nature biomedical engineering

PEER-REVIEW INFORMATION

Brachytherapy via a depot of biopolymer-bound 131I synergizes with nanoparticle paclitaxel in therapy-resistant pancreatic tumours

Corresponding author: Ashutosh Chilkoti

Editorial note

This document includes relevant written communications between the manuscript's corresponding author and the editor and reviewers of the manuscript during peer review. It includes decision letters relaying any editorial points and peer-review reports, and the authors' replies to these (under 'Rebuttal' headings). The editorial decisions are signed by the manuscript's handling editor, yet the editorial team and ultimately the journal's Chief Editor share responsibility for all decisions.

Any relevant documents attached to the decision letters are referred to as **Appendix #**, and can be found appended to this document. Any information deemed confidential has been redacted or removed. Earlier versions of the manuscript are not published, yet the originally submitted version may be available as a preprint. Because of editorial edits and changes during peer review, the published title of the paper and the title mentioned in below correspondence may differ.

Correspondence

Mon 21 Oct 2019
Decision on Article nBME-19-1528

Dear Prof Chilkoti,

Thank you for submitting to *Nature Biomedical Engineering* your Article, "^{131}I-ELP brachytherapy synergizes with paclitaxel to overcome resistance to radio-chemotherapy in multiple pancreatic tumor models". I regret that we are unable to consider it for external peer review.

As you may know, we screen manuscripts against our editorial criteria, and decline a substantial proportion of them without input from external referees. In such cases, even if reviewers were to certify the manuscript as technically correct, we feel that it would not be of outstanding interest to merit publication in *Nature Biomedical Engineering*. These editorial judgements are based on considerations of the degree of advance, broad implications, and breadth and depth of the work reported in the manuscript.

The topic of the Article is within the remit of the journal. However, in view of published work showing synergistic effects from combining brachytherapy and chemotherapy, including in the context of pancreatic cancer, and given the previous reports describing both formulations of the therapeutic agents used in the present study, we feel that the degree of advance of the work does not reach the high threshold that we look for in manuscripts that we consider for external peer review.

Please be assured that this editorial decision does not represent a criticism of the technical quality of the work. We hope that you will soon be able to see the manuscript published.

Best wishes,

João

Dr João Duarte
Senior Editor, *Nature Biomedical Engineering*

* Although we cannot offer to publish your manuscript in *Nature Biomedical Engineering*, the work may be appropriate for another journal published by Nature Research. Should you wish to explore suitable journals and transfer your manuscript to a journal of your choice, please use our manuscript transfer portal. This transfer link remains active until used. If you transfer your manuscript to Nature-branded journals or to the Communications journals, you will not have to re-supply manuscript metadata and files.

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Mon 24 Feb 2020
Decision on Article NBME-19-1528A-Z

Dear Prof Chilkoti,

Thank you again for submitting to *Nature Biomedical Engineering* your Article, "^{131}I-ELP brachytherapy synergizes with paclitaxel to overcome resistance to radio-chemotherapy in multiple pancreatic tumor models", and please accept my renewed apologies for the long delay in processing your manuscript. The manuscript has been seen by a total of six experts, but as I mentioned in previous emails three of those have not submitted a report. Thus we now have three reports which you will find at the end of this message. You will see that although the reviewers have some good words for the work, they articulate concerns about the degree of advance that this study represents over relevant published work, as well as some issues about how the data support the claims in the manuscript, and in this regard provide useful suggestions for improvement. We hope that with significant further work you can address the criticisms, increase the level of significance of the study, and convince the reviewers of its merits. In particular, we would expect that a revised version of the manuscript provides:

- Data on degradation of the implant in vivo;
- Additional data on biodistribution of the radionucleaotide;
- Additional background citations as highlighted by reviewers #2 and #3;
- Comparative studies in combining EBRT with CP-PTX studies according to the suggestions from reviewer #3, to ascertain the advantages of the approach over Cp-PTX;
- Qualification of the claims and addressing of the technical issues from the comments of reviewer #3;
- A reply to the questions of dosing effects from reviewer #2.

When you are ready to resubmit your manuscript, please upload the revised files, a point-by-point rebuttal to the comments from all reviewers, the (revised, if needed) reporting summary, and a cover letter that explains the main improvements included in the revision and responds to any points highlighted in this decision.

Please follow the following recommendations:

* Clearly highlight any amendments to the text and figures to help the reviewers and editors find and understand the changes (yet keep in mind that excessive marking can hinder readability).

* If you and your co-authors disagree with a criticism, provide the arguments to the reviewer (optionally, indicate the relevant points in the cover letter).

* If a criticism or suggestion is not addressed, please indicate so in the rebuttal to the reviewer comments and explain the reason(s).

* Consider including responses to any criticisms raised by more than one reviewer at the beginning of the rebuttal, in a section addressed to all reviewers.

* The rebuttal should include the reviewer comments in point-by-point format (please note that we provide all reviewers will the reports as they appear at the end of this message).

* Provide the rebuttal to the reviewer comments and the cover letter as separate files.

We hope that you will be able to resubmit the manuscript within 15 weeks from the receipt of this message. If this is the case, you will be protected against potential scooping. Otherwise, we will be happy to consider a revised manuscript as long as the significance of the work is not compromised by work published elsewhere or accepted for publication at *Nature Biomedical Engineering*.

We hope that you will find the referee reports helpful when revising the work. Please do not hesitate to contact me should you have any questions.

Best wishes,

João

Dr João Duarte
Senior Editor, *Nature Biomedical Engineering*

Reviewer #1 (Report for the authors (Required)):

This is an outstanding manuscript demonstrating brachytherapy using local implantation of biodegradable polymers containing beta-radiotherapy (131I). The paper is well written, and the experiments are well designed and excited. The paper is developing a localized radiotherapy which is sensitized with systemic chemotherapy, which the study finds is much more effective than external beam radiation.

Major comments:
(1) The concept is not very novel, a very similar paper with almost the same concept was published "Injectable Hydrogels for Localized Chemotherapy and Radiotherapy in Brain Tumors" (PMID: 29162424).
(2) despite the statement by the authors that the implant degrades way months after the decay of the isotope, not data was showed to demonstrate the biodegradation of the implant in vivo, and support this statement.
(3) no release profiles of the radionucleotide from the implant were demonstrated in vitro.
(4) no biodistribution of the radionucleaotide was shown to demonstrate the localized effect of the system.
(5) can the authors provide an explaination of why the IV PTX worked better than the intra-tumoral, this seems counter intuitive. This may be attributed to a problem in the release of PTX from the CP-PTX formulation. A control og free PTX intratumoral injection should be provided.

Reviewer #2 (Report for the authors (Required)):

In this study, Schaal et al. present novel and promising data showing that the combination of intratumoral I131 brachytherapy with paclitaxel induces impressive pancreatic tumor responses. They performed a series of rigorous studies to show that this combination appears significantly more effective than either IELP or chemotherapy alone, and also that IELP is more efficacious than external beam RT.

I am a clinical oncologist and not a laboratory scientist, and therefore my insight into the rigor and appropriateness of the experimental techniques is very limited. However, the experimental designs seemed robust and clearly reported.

From a clinical standpoint, and understanding very well the challenge of treating pancreatic cancer and the relative ineffectiveness of external beam radiation therapy for this indication, I believe the authors have made a strong case that this novel brachytherapy system may have uniquely potent effects, due in large part to synergy with chemotherapy which may be mediated by a number of differences vs. standard RT, particularly the ability to deliver a constant dose of radiation around-the-clock, and by advantageous effects on the tumor stroma.

My comments and questions are as follows:

1. The introductory background describing the role and evidence for EBRT in pancreatic cancer seems slightly outdated and potentially misleading. The LAP07 trial indicated that EBRT has no survival advantage in addition to chemotherapy for unresectable pancreatic cancer, and as such the role of EBRT in the routine first-line management of PC is diminished. The authors cite the LAP07 trial (citation #6) but do not seem to take heed of the failure to meet its primary endpoint. Similarly regarding SBRT approaches, while it is true that high doses per fraction have been attempted and found toxic, it is for this very reason that current approaches to EBRT intensification have focused on using multifraction regimens, commonly 5 fractions but in some cases using as many as 25 fractions. Overall, an updating and revising of this background regarding EBRT would be helpful.
2. In figure 2A, the trade name Abraxane should be replaced by nab-paclitaxel.
3. As noted above, I am not a laboratory scientist and as such have limited knowledge of the standards for in

vitro or animal-based study for pancreas cancer. Is the EBRT dose of 5Gy x 5 a reasonable approximation for the standard 50Gy in 25-28 fractions in humans? Otherwise, did the authors consider studying a higher EBRT dose in the mice to investigate the possibility that the apparent advantage of IELP over EBRT is simply due to a dose and/or fractionation effect?
4. Thinking ahead to a potential clinical trial in humans, a major practical obstacle and question is the appropriate dosing and placement and distribution of IELP in human pancreatic tumors. The authors do not appear to provide any information on how this was done in the mice (other than stating it was injected in the center of the tumor). Can more information about this be provided? In humans, the major limitation to EBRT dose is the tolerance of the GI hollow viscera, particularly duodenum. Portions of tumor that are relatively far from viscera can safely be given quite high doses of EBRT but the limiting issue is delivering ablative doses to tumor adjacent to the viscera. Is there any theoretical or experimental rationale to inform whether IELP radiation may have a better therapeutic ratio compared to EBRT when applied near GI viscera?

Reviewer #3 (Report for the authors (Required)):

In this study a biomaterials approach for delivering localized radionuclide therapy in treatment of pancreatic tumors was presented. Results showed that this approach was successful in slowing tumor growth and extending survival. Further work shed more light on the benefit of such an approach as it created significant alterations to the intercellular collagen and junctional proteins within the tumor microenvironment. The study also inferred therapeutic advantage because of the continuous exposure of the tumor cells to irradiation compared to strategies like external beam radiotherapy (EBRT). A key strength of this study is the demonstration of therapeutic efficacy across multiple models of pancreatic cancer. Also a significant amount of work was done to provide insights into why this approach may be better compared to EBRT. Another strength was the Bliss Independence isobolograms studies to show synergy. However, this reviewer has serious concerns about the claims made in this project which were not adequately supported by the results:

