nature portfolio

# Peer Review File

## Ternary Inulin Hydrogel with Long-Term Intestinal Retention for Simultaneously Reversing IBD and its Fibrotic Complication

# REVIEWER COMMENTS

**Reviewer #1 (Remarks to the Author):**

In this manuscript, a PPy/PFD@Inulin gel was designed to prevent and alleviate the detrimental effects associated with IBD and intestinal fibrosis. This article has detailed data and a rigorous demonstration process. The following concerns should be addressed:

In the introduction, it is claimed that high oxidative stress, flora imbalance, and damage to the intestinal barrier in the intestinal microenvironment may progress to more severe conditions such as intestinal fibrosis. As shown in Figures 6 and 7, the therapeutic effects of PPy/PFD@Inulin gel were comparable to PPy@Inulin gel treatment, especially for modulating flora imbalance, which is conducive to inhibiting intestinal fibrosis. Therefore, please explain more specifically the importance (necessity) of encapsulating PFD.

In vitro degradation experiments confirmed that the presence of Bifidobacterium longum in phosphate-buffered saline (PBS) accelerated gel degradation in Figure 2c. Please explain why Bifidobacterium longum was able to accelerate gel degradation.

In Figure 2c, the degradation rate of PPy/PFD@Inulin gel remained below 5% after 6 hours and below 15% after 12 hours, which was beneficial for escorting the PPy/PFD@Inulin gel to the intestine, as gastric emptying time is commonly only 3-4 hours. However, the cumulative release (%) of PFD molecules was up to 60% in pH 1.5 PBS within 4 hours (Figure 2d). This may directly result in lower PFD content in the neutral colon, affecting the progression of inhibiting intestinal fibrosis. Please explain this phenomenon.

PPy nanozymes exhibited excellent free radical scavenging activities at a low concentration of 60 μg/mL in Figure 3a-e. Why did the authors choose high concentrations (400 μg/mL) for further in vitro and in vivo experiments? In addition, please verify whether the cells were treated with LPS or $H_2O_2$ in Figure 3g and 3i?

More characterization data are needed to evaluate the stability of PPy/PFD@Inulin gel, such as XRD, FTIR, free radical scavenging rates, etc.

To visually verify that oral administration of the Cy5.5-PPy/PFD@Inulin gel exhibited a longer residence time in the colon than Cy5.5-PPy nanozymes, I suggest a comparison at the same time in Figure 2f. Additionally, some significant differences may be added in Figures 2, 7, 9, etc.

**Reviewer #2 (Remarks to the Author):**

This paper introduces the development of a versatile ternary inulin composite gel with exceptional injectable properties. The ternary hydrogel, which possesses multifunctionality including reactive oxygen and nitrogen species scavenging, intestinal flora regulation, and down regulating the TGF-

β/Smad signaling pathway. In addition to managing IBD, it can also help prevent and decrease the adverse effects of intestinal fibrosis associated with IBD complications. Furthermore, this hydrogel prepared from prebiotic inulin serves as a drug delivery platform, prolonging the action time of the active components in the intestine, while also modulating the intestinal microbiota, ultimately achieving a synergistic therapeutic effect. Inspired by the aforementioned multifunctional composite hydrogel, it could serve as a convenient and valuable platform for treating various other diseases. This work is innovative, but minor revision is needed to further improve this manuscript.

1. The author expresses in the article that the final synthesized composite hydrogel uses "PPy/PFD@Inulin gel", but the label in Fig. 1 uses "PPy/PFD gel", please keep it consistent.
2. It is suggested that the ordinate "absorbance" in Supplementary Fig. 2 and Supplementary Fig. 7 be modified to "absorbance (a.u.)" and the first letter of "transmittance" in Supplementary Fig. 3a should be capitalized.
3. It is recommended to supplement the significant differences between the experimental and control groups in the antioxidant data plot simulating the gastrointestinal environment in Fig. 2e.
4. It is recommended to re-check the data graph in the article, for example, the axes in Fig. 8i are not fully displayed.
5. In the EdU experiment shown in Fig. 9a, it is suggested to perform percentage counting of EdU-positive cells to more intuitively reflect the proliferation inhibition of CCD-18CO cells by PPy/PFD@Inulin gel.
6. In the article, the abbreviation of α-smooth muscle actin is expressed as "α-SMA". In the conclusion section, the expression of "α-SAM" is wrong, please modify it.
7. It is recommended to supplement the specific data descriptions of colon length in Fig. 5c, d and Fig. 8c, d within the article. This will provide a more detailed demonstration of the therapeutic effects associated with colon length.

**Reviewer #3 (Remarks to the Author):**

Cao and colleagues developed a ternary inulin hydrogel with long-term intestinal retention and investigated whether this hydrogel may have therapeutic potential for the treatment of IBD and its fibrotic complications. The manuscript presents an innovative approach to treating IBD and its associated complications, particularly intestinal fibrosis, by introducing a probiotic inulin hydrogel loaded with polypyrrole (PPy) nanozymes and pirfenidone (PFD). The manuscript outlines the synthesis, characterization, in vitro and in vivo biocompatibility, therapeutic efficacy, and underlying mechanisms of action of the PPy/PFD@inulin gel.

Overall, the manuscript is well written and uses clear and concise language. The described approach of combining the therapeutic effects of PPy nanozymes, PFD, and the probiotic benefits of inulin is an innovative approach and may allow targeting various pathogenic features of IBD, including oxidative stress, inflammatory cytokines, and intestinal dysbiosis. The manuscript provides compelling evidence for the therapeutic efficacy of the hydrogel in both prophylactic and delayed treatment models of DSS-induced colitis. However, the characterization of the mechanisms of action remains rather superficial and some conclusions are not fully supported by the experimental data. Therefore, I strongly recommend addressing several issues that could make this study more robust and impactful.

Major:

1. While the study outlines the beneficial effects on RONS scavenging, inflammation reduction and restoration of homeostatic microbiota, a deeper mechanistic insight into how the hydrogel components interact with the biological pathways would enhance the understanding of its therapeutic potential. The authors focus on very few inflammatory cytokines. It would be more helpful to perform a broader characterization, e.g. by bulk mRNA sequencing, and to study changes in immune cell infiltration.
2. Figure 7 suggests that intestinal barrier function is impaired, but the characterization is very superficial. Immunofluorescence staining against ZO-1 and Occluding can be very subjective - it would be good to provide Western blot of barrier proteins. I would also suggest adding an in vitro FITC dextran assay of intestinal barrier function. It would also be interesting to see if the colonic mucus layer is affected. One hypothesis could be that the hydrogel limits bacterial translocation. This could be assessed by mucus staining in combination with FISH of luminal bacteria.
3. It would be very helpful to also study the effect of the hydrogel on intestinal physiology without additional DSS treatment. How does the hydrogel affect feeding behavior? Does the treatment affect weight gain? How is intestinal transit time affected? Does the intestinal microbiota change? How is the intestinal barrier function affected by the hydrogel?
4. Figure 9d: Showing 1 replicate per condition is not very helpful. It is better to show fewer conditions with more replicates. Please also include the original Western blots for quantification in Figure 9e in the Supplement.

Minor:

1. Discussion of the scalability of the hydrogel synthesis process and any potential manufacturing and regulatory challenges would be beneficial to understanding the path to clinical application.
2. The manuscript could benefit from professional editing by a native speaker.

