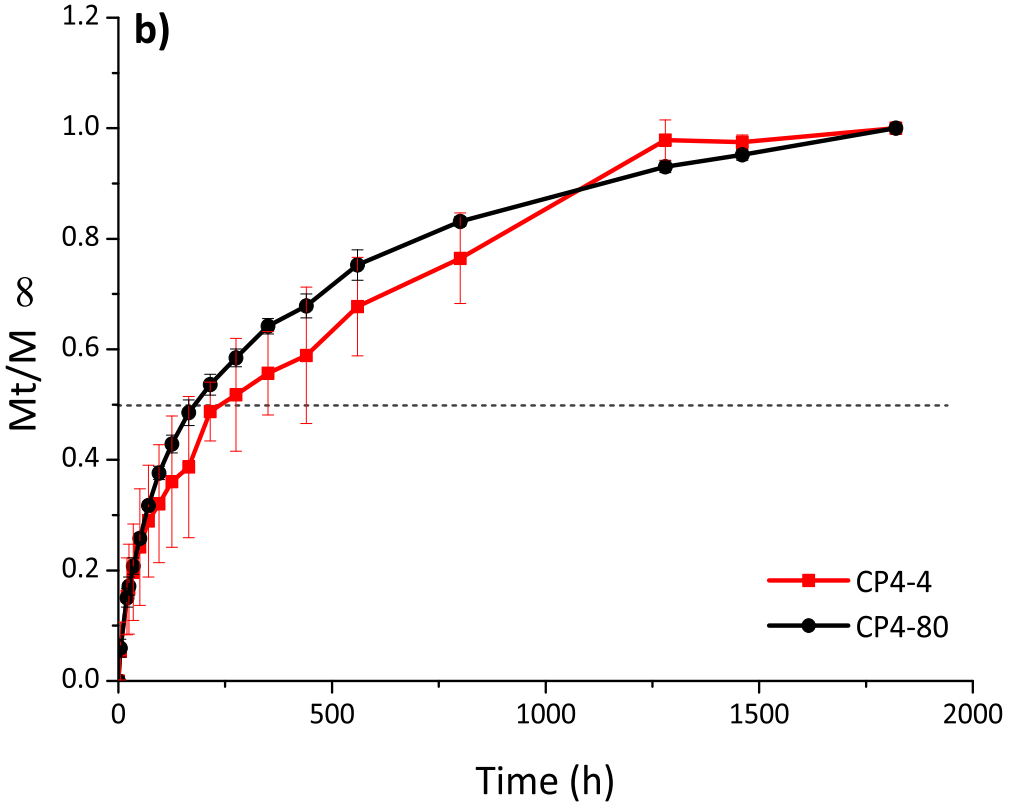

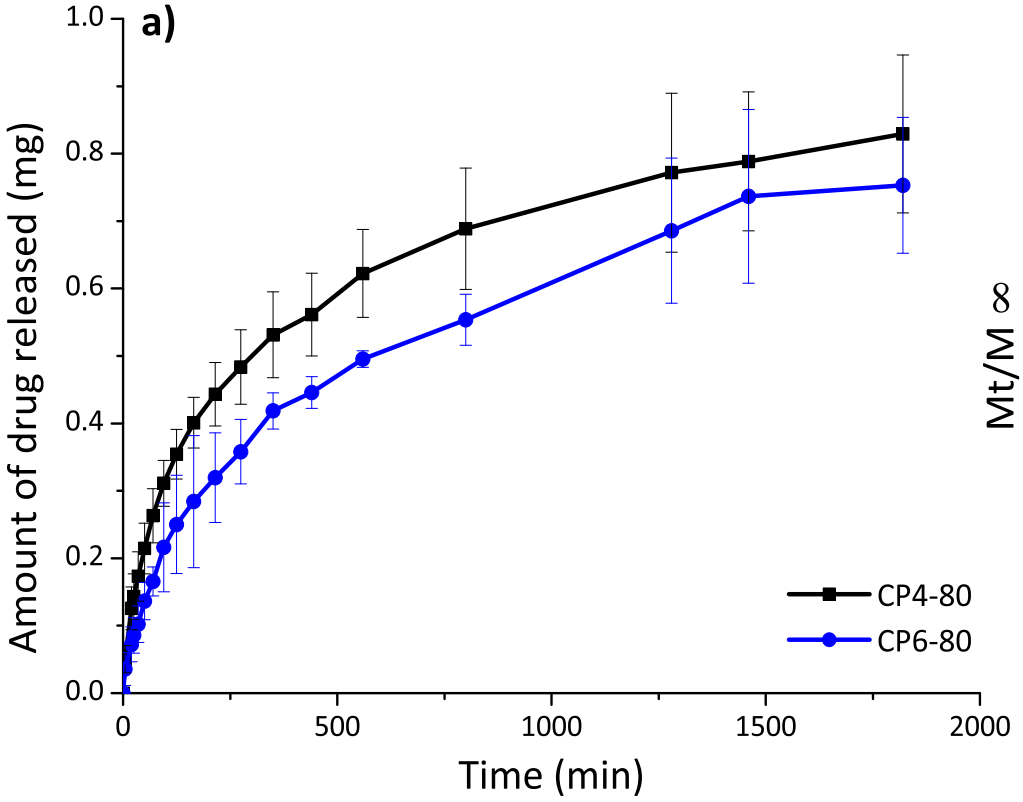

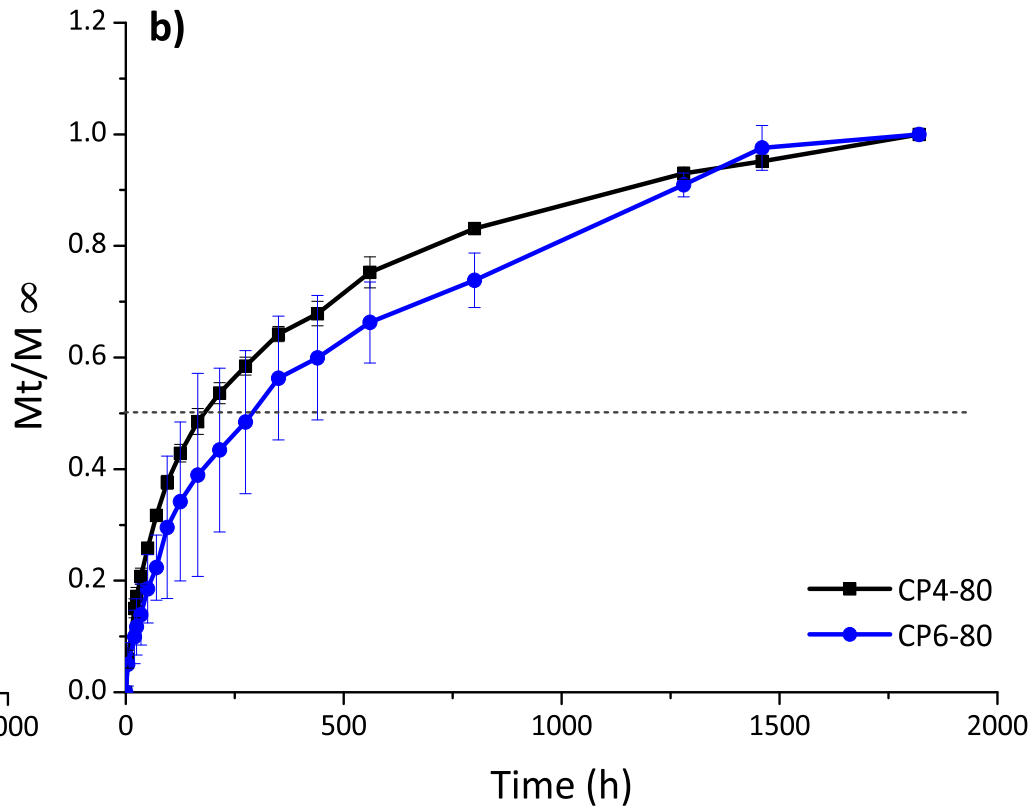

Table 1.-Porosimetry parameters of porous structure of CS-PVA hydrogels.

Hydrogel	Total intrusion volume (mL/g)	Total pore area (m^2/g)	Porosity (%)	Permeability (mdarcy)	Tortuosity
CP4-4	5.16	10.19	67.13	132.43	10.46
CP4-80	7.36	15.14	85.95	151.16	5.83
CP6-80	6.69	12.86	84.82	129.28	12.2

Table 2.- Encapsulation and release efficiences for CS-PVA hydrogels.

