Response to reviewers:

RE: JoVE55517R1 “Synthesis and Characterization of Charged Hydrogels for the Extended Delivery of Vancomycin”

Dear Dr. Nguyen,

Thank you for considering our manuscript entitled “Synthesis and Characterization of Charged Hydrogels for the Extended Delivery of Vancomycin” for publication in the Journal of Visualized Experiments. We appreciate the critiques and suggestions from the reviewers, and have found them useful in further refining the presented study. In sum, the reviewer comments focused on clarification of specific methodological steps. Also, the reviewers requested that additional sources be provided to assist in discussing this field, and to aid in placing this work within the field. We hope that the changes we have made will have sufficiently addressed the major concerns raised by the reviewers. We would like to thank the reviewers for their attention and critique of our manuscript, as their recommendations aided to improve the impact and rigor of this article. Below, we have sequentially noted our responses for each question or concern raised by the reviewers. Finally, we thank the Editor for careful consideration of this manuscript. We look forward to your response.

**Comment 1\_1:** Figure 3: What are the units of time on the X-axis? hours?

**Response 1\_1:** In figure 3, the X-axis units are time in minutes. The X-axis title has been updated to read “Time (min)”.

**Comment 1\_2:** Please include Gustafson CT, Boakye-Agyeman F, Brinkman CL, Reid JM, Patel R, Bajzer Z, et al. (2016) Controlled Delivery of Vancomycin via Charged Hydrogels. PLoS ONE 11(1): e0146401. doi:10.1371/journal.pone.0146401 as a numbered reference.

**Response 1\_2:** We have added the reference to the original article in the citation list, and cited this article in the introduction section (line 185). Additionally, each figure legend has been updated to read “Gustafson CT, *et al* 20”. The legends originally were written as “Gustafson CT, Boakye-Agyeman F, Brinkman CL, Reid JM, Patel R, Bajzer Z, et al. (2016) Controlled Delivery of Vancomycin via Charged Hydrogels. PLoS ONE 11(1): e0146401. doi:10.1371/journal.pone.0146401”

**Comment 2\_1:** The last keyword 'Staphylococcus aureus' was not mentioned throughout this manuscript and it should be deleted.

**Response 2\_1:** Thank you for the correction, we have removed this key word.

**Comment 2\_2:** The authors described in the introduction part, line 127, 'environmentally responsive biopolymers such as OPF would provide an ideal solution to control dosing of antibiotics for such applications', which means there are other environmentally responsive biopolymers suitable for this study. The authors should describe the reason(s) of choosing OPF instead of other biopolymers in this manuscript.

**Response 2\_2:** Thank you for noting that this point should be clarified. OPF, an environmentally responsive hydrogel that our group has characterized (please see references Dadsetan, *et al* 2009 and Dadsetan, *et al* 2010 [references #s 11 and 12]), was evaluated as a delivery vehicle for vancomycin. We chose to work with OPF because of the previously published work by our group and others that has demonstrated the utility of OPF in various tissue regenerative and drug delivery applications. Additionally, OPF properties are modular by co-polymerization steps which are relatively straightforward and amenable for synthesis as needed in surgical applications. We have added additional information describing this reasoning in lines 129-131 “This study focuses on the use of OPF due to its previously characterized biocompatibility in cellular and animal models, and facile synthesis and customization.”

**Comment 2\_3:** The authors should also mention why they chose SMA for this study.

**Response 2\_3:** Sodium methacrylate was selected to functionalize the OPF polymer hydrogels because we have shown previously that charged, structurally sound hydrogels could be produced through this synthetic process.

**Comment 2\_4:** Line 415 'Additionally, we recommend sequencing the order of the samples to be run on the HPLC, such that those known to contain less vancomycin would be run first.' should mentioned at line 285, method 5.5 in order to obtain better results.