**Response to referees:**

**Reviewer #1 (Remarks to the Author):**

In this manuscript, a PPy/PFD@Inulin gel was designed to prevent and alleviate the detrimental effects associated with IBD and intestinal fibrosis. This article has detailed data and a rigorous demonstration process. The following concerns should be addressed:

**Response:** We are very grateful to your nice and valuable comments.

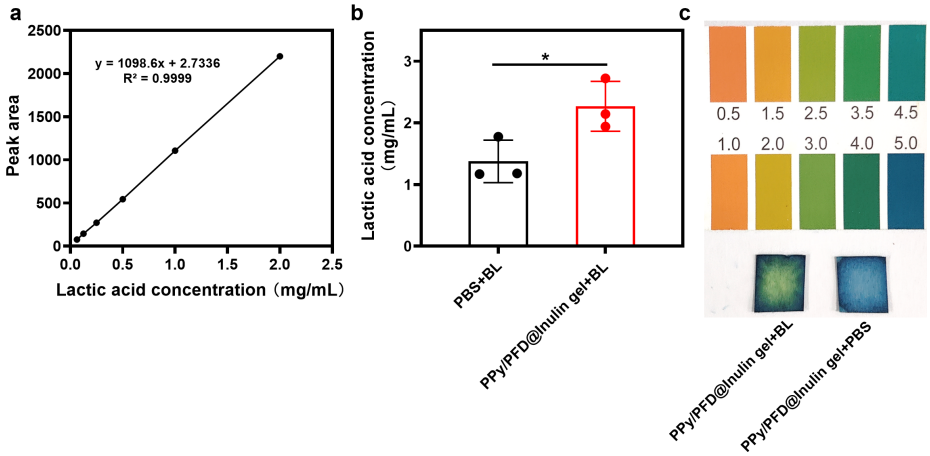
**Q1.** In the introduction, it is claimed that high oxidative stress, flora imbalance, and damage to the intestinal barrier in the intestinal microenvironment may progress to more severe conditions such as intestinal fibrosis. As shown in Figures 6 and 7, the therapeutic effects of PPy/PFD@Inulin gel were comparable to PPy@Inulin gel treatment, especially for modulating flora imbalance, which is conducive to inhibiting intestinal fibrosis. Therefore, please explain more specifically the importance (necessity) of encapsulating PFD.

**Response:** Figs. 6 and 7 predominantly present the outcomes of animal experiments of acute colitis (delayed model). The monotherapy group treated with PFD demonstrates that PFD's anti-inflammatory efficacy is largely insignificant within this model, thus rendering the therapeutic effect of PPy/PFD@Inulin gel is similar to that of PPy@Inulin gel. The primary objective of encapsulating PFD is to mitigate adverse symptoms associated with fibrosis in chronic colitis models, such as excessive collagen deposition leading to intestinal wall thickening and intestinal stricture. Additionally, Fig. 8 illustrates that PPy@Inulin gel effectively modulates inflammation, although its efficacy in addressing fibrosis is limited. By encapsulating PFD, the PPy/PFD@Inulin gel formulation can not only manage IBD but also avert the development of fibrosis.

**Q2.** In vitro degradation experiments confirmed that the presence of Bifidobacterium longum in phosphate-buffered saline (PBS) accelerated gel degradation in Figure 2c. Please explain why Bifidobacterium longum was able to accelerate gel degradation.

**Response:** We greatly appreciate your insightful advice. To elucidate the mechanism underlying the accelerated of the gel by Bifidobacterium, additional experiments have been incorporated, as shown in Supplementary Fig. 7. Initially, as depicted in Supplementary Fig. 7a and b, the concentration of lactic acid generated by BL in the PPy/PFD@Inulin gel exceeds that in PBS. This indicates that BL utilizes the

PPy/PFD@Inulin gel to enhance its proliferation, consequently elevating lactic acid production. Furthermore, the presence of lactic acid in the degradation environment leads to a reduction in pH levels, as demonstrated in Supplementary Fig. 7c, where the pH value approximates 3.0. This acidic environment significantly facilitates the degradation of the PPy/PFD@Inulin gel.




**Supplementary Fig. 7. a** The relationship between different concentrations of lactic acid and peak area was determined by high performance liquid chromatography. **b** Lactic acid concentration of PPy/PFD@Inulin gel in PBS and BL. **c** Degradation of PPy/PFD@Inulin gel in PBS and BL, pH in upper liquid.

**Q3.** In Figure 2c, the degradation rate of PPy/PFD@Inulin gel remained below 5% after 6 hours and below 15% after 12 hours, which was beneficial for escorting the PPy/PFD@Inulin gel to the intestine, as gastric emptying time is commonly only 3-4 hours. However, the cumulative release (%) of PFD molecules was up to 60% in pH 1.5 PBS within 4 hours (Figure 2d). This may directly result in lower PFD content in the neutral colon, affecting the progression of inhibiting intestinal fibrosis. Please explain this phenomenon.

**Response:** We deeply appreciate your good question. Based on the current findings, although the cumulative release of PFD molecules from the PPy/PFD@Inulin gel reached 60% within 4 hours, the free PFD achieved 60% release within 20 minutes and was completely released within 2 hours. The hydrogel drug delivery system extended the release duration by 12 times. The experimental data indicate that the therapeutic efficacy for intestinal fibrosis remains uncompromised, and the treatment outcome of the PPy/PFD@Inulin gel group surpasses that of the free PFD group.

**Q4.** PPy nanozymes exhibited excellent free radical scavenging activities at a low

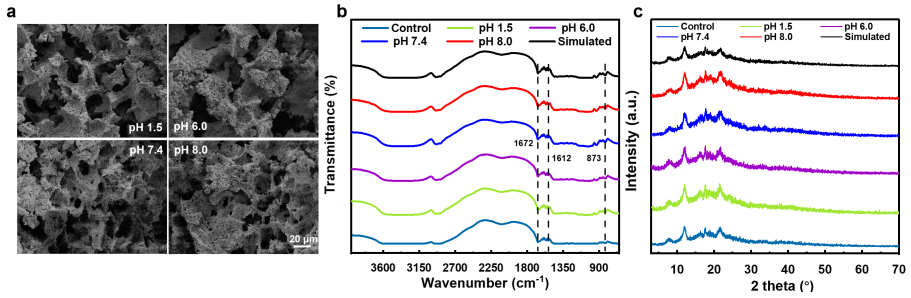
concentration of 60 μg/mL in Figure 3a-e. Why did the authors choose high concentrations (400 μg/mL) for further in vitro and in vivo experiments? In addition, please verify whether the cells were treated with LPS or $H_2O_2$ in Figure 3g and 3i?

**Response:** Thank you very much for your good question. PPy nanozymes demonstrated remarkable free radical (including DPPH, ABTS and $O_2\cdot^-$) scavenging efficacy at a low concentration of 60 μg/mL. However, due to modified experimental parameters, a higher concentration (400 μg/mL) was employed. Throughout the experiment, variables such as the concentration of $H_2O_2$ or incubation time influenced the utilized concentration of PPy nanozymes. We conducted assays with varying gradient concentrations of PPy nanozymes. Under the specified experimental conditions, a concentration of 400 μg/mL PPy nanozymes yielded optimal results.