Here are some examples:
- The study claims in the abstract that this new approach would surmount the limits of external beam radiation therapy (EBRT) when combined with systemic paclitaxel. However the comparative studies done with EBRT are not adequate to make this claim: In the results in figures 2 and 5: Comparative studies in combining EBRT with CP-PTX studies were not done fairly: no intra-tumoral administration of drug was done as was done for brachytherapy; no studies on more relevant orthotopic models; no doses were varied (Note that RT doses in humans do not necessarily correspond to dosage in animals). Also specifics about the irradiation like field size were not provided which can be a factor given difficulty to reproduce IMRT capability and small field dosimetry in animal studies. All these reasons seriously weaken the validity of such a strong claim
- The authors also claim in the abstract that “Impressively, the combination 131I-ELP brachytherapy and systemic paclitaxel induced complete regression in three genetically diverse subcutaneous tumor models — BxPc3- luc2, MIA PaCa-2, and AsPc-1 — as well as an orthotopic pancreatic tumor”. As figure 6 shows this claim is not completely true. From this figure, complete regression was only observed in one model, and this was only temporary. Long term survival while statistically significant was definitely not impressive compared to what has been observed in other preclinical studies, which were not adequately reviewed in this work.
- The authors also claim that these effects could not be replicated with EBRT combination therapy, demonstrating that 131I-ELP brachytherapy can provide a promising new radiotherapy alternative for overcoming resistance in pancreatic cancer. The fact that these effects could not be replicated might be because enough parameters were not varied for the animal studies with EBRT like dose variation of EBRT or drug combination etc. Furthermore, the authors completely failed to compare their studies with previous studies of pancreatic cancer with brachytherapy. This would be a relatively more fair or adequate comparison before comparison with EBRT. Many previous studies have already been done with brachytherapy for pancreatic cancer with apparently even better results in human than seen in this study for mice:
See https://www.ncbi.nlm.nih.gov/pubmed/28151849, https://www.ncbi.nlm.nih.gov/pubmed/1698755, https://www.ncbi.nlm.nih.gov/pubmed/1587751 and the references therein. The
authors need to address with some research evidence why they think their brachytherapy approach would succeed compared to these previous work. This would be the first level of comparison needed with adequate variation of parameters before comparison with EBRT.
- In the conclusion, the authors claim that “Utilizing new advances in material science and drug delivery, our results demonstrate unforeseen effects and exciting benefits that radiotherapeutic medicine can provide to

this field" Some of these effects in terms of therapeutic efficacy have already been seen in previous brachytherapy studies, e.g. using I-125, which the authors failed to review as is important for such a manuscript.
- In the discussion the authors state: "we hypothesize that the synergistic efficacy of 131I-ELP with paclitaxel could possibly prevent and even enable the treatment of metastatic disease when further combined with immune-oncological agents. This is because our data show that 131I-ELP provides highly potent radiation-induced cell death that is far superior to EBRT, which has been widely shown to help potentiate immune-oncological interventions. Such effects could be used to increase antigen display and trafficking or re-polarize the infiltrating immune cells within the tumor…. The result could be to potentiate an 'abscopal effect', whereby local treatment primes the immune system to detect and treat disseminated metastases." While it is ok to speculate in the discussion section, this claim is a stretch given that brachytherapy with continuous irradiation over many days may actually destroy infiltrating radiosensitive immune-cell populations needed to potentiate the abscopal effect. Again the comparison with EBRT is not fair as this study did not directly compare immunogenic cell death generated by brachytherapy and EBRT
- Overall many of the claims made in this study were exaggerated by use of words like "impressively", "unforeseen", "exciting", 'highly striking", " far outstrips", " our rigorous preclinical studies" " no other treatment combination in any of the preclinical literature has achieved a 100% ORR across these cell lines". The last claim again highlights the lack of adequate review of the literature as with previous brachytherapy work, and preclinical studies combining EBRT with immunoadjuvants.

Other concerns:
1. Methods: While the authors claim that they did rigorous preclinical studies, there are also significant concerns in the methods:
- Studies on Histochemical pathology of treated tumor used only 2 mice per cohort. This lacks enough rigor
- Toxicity studies were not that rigorous. Figure 7 shows that the following was investigated: Body weight over about 63 days: Serological α-amylase levels and off-target toxicities. No adequate toxicity studies were done to examine the systemic effects of the drugs e.g. in liver or kidney, and no longer term toxicities, apparently because the study also showed little impressive long term survival
- Not enough information was provided to justify rigor in the EBRT studies: e.g. what was the field size used for subcutaneous/orthotopic studies? Could the field size be a factor in influencing the outcomes for this animal studies where small field dosimetry is a factor compared to the delivery of IMRT clinical doses adopted for this study?
2. Not enough was done to justify why systemic delivery was more effective than intratumoral given the mechanism of action of the drug. The main benefit of systemic administration given the mechanism of action of the drug lies in the non-invasiveness of such an approach. But the Brachytherapy approach is already invasive, so it is confounding that i.v was better than i.t. administration of the drug. It will be helpful to clarify this.
3. Novelty: without adequate review and comparative investigation with other brachytherapy approaches that have gone through preclinical phase to clinical trials, the case for novelty of this work is weak.
4. Significance: issues on clinical Implementation of this approach needs to be better addressed including:
a. Which patients would benefit? It is not clear if this is for treatment of patients with localized pancreatic cancer or for patients with metastasis. If localized, the significance is less because most patients with pancreatic cancer are diagnosed with metastatic disease. This is partly why previous brachytherapy approaches have not been adopted. It would be good if the authors can elaborate on the specific patient populations they envision would benefit from this given the localized nature of this therapy approach.
b. Several endoscope-guided implantation techniques have indeed already been developed and employed clinically, but optimization to inject the ELP is still required. Would this follow previous approaches with brachytherapy for pancreatic cancer? What would be different.
c. The authors state "Despite all of our current surgical and radio-chemotherapeutic options, clinical evidence continues to stress the need for improved treatment modalities for local disease, as high recurrence rates and poor survival persist" Given that only temporal regression was observed in the best results of this study (figure 6). It is not clear how this approach would be better. It would be helpful to have the authors clarify the benefits and how it would be different from other brachytherapy approaches that have failed to be adopted following preclinical studies and clinical trials
d. Previous Brachytherapy studies (https://www.ncbi.nlm.nih.gov/pubmed/29384971, https://www.ncbi.nlm.nih.gov/pubmed/20101258) show major toxicity issues for pancreatic cancer. While the half-life for different brachytherapy sources should be considered, the approach proposed in this study may also face similar issues. This study does not address how this would be different in comparative investigations with these previously used brachytherapy approaches.

Wed 18 May 2022
Decision on Article NBME-19-1528B

Dear Prof Chilkoti,

Thank you for your patience in waiting for the feedback on your revised manuscript, "^{131}I-ELP brachytherapy synergizes with nanoparticle paclitaxel to overcome resistance to radio-chemotherapy in multiple pancreatic tumors", which has been seen by one of the original reviewers (Reviewer #2) and one more expert (Reviewer #4). Unfortunately, the other two reviewers from the previous round were unavailable, and it took me longer than expected to find an available expert with complementary expertise.

In their reports, which you will find at the end of this message, you will see that the reviewers acknowledge the substantial improvements to the work and that they raise a few additional points that I am sure you will be able to address by including the necessary caveats in the discussion.

When you are ready to resubmit your manuscript, please upload the revised files, a point-by-point rebuttal to the comments from all reviewers, the reporting summary, and a cover letter that explains the main improvements included in the revision and responds to any points highlighted in this decision.

We look forward to receive a further revised version of the work. Please do not hesitate to contact me should you have any questions.

Best wishes,

Pep

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Pep Pàmies
Chief Editor, *Nature Biomedical Engineering*

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Reviewer #2 (Report for the authors (Required)):

After a long (2 year) period, the authors present a comprehensively revised and expanded manuscript regarding their investigation of a novel intratumoral I131 brachytherapy system combined with paclitaxel in mouse models of pancreatic cancer. They have done an excellent job responding to the reviewers' critiques and in supplementing their experiments to address several uncertainties and concerns. Overall, I believe that the critiques have been well-addressed. I continue to believe that this is novel and potentially impactful work that could develop into a meaningful advance over currently available radiation and chemotherapies for this difficult disease. This of course would ultimately depend on whether the efficacy and safety observed in mice translates to humans, and also on the development of technical means to accurately deliver I-ELP to human patients. These matters are of course beyond the scope of this paper, though the authors do comment in the discussion and response to
reviewers regarding the potential means of technical delivery of I-ELP in humans.

The authors have updated and improved their discussion of the existing literature and role for external beam RT in pancreatic cancer. However, there is arguably still some missing nuance. They cite an NCDB analysis showing that survival improves with doses >4Gy, implying that hypofractionated SBRT inherently improves outcomes on the basis of hypofractionation. However, the relative efficacy of different radiation doses for pancreatic cancer is almost certainly due to higher biologically effective doses (BED) which is a function of fraction number, not only fraction size. There are more recent experiences with radiation regimens that use relatively modest fraction sizes (3-4.5Gy) but increase the number of fractions to achieve high BED (see Reyngold M et al., JAMA Oncology 2021). The apparent success and feasibility of these regimens also may provide additional context to the authors' statement that "increasing SBRT ablative doses above this threshold (25-33Gy in 3-5 fractions)
result in serious GI toxicity." It is true that increasing doses *to the GI luminal organs* results in toxicity, but new techniques indicate that it is increasingly feasible to escalate radiation dose to the tumor while still

limiting dose to the GI organs below toxicity thresholds.

I do not otherwise have substantive other comments or concerns regarding the revised manuscript. I commend the authors for performing additional experiments to investigate the potential impact that different external beam radiation prescriptions might have on their results, and their inclusion of this data meaningfully strengthens the conclusion that there is something fundamentally more efficacious about I-ELP brachytherapy vs. EBRT. The additional details and specifications regarding the animal irradiation is also appreciated.

Reviewer #4 (Report for the authors (Required)):

In this study, Schaal et al. developed a liquidly injectable biopolymer to deliver 131Iodine radionuclide as a form of brachytherapy. In particular, they present a series of data showing that the combination of intratumoral I131 brachytherapy with paclitaxel induces impressive pancreatic tumor responses. They performed a series of rigorous studies to show that this combination appears significantly more effective than either I-ELP or chemotherapy alone, and that I-ELP is more efficacious than external beam RT. Although a large amount of data have been provided, and the authors have carefully responded to comments of the previous reviewers, I still feel the novelty of this work is limited. Moreover, the clinical significance of this work may not be high enough for publication in Nature Biomedical Engineering.

- In the discussion the authors state:“This is because our data show that 131I-ELP provides highly potent radiation-induced cell death that is far superior to EBRT, which has been widely shown to help potentiate immune- oncological interventions.” Although these speculations are reasonable, it is not difficult to evaluate the immune cell infiltration and cytokine levels in tumors, and we hope that the authors can take them into consideration.

- The combination therapy with 131I-ELP brachytherapy and CP-PTX was promising in this work, but the selection of chemotherapy drugs was not emphasized in the paper. What effect will be produced when combined with other types of chemotherapy drugs?

- I-131 may not be the best radioisotope for brachytherapy, as its gamma emission energy is too high and its beta emission energy is not high enough. Y-90 may be a better radioisotope (higher beta energy and longer therapeutic distance) if beta particles play the key role in this system.

Wed 18 May 2022
Decision on Article NBME-19-1528B

Dear Prof Chilkoti,

Thank you for the latest version of your manuscript, "^{131}I-ELP brachytherapy synergizes with nanoparticle paclitaxel to overcome resistance to radio-chemotherapy in multiple pancreatic tumors". Having consulted with Reviewers #2 and #4 (whose comments you will find at the end of this message), I am pleased to write that we shall be happy to publish the manuscript in *Nature Biomedical Engineering*, provided that the points specified in the attached instructions file are addressed.

When you are ready to submit the final version of your manuscript, please upload the files specified in the instructions file.

Please do not hesitate to contact me should you have any questions.