**Response 2\_4:** We appreciate that this addition was suggested, as it is an important methodological step. We have updated Protocol Section 5.5 to read “5.5. Run all samples through the HPLC, ordering the samples such that solutions expected to contain less vancomycin are run first. This prevents contamination from higher concentration samples in the event that all vancomycin does not elute or rinse properly. Calculate signal peak area at 282 nm wavelength detection. Allow for 10 min per sample in order to remove any excess drug or polymer degradation materials, and to re-equilibrate the column prior to the next sample.”

**Comment 2\_5:** Figure 2 shows the change of swelling ratio for the system. However, whether the loaded vancomycin in the system would have influenced the swelling ratio was not tested or mentioned in the manuscript. This evaluation should be included in the study.

**Response 2\_5:** The reviewer notes an important point, that brings out a deficiency in our explanation of this part of our study. We will clarify our thoughts regardiing influences on the swelling ratio, and we respectfully ask the reviewer if this clarification adequately addresses Comment 2\_5 without additional experimental work. The uncharged (0%SMA) hydrogels have a swelling ratio much lower than the charged hydrogels in the vancomycin solution. The swelling of all hydrogel groups in water is not significantly different than the swelling of the hydrogels in the vancomycin solution. Thus, drug loading may be facilitated in part by the opening of the hydrogel mesh in a solution with lower ionic strength (e.g. water) than DPBS. In contrast, there is no difference in swelling between hydrogel groups (uncharged, charged) in the DPBS solution. This indicates that extended drug release, which is done into DPBS, is mediated only by incorporation of charges in the hydrogel matrix, and not by contraction / expansion of the hydrogel matrix. Thus, we did not consider it necessary to examine the influence of loading on swelling ratio, as there was no observed difference in swelling in DPBS between charged groups.

**Comment 3\_1:** Citations are poorly organized as some very important reviews are ignored to cite. Nothing now except I need them to add the following important references on hydrogels.

1. "Production lof Chitosan-based Hydrogels for Biomedical Applications", Chapter in Chitosan Based Biomaterials, Fundamentals, Volumer 1, Edited by J. Amber Jennings and Joel D. Bumgardner, Woodhead Publishing Series in Biomaterials, Chapter, 12, pp. 295-319 (2016).

2. "Polysaccharide-based Hydrogels as Biomaterials", in Polymeric Hydrogels as Smart Biomaterials, Springer Series on Polymers and Composite Materials, (Editor, Susheel Kalia), pp. 45-71 (2015).

3. "Polysaccharide-based Micro/nanohydrogels for Delivering Macromolecular Therapeutics", J. Controlled Release, 193 (2014) 162-173.

4. "Polymeric Hydrogels for Oral Insulin Delivery", J. Controlled Release, 165 (2013) 129-138.

5. "Developments in Polymeric Devices for Oral Insulin Delivery", Expert Opinion on Drug Delivery, 5 (2008) 403-415.

6. "Biochemical Pharmacology Novel Ultra-small Nanoparticles of PEGylated Deoxycholic Acid Conjugated Polyhydroxybutyrate Copolymers for Oral Insulin Delivery"

Nanomedicine, 10(2015)1569-1583.

7. "Ultra-small fluorescent bile acid conjugated PHB-PEG block copolymeric nanoparticles: synthesis, characterization and cellular uptake", Royal Society of Chemistry RSC Advances, 3(2013)7064-7070.

**Response 3\_1:** Thank you for the suggested sources, we found that it was helpful to read these materials, and have updated the manuscript to include references to several which were most relevant to this work. Specifically, the recommended book chapter from "Polysaccharide-based Hydrogels as Biomaterials" (Editor, Susheel Kalia), the article "Polysaccharide-based Micro/nanohydrogels for Delivering Macromolecular Therapeutics" by Ganguly K, *et al*, and the article “Polymeric Hydrogels for Oral Insulin Delivery” by Chaturvedi K, *et al* were referenced as citations #s 22, 27, and 28, respectively.

Kind regards,

Carl T. Gustafson