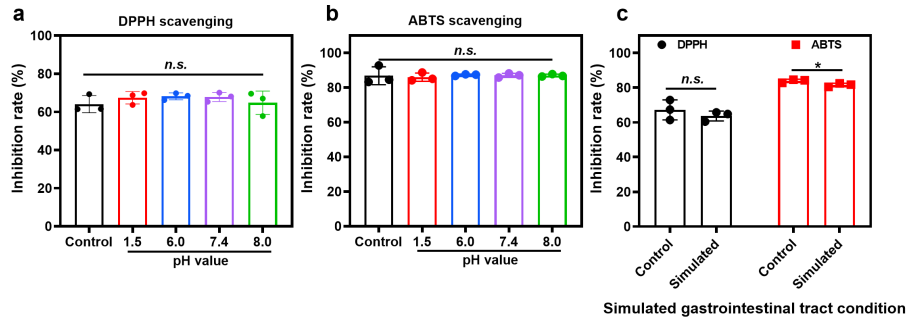
In Fig. 3g and 3i, cells were treated with $H_2O_2$, and the 0 + 3 mM $H_2O_2$ group in Fig. 3g caused a large number of cell deaths, while the 0 + 1 mM $H_2O_2$ group in Fig. 3i presented green fluorescence in the cells. In Supplementary Fig. 15, the cells were exposed to LPS, leading to substantial green fluorescence in the LPS-treated group due to the generation of intracellular ROS.

**Q5.** More characterization data are needed to evaluate the stability of PPy/PFD@Inulin gel, such as XRD, FTIR, free radical scavenging rates, etc.

**Response:** Thank you for your kind suggestion. Based on your recommendation, we supplemented assessments including XRD, FTIR, and free radical scavenging rates, thereby enhancing the robustness of our findings. Initially, after immersing the PPy/PFD@Inulin gel in PBS at varying pH levels and simulating gastrointestinal conditions, FTIR and XRD analyses proved that the structural integrity of the PPy/PFD@Inulin gel remained intact, as illustrated in Supplementary Fig. 6b and c. Subsequently, the antioxidant properties of the PPy/PFD@Inulin gel was evaluated by soaking it in PBS at different pH levels and also under simulating gastrointestinal environment. The DPPH and ABTS radical scavenging assays revealed that the antioxidant efficacy was nearly unchanged to the control group, as depicted in Supplementary Fig. 12.




**Supplementary Fig. 6. a** The SEM image of PPy/PFD@Inulin gel after treating for 6 h in pH 1.5, pH 6.0, pH 7.4, pH 8.0 solution. **b** FTIR of PPy/PFD@Inulin gel after simulated treatment in different pH PBS and simulated Gastrointestinal Environment. **c** XRD of PPy/PFD@Inulin gel after simulated treatment in different pH PBS and simulated Gastrointestinal Environment.




**Supplementary Fig. 12. a** At different pH levels (pH 1.5, 6.0, 7.4, and 8.0), the inhibition rates of DPPH and ABTS (**b**) by PPy/PFD@Inulin gel were determined. **c** After simulating the gastrointestinal environment, PPy/PFD@Inulin gel was used to determine its clearance rate of DPPH and ABTS. Data are presented as mean ± SD (n = 3 biological replicates).

**Q6.** To visually verify that oral administration of the Cy5.5-PPy/PFD@Inulin gel exhibited a longer residence time in the colon than Cy5.5-PPy nanozymes, I suggest a comparison at the same time in Figure 2f. Additionally, some significant differences may be added in Figures 2, 7, 9, etc.

**Response:** We deeply appreciate your kind suggestion. To visually confirm the prolonged retention of oral Cy5.5-PPy/PFD@Inulin gel in the colon compared to Cy5.5-PPy nanozymes, we have annotated the original fluorescence data in Fig. 2f. Additionally, significant analyses have been incorporated into Fig. 2c, Fig. 2e, Fig. 3e, Fig. 7b (now revised as Supplementary Fig. 22), Fig. 9b (now revised as Fig. 11b), and Fig. 9e, f (now revised as Fig. 11e, f).

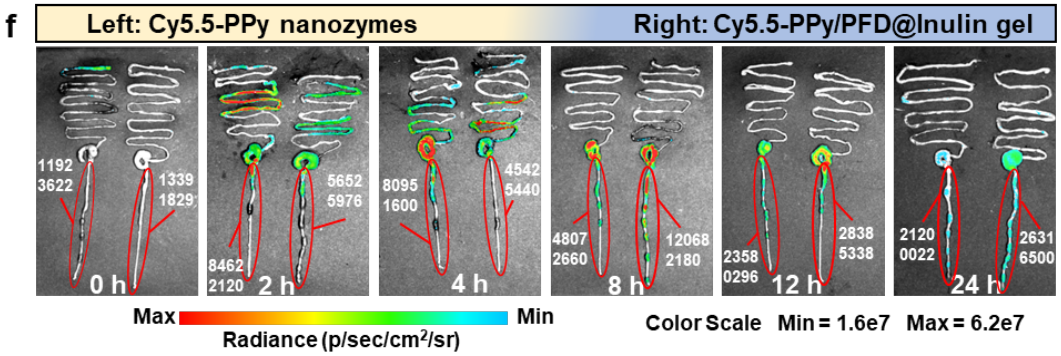



**Fig. 2 | f** Cy5.5-PPy nanozymes and Cy5.5-PPy/PFD@Inulin gel were administered orally to mice. The gastrointestinal tract was observed for 24 h.

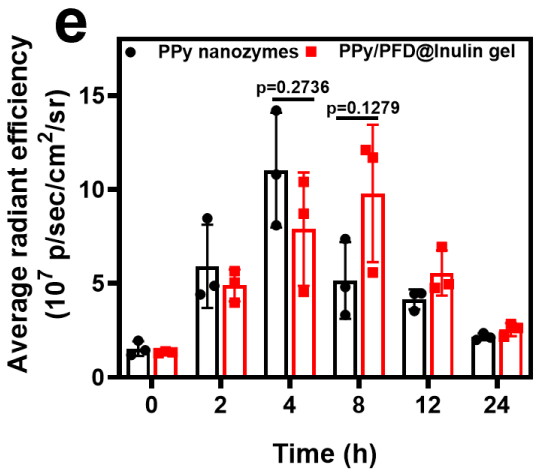



**Fig. 2 | e** Cy5.5-PPy nanozymes and Cy5.5-PPy/PFD@Inulin gel were administered orally to mice. The mean fluorescence intensity in the colon was quantified.

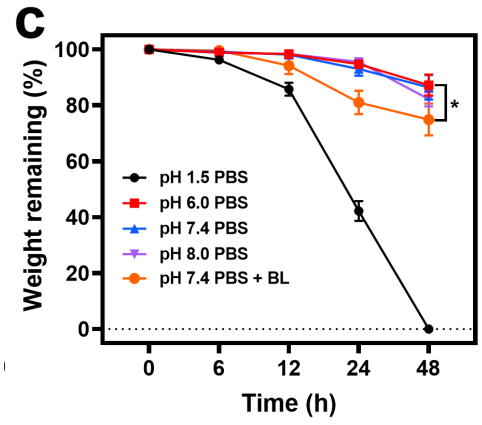



**Fig. 2 | c** In vitro degradation kinetics of the PPy/PFD@Inulin gel were studied under different pH levels and the presence of Bifidobacterium longum.