Best wishes,

Pep

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Pep Pàmies
Chief Editor, *Nature Biomedical Engineering*

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Reviewer #2 (Report for the authors (Required)):

The authors have acknowledged my previous minor comment about fraction number and fraction size as both being relevant to achieving tumoricidal effect with external beam radiation therapy and revised the manuscript accordingly. I do not have any further substantive comments regarding this manuscript.

Reviewer #4 (Report for the authors (Required)):

Although I still have doubt regarding the novelty of this work, I would let the editor to make the final decision.

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Nature Biomedical Engineering is a Transformative Journal. Authors may publish their research with us through the traditional subscription access route, or make their paper immediately open access through payment of an article-processing charge. More information about publication options is available.

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Rebuttal 1

Reviewer 1

This is an outstanding manuscript demonstrating brachytherapy using local implantation of biodegradable polymers containing beta-radiotherapy (^{131}I). The paper is well written, and the experiments are well designed and excited. The paper is developing a localized radiotherapy which is sensitized with systemic chemotherapy, which the study finds is much more effective than external beam radiation.

Major comments:

1. *The concept is not very novel, a very similar paper with almost the same concept was published "Injectable Hydrogels for Localized Chemotherapy and Radiotherapy in Brain Tumors" (PMID: 29162424).*
   - The reviewer is correct in that the delivery biopolymer system used in this paper is not novel at the time of submission. We explicitly acknowledged in the introduction of the paper that we previously published our efforts to engineer this biopolymer system and optimize its stability for intratumoral radionuclide therapy.
     The novelty and impact of our paper center around the following three findings:
     1. The unexpected level of synergy observed for ^{131}I-ELP brachytherapy combined with paclitaxel nanoparticle **was not** observed for the combination of external beam radiation therapy with paclitaxel nanoparticles.
     2. The underlying mechanism that drives this synergy, particularly the ability of the ^{131}I-ELP to differentially modify the tumor microenvironment compared to external beam radiation.
     3. Achieving complete regression *in multiple pancreatic tumor models* with our combination therapy — models that are known to be highly resistant to all types of therapeutics—is, unprecedented, we submit, as we show via a thorough review of literature of 300 extant studies that we have documented in the ancillary document that we have provided with this revised manuscript.
   - The authors were not aware of the study the reviewer cited but appreciate the reviewer's inclusion as it is a highly relevant system for comparison. There are several key differences in our injectable biopolymer system as opposed to the chitosan hydrogel system in the citd manuscript that differentiate our technology and allow for it to be used more effectively to treat pancreatic cancer.
     - First, the chitosan hydrogel system was designed to fill a resected surgical cavity. The higher viscosity of the system and finely tuned control release design of the system prevents it from being injected into a tumor or tumor bed. Because of this, de la Putente et. al. conducted their preclinical testing by surrounding a 2 mm tumor with a 4 mm shell of their radioactive hydrogel. There are major problems with his approach—first, this approach cannot, we believe, be translated to the clinic as it will be impossible to encase a tumor with a hydrogel. Second, another major problem with this approach is that the average penetration depth of the β-particles emitted by ^{131}Iodine is only 0.4 mm, with a maximum distance of 2 mm. Because of this emission geometry, most of the

radiation dose delivered in their hydrogel is absorbed within the hydrogel itself and not by the tumor. For tumors that are larger than 2 mm, not enough β-particle radiation would penetrate into the tumor from the hydrogel to provide an effective therapeutic dose.

In contrast, our ^{131}I-ELP brachytherapy system is designed to be directly injected into the tumor interstitium. The ELP phase transitions into an insoluble coacervate by body heat and is further stabilized radiation crosslinking of the coacervate into a hydrogel. It does not require extrinsic crosslinking agents. The thermally stabilized coacervation of ^{131}I-ELP allows it to be injected into intact tumors or into the tumor bed following surgical resection. With this approach, the ^{131}Iodine radionuclide is perfused and stabilized throughout the tumor interstitium, ensuring the β-particle emissions are absorbed primarily by the tumor.

- Second, although the injectable chitosan hydrogel is capable of both ^{131}I radiotherapy and TMZ chemotherapy delivery, the paper did not explore nor achieve synergistic benefits in the treatment of tumors. In contrast, the main objective of our paper was to explore the potential synergy between radiation therapy and chemotherapy, and we demonstrate in multiple pancreatic tumor models that we can overcome the intrinsic radiation and chemotherapy resistance of pancreatic cancer with our approach, leading to significant tumor regression and prolonged survival in multiple pancreatic tumor models.

- Third, our data shows more compelling therapeutic efficacy than Putente, et al. We acknowledge that a direct comparison of efficacy is fraught with complications as our manuscript focused on pancreatic cancer, while Putente et al. focused on the treatment of glioblastoma tumors. These tumors differ both in anatomical location and delivery challenges, but both tumors rapidly proliferate and possess high radiation resistance. As the reviewer is mostly likely aware, the α/β ratios is typically used to characterize the intrinsic radiation resistance of a tumor (α) and its repair capacity (β). Glioblastoma typically exhibit an average α/β ratio of 8 Gy (5.0-10.8 range)[1]. Multicenter studies for stereotactic body radiotherapy, meanwhile, put the α/β ratio of pancreatic cancer at 10 Gy[2]. This is important in considering the results between our two systems. Thus, given the similar α/β ratios, it is not unreasonable to compare the efficacy and safety of the two systems.

  The chitosan hydrogel system did control tumor growth but only extended survival to 32 days (Putente, Figure 6C). Our ^{131}I-ELP combination therapy in contrast, completely ablated tumors and led to long-term survivors in four different tumors models of pancreatic cancer with different genetic profiles as well as orthotopic tumors and exhibited no adverse effects. These results stand in stark contrast to each other.

[1] M Mason, N Laperriere, W Wick, DA Reardon, A Malmstrom, E Hovey, M Weller, JR Perry. (2016) *Neuro-Oncology Practice*. **3**(2) DOI:10.1093/nop/npv027

[2] PW Prior Jr, X Chen, WA Hall, BA Erickson, A Li. (2018). "Estimation of the Alpha-beta ratio for chemoradiation of locally advanced pancreatic cancer". *Int J Rad Onc Bio Physics.* **102**(3) DOI: https://doi.org/10.1016/j.ijrobp.2018.06.250

- Our studies showed that our ^{131}I-ELP radiotherapy system provided better synergistic and therapeutic efficacy when combined with a systemic chemotherapy approach over local chemotherapy. This result was counter intuitive as, similar to the results of de la Putente et al., we expected the higher potential bioavailability resulting from local delivery of chemotherapy to result in greater anti-tumor activity. However, our results repeatedly showed that systemic chemotherapy is the preferred route of administration of paclitaxel for combination therapy for Abraxane (nab-paclitaxel) and the CP-PTX nanoparticles.

2. *Despite the statement by the authors that the implant degrades way months after the decay of the isotope, not data was showed to demonstrate the biodegradation of the implant in vivo and support this statement.*
   - Originally, this data was included in the supplemental information of our manuscript in Table S3. However, we agree with the reviewer's comment that this is a critical design feature that should be addressed in the main body of our manuscript. To this end, we have now included information from several new studies that we carried out to address this comment, including an orthotopic biodistribution study to further clarify the degradation of the ^{131}I-ELP depot. The manuscript now includes the following new data:
     - The *in vivo* whole-body retention of ^{131}Iodine from our initial pilot study in Figure 1, examining co-administration or separate injection of CP-PTX with the ^{131}I-ELP at a dose of 1.3μCi/mg.
     - The tumor-specific retention and circulating blood release profile for ^{131}I when intratumorally injected into an orthotopic pancreatic tumor mouse model. This data has now been included in Figure 1.
     - Depot stability profile of our final optimized therapeutic regimen (10 μCi/mg) used to treat orthotopic BxPc3-luc2 pancreatic tumors. This data is now included as a sub-panel in Figure 8.
     - Finally, Table S3 of Supplemental Information summarizes the complete evaluation of ^{131}I-ELP stability across all our *in vivo* studies. This includes analysis of the depot degradation half-life, as well as its effective ^{131}I therapeutic half-life.

3. *No release profiles of the radionucleotide from the implant were demonstrated in vitro. (4) no biodistribution of the radionucleotide was shown to demonstrate the localized effect of the system.*
   - As mentioned above, to address the reviewers' concern, we performed experiments to evaluate the release profile of the ^{131}I-ELP brachytherapy depot. The release kinetics were measured after intratumoral administration of ^{131}I-ELP to form a dept in an orthotopic pancreatic tumor mouse model to make the experiment —and ensuing data— as clinically relevant as possible. The tumor retention of ^{131}I as well as the kinetic release profile of ^{131}I into blood have now been included as part of Figure 1 in the revised manuscript.

4. *Can the authors provide an explanation of why the IV PTX worked better than the intra-tumoral, this seems counter intuitive. This may be attributed to a problem in the release of PTX from the CP-PTX formulation. A control of free PTX intratumoral injection should be provided.*

   - This is an excellent question, as we agree that it should intuitively follow that the intra-tumoral delivery of CP-PTX should increase the chemotherapy's bioavailability at the tumor site. However, we found that the co-infusion of the CP-PTX with the ^{131}I-ELP disrupted the stability of the ^{131}I-ELP depot. This data is now included in Figure 1. We believe these results are better understood in the context of our dose-escalation experiments with CP-PTX and radioactivity. In these studies, we found that the radiation dose had a major impact on tumor response. In contrast, no discernible difference in tumor response was observed when the paclitaxel dose was increased. Thus, the benefit of increasing the bioavailability of the CP-PTX by co-infusion into tumor compared to systemic administration was offset by the increased loss of radioactivity from the tumor upon co-infusion.

     To answer the reviewer's question regarding the release of PTX from CP-PTX, we rechecked our *in vitro* quality assurance tests on the batches of CP-PTX used in our studies and found no discrepancies between them and previous batches whose release had been extensively characterized and that have proven efficacious in treating several other PTX sensitive tumors in mouse models.

     J Bhattacharyya, et al. (2015) *Nat. Commun*. **6**:7939. PMID: 26239362

     To fully understand the underlying causes of this counter-intuitive result, a much more extensive set of studies would need to be performed. However, a review of other localized delivery systems for paclitaxel in the literature have not shown a strong therapeutic response in treating pancreatic tumors, which is consistent with our results. For example, a chitosan-based hydrogel system was developed for sustaining controlled release of paclitaxel and used to intratumorally treat EMT-6 tumors in mice. Even at a dose of 40/mg/kg locally, this intratumoral treatment only mildly inhibited tumor growth (E Ruel-Gariepy, et al. 2004. *European Journal of Pharmaceutics and Biopharmaceutics*, **57**: 53-63 https://doi.org/10.1016/S0939-6411(03)00095-X).

