nature portfolio

Peer Review File

Sprayable Anti-Adhesive Hydrogel for Peritoneal Macrophage Scavenging in Post-Surgical Applications

Reviewer #1 (Remarks to the Author):

The manuscript reported a sprayable, rapid-forming hydrogel composed of sHA, chitosan, and PF127 as adhesion barrier for therapeutic applications in post-surgical adhesions. The sHA of hydrogel played a crucial role in mitigating inflammation and promoting M2 polarization in peritoneal macrophages, which was evidenced in a rat ischemic button model via the customized dual-sprayer. The manuscript can be improved after addressing the following questions.

1. The title reads like a review instead of a research article, which requires specific descriptions.
2. In Figure 2e and 2g, why did the oscillatory strain sweep (G”>G’) and strain sweep (G’>G”) of sHAChiF at 25 ºC opposite result? Is sHAChiF a hydrogel at 25 ºC?
3. On the page 23, the Chi was solubilized by Pluronic® F127 in HCl solution (0.1 M) since Chi was insoluble in neutral pH in the preparation of sHAChiF. What are the pH of Chi/F127 and the final pH of the hydrogel? If it is still very low, does it induce serious inflammation?
4. On page 18, why was the quantitative measurement of peritoneal macrophage M1/M2 taken from day 7 after surgery while the quantification of pro-inflammatory cytokines taken from day 4 after surgery? It is better to do on the same day.
5. The thermo-gelation mechanism of F127 in Fig. 2a is not clear. How can the micelles form the 2ed network.
6. The significance of circles and triangles in Fig. 2c and d was not mentioned. The screening results in Figure 2d looks inconsistent with the concentration of polymers.
7. Some details should be provided. What is the degree of sulfation of sHA in DS 1, 2, and 3? The experimental procedure to measure the adhesion score is unclear. How about the toxicity of F127 towards NIH-3T3 fibroblasts? A simple temperature sweep experiment of sHAChiF could provide clear information about thermogelation.

Reviewer #2 (Remarks to the Author):

The manuscript reports the development of new anti-adhesive barriers composed of sulphonated hyaluronic acid, chitosan and pluronic F127. Although mixed materials of alginate and F127 have been clinically studied, the anti-inflammatory effect of sHA has been successfully introduced. My comments to authors are as follows.

(i)
NASAIDs or steroids have been widely studied and showed the efficacy to anti-peritoneal adhesion in several studies. But side effects are a concern to use anti-inflammatory drugs to increase the risk of infection, so they are not used clinically. Authors should carefully analyse the anti-inflammatory mechanism between previous drugs and current materials to show the potential of this material.

(ii)
Chitosan is not rapidly degraded in the peritoneum. Chitosan is not yet clinically available in the peritoneum. Degradation in peritoneum over one to two months should be carefully monitored.

(iii)
The ischaemic button model is not a standard model, and the cecal abrasion and peritoneal

sidewall defect models are not desirable. Ischaemic button model is not as severe adhesion model, so the data of cecal abrasion and peritoneal sidewall defect model should be added.

Reviewer #3 (Remarks to the Author):

See attached documents.

Reviewer #3 Attachment on the following page

In this manuscript, the authors report a sprayable, biosafe, anti-inflammatory sHAChiF hydrogel for preventing postoperative peritoneal adhesion. In a rat ischemic button model, sHAChiF hydrogel could mitigate inflammation, promote M2 polarization and persist during the post-surgical adhesion progression period, showing great protection against post-surgical adhesion. The study has been generally well conducted, but the manuscript needs improvements in terms of additional analyses and data presentation.

1. How to prove that sHA and Chi form the first-level network first and then pluronic® F127 forms the second-level network instead of three components forming sHAChiF hydrogel at the same time? In other words, the formation of a secondary network by pluronic® F127 requires more chemical means to prove.
2. . At 25 ℃ and 37 ℃, the time required for the formation of sHAChiF hydrogel is not mentioned.
3. It is well known that strongly acidic conditions are harmful to humans. The concentration of acetic acid solvent and hydrochloric acid solvent for spraying are not mentioned. Meanwhile, the question of what effect it has on the rheology, biocompatibility and optimal gel conditions of the sHAChiF hydrogel deserves discussion.
4. The levels of collagens in Masson’s trichrome staining need to be quantified and compared in all groups. At the same time, it would be better if the adhesion areas are marked in the staining results.
5. Figure captions appear misplaced throughout the article. For example, in “The sHA/Chi/F127 mixture rapidly developed an opaque gel structure upon mixing at 25 ℃ (Fig. 1b)”, its picture should correspond to Fig 2b.

Reviewer #1

We thank Reviewer #1 for the constructive comments on our manuscript. We have made every attempt to fully address these comments in the revised manuscript. we believe these revisions have resulted in a significantly improved manuscript. Reviewer #1's original comments are listed below followed by our response to each comment.

※ Please note that in this revision letter, figures labeled as "Figure R1", "Figure R2", etc., and tables labeled as "Table R1", "Table R2", etc. are used for explanatory purposes only and are not part of the actual manuscript. This labeling helps to clearly distinguish these figures and tables from those included in the manuscript.

Table R1. Table of revisions (Reviewer #1)

Comment #	Reviewer #1's comments	Author's responses	Location in manuscript
1	The title reads like a review instead of a research article, which requires specific descriptions.	Agree. The title was changed.	Page 1 Line 1-2
2	In Figure 2e and 2g, why did the oscillatory strain sweep (G"□G') and strain sweep (G'□G") of sHAChiF at 25 ºC opposite result? Is sHAChiF a hydrogel at 25 ºC?	Supporting description provided.	Page 6 Line 136-138
3	On the page 23, the Chi was solubilized by Pluronic® F127 in HCl solution (0.1 M) since Chi was insoluble in neutral pH in the preparation of sHAChiF. What are the pH of Chi/F127 and the final pH of the hydrogel? If it is still very low, does it induce serious inflammation?	Supporting description provided.	Page 22 Line 544-546
4	On page 18, why was the quantitative measurement of peritoneal macrophage M1/M2 taken from day 7 after surgery while the quantification of pro-inflammatory cytokines taken from day 4 after surgery? It is better to do on the same day.	Agree. Supporting data provided.	Supplementary Information: Supplementary Fig. 10; Page 9
5	The thermo-gelation mechanism of F127 in Fig. 2a is not clear. How can the micelles form the 2ed network.	Supporting data provided.	Supplementary Information: Supplementary Fig. 3; Page 5