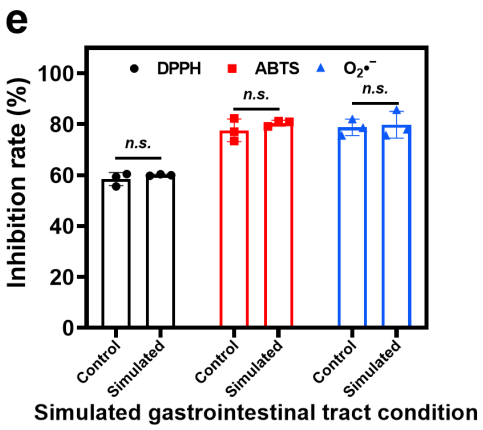
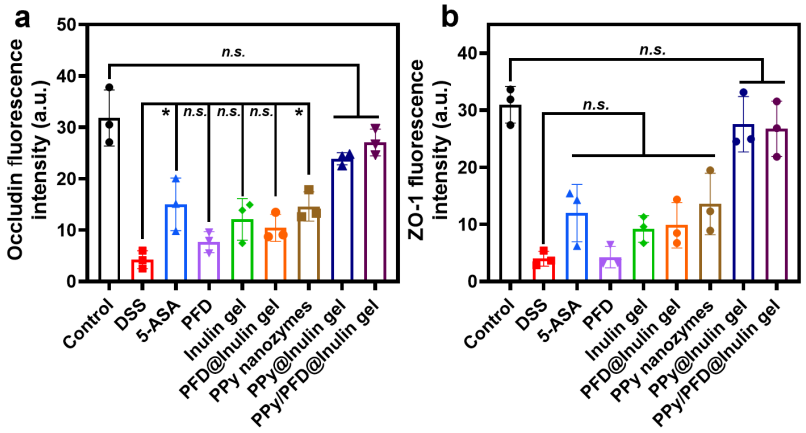



**Fig. 3 | e** PPy nanozymes retention activity after treatment in a simulated gastrointestinal environment was retained.




**Supplementary Fig. 22.** The relative fluorescence intensity of Occludin (**a**) and ZO-1 (**b**) proteins in colon. Data are presented as mean ± SD (n = 3 biologically independent samples).

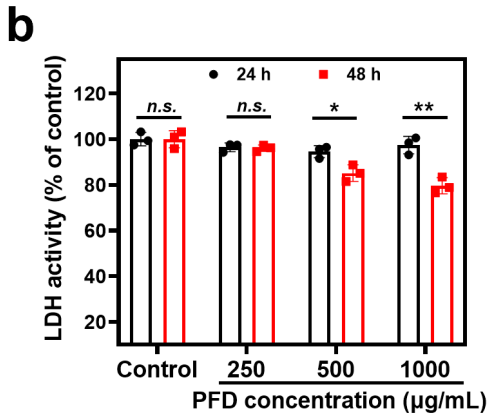



**Fig. 11 | c** Relative LDH activity of CCD-18Co cells.

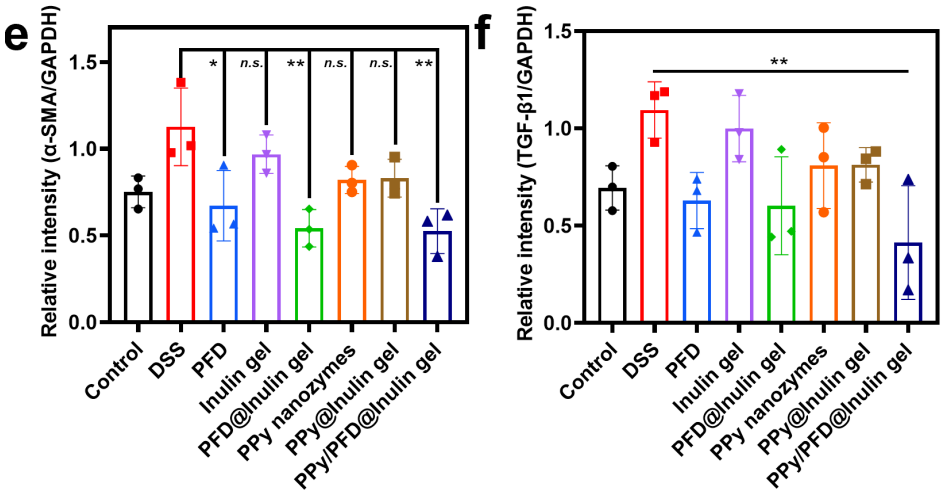



**Fig. 11 | e, f** Quantitative Western blot analyses were performed.

**Reviewer #2 (Remarks to the Author):**

This paper introduces the development of a versatile ternary inulin composite gel with exceptional injectable properties. The ternary hydrogel, which possesses multifunctionality including reactive oxygen and nitrogen species scavenging, intestinal flora regulation, and down regulating the TGF-β/Smad signaling pathway. In addition to managing IBD, it can also help prevent and decrease the adverse effects of intestinal fibrosis associated with IBD complications. Furthermore, this hydrogel prepared from prebiotic inulin serves as a drug delivery platform, prolonging the action time of the active components in the intestine, while also modulating the intestinal microbiota, ultimately achieving a synergistic therapeutic effect. Inspired by the aforementioned multifunctional composite hydrogel, it could serve as a convenient and valuable platform for treating various other diseases. This work is innovative, but minor revision is needed to further improve this manuscript.

**Response:** We are very grateful to your kind comments.

**Q1.** The author expresses in the article that the final synthesized composite hydrogel uses "PPy/PFD@Inulin gel", but the label in Fig. 1 uses "PPy/PFD gel", please keep it consistent.

**Response:** Thank you for your kind reminding. We have reviewed the full text to ensure that "PPy/PFD gel" is unified into "PPy/PFD@Inulin gel".

**Q2.** It is suggested that the ordinate "absorbance" in Supplementary Fig. 2 and

Supplementary Fig. 7 be modified to "absorbance (a.u.)" and the first letter of "transmittance" in Supplementary Fig. 3a should be capitalized.

**Response:** Thank you for your kind suggestion. We have modified the problem of the ordinate "absorbance (a.u.)" in Fig.2 and Fig.7 and the lowercase of the initials of the Supplementary Fig.3a ordinate.

**Q3.** It is recommended to supplement the significant differences between the experimental and control groups in the antioxidant data plot simulating the gastrointestinal environment in Fig. 2e.

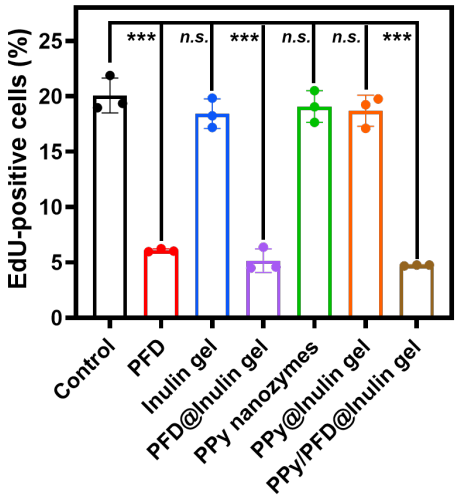
**Response:** Thank you for your kind suggestion, we have added the significant difference between the experimental group and the control group in the antioxidant data map simulating the gastrointestinal environment, so that readers can see more intuitively whether there is a difference between the experimental group and the control group.

**Q4.** It is recommended to re-check the data graph in the article, for example, the axes in Fig. 8i are not fully displayed.

**Response:** Thank you for your careful examination. We apologize for our carelessness, and according to your opinion, we have corrected it so that the axis of figure 8i is fully displayed.