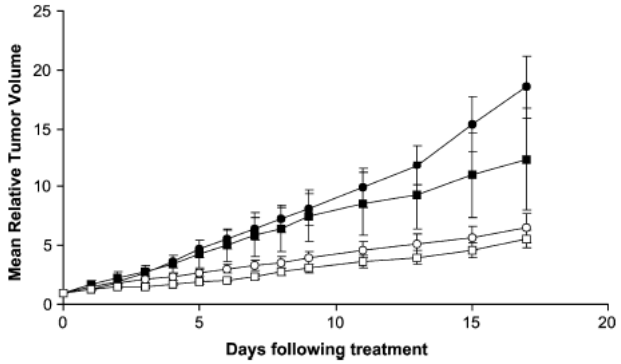

Fig. 5. In vivo anti-tumor effect for treatments initiated on day 4 after tumor inoculation. Saline 0.2 mL/day IV × 4 days (closed circles, $n = 8$), Taxol® 10 mg/kg/day IV × 4 days (open circles, $n = 8$), C/GP solution 10 μL intratumoral (closed squares, $n = 8$) and paclitaxel/C/GP solution (64 mg/mL paclitaxel) 10 μL (40 mg/kg paclitaxel) intratumoral (open squares, $n = 8$).

Another example is a poly(lactic-co-glycolic) biodegradable, drug eluting mesh that was developed for sustained local release of paclitaxel over 60 days and was used to treat patient-derived PDAC orthotopic tumors in mice. (L Indolfi, et al. 2016. *Biomaterials*. **93: 71-82** https://doi.org/10.1016/j.biomaterials.2016.03.044). In these studies, tumors were explicitly pre-evaluated to ensure sensitivity to paclitaxel treatment. As seen below, the PLGA drug eluting system resulted in tumor growth inhibition in the different xenografts. While PDAC-6 showed differentiation between delivery vectors, i.v. and intratumoral treatment were nearly indistinguishable in the PDAC-3 tumors at timepoints evaluated in our pilot study (11 days). In contrast, our BxPc3 and AsPc-1 models were selected explicitly as they have demonstrated high resistance to radiation and chemotherapy, a phenotype that mirrors clinical phenotypes of pancreatic cancer.

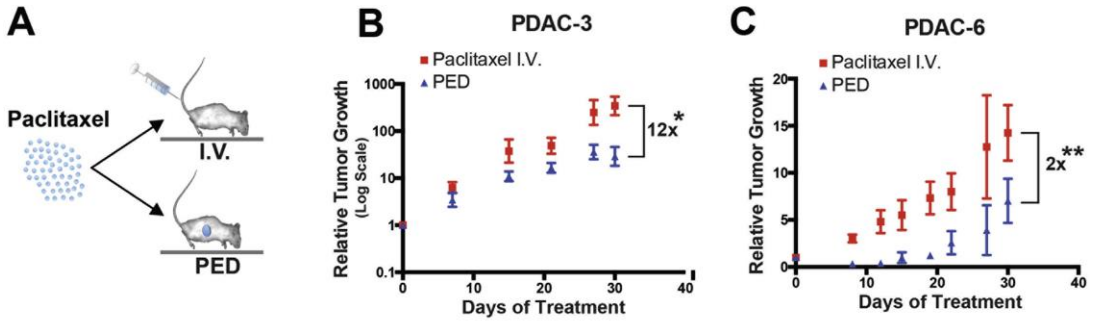

Fig. 5. Response to treatment is highly improved by targeted delivery of chemotherapy. (**A**) Schematic representation of the *in vivo* model design used to compare the efficacy of paditaxel delivered intravenously (intravenous) to the paditaxel eluting device (PED) treatment. (**B-C**) Relative tumor growth curves of PDAC-3 and PDAC-6 mouse xenografts after either paclitaxel intravenous or PED treatment (data are displayed as mean ± s.e.m). (**D**) Representative images of tumor masses isolated from PDAC xenografts after treatment with

Finally, we believe that further development and fine tuning of the co-administration system is outside the scope of the paper. Instead, our focus is to identify the conditions that best achieve synergy between brachytherapy and chemotherapy, understand the underlying mechanisms, and compare our results to external beam radiation. In terms of clinical adoption, we also believe that our findings make our ^{131}I-ELP system more amenable to being combined with existing, clinically approved intravenous chemotherapy regimens, such as nab-paclitaxel.

Reviewer 2

In this study, Schaal et al. present novel and promising data showing that the combination of intratumoral I131 brachytherapy with paclitaxel induces impressive pancreatic tumor responses. They performed a series of rigorous studies to show that this combination appears significantly more effective than either I-ELP or chemotherapy alone, and that I-ELP is more efficacious than external beam RT.

I am a clinical oncologist and not a laboratory scientist, and therefore my insight into the rigor and appropriateness of the experimental techniques is very limited. However, the experimental designs seemed robust and clearly reported.

From a clinical standpoint, and understanding very well the challenge of treating pancreatic cancer and the relative ineffectiveness of external beam radiation therapy for this indication, I believe the authors

have made a strong case that this novel brachytherapy system may have uniquely potent effects, due in large part to synergy with chemotherapy which may be mediated by a number of differences vs. standard RT, particularly the ability to deliver a constant dose of radiation around-the-clock, and by advantageous effects on the tumor stroma.

My comments and questions are as follows:

1. The introductory background describing the role and evidence for EBRT in pancreatic cancer seems slightly outdated and potentially misleading. The LAP07 trial indicated that EBRT has no survival advantage in addition to chemotherapy for unresectable pancreatic cancer, and as such the role of EBRT in the routine first-line management of PC is diminished. The authors cite the LAP07 trial (citation #6) but do not seem to take heed of the failure to meet its primary endpoint. Similarly regarding SBRT approaches, while it is true that high doses per fraction have been attempted and found toxic, it is for this very reason that current approaches to EBRT intensification have focused on using multi-fraction regimens, commonly 5 fractions but in some cases using as many as 25 fractions. Overall, an updating and revising of this background regarding EBRT would be helpful.

- We appreciate the clinical insights of the reviewer and have updated our introduction to better describe the clinical outcomes of external beam radiation therapy (EBRT) for the treatment of pancreatic cancer. We have added new citations to new trials exploring EBRT dose escalation and multi-fraction regimens to improve patient responses.

  To summarize, NCCN guidelines still recommend EBRT combined with chemotherapy, even with the results from the LAP07 trial. The reviewer is correct that the LAP07 trial, unlike other previous trials, failed to meet the primary endpoint of improved overall survival when receiving 54Gy of EBRT (1.8 Gy fractions) combined with capecitabine compared to patients receiving gemcitabine alone. Survival was 16.5 months for EBRT + capecitabine compared to 15.2 months for gemcitabine alone. However, the LAP07 data still showed reduced rates of local disease progression and a trend to improved progression-free survival. Subsequent radiotherapy trials explored the use of stereotactic beam radiation therapy (SBRT) to improve upon the local control benefits when higher dose fractions are used for treatment [1]. The National Cancer Data Base was reviewed to retrospectively examine the impact of SBRT, defined as fractionated doses >4 Gy to treat local pancreatic cancer patients [2]. In addition to finding improved overall survival through multivariate analysis, a lower proportion of SBRT treated patients developed T4 tumors and were less likely to be node positive. However, SBRT delivered in ablative doses of 1-3 fractions were associated with serious GI toxicity [3,4]. Subsequent trials established that hypofractionated regimens delivering 25-33 Gy in 3-5 fractions resulted in minimal acute and late toxicity [5,6]. These regimens, however, only resulted in a modest increase in overall survival as compared to conventional radiotherapy, with a 2-year survival rate of 21.7% vs 16.5%, p = 0.0014 [1,2].

  We have amended the introduction of the revised manuscript to include this updated clinical trial data.

1. M Reyngold, et al. (2019). *Radiation Oncology*. **14**:95 https://doi.org/10.1186/s13014-019-1309-x
2. J Zhong, et al. (2017). *Cancer*. **123**(18): 3486-3493. DOI: 10.1002/cncr.30706
3. D Schellenbert, et al. (2008) *Int J Radiat Oncol Biol Phys.* **72**(3): 678-86. DOI: 10.1016/j.ijrobp.2008.01.051

4. M Hoyer, et al. (2005) *Radiother Oncol*. **76**(1): 48-53 DOI: 10.1016/j.radonc.2004.12.022
5. JM Herman, et al. (2015) *Cancer* **121**(7):1128-37 DOI: 10.1002/cncr.29161
6. JJ Park, et al. (2017) *Acta Oncol.* **56**(12):1746-1753 https://doi.org/10.1080/0284186X.2017.1342863

2. In figure 2A, the trade name Abraxane should be replaced by nab-paclitaxel.

- We have updated all our figures and text to remove the tradename. All instances now refer to it as nab-paclitaxel. It should be noted that the nab-paclitaxel specifically purchased and used in these studies was Abraxane®. This is now only mentioned in the methods.

3. As noted above, I am not a laboratory scientist and as such have limited knowledge of the standards for in vitro or animal-based study for pancreas cancer. Is the EBRT dose of 5Gy x 5 a reasonable approximation for the standard 50Gy in 25-28 fractions in humans? Otherwise, did the authors consider studying a higher EBRT dose in the mice to investigate the possibility that the apparent advantage of IELP over EBRT is simply due to a dose and/or fractionation effect?

- For our original selection of the EBRT dose scheme, we consulted and relied on the clinical and translational expertise of our collaborators and co-authors Dr. David G. Kirsch and Dr. Jeremy Brownstein, radiation oncologists at the Duke Cancer Institute. The 5Gy x 5 fraction dose scheme was selected based on several factors.
  - First, the aggressiveness of pancreatic cell line xenografts in immunocompromised mice often cause them to be non-responsive to 2Gy fractions of EBRT. This causes the tumors to grow too rapidly for the full course of EBRT (25 fractions) to be administered[7,8]. In fact, most fractions of this regimen cannot be administered to mice in these tumor models as the mice have to be euthanized because of their tumor burden. Thus, we decided that the best course would be to select a fractionated EBRT regimen that could be fully administered to the mice with these tumor xenografts and that approximated the clinical standard of care for humans. A regimen that only utilized 5 fractions could be completed in 1.5 weeks and is well within the tumor growth profile of BxPc3, AsPc-1, and MIA PaCa-2.
    7. MA Morgan, et al. (2008) *Clin Cancer Res*. **14**(16): 5142-5149.
    8. A Sugyo, et al. (2015) *PLOS One*. **10**(4): e0123761.
  - Second, clinical studies by the Swedish Rectal Cancer Trial examining the 5Gy x 5 dose schema in rectal cancer found that this short radiation course was equivalent in response to the 28 dose EBRT standard regimen[9,10]. This agreed with the work done by Bujkot et al who also saw that 5Gy x 5 fraction radiotherapy showed similar efficacy as standard EBRT chemoradiation therapy in a randomized trial[11]. Based on this clinical data, we concluded that using a 5Gy x 5 dose scheme was an appropriate EBRT dose scheme that would ensure that the mice would receive the full EBRT treatment for comparison against our ^{131}I-ELP brachytherapy.
    9. TS Hong, H Mamon. (2011) *Oncologist.* **16**(5): 717-721.
    10. B Cedermark, et al. (1997) *N Engl J Med.* **336**(14): 980-987.
    11. K Bujko, et al. (2006) Br J Surg. **93**(10): 1215-1223.
  - Third, we wanted to ensure that the EBRT dose schema we selected was actively being used in a clinical setting to treat locoregional pancreatic tumors. In the case of 5Gy x 5

fraction EBRT, we found multiple instances where this fractionation treatment has been used in patients.