6	The significance of circles and triangles in Fig. 2c and d was not mentioned. The screening results in Figure 2d looks inconsistent with the concentration of polymers.	Supporting description provided.	Page 5 Line 108-110 Page 5 Line 114-115
7	Some details should be provided. What is the degree of sulfation of sHA in DS 1, 2, and 3? The experimental procedure to measure the adhesion score is unclear. How about the toxicity of F127 towards NIH-3T3 fibroblasts? A simple temperature sweep experiment of sHAChiF could provide clear information about thermogelation.	Supporting data and description provided.	Page 12 Line 305-307 Supplementary Information: Supplementary Fig. 3a; Page 5

Reviewer #1 - Comment #1

The title reads like a review instead of a research article, which requires specific descriptions.

Author's response

We thank you for all your valuable and positive comments. To emphasize the functionality of sHA, we changed the title.

Reviewer #1 - Comment #2

In Figure 2e and 2g, why did the oscillatory strain sweep (G"□G') and strain sweep (G'□G") of sHAChiF at 25 ºC opposite result? Is sHAChiF a hydrogel at 25 ºC?

Author's response

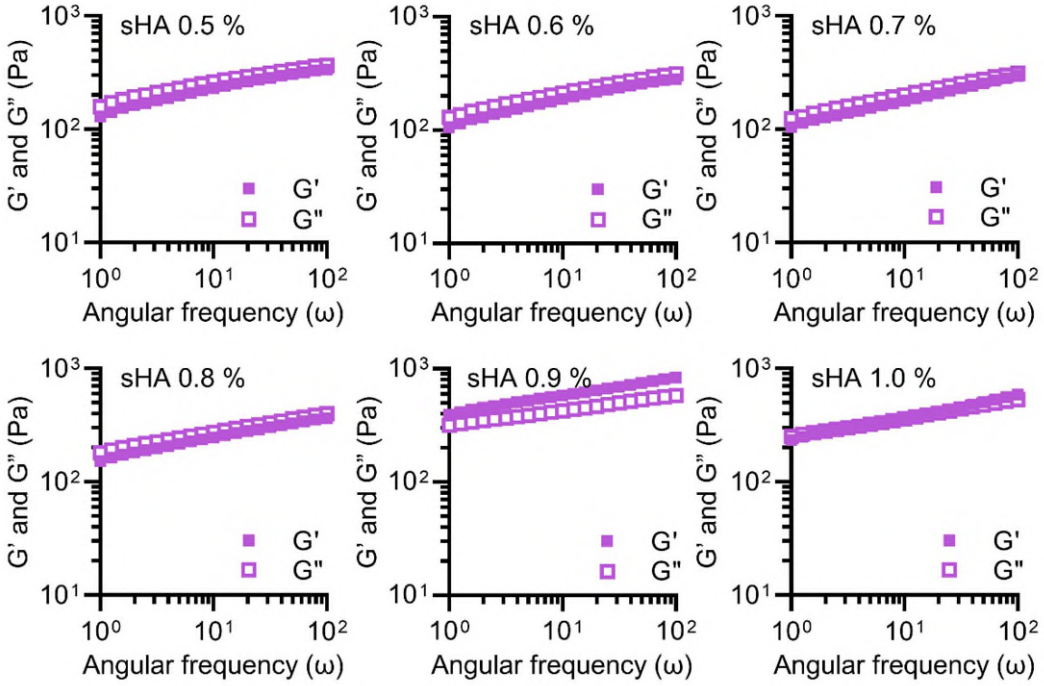


Fig. R1: Rheological properties of sHAChiF at 25 ℃ with different sHA concentrations from 0.5 % to 1.0 %. The concentrations of Chi and F127 were 1 % and 20 %, respectively.

We appreciate the reviewer's constructive comment. At 25 ℃, the sHAChiF hydrogel exhibited a viscous semi-gel state similar to petroleum jelly such as Vaseline®. Petroleum jelly is characterized by rheological properties where the values of G' and G" are close to each other[1]. The sHAChiF hydrogels displayed similar rheological results across various formulations (Fig. R1).

This inherent feature of the sHAChiF hydrogels helps with spreading. Meanwhile, this is the reason why an additional network is required for more stability. After spreading, the thermogelation of the sHAChiF hydrogels by body temperature could solve the stability problem. We added sentences describing this rheological behavior of the sHAChiF hydrogel on the Page 6 Line 136-138.

In the Page 6 Line 136-138:

At 25 ℃, the *G'* and the *G"* values were close to each other, indicating a viscous semi-gel state similar to petroleum jelly[27]. At 37 ℃, the G' value exceeded the G" value, indicating that the sHAChiF maintained its gel-like state.

Reviewer #1 - Comment #3

On the page 23, the Chi was solubilized by Pluronic® F127 in HCl solution (0.1 M) since Chi was insoluble in neutral pH in the preparation of sHAChiF. What is the pH of Chi/F127 and the final pH of the hydrogel? If it is still very low, does it induce serious inflammation?

Author's response

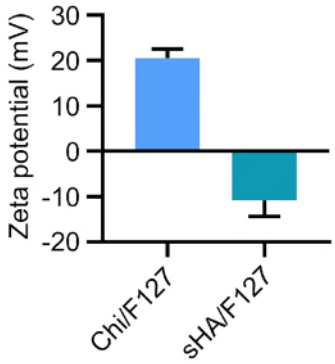


Fig. R2: Zeta potential of the Chi/F127 and sHA/F127.

We thank the reviewer for this insightful comment and fully acknowledge the reviewer's concern. The method for Chi neutralization and solubilization is described in the Methods section, specifically under 'Preparation of sHAChiF' (Page 22 Line 544-546). We neutralized Chi solution prior to its use. Two issues were identified in the Chi neutralization process.