**Q5.** In the EdU experiment shown in Fig. 9a, it is suggested to perform percentage counting of EdU-positive cells to more intuitively reflect the proliferation inhibition of CCD-18CO cells by PPy/PFD@Inulin gel.

**Response:** Thank you for your kind suggestion. We have analyzed the fluorescence staining image of EdU and counted the positive cells, which makes it more intuitive to see the inhibition of PPy/PFD@Inulin gel on the proliferation of CCD-18CO cells.




**Supplementary Fig. 19.** Quantification of EdU positive cells in the EdU test.

**Q6.** In the article, the abbreviation of α-smooth muscle actin is expressed as "α-SMA". In the conclusion section, the expression of "α-SAM" is wrong, please modify it.

**Response:** Thank you for your careful examination. We apologize for our carelessness, and according to your opinion, we have made corrections to make "α-SMA" consistent in the full text.

**Q7.** It is recommended to supplement the specific data descriptions of colon length in Fig. 5c, d and Fig. 8c, d within the article. This will provide a more detailed demonstration of the therapeutic effects associated with colon length.

**Response:** Thank you for your kind suggestion. We have added the description of colon length in Fig.5c, d and Fig.8c, d and used the data to express the therapeutic effect of the PPy/PFD@Inulin gel, which makes the content of the article more substantial.

**Reviewer #3 (Remarks to the Author):**

Cao and colleagues developed a ternary inulin hydrogel with long-term intestinal retention and investigated whether this hydrogel may have therapeutic potential for the treatment of IBD and its fibrotic complications. The manuscript presents an innovative approach to treating IBD and its associated complications, particularly intestinal fibrosis, by introducing a probiotic inulin hydrogel loaded with polypyrrole (PPy) nanozymes and pirfenidone (PFD). The manuscript outlines the synthesis,

characterization, in vitro and in vivo biocompatibility, therapeutic efficacy, and underlying mechanisms of action of the PPy/PFD@inulin gel.

Overall, the manuscript is well written and uses clear and concise language. The described approach of combining the therapeutic effects of PPy nanozymes, PFD, and the probiotic benefits of inulin is an innovative approach and may allow targeting various pathogenic features of IBD, including oxidative stress, inflammatory cytokines, and intestinal dysbiosis. The manuscript provides compelling evidence for the therapeutic efficacy of the hydrogel in both prophylactic and delayed treatment models of DSS-induced colitis. However, the characterization of the mechanisms of action remains rather superficial and some conclusions are not fully supported by the experimental data. Therefore, I strongly recommend addressing several issues that could make this study more robust and impactful.

**Response:** We greatly appreciate your kind and valuable comments.

Major:

**Q1.** While the study outlines the beneficial effects on RONS scavenging, inflammation reduction and restoration of homeostatic microbiota, a deeper mechanistic insight into how the hydrogel components interact with the biological pathways would enhance the understanding of its therapeutic potential. The authors focus on very few inflammatory cytokines. It would be more helpful to perform a broader characterization, e.g. by bulk mRNA sequencing, and to study changes in immune cell infiltration.

**Response:** Thank you for your kind suggestion.

To further elucidate the protective efficacy of PPy/PFD@Inulin gel on DSS-induced colitis in murine models, RNA sequencing (RNA-seq) was employed for a comprehensive quantitative assessment of the gene expression landscape in colonic tissues. Initially, the heatmap depicted in Fig. 9a strikingly contrasts the gene expression profiles between the DSS-treated and PPy/PFD@Inulin gel-treated groups. Principal Component Analysis (PCA) provided further stratification, distinctly segregating the DSS group from both the control and PPy/PFD@Inulin gel groups (Fig. 9b). Volcano plot and Venn analysis identified 511 differentially expressed genes (DEGs) (228 upregulated, 283 downregulated) between the PPy/PFD@Inulin gel and DSS-treated groups (Fig. 9c and Supplementary Fig. 26). Additionally, 981 DEGs (451 upregulated, 530 downregulated) were delineated between the control and DSS groups, while 211 DEGs (57 upregulated, 154 downregulated) were found between the control

and PPy/PFD@Inulin gel groups (Supplementary Figs. 25 and 26).

To ascertain their functional relevance, Gene Ontology (GO) analysis was conducted on DEGs between the PPy/PFD@Inulin gel and DSS groups, revealing significant involvement in biological signaling pathways such as neutrophil migration, bacteria response, and inflammatory response (Fig. 9d). KEGG pathway analysis further indicated that these DEGs are implicated in inflammatory pathways including the IL-17 signaling pathway, cytokine-cytokine receptor interaction, TNF and JAK/STAT signaling pathways (Fig. 9e). The JAK-STAT pathway plays a crucial role in modulating both adaptive and innate immune responses, particularly in IBD. The ImmuCC algorithm was employed to profile the infiltration of 23 types of immune cell types within the colonic tissues of these three groups. The bar graph in Fig. 9f and heatmap in Fig. 9g illustrated the differential abundance of immune cell infiltrates among the groups. Compared to the healthy control and PPy/PFD@Inulin gel groups, the DSS group showed elevated levels of activated dendritic cells, M0 macrophages, and M1 macrophages in the colonic mucosa.