Sample	Diflunisal loaded		Diflunisal released
	mg/g hydrogel	Encapsulation	% released respect to loaded
CP4-4	3.05± 0.09	71	79 ± 3.33
CP4-80	3.22 ± 0.47	73	86 ± 0.4
CP6-80	3.19 ± 0.05	68	80 ± 3.9

Table 3.-Kinetic parameters of diflunisal release from chitosan-PVA hydrogels.

Sample	Korsmeyer-Peppas			Higuchi		First Order		Zero Order	
	k_{KP} x 10^2 (min^{-n})	n	R^2	k_H x 10^2 ($min^{-0.5}$)	R^2	k_1 x 10^2 (min^{-1})	R^2	k_0 x 10^2 (min^{-1})	R^2
CP4-4	4.3 ± 0.39	0.44 ± 0.02	0.99	3.1 ± 0.1	0.982	0.29 ± 0.03	0.803	0.18 ± 0.02	0.544
CP4-80	3.6 ± 0.33	0.50 ± 0.02	0.99	3.7 ± 0.1	0.994	0.42 ± 0.03	0.894	0.27 ± 0.02	0.698
CP6-80	2.3 ± 0.24	0.54 ± 0.02	0.99	2.9 ± 0.1	0.989	0.27 ± 0.02	0.925	0.17 ± 0.01	0.767

= kinetic constant; *n* = diffusion constan

Materials:

Name	Company	Catalog numb	Comments
Chitosan medium molecular weight	Sigma-Aldrich	448877	Mw determined by capillary viscometry (637,000 Da) and deacetylation degree of 70%
Diflunisal (2'-4'-difluoro-4-hydroxy-3-biphenyl-carboxylicacid)	Merck		
Glacial acetic acid	Sigma-Aldrich	1005706	
Poly(vinil alcohol)	Sigma-Aldrich	341584	Mw 89,000-98,000, 99+% hydrolyzed

Equipment:

Name	Company	Comments
Cressington Sputter Coater 108 auto	TED PELLA INC	
Cryodos Lyophilizator	Telstar	
Falcon tubes	Thermo Fisher	
FT-IR spectroscopy	Nicolet iS50	in ATR mode
Lyophilizator	LABCONCO	
Micromeritics Autopore IV 9500	Micromeritics	
Scanning electron microscope	Pemtron SS-300LV	
UV-visible spectrophotometer	Agilent 8453	

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Title of Article: Freeze-thawing method to prepare chitosan-PolY (vinil alcohol) hydrogels without crosslinking agents and diflunisal release studies.

Author(s): María D. Figueroa-Pizano, Itzar Vélaz, María E. Martínez-Barbosa

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Department:	Departamento de Investigación en Polímeros y Materiales
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May 30th 2019

Dear Phillip Steindel, Ph. D.

JoVE Review Editor

Please, find enclosed the revised manuscript (with all changes remarked in red) for the paper entitled "**Freeze-thawing method to prepare chitosan-poly (vinyl alcohol) hydrogels without crosslinking agents and diflunisal release studies**".

First of all, we would like to thank the amendments suggested from you and the reviewers. We have taken into account all the corrections received because we have found them very useful. We think the overall quality of the article has been improved and we hope it is a suitable one for its publication.

Next, we include a separate point-by-point response detailing how the revision has been made.

Thank you for your attention.

Best regards,

María Elisa Martínez-Barbosa, Ph.D.

Editorial Comments:

Changes to be made by the author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Grammar issues have been corrected as follows:

a. The word "Poly(vinyl alcohol)" was changed by "Poly (vinyl alcohol)" overall the manuscript.
b. In line 53: "tridimensional" was changed by "three-dimensional".
c. In line 55: "physiologic" was changed by "physiological".

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d. In line 81: “bounds” was changed by “bonds”.
e. In line 89: “source with positive charge” was changed by “sources with positive charges”.
f. In line 93: “cellular” was changed by “cell”.
g. In line 162: “samples were in vacuum” was changed by “samples under vacuum”.
h. In line 179: “by” was changed by “with”.

2. **Keywords: Please provide at least 6 keywords or phrases.**
The word “Porosimetry” was added to Keywords.