12. M Palta, et al. (2016) *Int J Rad Oncology Biology Physics.* **96**: S204-S205
13. MD Chuong, et al. (2013) *Int J Radiat Oncol Biol Phys.* **86**:516:522
14. JM Herman, et al. (2015) *Cancer*. **121**: 1128-1137

- Nevertheless, we appreciate the reviewer's concern regarding the EBRT dose regimen. We thus went back and calculated the biological equivalent dose (BED) of a variety of EBRT dose regimens for pancreatic tumors with an α/β ratio of 10.

Fraction per Dose	# Fractions	Total Dose (Gy)	BED (α/β=10)	In Study
5 Gy	x5	25	37.5 Gy	Y
2 Gy	x25	50	60 Gy	
3 Gy	x15	45	58.5 Gy	Y
5 Gy	x10	50	75 Gy	
25 Gy	x1	25	87.5 Gy	
10 Gy	x5	50	100 Gy	Y
15 Gy	x5	75	187.5 Gy	

Despite the clinical literature, we did find that the BED calculation for the 5 Gy x 5 fraction regimen to be lower than the standard 25 fraction approach. **Thus, to directly and explicitly address this concern raised by the reviewer, we have now included additional tumor regression studies in BxPc3 xenografted mice that examine the efficacy and synergy of two additional dose regimens.** We selected a BED equivalent regimen (3Gy x 15 fractions) and a EBRT dose scheme with a higher BED (10Gy x 5 fractions). These results are now included in the revised manuscript.

4. Thinking ahead to a potential clinical trial in humans, a major practical obstacle and question is the appropriate dosing and placement and distribution of IELP in human pancreatic tumors. The authors do not appear to provide any information on how this was done in the mice (other than stating it was injected in the center of the tumor). Can more information about this be provided? In humans, the major limitation to EBRT dose is the tolerance of the GI hollow viscera, particularly duodenum. Portions of tumor that are relatively far from viscera can safely be given quite high doses of EBRT, but the limiting issue is delivering ablative doses to tumor adjacent to the viscera. Is there any theoretical or experimental rationale to inform whether IELP radiation may have a better therapeutic ratio compared to EBRT when applied near GI viscera?

- This is a very important question, and it is something we have thought extensively about. We believe one of the chief advantages of our liquid biopolymer system is that it can be injected using ultra-sound guided endoscopy methods that are currently established and used to deliver local chemotherapy to pancreatic tumors.

15. AS Rosemurgy, et al. (2017) *J Pancreat Cancer.* **3**(1): 58-65.
16. BM Yan, J Van Dam. (2008) *Can J Gastroenterol*. **22**(4): 405-410.

For our preclinical studies in mice, however, endoscopic methods were impractical due to the small size of the mice. Instead, we used a survival surgery procedure to access the pancreas of the mouse and locate the tumor at the time of treatment. We then inserted a catheterized syringe into the tumor and injected a fixed volume of ^{131}I-ELP into the center of the tumor

utilizing a micro-syringe pump. We are currently optimizing a proof-of-concept study for evaluating US-guided endoscopic delivery of our ^{131}I-ELP in a large —porcine— animal model as this will be critical for clinical translation but that study is still in the IRB approval stage and cannot be included in this study.

- Your concern about the dose tolerance of the GI hollow viscera is one of the main reasons that we thought that ^{13}I-ELP brachytherapy may provide a therapeutic advantage over traditional EBRT. This is because the radionuclide we have used —^{131}Iodine— is a β-particle emitting radionuclide, which has a maximum penetration depth of ~2 mm in tissue, with an average range of 0.4 mm. Because of this low penetration depth, our brachytherapy system provides focal radionuclide therapy. The rapid phase transition of the ELP ensures that the biopolymer retains the radionuclide within the site of the injection, ensuring that the radiation exposure is limited to the tumor. This contrasts with the highly penetrative radiation found in EBRT or the gamma rays employed in traditional brachytherapy seeds. The narrow margins of ^{131}I β-particle emissions also spare the GI viscera while fully exposing the pancreatic tumor to the radionuclide dose. This results in a better therapeutic index for the patient and surrounding viscera.

Reviewer 3

In this study a biomaterials approach for delivering localized radionuclide therapy in treatment of pancreatic tumors was presented. Results showed that this approach was successful in slowing tumor growth and extending survival. Further work shed more light on the benefit of such an approach as it created significant alterations to the intercellular collagen and junctional proteins within the tumor microenvironment. The study also inferred therapeutic advantage because of the continuous exposure of the tumor cells to irradiation compared to strategies like external beam radiotherapy (EBRT). A key strength of this study is the demonstration of therapeutic efficacy across multiple models of pancreatic cancer. Also, a significant amount of work was done to provide insights into why this approach may be better compared to EBRT. Another strength was the Bliss Independence isobolograms studies to show synergy. However, this reviewer has serious concerns about the claims made in this project which were not adequately supported by the results:

Here are some examples:

1. *The study claims in the abstract that this new approach would surmount the limits of external beam radiation therapy (EBRT) when combined with systemic paclitaxel. However the comparative studies done with EBRT are not adequate to make this claim: In the results in figures 2 and 5: Comparative studies in combining EBRT with CP-PTX studies were not done fairly: no intra-tumoral administration of drug was done as was done for brachytherapy; no studies on more relevant orthotopic models; no doses were varied (Note that RT doses in humans do not necessarily correspond to dosage in animals). Also, specifics about the irradiation like field size were not provided which can be a factor given difficulty to reproduce IMRT capability and small field dosimetry in animal studies. All these reasons seriously weaken the validity of such a strong claim*
    - Intratumoral administration of the CP-PTX was conducted in our initial pilot study using orthotopic tumors but these results were included in the Supplemental section of the

original manuscript submission, and it is hence understandable that the reviewer missed them. We tested both intratumoral CP-PTX treatment as a monotherapy and as a mixture with ^{131}I-ELP in a single infusion in our original study. Both treatments were found to be not as effective as ^{131}I-ELP co-administered with systemic CP-PTX therapy. These studies were originally included in our Supplemental Information, but we have now reworked the manuscript so that these results are clearly presented in Figure 1. Figure 1 also presents new data on the depot stability of ^{131}I-ELP by itself or when injected as a mixture with CP-PTX. The results show that ^{131}I-ELP stability is significantly lower in the mixture, which is most likely the cause of the significant difference in observed tumor response of *i.v.* versus *i.t.* CP-PTX when combined with ^{131}I-ELP brachytherapy. We hope the revised structure of the manuscript will help clear up some of the confusion regarding this point.

- *I.t.* drug administration of CP-PTX was done for brachytherapy but found to be ineffective. Instead, the conventional method of systemic paclitaxel therapy plus radiation was found to be most advantageous when I-131 brachytherapy was used. All other studies use systemic paclitaxel co-treatment with both system from that point on.

- We are confused by the reviewer's statement regarding not using orthotopic models as we used an orthotopic BxPc3 pancreatic tumor model multiple times in key studies. An orthotopic BxPc3 pancreatic tumor model was used to assess biodistribution. It was used in the H&E characterization of the tumor microstroma after either brachytherapy or EBRT treatments, both as monotherapies and as combination therapies. The orthotopic BxPc3 model was also used to assess the therapeutic combination of ^{131}I-ELP with CP-PTX and demonstrated 100% CR responses in all mice. 2 mice remained cured by H&E evidence after 150 days.

- The reviewer is correct that we only used one EBRT dose regimen based on standard of care in the clinic. In our revised manuscript, we have gone back and conducted additional mouse xenograft therapy studies using multiple EBRT dose regimens to ensure fair comparison. We consulted with our clinical co-authors and selected dose schemes that were clinically relevant in the field of pancreatic cancer but delivered higher biologic effective doses (BED). The details are already provided in the previous response to reviewer 1, but to recapitulate, we have now included additional tumor regression studies in BxPc3 xenografted mice that examine the efficacy and synergy of two additional dose regimens — a BED equivalent regimen (3Gy x 15 fractions) and a EBRT dose scheme with a higher BED (10Gy x 5 fractions). These results are now included in the revised manuscript. These new studies show that these additional EBRT combination therapy trials still did not achieve the efficacy observed with our ^{131}I-ELP brachytherapy system.

- We have amended our methods section to include more information about the micro-irradiator settings and conditions, including field size.

2. *The authors also claim in the abstract that "Impressively, the combination 131I-ELP brachytherapy and systemic paclitaxel induced complete regression in three genetically diverse subcutaneous tumor models — BxPc3- luc2, MIA PaCa-2, and AsPc-1 — as well as an orthotopic pancreatic tumor". As figure 6 shows this claim is not completely true. From this figure, complete regression was only observed in one model, and this was only temporary. Long term survival while statistically significant was definitely not impressive compared to what has been observed in other preclinical studies, which were not adequately reviewed in this work.*

   - The comment of the reviewer is unclear. We observed a 100% —complete— response in the BxPc3-luc2 xenograft model, the MIA PaCa-2 xenograft model, and the BxPc3-luc2 orthotopic model. Only the AsPc-1 xenograft model did not exhibit a 100% complete response rate – only 28.5% of tumors had complete responses. However, 100% of the AsPc-1 tumors did have a partial response. We apologize if this was unclear in our presentation of the data from the regression plots, which are average responses. We have now included tumor response waterfall plots to make this clear to readers.

   - As for our claims that these results were impressive, **we have reviewed over 300+ articles and 1000+ unique monotherapy and combination treatment regimens in preclinical models of pancreatic tumors.** This comprehensive survey of the literature shows that achieving PR is very rare in these models, especially for BxPc3 and AsPc-1, let alone tumor CR. Obviously we could not include these as a reference within a research article. We are happy to provide this analysis to the editors & reviewers and have now included a detailed excel sheet review of all these studies as part of our SI materials. We hope these will help alleviate concerns about our claims.

     It is our opinion that comparing survival times between publications often does allow accurate comparison between therapies. This is because absolute survival time is confounded by the age of the animals, strain of the animals, site of tumor inoculation, size of tumor at treatment, and different growth kinetics of tumors in different laboratories. We believe that tumor response and fold improvement in survival over controls tend to be more reliable indicators. As noted above, we have reviewed over 300 preclinical articles examining the relative response of pancreatic tumor xenografts to many different types of treatments. In our review, our results stand out. Below is the summary of results we found for the specific cell lines we used (our full review and analysis can be found in the supplementary materials of our resubmission):
     - MIA PaCa-2: Of 170 treatments tested, 15 were found to induce a CR response., and only 8 of those 15 treatments achieved a 100% CR rate.
     - BxPc3: Of 183 treatments tested, only 10 induced a CR response. Only 3 achieved a 100% CR rate.
     - AsPc-1: Of 125 treatments tested, only 2 induced a CR response. None achieved a 100% CR rate.
   - To address the reviewer's point, however, we have removed the term 'impressively' as well as other superlative descriptors from our manuscript.