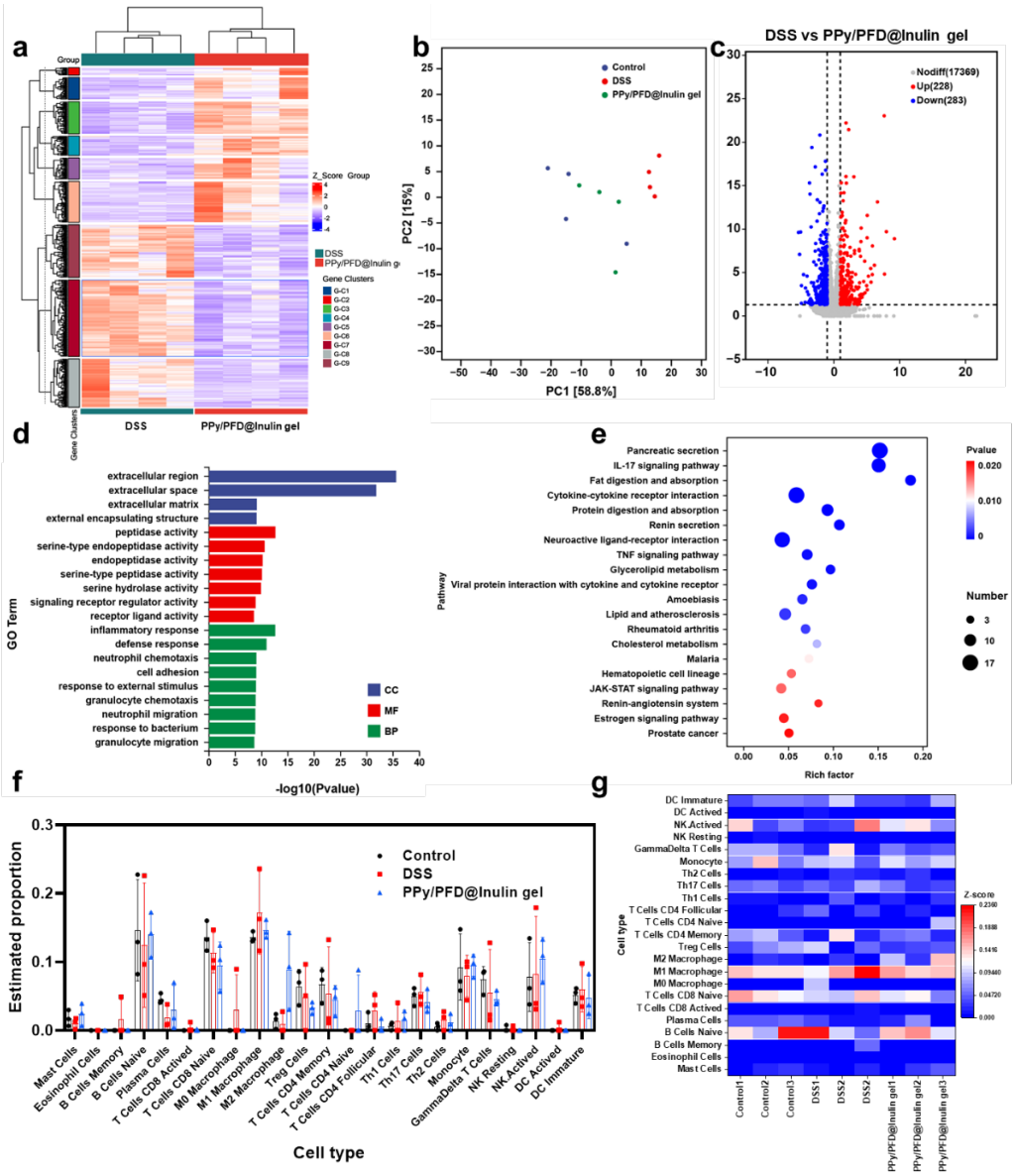



**Fig. 9 | RNA sequencing analysis of colon tissues treated with PPy/PFD@Inulin gel. a** Heat map of the total differential metabolite clustering in the DSS group and PPy/PFD@Inulin gel group. **b** PCA of the colon tissue gene expression values in the Control DSS, and PPy/PFD@Inulin gel. **c** Volcano plot of the differential genes in the colon tissues of the Control DSS, and PPy/PFD@Inulin gel. **d** GO enrichment analysis of DEGs. BP represents the "biological process"; CC represents the "cellular component"; MF represents the "molecular function". **e** KEGG pathway enrichment analysis of DEGs. **f** The histogram shows the difference of infiltrated immune cells among control group, DSS group and PPy/PFD@Inulin gel group. **g** Heat map of the difference of infiltrating immune cells among control group, DSS group and PPy/PFD@Inulin gel group. (n=4 biologically independent samples for (**a-e**), n=3 biologically independent samples for (**f** and **g**)).

**Q2.** Figure 7 suggests that intestinal barrier function is impaired, but the characterization is very superficial. Immunofluorescence staining against ZO-1 and Occluding can be very subjective - it would be good to provide Western blot of barrier proteins. I would also suggest adding an in vitro FITC dextran assay of intestinal barrier function. It would also be interesting to see if the colonic mucus layer is affected. One hypothesis could be that the hydrogel limits bacterial translocation. This could be assessed by mucus staining in combination with FISH of luminal bacteria.

**Response:** We are very grateful to your kind suggestions.

Western blot images of ZO-1 and occluding are provided in Fig. 8c and Supplementary Fig. 21. As shown in Fig. 8b and Supplementary Fig. 21, the Western blotting analysis confirmed that mice with colitis showed impaired epithelial tight junctions characterized by decreased expression of ZO-1 and occluding proteins. Treatment with PPy/PFD@Inulin gel significantly increased the expression of these proteins.

Damage to intestinal mucosal barrier typically results in elevated serum FITC-dextran levels post-gavage. By orally administering FITC-dextran to mice across different experimental groups and subsequently quantifying serum FITC-dextran using fluorescence imaging and a microplate reader, the DSS group exhibited significantly higher fluorescence intensity in the colonic region compared to the control group (Fig. 8a, b). Intervention with PPy/PFD@Inulin gel markedly decreased colonic permeability to FITC-dextran. Microplate reader measurements corroborated these findings, indicating that serum FITC-dextran levels in PPy/PFD@Inulin gel-treated mice approached those of the control group (Supplementary Fig. 20).

Fluorescent in situ hybridization (FISH) imaging demonstrated high Muc2 protein levels, a major component of intestinal mucus barrier, in the control group, which effectively shielded epithelial cells from bacterial invasion (Supplementary Fig. 23). In contrast, the DSS-treated group exhibited significantly increased bacterial invasion.

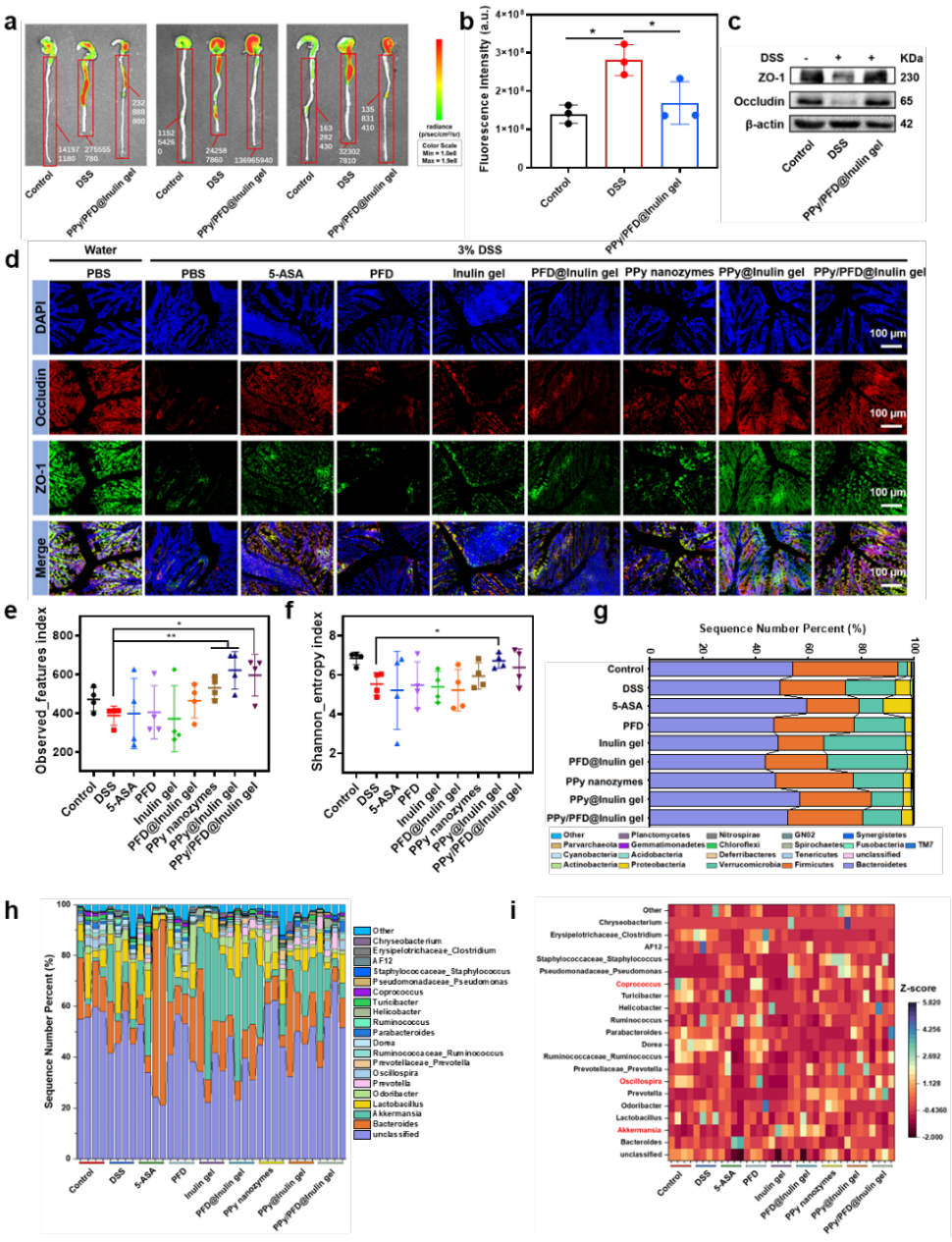
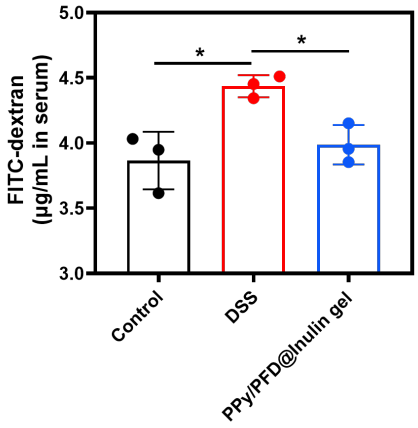
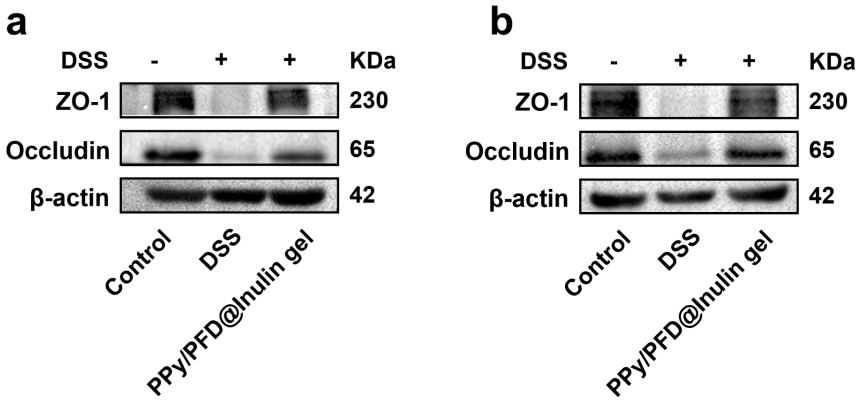