3. **3. 1.2: How are the solutions mixed? On a stirrer?**
The phrase “mix both solutions 1:1 until” was changed to “mix both solutions 1:1 using a magnetic stirrer” (line 128).

4. **4. 6.3: Please describe how to calculate the encapsulation efficiency.**
The determination of Encapsulation Efficiency (EE) is explained in lines 185-190, and the Equation 3 was added.

5. **JoVE is a methods-based journal. Thus, the discussion section of the article should be focused on the protocol and not on the representative results. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:**

a) Critical steps in the protocol

b) Modifications and troubleshooting of the method

c) Limitations of the method

d) The significance of the method with respect to existing/alternative methods

e) Future applications or directions of the method

As recommended, in this section (lines 322-368), some phrases concerning the results were eliminated. Indeed, some paragraphs were added focusing the discussion on the critical steps in the protocol, the significance and modifications of the method as well as the limitations. The corresponding citations were inserted.

Reviewers' comments:

Reviewer #1:

Major Concerns:

1. **The Figure 1 should include the chitosan and PVA FTIR curves as controls.**
   In Figure 1 (renumbered like Figure 2) the FT-IR of pure chitosan and PVA were included as controls.
2. **In the Figure 2, the scale bar should be included in the SEM pictures. Also, the CP4-80 picture is duplicated. One of the CP4-80 SEM image should be removed. Moreover, the manuscript states that the CP4-4 hydrogel has bigger pores than the CP4-80 hydrogels. However, the CP4-4, CP4-20 and CP4-80 hydrogels seems very similar in SEM.**
   In Figure 2 (renumbered like Figure 3) the scale bars are automatically included at the bottom (outside) of each SEM micrograph. Indeed, this figure was restructured in order to eliminate CP4-80 SEM duplication and to conserve the comparison lines (effect of the temperature, left to right; effect of the number of freezing cycles, top to bottom). Finally, porosimetry curves (from the original Figure 3) were inserted in the same Figure (Figures 3-a, 3-b).

   Concerning the results, it is true that it is not easy to appreciate the differences between hydrogels CP4-4, CP4-20 and CP4-80 from SEM Images, however these differences can be batter appreciated by the porosimetry results.

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   3. Balaji, S. *et al.* Preparation and comparative characterization of keratin-chitosan and keratin-gelatin composite scaffolds for tissue engineering applications. *Materials Science and Engineering C*. **32** (4), 975–982, doi: 10.1016/j.msec.2012.02.023 (2012).

4. **The release profiles were fitted into different models. However, the model equations were missing in the manuscript. In the Table 3, the n and k values were not defined.**

Moreover, it is not clear how the conclusion of diffusion-controlled release mechanism was made based on the result of fitting.

In section 6.5 the model equations were described (Eq. 4-7). Indeed, in this section, the *n* and *k* values were defined (Table 3). Moreover, we describe how to interpret the *n* values obtained and the procedure to follow to conclude the predominant release mechanisms (lines 202-206).

5. **The manuscript mentioned in multiply places about the crystalline zones formed due to the PVA chain interaction during freeze-thawing process. However, this was not strongly supported by the SEM images of the hydrogels.**

   To our knowledge, the crystalline zones formed by polymeric chains couldn't be observed by SEM, even nor by TEM, due either by the resolution needed and also because the polymeric material hasn't the contrast enough. Other techniques can be used for that purpose. However, this characterization was not the objective of our study. In fact, according to the literature, these crystalline zones in the PVA hydrogels are well characterized by Hassan, C.M. and Peppas, N.A. in Structure and Applications of Poly (vinyl alcohol) Hydrogels Produced by Conventional Crosslinking or by Freezing / Thawing Methods. *Advances in Polymer Science*. **153**, 37–65, doi: 10.1007/3-540-46414-X_2 (2000).

   Minor Concerns:

   1. In Line 181, section 6.3: "Measure the absorbance at 252 nm of the supernatant solutions at 252 nm" has two "at 252 nm".

   Thank you, the second "at 252 nm" has been deleted (line 185).

   Moreover, this section did not mention about the instrument has been used.

   As required in the "Standard Manuscript Template", all the instruments specifications are enlisted in the "Table of Materials" (attached Excel document).

   The encapsulation efficiency is not defined in the manuscript.