First, the stable solubilization of Chi at neutral pH was a challenge. We solubilized Chi using F127 in HCl solution (0.1 M) and adjusted the pH to 7 using F127 in PBS. Although Chi typically precipitates at neutral pH, F127 helped Chi maintain its liquid phase.

Second, maintaining the cationic charge of Chi was crucial. The zeta potential of Chi/F127 at pH 7 was measured to be +20.56 ± 2.01 mV, consistent with previous reports indicating that Chi solutions retain a cationic charge at neutral pH[2,3] (Fig. R2).

Consequently, this solubilization method effectively resolved the insolubility issue of Chi at neutral pH, ensuring a stable electrostatic potential and making Chi/F127 safe for use as a biomaterial.

Reviewer #1 - Comment #4

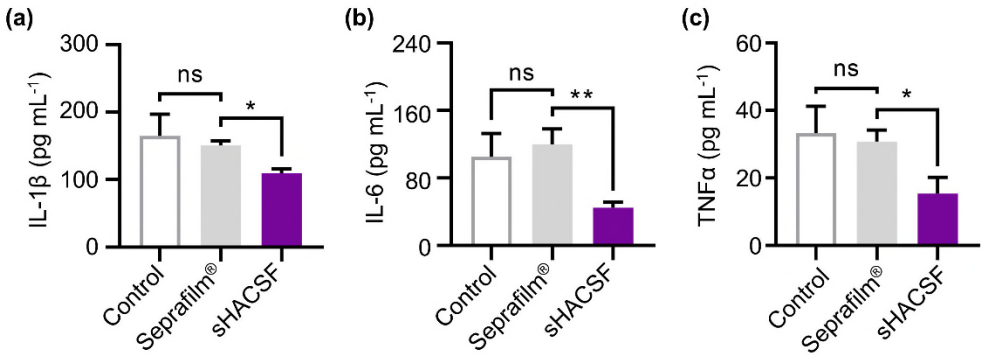
On page 18, why was the quantitative measurement of peritoneal macrophage M1/M2 taken from day 7 after surgery while the quantification of pro-inflammatory cytokines taken from day 4 after surgery? It is better to do on the same day.

Author's response

Thank you for the constructive comment. In the main figure, we aimed to show inflammatory state of the model at a mid-point (4 d) of the experiment cycle (7 d).

We newly added the cytokine analysis data on 7 d post-surgery in Supplementary Fig. 10. Similar to the cytokine profile at d 4, the data from d 7 displayed reduced levels of inflammatory cytokines.

In the Supplementary Fig. 10 (Supplementary Information: Page 9):



Supplementary Fig. 10. Quantification of pro-inflammatory cytokines in rat ischemic button model on d 7 after surgery. (a) IL-1β. (b) IL-6. (c) TNF-α. n = 4, mean ± SD. ns: not significant, *p < 0.05, and **p < 0.01.

Reviewer #1 - Comment #5

The thermo-gelation mechanism of F127 in Fig. 2a is not clear. How can the micelles form the 2nd network.

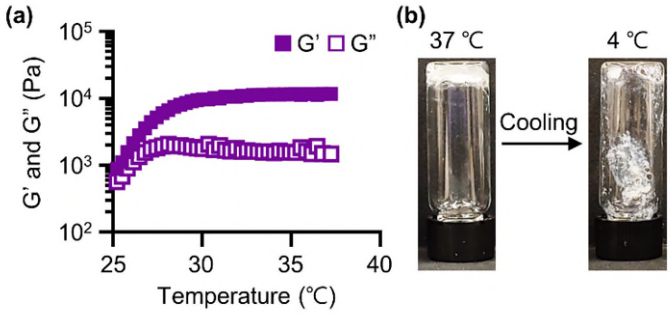
Author's response

We appreciate the reviewer for the To provide evidence for the thermogelation of sHAChiF, we conducted a temperature sweep test.

We prepared sHAChiF hydrogel 10 min before the measurement to exclude any mechanical enhancement from polyelectrolyte complex formation. We then measured the storage modulus (G') and loss modulus (G") of the sHAChiF hydrogel as the temperature changed from 25 ℃ to 37 ℃. The G' value saturated at 30.05 ℃, showing a 6.47-fold increase compared to the G' at 25 ℃ (Supplementary Fig. 3a). This increase in mechanical strength is attributed to the gradual aggregation of F127 as the temperature rises.

Additionally, we observed that the F127 network weakened upon cooling. The gelated sHAChiF hydrogel flowed down when the vial of sHAChiF was cooled from 37 ℃ to 4 ℃ and inverted (Supplementary Fig. 3b). The gel did not become completely liquid, demonstrating that the sHAChiF hydrogel consists of two networks: the primary network formed by the polyelectrolyte complex of sHA and Chi, and the secondary network formed by the thermogelation of F127.

In the Supplementary Fig. 3 (Supplementary Information: Page 5):



Supplementary Fig. 3. Thermogelation of sHAChiF. (a) Temperature sweep test of sHAChiF performed from 25 ℃ to 37 ℃. (b) Inverted vial test of sHAChiF from 37 ℃ to 4 ℃.

Reviewer #1 - Comment #6

The significance of circles and triangles in Fig. 2c and d was not mentioned. The screening results in Figure 2d looks inconsistent with the concentration of polymers.

Author's response

We appreciate this constructive comment. We added the previously omitted description of the significance of circles and triangles on Page 5 Line 108-110.

The screening results displayed a diagonal pattern, indicating that successful gelation was achieved at an optimal balance between the concentrations of Chi and sHA. Excessive sHA accelerated precipitation and caused gelation to fail at 25 ℃. Therefore, a sufficient Chi concentration was a prerequisite for successful sHAChiF gelation. We newly added this description on Page 5 Line 114-115.

In the Page 5 Line 108-110:

The symbols in Fig. 2c, d denote the outcomes of the test. Specifically, the circle indicates successful gelation, whereas the triangle represents gelation failure.