**Fig. 8 | The repair effect of PPy/PFD@Inulin gel on colon epithelium and intestinal flora regulation. a, b** Fluorescence images and fluorescence quantification detected by imaging of small animals 2 h after oral administration of 4 kDa FITC-dextran. **c** Western blotting analysis of occludin and ZO-1 expression of protein extracts from colon tissue. **d** Immunofluorescence staining ZO-1 (green) and occludin (red) proteins, and the nucleus was stained with DAPI (blue). **e, f** Estimation of the gut microbial abundance by the number of OTUs (**e**) and indices of community α-diversity determined by Shannon (**f**). **g** Composition analysis of gut microbiota at the phylum level in different experimental groups. **h, i** Relative abundance of gut microbes at genus level in mice (**h**) and heatmap showing normalized Z-score values of microbial

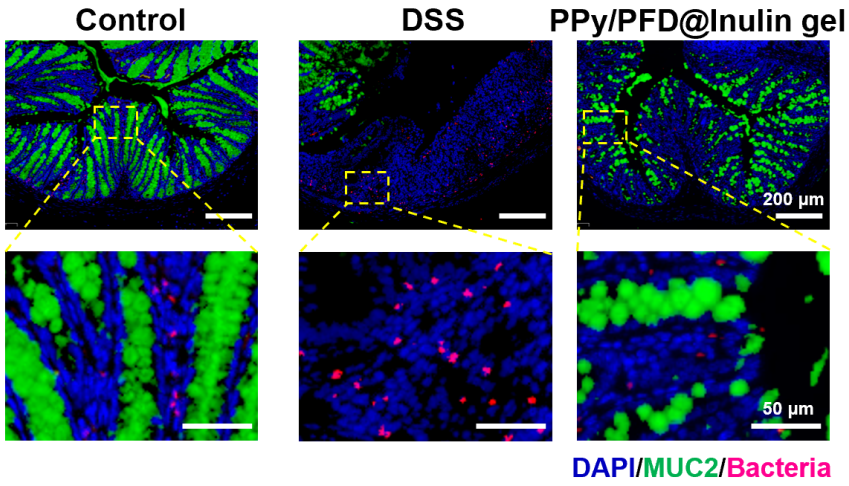
abundance at genus level (**i**). Data are presented as mean ± SD (n=3 biologically independent samples for (d), n=4 biologically independent samples for (e and f)). P > 0.05 (n.s.), *p < 0.05, **p < 0.01, ***p < 0.001, statistical analysis was evaluated with two-tailed Student's t tests.




Supplementary Fig. 20. Concentration of FITC-dextran in serum of mice. Data are presented as mean ± SD (n = 3 biologically independent samples).




Supplementary Fig. 21. Western blotting analysis of Occludin and ZO-1 expression of protein extracts from colon tissue.




Supplementary Fig. 23. FISH (EUB338) combined with immunofluorescence imaging (anti-Muc2 antibody) analysis of the colon sections.

**Q3.** It would be very helpful to also study the effect of the hydrogel on intestinal physiology without additional DSS treatment. How does the hydrogel affect feeding behavior? Does the treatment affect weight gain? How is intestinal transit time affected? Does the intestinal microbiota change? How is the intestinal barrier function affected by the hydrogel?

**Response:** We are very grateful to your kind suggestions.

We added some experiments, as shown in Fig. 5 and Supplementary Fig. 9, to explain the effects of hydrogels on intestinal physiology.

Firstly, over the 9-day period, the experimental group of mice exhibited reduced food intake compared to the normal group, attributed to the hydrogel occupying gastric volume and thus decreasing food consumption (Fig. 5b).

Secondly, oral administration of PPy/PFD@Inulin gel to normal mice did not affect weight gain, as shown in Figure 5c.

Thirdly, because the hydrogel is more viscous, the stomach emptying time is longer. Previous studies have indicated that gel-state polysaccharide fibers could extend gastric emptying (owing to thickening), adhere to the intestinal mucosa and prolong the intestinal residence time. A lap-shear adhesion test was used to evaluate the bio-adhesion of PPy/PFD@Inulin gel (Supplementary Fig. 9). Interestingly, PPy/PFD@Inulin gel adhered to pig skin tissue and endured much larger deformation than PPy nanozymes, which can be attributed to its preparation in gel form.