3. *The authors also claim that these effects could not be replicated with EBRT combination therapy, demonstrating that 131I-ELP brachytherapy can provide a promising new radiotherapy alternative for overcoming resistance in pancreatic cancer. The fact that these effects could not be replicated might be because enough parameters were not varied for the animal studies with EBRT like dose variation of EBRT or drug combination etc. Furthermore, the authors completely failed to compare their studies with previous studies of pancreatic cancer with brachytherapy. This would be a relatively more fair or adequate comparison before comparison with EBRT. Many previous studies have already been done with brachytherapy for pancreatic cancer with apparently even better results in human than seen in this study for mice: See https:/www.ncbi.nlm.nih.gov/pubmed/28151849, https://www.ncbi.nlm.nih.gov/pubmed/1698755, https://www.ncbi.nlm.nih.gov/pubmed/1587751 and the references therein. The authors need to address with some research evidence why they think their brachytherapy approach would succeed compared to these previous work. This would be the first level of comparison needed with adequate variation of parameters before comparison with EBRT.*

   - We considered the point the reviewer raised about not considering enough parameters or EBRT variables for cross comparing with our technology. As recommended by the reviewers, we have now carried out new experiments to expand the dose regimens of EBRT to include various fractionation schemes and BED to better compare our brachytherapy approach with EBRT. The rationale of the EBRT treatment regimens originally chosen are already described in response to Reviewer 2. We then completed two additional EBRT survival therapy studies in BxPc3 tumor xenografts, with and without CP-PTX. Our studies ensured that the new EBRT treatments matched or exceeded the BED of the standard EBRT regimen of 25 fractions of 2 Gy. This new data has been added in Figure 3e-h of the revised manuscript, with BED values provided in the methods section.

   - We have reviewed the preclinical literature for comparative data on ^{125}Iodine brachytherapy studies for comparison with this study. **We were unable to find any such papers from other groups** and request the reviewer to point us to the studies they have found in their review. The only data we know of that we can compare to this study is our previous study (Liu, et al. *Cancer Research* 2012) where we examined the use of the different isotopes to treat prostate tumors. In these studies, we found that ^{125}I only led to mild growth inhibition, whereas ^{131}I was highly efficacious. Given the much higher intrinsic resistance of pancreatic tumors to brachytherapy compared to prostate tumors, we did not believe that it was necessary to include a ^{125}I-labeled control.

   - The only studies we were able to find are clinical trials where ^{125}I seed brachytherapy was examined for use in locoregional pancreatic tumors. The bulk of these studies were done in the late 1990's. In line with the articles the reviewer provided, the main benefit observed was pain relief for patients. When used in combination with other treatments, overall survival (OS) improved to ~11.8 months. These results were not advantageous enough for the treatment to become standard practice. Instead, the NCCN guidelines clearly cite the use of EBRT and concurrent chemotherapy (Abraxane and gemcitabine) for enhancing patient survival. As the purpose of this paper was to evaluate our ^{131}I-ELP

based on this, we believed the best standards of care for comparison were the NCCN recommended treatments of EBRT.

4. *In the conclusion, the authors claim that "Utilizing new advances in material science and drug delivery, our results demonstrate unforeseen effects and exciting benefits that radiotherapeutic medicine can provide to this field" Some of these effects in terms of therapeutic efficacy have already been seen in previous brachytherapy studies, e.g., using I-125, which the authors failed to review as is important for such a manuscript.*
    - Based on the reviewer's feedback, we did consider amending the introduction to mention the history of seed brachytherapy withing the context of pancreatic cancer, and what differentiates ^{131}I-ELP from this technology. However, given reviewer feedback about length of the introduction and citation limits, we believe this would distract from the primary purpose of the paper. **We are happy to reconsider and add this if the Editor and Reviewer believe it necessary.**

5. *In the discussion the authors state: "we hypothesize that the synergistic efficacy of 131I-ELP with paclitaxel could possibly prevent and even enable the treatment of metastatic disease when further combined with immune-oncological agents. This is because our data show that 131I-ELP provides highly potent radiation-induced cell death that is far superior to EBRT, which has been widely shown to help potentiate immune- oncological interventions. Such effects could be used to increase antigen display and trafficking or re-polarize the infiltrating immune cells within the tumor…. The result could be to potentiate an 'abscopal effect', whereby local treatment primes the immune system to detect and treat disseminated metastases." While it is ok to speculate in the discussion section, this claim is a stretch given that brachytherapy with continuous irradiation over many days may actually destroy infiltrating radiosensitive immune-cell populations needed to potentiate the abscopal effect. Again, the comparison with EBRT is not fair as this study did not directly compare immunogenic cell death generated by brachytherapy and EBRT*
    - We agree the topic is an area of speculation and it is a hypothesis we are actively investigating. Based on the histological evidence comparing EBRT and ELP brachytherapy, we thought our comparison against EBRT was fair. When we evaluated TUNEL staining, there was a clear and significant difference in the cell death between the two radiation therapies. TUNEL detects apoptotic cell death, which is a critical route of immunogenic cell death and antigen display that has been studied in the immunology field. Our results would suggest that the ICD generated with our ^{131}I-ELP is greater than that of EBRT. However, we also agree that this is still just a hypothesis driven by our histology. Whether or not this may prove beneficial for abscopal therapies remains uncertain, especially as the continuous irradiation of the ^{131}Iodine may have a negative impact on infiltrating immune cells. We have modified the wording on this claim to indicate:
        - "The reduction of intratumoral barriers and stroma could improve immune cell infiltration within the tumor environment or re-polarize the infiltrating immune cells within the tumor. These results could potentiate an "abscopal effect", whereby local treatment primes the immune system to detect and treat disseminated metastases. However, the impact of the ^{131}I-ELP on immune cells

would have to be explored. The results herein suggest that combining ^{131}I-ELP and *i.v.* PTX with immunomodulators may be a worthwhile line of investigation, and one that will be pursued in future studies.".

6. *Overall many of the claims made in this study were exaggerated by use of words like "impressively", "unforeseen", "exciting", 'highly striking", "far outstrips", "our rigorous preclinical studies" " no other treatment combination in any of the preclinical literature has achieved a 100% ORR across these cell lines". The last claim again highlights the lack of adequate review of the literature as with previous brachytherapy work, and preclinical studies combining EBRT with immunoadjuvants.*
    - We apologize and have removed these qualifiers from the wording in the revised manuscript.
    - As mentioned in a previous response, these claims were made after we conducted an exhaustive review of the preclinical literature examining the efficacy of different treatment regimens against different pancreatic tumor lines. **We reviewed over 300 articles containing over 1000+ different combination treatment regimens. The review covered standard EBRT, stereotactic beam radiation, targeted radionuclide therapy, standard of care chemotherapeutics, gene therapy, immunotherapy agents, and novel nanoparticle systems.** We looked at the 3 cell lines we used in our manuscript, as well as 118 other human, mouse, and rat pancreatic tumor cell lines. We have continued to update our literature review with new articles and treatment results since our submission.
    - We understand the reviewer did not see these results, but we are happy to provide the excel spreadsheet summarizing all the articles and treatments in our response and our Supplemental Materials.
        - Of 1102 treatments,
            - Only 53 treatments (4.8%) achieved a complete response (any mice) across all 121 different tumor lines.
            - Only 7 treatments (0.6%) achieved a complete response (any mice) in two different tumor models.
            - Only 23 achieved a 100% CR rate in any tumor model (2.1%).
            - None achieved a 100% CR rate in multiple tumor lines of different genotypes.
        - In orthotopic pancreatic tumor models (*n*=266)
            - Only 19 treatments (7.1%) achieved a PR or CR
            - Only two treatments (0.7%) achieved a complete response in any animal

Other concerns:

1. *Methods: While the authors claim that they did rigorous preclinical studies, there are also significant concerns in the methods:*
    a. *Studies on Histochemical pathology of treated tumor used only 2 mice per cohort. This lacks enough rigor*

    Animal cohort size of *n*=3-5 were used for all pathology studies. We have reviewed all our data graphs quantitating our blinded IHC analysis. We have ensured that all data points are included and individually shown so that readers can clearly see the *n*. We did

find an error in the Methods section where one of the groups was incorrectly listed with an n = 2. We have updated it to 3, which is shown in our data plots. We also reviewed our results with an IACUC representative as well as our pathology expert to assess whether we should try to raise the *n* to 6 for all groups. Based on the statistical significance seen with our current animal numbers, they did not believe that the need for additional animals were justified.

b. *Toxicity studies were not that rigorous. Figure 7 shows that the following was investigated: Body weight over about 63 days: Serological α-amylase levels and off-target toxicities. No adequate toxicity studies were done to examine the systemic effects of the drugs e.g., in liver or kidney, and no longer term toxicities, apparently because the study also showed little impressive long term survival*

   The reviewer is correct that the only systemic toxicity assessments done during the therapy studies were the body weight and the α-amylase level monitoring. α-amylase was used to assess acute kidney injury. Long-term kidney and liver toxicity studies from radionuclides typically require > 9 months of survival. As our studies were limited to 120 days, this assessment was not feasible.

   However, we did perform a biodistribution study and dosimetric analysis of our ^{131}I-ELP depots in the original paper. These results showed extremely low levels of ^{131}Iodine accumulation in either the liver or kidneys through 9 days. Moreover, the dosimetric analysis indicated that overall radiation exposure for all off-target organs was less than a cGy based on the animal dosing. This compared very favorably to the 23 Gy renal safety threshold typically observed in clinical settings. Previously, we had included this data graphically in Figure 8. However, to better emphasize these findings, we have now included this biodistribution data, with MIRD dose calculations for all tissues, in Table 1 of the revised manuscript.

c. *Not enough information was provided to justify rigor in the EBRT studies: e.g., what was the field size used for subcutaneous/orthotopic studies? Could the field size be a factor in influencing the outcomes for this animal studies where small field dosimetry is a factor compared to the delivery of IMRT clinical doses adopted for this study?*

   We have updated our methods section to include additional information regarding the micro-irradiator settings and field size. We consulted with Dr. Kirsch regarding the settings to be used when we performed two additional EBRT combination therapy experiments and confirmed that the field size and factor are consistent with current practice for preclinical translation work.

2. Not enough was done to justify why systemic delivery was more effective than intratumoral given the mechanism of action of the drug. The main benefit of systemic administration given the mechanism of action of the drug lies in the non-invasiveness of such an approach. But the Brachytherapy approach is already invasive, so it is confounding that i.v was better than i.t. administration of the drug. It will be helpful to clarify this.

Reviewer 1 had a similar question regarding the results comparing intravenous and intratumoral delivery of the CP-PTX. Please find our response under Reviewer 1 - Question 4.

3. Novelty: without adequate review and comparative investigation with other brachytherapy approaches that have gone through preclinical phase to clinical trials, the case for novelty of this work is weak.

The only brachytherapy technologies that have been tested in the clinic for pancreatic cancer are those that use low energy gamma emitters. As the reviewer noted himself/herself, there have been several clinical trials in the late 1990s that evaluated the potential of the seed technology when layered with external radiation and concurrent chemotherapy. The ^{125}I seeds demonstrated an OS of 9 months alone, and 12 months when used in combination with other treatments. The primary benefits appeared to be pain relief for patients and locoregional growth inhibition.