In the Page 5 Line 114-115:

In contrast, higher sHA concentrations led to gelation failure by precipitation of the complex.

Reviewer #1 - Comment #7

Some details should be provided. What is the degree of sulfation of sHA in DS 1, 2, and 3? The experimental procedure to measure the adhesion score is unclear. How about the toxicity of F127 towards NIH-3T3 fibroblasts? A simple temperature sweep experiment of sHAChiF could provide clear information about thermogelation.

Author's response

Thank you for your meticulous review. We categorized the comments and provided a detailed response to each category below.

1) Degree of sulfation

We omitted the description of the degree of sulfation. DS 1, 2, and 3 mean that 25-50 %, 50-75 %, and 75-100 % of the hydroxyl groups per HA unit were substituted by sulfate groups, respectively. We added this description to Page 12 Line 305-307.

2) Measurement of the adhesion score

The peritoneal adhesion index (PAI) is a commonly used method to score adhesion severity. The scoring criteria of the PAI are based on the difficulty of adhesion removal by dissection. Articles reporting post-surgical adhesion have predominantly used these criteria[4–6].

3) Cytotoxicity of F127

Pluronic F127 is an FDA-approved material and has been extensively studied and found to be safe for use[7]. Due to its long-proven safety, we didn't independently verify the biocompatibility of F127 alone. Instead, we assessed the biocompatibility of sHAChiF, which contained F127 (20%), and demonstrated that the safety of sHAChiF adequately represented the biocompatibility of the F127.

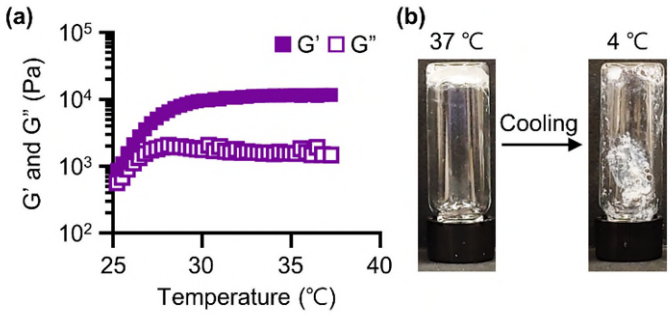
4) Thermogelation

In response, we newly added temperature-sweep test in Supplementary Fig. 3a. The elastic modulus increased along the temperature increase, which was the evidence of thermogelation.

In the Page 12 Line 305-307:

The DS of sHA was detailed as DS 1 (25-50 %), DS 2 (50-75 %), and DS 3 (75-100 %) to determine whether the anionic charge of sHA could influence the behavior of peritoneal macrophages.

In the Supplementary Fig. 3 (Supplementary Information: Page 5):



Supplementary Fig. 3. Thermogelation of sHAChiF. (a) Temperature sweep test of sHAChiF performed from 25 ℃ to 37 ℃. (b) Inverted vial test of sHAChiF from 37 ℃ to 4 ℃.

Again, we are grateful for the opportunity to strengthen our manuscript through your valuable comments and questions. We have diligently addressed your feedback and hope that these revisions persuade you to accept our submission.

Reviewer #2

We would like to thank Reviewer #2 for the effort and time he/she put in. We have addressed all the comments offered by Reviewer #2. We honestly feel that his/her insightful suggestions helped us in improving the manuscript. Below, we outline how we have handled each of Reviewer #2's comments.

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Table R2. Table of revisions (Reviewer #2)

Comment #	Reviewer #2's comments	Author's responses	Location in manuscript
1	NASAIDs or steroids have been widely studied and showed the efficacy to anti-peritoneal adhesion in several studies. But side effects are a concern to use anti-inflammatory drugs to increase the risk of infection, so they are not used clinically. Authors should carefully analyze the anti-inflammatory mechanism between previous drugs and current materials to show the potential of this material.	Agree. Supporting description provided.	N/A
2	Chitosan is not rapidly degraded in the peritoneum. Chitosan is not yet clinically available in the peritoneum. Degradation in peritoneum over one to two months should be carefully monitored.	Agree. Supporting data provided.	Supplementary Information: Supplementary Fig. 7; Page 7
3	The ischemic button model is not a standard model, and the cecal abrasion and peritoneal sidewall defect models are not desirable. Ischemic button model is not as severe adhesion model, so the data of cecal abrasion and peritoneal sidewall defect model should be added.	Agree. Supporting data provided.	Supplementary Information: Supplementary Fig. 8; Page 8

Reviewer #2 - Comment #1

NASAIDs or steroids have been widely studied and showed the efficacy to anti-peritoneal adhesion in several studies. But side effects are a concern to use anti-inflammatory drugs to increase the risk of infection, so they are not used clinically. Authors should carefully analyze the anti-inflammatory mechanism between previous drugs and current materials to show the potential of this material.

Author's response

We appreciate the reviewer's constructive comment. As the reviewer noted, the risk of infection is a potential limitation of using anti-inflammatory drugs as adhesion barrier, despite their effectiveness in reducing post-surgical adhesion. However, sHAChiF offers some safety advantages over other drugs and materials.

1) Selectivity of the sHA

   Unlike conventional drugs such as NSAIDs and steroids, the sHA acts selectively on macrophages. Macrophages capture and uptake the sHA through scavenger receptor[8] and CD44[9]. Specifically, 86.35 ± 4.89 % of peritoneal cells that took up the sHA was LPMs, as we demonstrated in Fig. 5j. This selective action would increase the safety of sHAChiF compared to NSAIDs or steroids, which are taken up by various cells and act systemically.

2) Anti-inflammation mechanism of the sHA

   sHA attenuates inflammation through the expression of anti-oxidative proteins SOD2 (Superoxide dismutase 2) and SOD3[10]. During inflammatory stimuli, SOD2 and SOD3 levels increase in macrophages treated with sHA, and these proteins inhibit pro-inflammatory signaling mediated by NF-kB. This mechanism could help mitigate pathological inflammation during infection, which generates ROS and damages tissue[11,12].