   The Encapsulation Efficiency (EE) was defined by Eq. 3, lines 193.

   2. It is better to include the structures of chitosan, PVA and diflunisal in the manuscript.

   The structures of chitosan, PVA and diflunisal were included in Figure 1 and cited in lines 78, 92, and 116, respectively. Therefore, all the Figures have been renumbered.

Reviewer #2:

Major Concerns:

1. **The FTIR-ATR spectra did not bring any relevant information.**

FT-IR is a basic characterization for polymeric materials in order to put in evidence the components present in the samples. In this case, even if it is a basic characterization, the FT-IR spectra have not been eliminated, because required by other reviewer, FT-IR spectra of pure chitosan and PVA were added to the Figure 2 as controls.

2. **The gel fraction % is necessary**

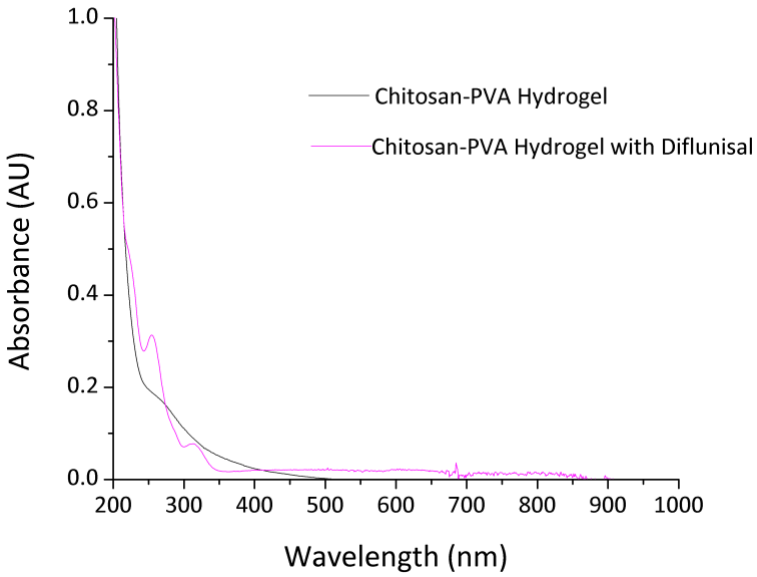
Generally, the gel fraction determination it is made in order to know the percentage of gel remaining after a swelling process, which is related to a degradation process or a dissolution of the polymers, in the short time. This determination it could be made under different conditions depending the purpose of the study, for example a) swelling the hydrogel in a solvent inert to all the hydrogel components, b) swelling the hydrogel in a solvent selective to one of the hydrogel components. However, this characterization was not the objective of our study. In our case, it could be observed (in the swelling graphs) that this could be happened at environ 120 hours of swelling. However, our drug charge and drug release studies were carried out at maximum 30 hours.

3. **The drug release and the drug charge are not so clear, how did you do that? With dialysis? Filtered? How did you know that Chi exudates are not present? Or PVA?**

In order to explain better the drug release and the drug charge, Section 6 has been restructured (lines 176-217).

Please, show the UV/Vis Spectrum.

In the next figure are shown the UV/Vis Spectrum of buffers solutions after immersion of: a) chitosan-PVA hydrogels without drug , b) chitosan-PVA hydrogels with diflunisal. It could be observed that no chitosan or PVA signals were detected by Uv-Vis, neither another signal that could present any interference in the diflunisal determination.



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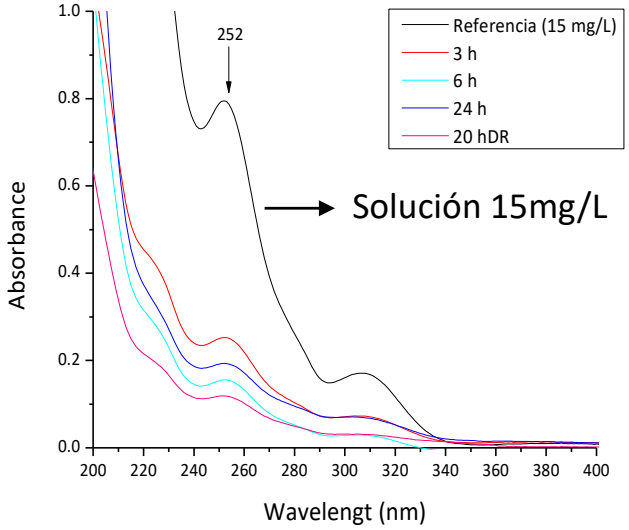

4. **<u>In the Discussion part it is not clear the differences obtained among the processes condition used. Please, Put references in this part.</u>**

As recommended, in this section (lines 322-368), some phrases concerning the results were eliminated. Indeed, some paragraphs were added focusing the discussion on the critical steps in the protocol, the significance and modifications of the method as well as the limitations. The corresponding citations were inserted.