What is unique about our technology is that it employs a β-particle radionuclide that cannot be delivered with seed technology. The seed's titanium casing blocks the penetration of the β-particles into the tumor tissue. As such, only low gamma energy radionuclides are used with seeds. A major novelty of our brachytherapy approach is the ability to successfully deliver and stabilize β-emitting radionuclides. The β-particle therapy imparted by ^{131}I-ELP is more focal and delivers higher energy radiation. The average energy of ^{131}I β-particles is 192 keV. ^{125}Iodine, meanwhile, emits an average gamma energy of 18 keV. This results in the ability to not just inhibit tumor growth, but also to ablate the tumor and cause its reduction. To our knowledge, there is no other system that utilizes a β-particle emitting brachytherapy technology that is suitable for comparison.

We have previously compared the γ -emitting ^{125}I-ELP, which is similar in therapeutic power to seed technology, with the β-emitting ^{131}I-ELP in our lab. The γ-rays of ^{125}I were even unable to effectively control tumor growth in a much easier to treat –than pancreatic cancer— prostate cancer xenograft model. ^{131}I-ELP, in contrast, was highly therapeutic. Furthermore, as ^{125}I seed technology has not been effective in the clinical pancreatic oncology field, we decided to focus on the comparing ^{131}I-ELP to the best practices and standards of care for pancreatic cancer, which is EBRT.

4. Significance: issues on clinical Implementation of this approach needs to be better addressed including:

a. Which patients would benefit? It is not clear if this is for treatment of patients with localized pancreatic cancer or for patients with metastasis. If localized, the significance is less because most patients with pancreatic cancer are diagnosed with metastatic disease. This is partly why previous brachytherapy approaches have not been adopted. It would be good if the authors can elaborate on the specific patient populations, they envision would benefit from this given the localized nature of this therapy approach.

The intended patient population indication is those with loco-regional pancreatic tumors. Currently, this represents 30% of patients with pancreatic cancer. Unfortunately, the 5-year survival for this patient population is < 11.5%. This partly arises because the current standard of care, EBRT combined with chemotherapy, can only affect growth inhibition. This extends survival, but eventual progression eventually leads to metastases and patient death.

We have re-written the introduction to try to make the clinical need and patient population who could benefit from this brachytherapy approach clearer.

b. *Several endoscope-guided implantation techniques have indeed already been developed and employed clinically, but optimization to inject the ELP is still required. Would this follow previous approaches with brachytherapy for pancreatic cancer? What would be different.*

This would follow a different procedure than seed brachytherapy. While ^{131}I-ELP can be delivered via a catheter and ultrasound-guided endoscopy, it is a liquid until injected into the tumor. Thus, clinical methods for its employment would be similar to catheterized injections of chemotherapeutics. The special applicators and after loaders common to seed brachytherapy would not be required.

c. The authors state, "Despite all of our current surgical and radio-chemotherapeutic options, clinical evidence continues to stress the need for improved treatment modalities for local disease, as high recurrence rates and poor survival persist" Given that only temporal regression was observed in the best results of this study (figure 6). It is not clear how this approach would be better. It would be helpful to have the authors clarify the benefits and how it would be different from other brachytherapy approaches that have failed to be adopted following preclinical studies and clinical trials

That is a very good question. As powerful as animal studies can be, the translation of mouse model results into humans is never a direct correlation. It is important to remember this works both ways. Survival times observed in mice never correlate with human survival time. This is partly due to allogenic scaling between species, but also due to different genetics, tumor heterogeneity, and metabolism of humans. It also arises because mouse xenografts require immunocompromised animals. The lack of an immune system in immunocompromised mice allows engraftment of human tumors in these mouse models, but it also means that most tumors grow exceedingly fast with none of the immune checkpoints found in humans to control tumor growth. We fully expect our ^{131}I-ELP to provide a better therapeutic advantage in humans with intact immune systems. Disregarding the potential for abscopal cross-effect, we suggest that the tumor ablating ability of the ^{131}I radionuclide will help limit metastases and enable to the immune system to better control the debulked tumor mass.

Second, while our current standards of care are not perfect, that does not mean they haven't advanced the survival of pancreatic patients over the years. This is why comparing our ^{131}I-ELP to the standards of care was critical to evaluate the clinical

potential of the technology. If it didn't achieve meaningfully better results in tumor models, it wouldn't be worth advancing. And the striking difference in tumor responses achieved with ^{131}I-ELP compared to different fractionation regimens of EBRT demonstrate its benefit. It can ablate and cause complete reduction of tumors that EBRT is not able to. This shows that it has a higher therapeutic potential than the current standard of care —EBRT— but that it can also fit within the current paradigm of combination radio-chemotherapy. Furthermore, our own comprehensive review of the preclinical literature comprising 300 studies demonstrated that the tumor responses we saw were not common in many of the new technologies being developed preclinically. This clear improvement in anti-tumor efficacy, combined with the lack of options for pancreatic cancer patients, is why we believe ^{131}I-ELP could offer a treatment for pancreatic cancer.

d. Previous Brachytherapy studies (https://www.ncbi.nlm.nih.gov/pubmed/29384971, https://www.ncbi.nlm.nih.gov/pubmed/20101258) show major toxicity issues for pancreatic cancer. While the half-life for different brachytherapy sources should be considered, the approach proposed in this study may also face similar issues. This study does not address how this would be different in comparative investigations with these previously used brachytherapy approaches.

We thank the reviewer for directing us to an excellent review of the issues associated with percutaneous seed technology. We believe this review also highlights many of the advantages that our liquid biopolymer approach provides over traditional titanium seed technologies, which are:

First, as a soluble liquid the ^{131}I-ELP conjugate can be delivered through an ultrasound-guided endoscope and catheter. This eliminates the need for intraoperative implantation methods, which is common in the use of traditional titanium seeds. As ^{131}I-ELP can be perfused into the tumor as a liquid through a single or small set of injection sites, it greatly reduces the trauma to the pancreatic tissue and subsequent inflammation that is common in seed brachytherapy procedures that implant >20 seeds[1]. By eliminating the physical implants of seed brachytherapy, it also would likely have reduced rates of infection. Additionally, titanium seeds have the drawback of being able to migrate, which leads them to become entrapped in other tissues. This happens rarely but has been observed in prostate cancer[2].

Second, the ELP biopolymer system is elastin-based and fully biocompatible as it has been injected into ~500 patients in multiple clinical trials (NCT01835730, NCT02581657, NCT01236404) with no severe adverse events. Because the stability of the ELP brachytherapy depot is concentration dependent, it ensures that the majority — >95%— of the injected depot is retained within the site of injection — the tumor. Any low level of ELP that diffuses out of the tumor, however, is rapidly cleared as demonstrated by the biodistribution data we show in Table 1 of the revised manuscript. Previous work by Liu, et al[3]. also shows that the ELP is degradable, preventing it from blocking or becoming entrapped in other tissues.

1. Lv, Wei-Fu, et al. (2017) *Medicine (Baltimore)*. **96**(52): e9535. (PMID: 29384971)
2. Nakano, M. et al. (2015) Radiat Oncol. 10: 228. (doi: 10.1186/s13014-015-0532-3)
3. Liu, W et al. (2006) J Control Release. 116(2): 170. (doi: 10.1016/j.jconrel.2006.06.026)

Finally, the use of the ELP biopolymer allows a wider range of therapeutically relevant radionuclides to be used for brachytherapy. By using ^{131}I-ELP, patients only deal with a half-life of 8 days, not the 60-day half-life observed with ^{125}I seeds. As mentioned earlier, the use of ^{131}I also means that the primary β-particle radiation is absorbed within a maximum distance of 2 mm, which minimizes the risk of toxicity to surrounding healthy tissue.

Rebuttal 2

Reviewer Feedback on NBME-19-1528B

Transmitted to Authors on May 18, 2022

Reviewer #2 (Report for the authors (Required)):

After a long (2 year) period, the authors present a comprehensively revised and expanded manuscript regarding their investigation of a novel intratumoral I131 brachytherapy system combined with paclitaxel in mouse models of pancreatic cancer. They have done an excellent job responding to the reviewers' critiques and in supplementing their experiments to address several uncertainties and concerns. Overall, I believe that the critiques have been well-addressed. I continue to believe that this is novel and potentially impactful work that could develop into a meaningful advance over currently available radiation and chemotherapies for this difficult disease. This of course would ultimately depend on whether the efficacy and safety observed in mice translates to humans, and also on the development of technical means to accurately deliver I-ELP to human patients. These matters are of course beyond the scope of this paper, though the authors do comment in the discussion and response to reviewers regarding the potential means of technical delivery of I-ELP in humans.

The authors have updated and improved their discussion of the existing literature and role for external beam RT in pancreatic cancer. However, there is arguably still some missing nuance. They cite an NCDB analysis showing that survival improves with doses >4Gy, implying that hypofractionated SBRT inherently improves outcomes on the basis of hypofractionation. However, the relative efficacy of different radiation doses for pancreatic cancer is almost certainly due to higher biologically effective doses (BED) which is a function of fraction number, not only fraction size. There are more recent experiences with radiation regimens that use relatively modest fraction sizes (3-4.5Gy) but increase the number of fractions to achieve high BED (see Reyngold M et al., JAMA Oncology 2021). The apparent success and feasibility of these regimens also may provide additional context to the authors' statement that "increasing SBRT ablative doses above this threshold (25-33Gy in 3-5 fractions) result in serious GI toxicity." It is true that increasing doses *to the GI luminal organs* results in toxicity, but new techniques indicate that it is increasingly feasible to escalate radiation dose to the tumor while still limiting dose to the GI organs below toxicity thresholds.

Author Response

We fully agree with the reviewer's feedback and have revised our introduction to include the Reyngold paper and to emphasize that a higher BED can be achieved by increasing both the fraction size and fraction number. We did not mean to imply otherwise and have rewritten the introduction accordingly. Please find our revisions in the 3rd paragraph of the Introduction section. We hope this better captures the nuance of EBRT dosing and hypofractionation.

We feel that this aligns well with what we believe to be an advantage of the ^{131}I-ELP system. It allows for continuous radiation dosing, which is the upper limit of fraction number, and allows for a lower dose rate to be employed by ^{131}I-ELP.

I do not otherwise have substantive other comments or concerns regarding the revised manuscript. I commend the authors for performing additional experiments to investigate the potential impact that different external beam radiation prescriptions might have on their results, and their inclusion of this data meaningfully strengthens the conclusion that there is something fundamentally more efficacious about I-ELP brachytherapy vs. EBRT. The additional details and specifications regarding the animal irradiation are also appreciated.

Reviewer #4 (Report for the authors (Required)):

In this study, Schaal et al. developed a liquidly injectable biopolymer to deliver 131Iodine radionuclide as a form of brachytherapy. In particular, they present a series of data showing that the combination of intratumoral I131 brachytherapy with paclitaxel induces impressive pancreatic tumor responses. They performed a series of rigorous studies to show that this combination appears significantly more effective than either I-ELP or chemotherapy alone, and that I-ELP is more efficacious than external beam RT. Although a large amount of data has been provided, and the authors have carefully responded to comments of the previous reviewers, I still feel the novelty of this work is limited. Moreover, the clinical significance of this work may not be high enough for publication in Nature Biomedical Engineering.

- In the discussion the authors' state: "This is because our data show that 131I-ELP provides highly potent radiation-induced cell death that is far superior to EBRT, which has been widely shown to help potentiate immune- oncological interventions." Although these speculations are reasonable, it is not difficult to evaluate the immune cell infiltration and cytokine levels in tumors, and we hope that the authors can take them into consideration.