3) Necessity of anti-microbial prophylaxis

   Regardless of the use of anti-inflammatory adhesion barrier, the risk of pathological exacerbation by infection should be controlled through independent strategies. Anti-microbial prophylaxis before and after surgery is important to prevent incision infection and support healing[13,14]. If infection occurs, an adhesion barrier without anti-inflammatory function may promote post-surgical adhesion through inflammation progression[15].

4) Drug loading ability of the sHAChiF

   To address the potential limitation of sHAChiF hydrogel, anti-microbial drug loading into the sHAChiF hydrogel is a viable option. F127 is a well-known material for drug delivery, enabling the solubilization of hydrophobic and hydrophilic molecules because of its amphiphilic nature[16]. sHAChiF hydrogel with anti-microbial drugs would be a solution of the infection issue.

The efficacy of the sHAChiF hydrogel with anti-microbial drugs can be demonstrated using an intraperitoneal microbial contamination model[17], which generates ischemic button on the mouse peritoneal wall with cecal ligation and puncture. However, we didn't conduct the experiment as it might distract from the main focus of our study: (1) Gelability of sHA through polyelectrolyte complex formation, and (2) Potential biomedical application of sHA as an adhesion barrier by targeting peritoneal macrophages. If necessary, we can perform additional experiments to demonstrate the enhanced therapeutic strategy of sHAChiF hydrogel with anti-microbial drugs.

Reviewer #2 - Comment #2

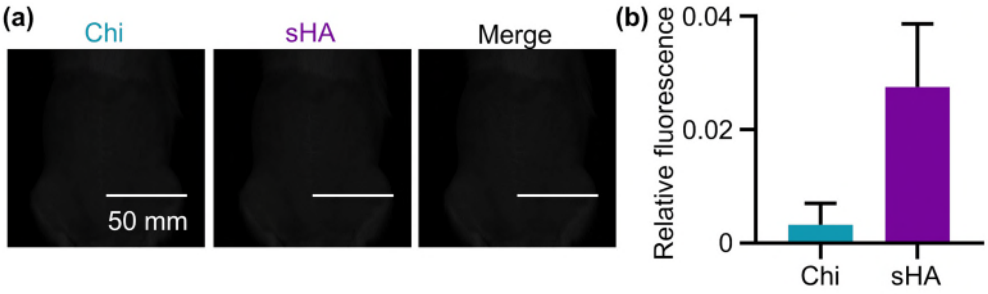
Chitosan is not rapidly degraded in the peritoneum. Chitosan is not yet clinically available in the peritoneum. Degradation in peritoneum over one to two months should be carefully

monitored.

Author's response

This is an excellent comment, which was appreciated by the authors. We tracked the retention test until 6 months. After 6 months, 99.67 ± 0.37 % of Chi and 97.24 ± 1.11 % of sHA were cleared. We newly added this data of long-term in vivo retention in Supplementary Fig. 7.

In the Supplementary Fig. 7 (Supplementary Information: Page 7):



Supplementary Fig. 7. Long-term in vivo retention of sHAChiF in rat ischemic button model. (a) Representative IVIS images of fluorescence by $Chi^{Cy5.5}$ and $sHA^{Cy7.5}$ in dual-sprayable hydrogels, 6 month after surgery (Scale bar = 50 mm). (b) Fluorescence intensity of $Chi^{Cy5.5}$ and $sHA^{Cy7.5}$ in sHAChiF, 6 month after surgery, normalized by fluorescence at d 0 (n = 4, mean ± SD).

Reviewer #2 - Comment #3

The ischemic button model is not a standard model, and the cecal abrasion and peritoneal sidewall defect models are not desirable. Ischemic button model is not as severe adhesion model, so the data of cecal abrasion and peritoneal sidewall defect model should be added.

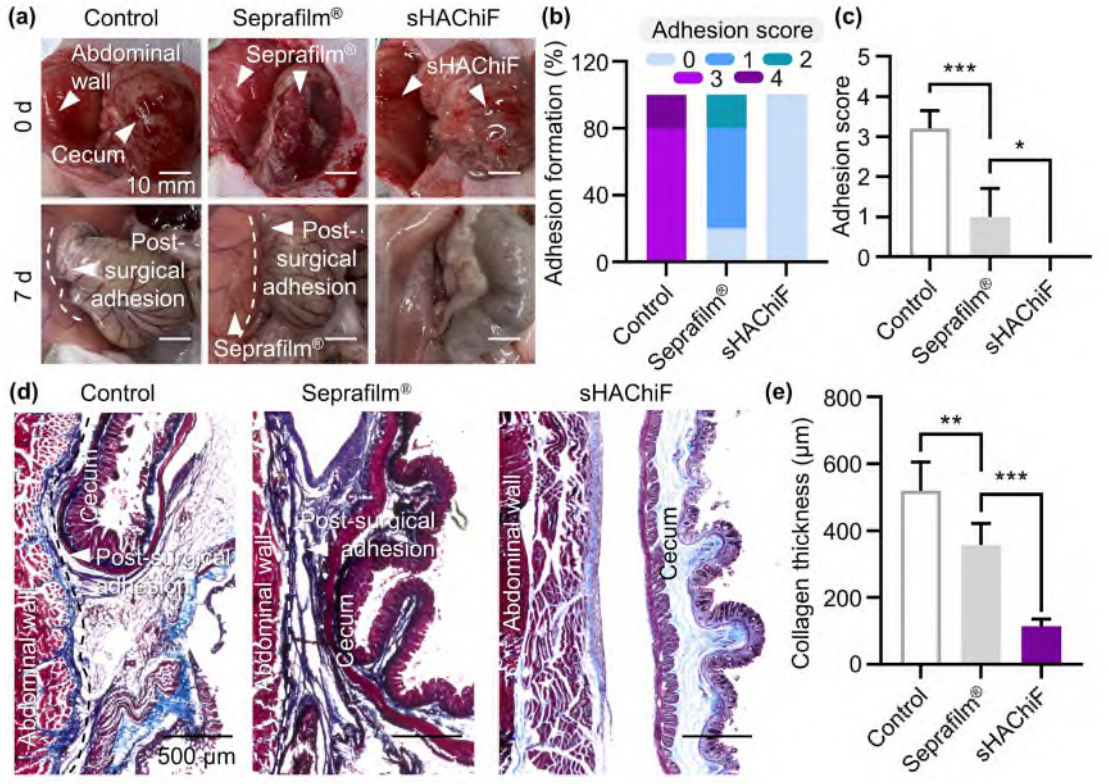
Author's response

Thank you for the constructive comment. We conducted additional experiments to demonstrate the therapeutic potential of the sHAChiF hydrogel as an adhesion barrier. We generated a rat cecal abrasion model by abrasion defects on the abdominal wall and cecum. Each end point of the abdominal wall and cecum was ligated to prevent modeling failure due to the peristaltic movement of the intestine.

