UNIVERSIDAD DE SONORA

Departamento de Investigación en Polímeros y Materiales

"El saber de mis hijos hará mi grandeza"

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.

3. **In the Table 1, how many replicates of measurements were conducted? The standard deviation should be included in the data.**
   Porosity measurements were done once, as shown in several works including similar studies, for example:

   1. Morgado, P.I. *et al.* Poly(vinyl alcohol)/chitosan asymmetrical membranes: Highly controlled morphology toward the ideal wound dressing. *Journal of Membrane Science*. **469**, 262–271, doi: 10.1016/j.memsci.2014.06.035 (2014).

   2. Temtem, M., Barroso, T., Casimiro, T., Mano, J.F., Aguiar-Ricardo, A. Dual stimuli responsive poly(N-isopropylacrylamide) coated chitosan scaffolds for controlled release prepared from a non residue technology. *Journal of Supercritical Fluids*. **66**, 398–404, doi: 10.1016/j.supflu.2011.10.015 (2012).

   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**

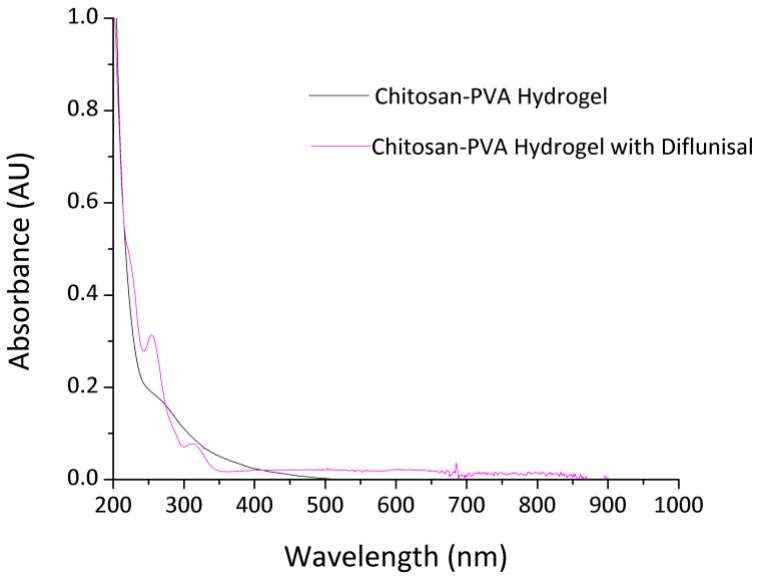
Generally, the gel fraction determination it is made in order to know the percentage of gel remaining after a swelling process, which is related to a degradation process or a dissolution of the polymers, in the short time. This determination it could be made under different conditions depending the purpose 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?**

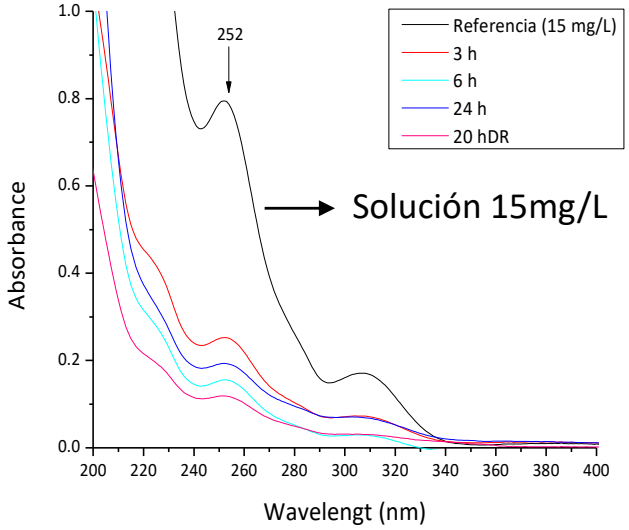
In order to explain better the drug release and the drug charge, Section 6 has been restructured (lines 176-217).

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.



Also, are presented the UV-Vis spectrums obtained during the drug loading studies for one of the sample, were neither interference of hydrogel components was presented.



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.