Revised manuscript entitled: "**Freeze-thawing method to prepare chitosan-poly (vinyl alcohol) hydrogels without crosslinking agents and diflunisal release studies"**.

Answers to Reviewer #1

First of all, we would like to thank the amendments suggested. We have taken into account all the corrections received because we have found them very useful.

Considering the requested corrections, we have made the following changes in the manuscript; all of them are highlighted in red in the document:

Major Concerns:

1. **The Figure 1 should include the chitosan and PVA FTIR curves as controls.**
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Answers to Reviewer #2

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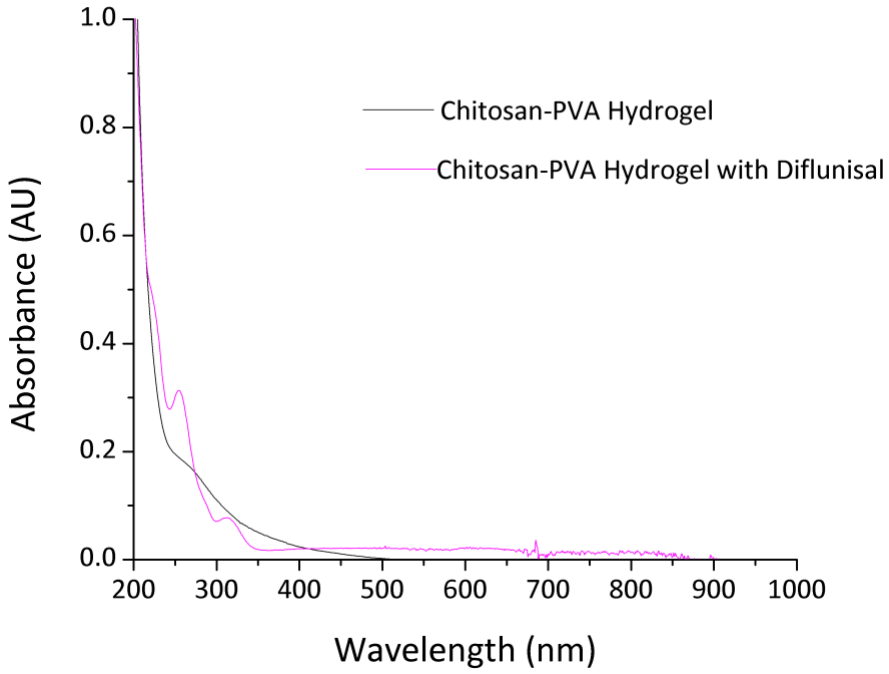

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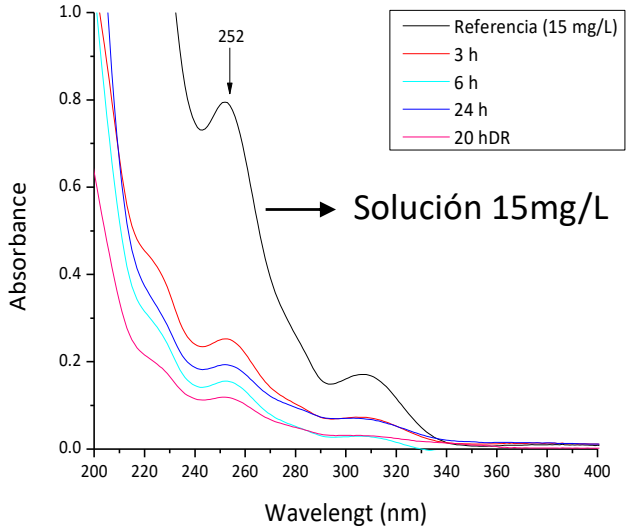

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