Seven days after surgery, we analyzed the experimental groups (n = 5). Remarkably, post-surgical adhesion didn't occur in any of the rats treated with sHAChiF. In contrast, the control group exhibited severe post-surgical adhesions, which were stronger than those observed in the previous ischemic button experiments. The Seprafilm® group showed reduced post-surgical adhesion compared to the control group.

As the reviewer noted, those results indicated that the cecum abrasion model was a more severe adhesion model and that the results between groups contrast more sharply than in the ischemic button model. Despite the harsh condition of the cecum abrasion model, the sHAChiF hydrogel demonstrated its therapeutic potential as an adhesion barrier.

In the Supplementary Fig. 8 (Supplementary Information: Page 8):



Supplementary Fig. 8. Prevention of peritoneal adhesion in rat cecal abrasion model using sHAChiF. (a) Representative images of rat cecal abrasion model on d 0 and d 7 after surgery (Scale bar = 10 mm). (b) Adhesion formation rates with adhesion scores of sHAChiF per rat (n = 5, mean ± SD). (c) Quantification of the adhesion scores of dual-sprayable hydrogels (n = 5, mean ± SD). (d) Representative Masson's trichrome staining images of adhesive tissues on d 7 after surgery (Scale bar = 500 μm). (e) Quantification of collagen thickness of adhesive tissues on d 7 after surgery (n = 5, mean ± SD). ns: not significant, *p < 0.05, **p < 0.01, and ***p < 0.001.

We sincerely appreciate the opportunity to improve our manuscript with your insightful comments and questions. We have diligently worked to incorporate your feedback and hope that these revisions meet your approval for acceptance.

Reviewer #3

Thank you for the opportunity to revise our paper for the Nature communications. We are grateful for your careful and considered comments and have tried to respond to each comment. We believe the additional analyses discussed above have helped us to substantially improve our manuscript. Where appropriate, we have grouped related comments from each referee.

※ Please note that in this revision letter, figures labeled as "Figure R1", "Figure R2", etc., and tables labeled as "Table R1", "Table R2", etc. are used for explanatory purposes only and are not part of the actual manuscript. This labeling helps to clearly distinguish these figures and tables from those included in the manuscript.

Table R3. Table of revisions (Reviewer #3)

Comment #	Reviewer #3's comments	Author's responses	Location in manuscript
1	How to prove that sHA and Chi form the first-level network first and then pluronic® F127 forms the second-level network instead of three components forming sHAChiF hydrogel at the same time? In other words, the formation of a secondary network by pluronic® F127 requires more chemical means to prove.	Supporting data provided.	Supplementary Information: Supplementary Fig. 3; Page 5
2	At 25 ℃ and 37 ℃, the time required for the formation of sHAChiF hydrogel is not mentioned.	Supporting data provided.	Supplementary Movie 2
3	It is well known that strongly acidic conditions are harmful to humans. The concentration of acetic acid solvent and hydrochloric acid solvent for spraying are not mentioned. Meanwhile, the question of what effect it has on the rheology, biocompatibility and optimal gel conditions of the sHAChiF hydrogel deserves discussion.	Supporting description provided.	Page 22 Line 545
4	The levels of collagens in Masson's trichrome staining need to be quantified and compared in all groups. At the same time, it would be better if the adhesion areas are marked in the staining results.	Agree. Supporting data provided.	Supplementary Information: Supplementary Fig. 8; Page 8

5	Figure captions appear misplaced throughout the article. For example, in "The sHA/Chi/F127 mixture rapidly developed an opaque gel structure upon mixing at 25 ℃ (Fig. 1b)", its picture should correspond to Fig 2b.	Agree. Errors were revised.	Whole

Reviewer #3 - Comment #1

How to prove that sHA and Chi form the first-level network first and then pluronic® F127 forms the second-level network instead of three components forming sHAChiF hydrogel at the same time? In other words, the formation of a secondary network by pluronic® F127 requires more chemical means to prove.

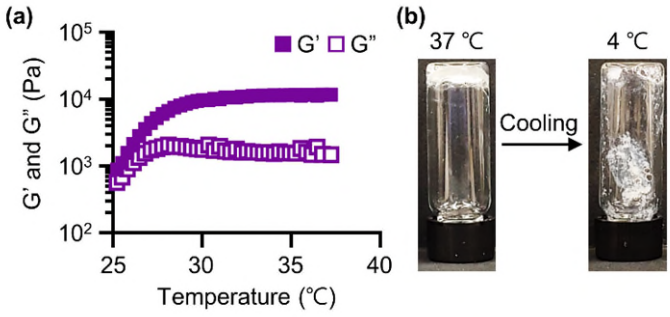
Author's response

We thank you for this constructive comment, and fully agree with your opinion. The Pluronic® F127 (PEO_{100}-PPO_{65}-PEO_{100}) is a well-known polymer capable of thermogelation. The amphiphilic triblock copolymer forms micelle structures and aggregates at certain temperature.

We added two new experiments to prove the thermogelation of sHAChiF in Supplementary Fig. 3. First, the temperature sweep test showed that the storage modulus (G') gradually increased with the temperature change from 25 ℃ to 37 ℃, with saturation at 30.05 ℃ (Supplementary Fig. 3a).