Author Response

The reviewer raises valid questions, and we agree that this is a priority area for investigation. However, examining the immune cell infiltration and cytokine levels will not provide translationally meaningful information in the context of the studies and tumor models used in this manuscript. This is due to several factors.

We chose to evaluate our combination therapy in human tumor models that were well studied and already found to be highly resistant to known therapeutics. We deemed human tumor lines critical for our studies to ensure the tumor model properly recapitulated the dense desmoplastic stromal found in clinical pancreatic cancer. To use these cell lines, our studies required us to xenograft the human tumor cell lines into immunocompromised mice. As such, all four of our animal models do not have fully functioning immune system; for this reason, we cannot properly evaluate immune cell infiltration and changes in intratumoral cytokine levels in these models.

To perform the experiments suggested by the reviewer, we would have to establish a fifth animal tumor model with a syngeneic tumor cell line, ensure it develops the proper desmoplastic stromal environment, and subsequently evaluate its therapeutic response to each agent alone and in combination matches our earlier studies. Only then could we interrogate the immunological microenvironment and put them in proper context with our earlier results. But, even then, the results would be circumspect for another reason.

The second major factor is that paclitaxel interacts with the murine MD-2/TLR-4 receptor to induce a strong immunological stimulatory response. This interaction, however, is not homologous for human TLR-4 receptors. In humans, paclitaxel does NOT induce a TLR-4 immune stimulation. Thus, any results we would achieve in a syngeneic rodent model would not provide clinical meaningful insights. Most likely, they would lead us to incorrect translational conclusions.

1. McConkey, D.J. et al. in Pancreatic Cancer 457-469 (Springer, New York, NY, 2010).
2. Deer, E.L. et al. Phenotype and genotype of pancreatic cancer cell lines. Pancreas 39, 425-435 (2010)

We have not modified the manuscript further on this point as an explanation for these complications is already included in the 3rd paragraph Results section, partially in response to the

previous round of reviewer comments. We hope the reviewer and editor can appreciate that these factors would require higher order animal models to accurately assess, and thus make them excessively difficult and time intensive. Moreover, we would argue that the lack of an immune factor makes the level of synergy observed in our results that more impressive.

- The combination therapy with 131I-ELP brachytherapy and CP-PTX was promising in this work, but the selection of chemotherapy drugs was not emphasized in the paper. What effect will be produced when combined with other types of chemotherapy drugs?

Author Response

We have now updated the introduction to better describe why paclitaxel was selected for our studies. As the reviewer's response indicates, ^{131}I-ELP could reasonably be paired with many other chemotherapy drugs that are known to be radiosensitizers. While we considered extensively testing the interaction of ^{131}I-ELP with many chemotherapy agents, we instead decided that the novelty of our approach was in our liquid brachytherapy system and should instead use a chemotherapeutic already approved by the FDA for the treatment of pancreatic cancer. Following the NCCN guidelines, patients either receive a regimen of FOLFIRINOX – a four drug combination regimen of folinic acid, fluorouracil, irinotecan hydrocholoride, and oxaliplatin – or the combination of nab-paclitaxel with gemcitabine. While FOLFIRINOX is considered the more potent regimen, it is NOT the chemotherapy regimen prescribed in combination with EBRT therapy. In that situation, gemcitabine plus nab-paclitaxel is the chemotherapy regimen that is used. Thus, we chose the chemotherapeutic that aligned with the context in which ^{131}I-ELP would be implemented – a replacement for EBRT.

To prove a meaningful advantage of our ^{131}I-ELP system, we thought it was important to limit the synergistic interactions to a single agent. Both gemcitabine and paclitaxel are known radiosensitizers. Their mechanisms of action, though, differ considerably. Gemcitabine is a nucleoside analog that primarily acts to impair DNA synthesis by blocking the cell cycle process at the G1/S phase[3,4]. Paclitaxel meanwhile is a microtubule stabilizer, which inhibits cell progression in the late G2/M phase[5]. Both drugs act on nucleotide damage and repair processes that overlap with the mechanism of action of radioactivity. When deciding between the two, we selected paclitaxel for three reasons. One, radiation-induced DNA damage is maximized in the late G2/M phase of the cell cycle. This is phase of the cell cycle that is similarly impacted by paclitaxel's pharmacology, and thus we hypothesized that it may better enhance the direct tumor-killing power of ^{131}I-ELP. Moreover, the continuity of the ^{131}I-ELP irradiation would eliminate the temporal disadvantage of pairing paclitaxel with EBRT. Second, acquired resistance to gemcitabine for pancreatic cancer is very rapid, sometimes as quick as weeks[4,6]. Thus, we selected the chemotherapy agent that gave us a wider window to work within. Finally (and practically), we had recently published on a novel paclitaxel micelle formulation (CP-PTX) that outperformed nab-paclitaxel in preclinical models. Initially, we thought that this improved formulation could likewise enhance the potential synergy between ^{131}I-ELP and paclitaxel. Only later did we find that the synergistic results for CP-PTX were similar to that of nab-paclitaxel (Abraxane).

3. E Mini, S Nobili, B Caciagli, I Landini, T Mazzei. (2006) Cellular pharmacology of gemcitabine. *Ann Oncol.* **17**(Supp 5): v7-v12.
4. S Zeng, M Pettler, B Lan, R Gruetzmann, C Pilarsky, H Yang. (2019). Chemoresistance in pancreatic cancer. *Int. J. Mol. Sci.* **20**(18)**:**4504.
5. TB Foland, WL Dentler, KA Suprenant, ML Gupta Jr, RH Himes (2005). Paclitaxel-induced microtubule stabilization causes mitotic block and apoptotic-like cell death in a paclitaxel-sensitive strain of *Saccharomyces cerevisiae*. *Yeast*. **22**(12):971-978.
6. M Amrutkar, IP Gladhaug. (2017). Pancreatic cancer chemoresistance to gemcitabine. *Cancers (Basel)*. **9**(11):157.

In our revised manuscript, we have now updated the last paragraph of the introduction to better describe the logic of why paclitaxel was selected over gemcitabine for investigation in our studies.

Finally, the reviewer's question about how ^{131}I-ELP would interact with other types of chemotherapy drugs is a very good question. One would reasonably expect that pairing ^{131}I-ELP with gemcitabine, the other FDA approved chemotherapeutic for pancreatic cancer in combination with EBRT, would also improve therapeutic outcomes. As an antimetabolite agent, gemcitabine works to inhibit the G1/S cellular phase repair of DNA damage caused by ionizing radiation. This should lead to greater tumor cell death as the cells would recover from the brachytherapy radiation damage. Likewise, our findings about how ^{131}I-ELP impacts the tumor microenvironment may slow or prevent the acquired tumor resistance commonly associated with gemcitabine. Ultimately, the combination of gemcitabine and paclitaxel with ^{131}I-ELP may prove the most synergistic and beneficial. However, because of the depth of studies required to explore the pharmacology of the interactions of ^{131}I-ELP with each drug and the nature of factorial expansion for studying multi-drug combinations, we had to limit the scope of our investigation for this manuscript to a single agent. We agree that follow on studies with other types of chemotherapy would be highly intriguing in future research endeavors.

- I-131 may not be the best radioisotope for brachytherapy, as its gamma emission energy is too high, and its beta emission energy is not high enough. Y-90 may be a better radioisotope (higher beta energy and longer therapeutic distance) if beta particles play the key role in this system.

Author Response

The reviewer's suggestion for using ^{90}Yttrium is very interesting. Ultimately, the observed synergy achieved using ^{131}Iodine should be fully translatable with ^{90}Yttrium. However, the authors view the higher energy and longer penetration distance of the ^{90}Y β-particle as a possible liability. The average penetration depth of ^{131}Iodine β-particles is ~0.4mm with a maximum distance of ~2mm[7]. This provides a high degree of anatomical precision for tumor irradiation, where the ELP biopolymer can be used to better control the spatial delivery of the radiation. ^{90}Yttrium has an average tissue penetration range of ~2.5mm and a maximum range of ~11mm[8]. In conventional brachytherapy, where seed sources are spaced apart, the longer radiation penetration range compensates for the heterogeneous spatial arrangement. With the ELP liquid biopolymer system, the brachytherapy is able to be tuned to more homogenously distribute the radionuclide throughout the tumor interstitium. This makes the need for higher energy isotopes with longer range less relevant. In fact, the higher range of ^{90}Y β-particles may lead to higher irradiation levels of healthy pancreatic tissues or other neighboring organs. Such exposure may also further exasperate by synergistic interactions with paclitaxel in those tissues. For these reasons, we believe ^{131}I provides a more advantageous radiation profile when used with liquid ELP brachytherapy.

7 EB Silberstein, et al. (2012) The SNMMI practice guideline for therapy of thyroid disease with ^{131}I 3.0. *J Nucl Med*. **53**(10) 1633-1651.

8 YC Kim, YH Kim, SH Uhm, YS Seo EK Park SY Oh, E Jeong, S Lee, JG Chloe (2010). Radiation safety issues in Y-90 microsphere selective hepatic radioembolization therapy: possible radiation exposure from patients. *Nucl Med Mol Imaging*. **44**(4):252-260.

A second advantage of using ^{131}Iodine is that it can be directly conjugated to the recombinant biopolymer ELP without any chemical modifiers or chelators. This means that the pharmacokinetic biodistribution of the radionuclide is limited to the ELP conjugate, which we have shown to be highly stable. If ^{131}I is cleaved from the ELP, it has low biological incorporation other tissues other

than the thyroid, which is easily blocked with over-the-counter supplements. An Yttrium approach, however, would require a chelator to be pre-conjugated to the ELP. This may impact the nanostructure and diffusion profile of the ELP micelles, which served as the basis of the biopolymer and dictate's its *in vivo* stability. The labeling method of ^{90}Y in this application would also be very different than the glass spheres used for liver embolization. The ^{90}Y would be exposed to the biological environment, which may lead to the ^{90}Y to be released through substitution reactions and inadvertent tissue accumulation would have to be closely studied and dosimetrically measured. This is not to say it could not be engineered, just that it is not a 1:1 switch.

Finally, the high energy gamma emission of ^{131}Iodine is always a concern in all its applications. However, it's dosimetry and risk profile are well established based on long term use in the field of treating Grave's disease and thyroid cancer. It is also being used in many targeted radiopharmaceutical compounds being developed in the clinic. One such example is Azedra, which is an FDA approved ^{131}Iodine therapeutic for the treatment of pheochromocytoma and paraganglioma. The gamma ray of ^{131}I can provides a theragnostic advantage for ^{131}I-ELP brachytherapy as it allows clinicians to accurately quantify the biological exposure and dosimetry of the injections. The exposure dose can then be titrated in accordance with these assessments.

It is also worth mentioning that no signs of chronic radiation toxicity or exposure was observed macroscopically or histologically in the mice on our studies that lived over 200 days. The authors then euthanized the mice and pathologically examined the whole pancreas (healthy tissue and treated tissues) of these mice. No signs of toxicity were observed in the H&E specimens of the pancreas or other organs. Also, no mouse ever developed secondary tumor lesions in any study, either proximal or disseminated.