Second, we conducted an inverted vial test at 4 ℃. After performing the inverted vial test at 37 ℃, we cooled the sample to 4 ℃ and repeated the vial inversion. We observed that the gelled sHAChiF hydrogel flowed down, whose status was not completely liquid. This data indicates that a network formed by thermogelation exists, along with another network independent of thermogelation.

In the Supplementary Fig. 3 (Supplementary Information: Page 5):



Supplementary Fig. 3. Thermogelation of sHAChiF. (a) Temperature sweep test of sHAChiF performed from 25 ℃ to 37 ℃. (b) Inverted vial test of sHAChiF from 37 ℃ to 4 ℃.

Reviewer #3 - Comment #2

At 25 ℃ and 37 ℃, the time required for the formation of sHAChiF hydrogel is not mentioned.

Author's response

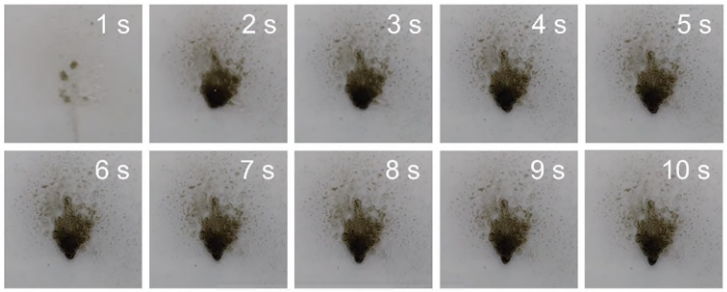


Fig. R3. Spraying and Gelation of the sHAChiF hydrogel.

We appreciate the Reviewer's insightful comment. The gelation of the sHAChiF hydrogel occurred immediately after blending the sHA/F127 and Chi/F127. Consequently, it was challenging to measure the gelation time of the sHAChiF hydrogel using robust analytic techniques such as rheology. Instead of measuring the gelation time, we included a new movie applying the sHAChiF hydrogel to a heated whiteboard (Supplementary Movie 2).

We set up a whiteboard and heated it to 37 ℃ by attaching a heating pad for a realistic application scenario. Then, we added black dye to each solution for contrast and sprayed the mixture onto the whiteboard. After spraying, the sHAChiF flowed down slightly but stopped within 5 s.

For a more accurate measurement of the gelation time of sHAChiF, a precise experimental setup would be required. For example, the rheometer should begin measuring the gel's mechanical changes immediately after blending. However, setting up such an experiment is currently extremely challenging. This is a technical limitation of our study.

Reviewer #3 - Comment #3

It is well known that strongly acidic conditions are harmful to humans. The concentration of acetic acid solvent and hydrochloric acid solvent for spraying are not mentioned. Meanwhile, the question of what effect it has on the rheology, biocompatibility and optimal gel conditions of the sHAChiF hydrogel deserves discussion.

Author's response

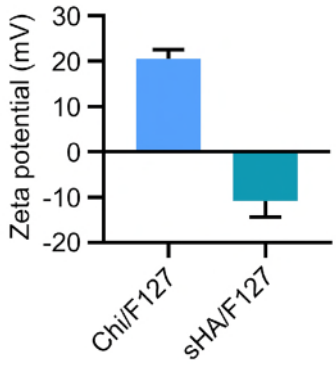


Fig. R2: Zeta potential of the Chi/F127 and sHA/F127.

We thank the Reviewer for this comment and fully acknowledged the issue regarding the safety of using acids to fabricate sHAChiF. We added information on the concentration of acetic acid

in the Chi/F127 solution (Page 22 Line 544).

As described in the Methods section (Page 22 Line 544-546), we addressed the safety issue of Chi/F127 by neutralizing it. We firstly solubilized Chi into the F127 in HCl solution (0.1 M) and then adjusted the pH to 7.0 using F127 in PBS. Furthermore, we confirmed whether the neutral pH remove the cationic charge of the Chi/F127 solution (Fig. R2; the same description is in the revision letter on Page 5).

In the Page 22 Line 545:

Because of the insolubility of Chi in neutral pH, the Chi was solubilized by Pluronic® F127 (19 or 20 %) in HCl solution or acetic acid (0.1 M) first and the pH was adjusted by Pluronic® F127 (19 or 20 %) in PBS.

Reviewer #3 - Comment #4

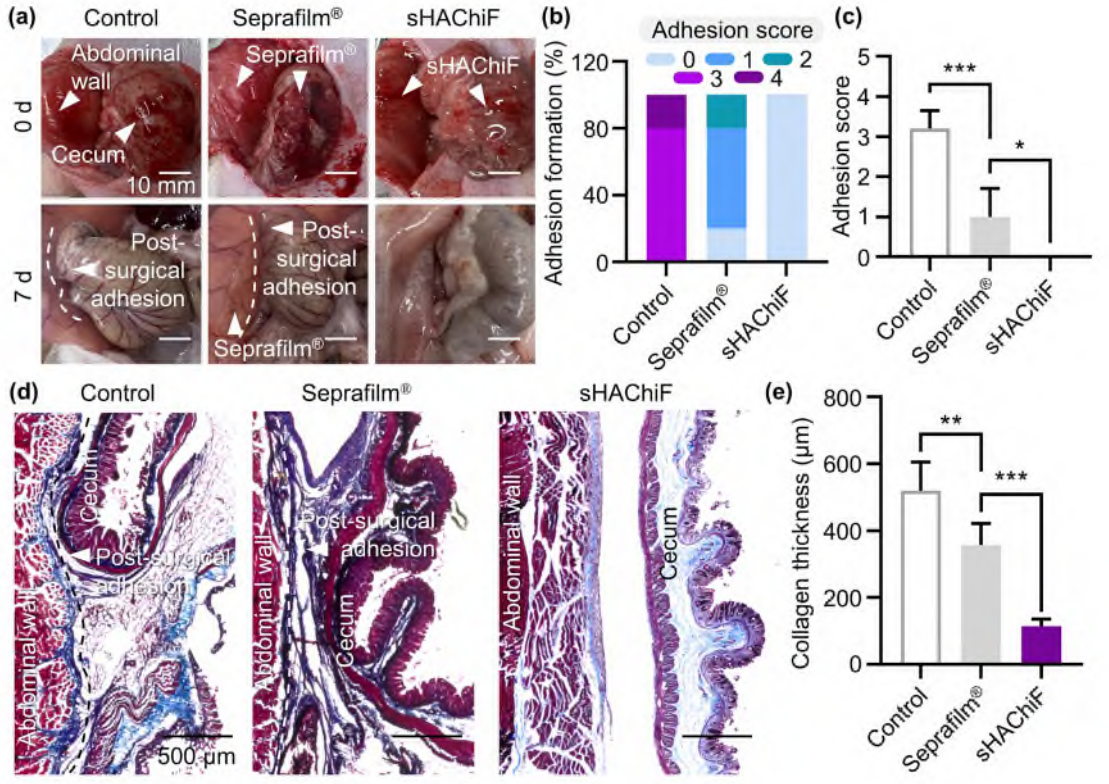
The levels of collagens in Masson's trichrome staining need to be quantified and compared in all groups. At the same time, it would be better if the adhesion areas are marked in the staining results.

Author's response

We agree with the reviewer's comment. Quantification of the histological results would provide more supportive evidence. However, in the ischemic button model, the variation in collagen deposition was high because some buttons were attached to fat tissue while others were attached to other organs such as the liver or cecum.

To obtain more reliable results, we newly added another post-surgical adhesion model known as the cecal abrasion model. This model fixes the location of the cecum and abdominal wall, simplifying the analysis. We quantified the collagen thickness of the samples and analyzed the adhesion formation between the cecum and the abdominal wall. The results showed that sHAChiF significantly reduced post-surgical adhesion, with collagen thickness being lower than in the control and Seprafilm® groups.

In the Supplementary Fig. 8 (Supplementary Information: Page 8):



Supplementary Fig. 8. Prevention of peritoneal adhesion in rat cecal abrasion model using sHAChiF. (a) Representative images of rat cecal abrasion model on d 0 and d 7 after surgery (Scale bar = 10 mm). (b) Adhesion formation rates with adhesion scores of sHAChiF per rat (n = 5, mean ± SD). (c) Quantification of the adhesion scores of dual-sprayable hydrogels (n = 5, mean ± SD). (d) Representative Masson's trichrome staining images of adhesive tissues on d 7 after surgery (Scale bar = 500 μm). (e) Quantification of collagen thickness of adhesive tissues on d 7 after surgery (n = 5, mean ± SD). ns: not significant, *p < 0.05, **p < 0.01, and ***p < 0.001.

Reviewer #3 - Comment #5

Figure captions appear misplaced throughout the article. For example, in "The sHA/Chi/F127 mixture rapidly developed an opaque gel structure upon mixing at 25 ℃ (Fig. 1b)", its picture should correspond to Fig 2b.

Author's response

Thank you for your careful reading. We revised overall errors in the manuscript.

We are grateful for the opportunity to improve our manuscript based on your valuable comments and questions. We have carefully addressed your feedback and hope these revision will persuade you to accept our submission.

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Reviewer #1 (Remarks to the Author):

The authors have addressed the comments from the reviewers. I suggest its publication as it is.

Feedback on Reviewer #3's comments:

I have carefully reviewed the responses to the comments from the reviewer 3. The authors have mostly addressed the comments. I have only one question as below. Otherwise, it is ready for publication.

Comments: Chi is not solution at high pH. After adjusting the pH to 7.0, does Chi become insoluble and precipitate from the solution? This may induce the inhomogeneous formation of polymer networks. If not, why?

In addition, the authors should mention the final pH (...the pH was adjusted to be 7.0 by F127...) in the line 545 on Page 22.

Reviewer #2 (Remarks to the Author):

The manuscript was well revised. I hope further progress of the authors' research.

Reviewers' comments

We thank Reviewers for the constructive comments on our manuscript. Reviewers' original comment is listed below followed by our response to the comment.

※ Please note that in this revision letter, figures labeled as "Figure R1", "Figure R2", etc., and tables labeled as "Table R1", "Table R2", etc. are used for explanatory purposes only and are not part of the actual manuscript. This labeling helps to clearly distinguish these figures and tables from those included in the manuscript.

Table R1. Table of revisions (Editor)

Comment #	Reviewers' comments	Author's responses	Location in manuscript
1	Chi is not solution at high pH. After adjusting the pH to 7.0, does Chi become insoluble and precipitate from the solution? This may induce the inhomogeneous formation of polymer networks. If not, why?	Supporting description provided.	N/A
2	In addition, the authors should mention the final pH (...the pH was adjusted to be 7.0 by F127...) in the line 545 on Page 22.	The sentence was revised.	Page 22 Line 550

Reviewers' comments - Comment #1

Chi is not solution at high pH. After adjusting the pH to 7.0, does Chi become insoluble and precipitate from the solution? This may induce the inhomogeneous formation of polymer networks. If not, why?

Author's response

We appreciate your valuable comments. As you noted, chitosan (Chi) solutions typically precipitate at neutral pH. However, when F127 was added, the Chi/F127 solution maintained its liquid state even after pH adjustment to 7.0. F127 has been employed to solubilize insoluble molecules, such as curcumin[1–4]. Due to its amphiphilic nature, F127 can stabilize the hydrophobicity of insoluble molecules. In this context, F127 interacts with Chi in the Chi/F127 solution, preventing Chi from precipitating as the pH increases.

Reviewers' comments - Comment #2

In addition, the authors should mention the final pH (...the pH was adjusted to be 7.0 by F127...) in the line 545 on Page 22.

Author's response

Thank you for your careful reading. We revised the sentence in the manuscript.

In the Page 22 Line 550:

Chi was solubilized by Pluronic® F127 (19 or 20 %) in HCl solution or acetic acid (0.1 M) first

and the pH was adjusted to be 7.0 by Pluronic® F127 (19 or 20 %) in PBS.

We are grateful for the opportunity to improve our manuscript based on your valuable comments and questions.

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