Then, after oral administration of PPy/PFD@Inulin gel, there are some changes in

intestinal flora, as shown in Fig. 5d-j. The potential of PPy/PFD@Inulin gel to modulate the abundance of gut microbiota in normal mice was studied using 16S rRNA gene sequencing Through alpha diversity metrics including observed species, Simpson, and Shannon indices, oral administration of PPy/PFD@Inulin gel increased the abundance and diversity of gut microbiota in mice (Fig. 5d-f). The Venn diagram in Fig. 5g illustrates the enhanced richness of microbial communities in response to PPy/PFD@Inulin gel. Principal Coordinate Analysis (PCoA) based on Bray-Curtis distances also showed partial overlap yet slight differentiation between OTUs from mice treated with PPy/PFD@Inulin gel and those from the healthy control group (Fig. 5h). Additionally, at the phylum level, *Verrucomicrobia* significantly increased in mice treated with PPy/PFD@Inulin gel, consistent with the heatmap at the genus level, indicating a notable rise in *Akkermansia* abundance (Fig. 5i, j). Consistent with the previous reports that oral administration of inulin substantially increased the abundance of *Akkermansia* (known for its association with the maintenance of intestinal barrier function. To sum up, oral PPy/PFD@Inulin gel can regulate the abundance of intestinal flora and increase the content of Akkermansia beneficial bacteria in mice.

Finally, PPy/PFD@Inulin gel can eliminate excessive RONS, alleviating damage to the intestinal barrier caused by RONS. The gel may modulate the composition of the microbiota and their metabolic products, promoting mucosal immune homeostasis and intestinal epithelial barrier repair, and interacting with the mucosal immune system. For instance, the gel increases the abundance of *Akkermansia*, which can protect intestinal barrier functions and reduce levels of inflammatory cytokines through specific metabolites and membrane proteins interacting with host cells or receptors, thereby maintaining intestinal health.

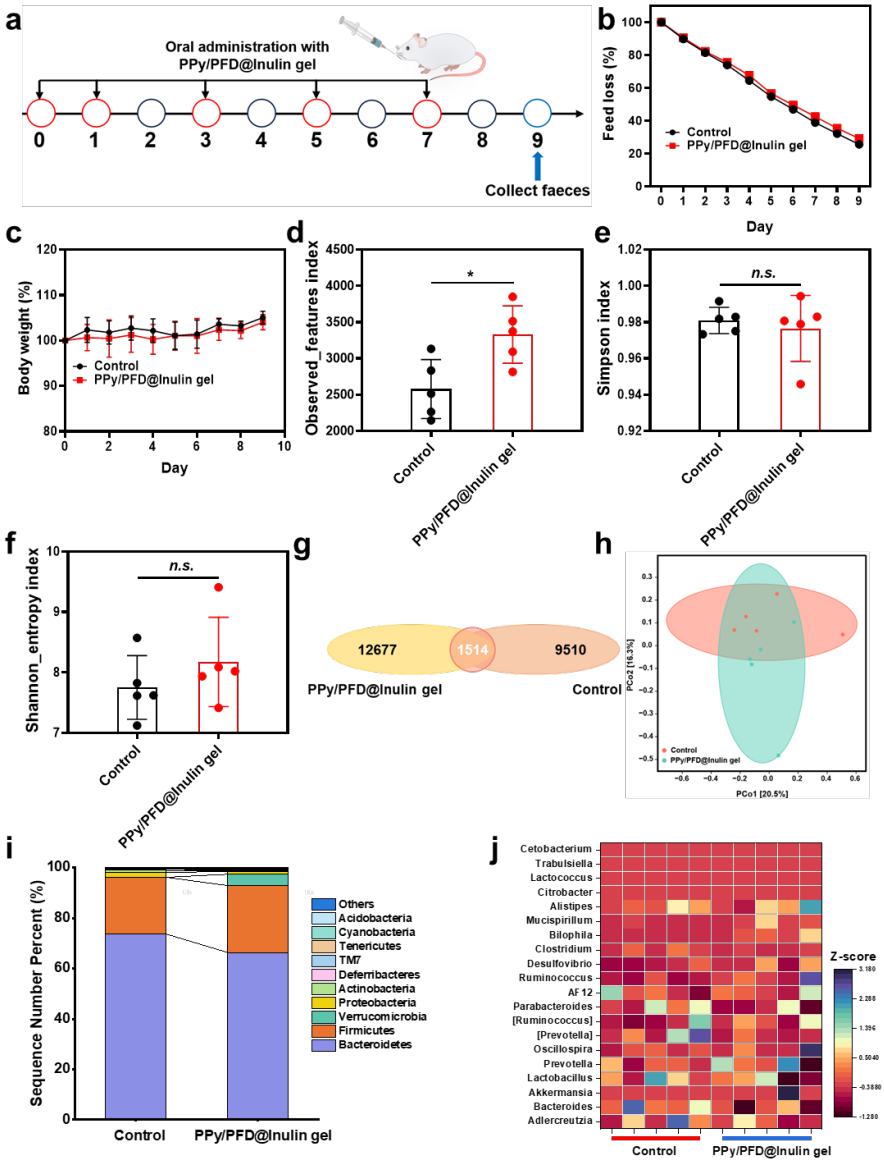
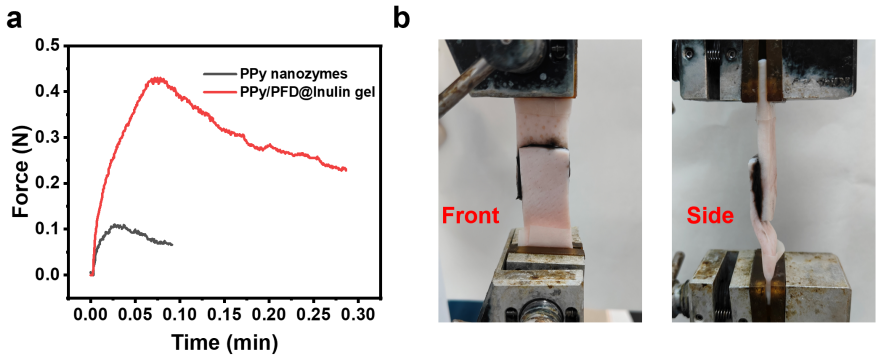


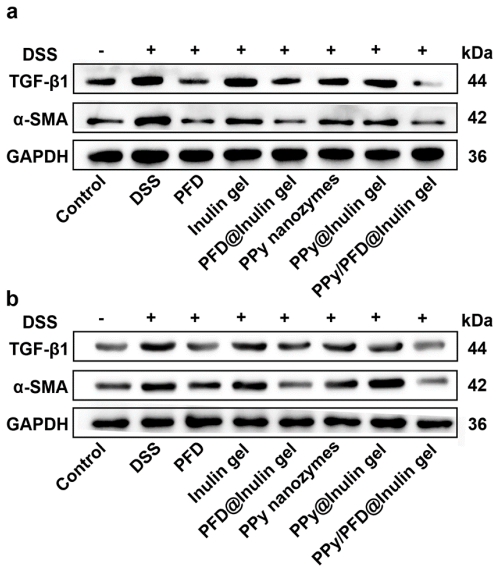

**Fig. 5 | Effect of hydrogel on intestinal physiology. a** Roadmap for experimental design. **b** The amount of feed loss in 9 days. **c** In 9 days, the body weight of mice was recorded. **d-f** Estimation of the gut microbial abundance and indices of community α-diversity determined by the number of observed operational taxonomic units (OTUs, **d**), Simpson (**e**), and Shannon (**f**). **g** Venn diagram illustrates the numbers of ASV/OTU in the control and PPy/PFD@Inulin gel. **h** Beta diversity is illustrated of the PCoA, based on Bray-Curtis distances. **i** Composition analysis of gut microbiota at the phylum level in different groups. **j** Heat map of intestinal microorganisms in mice at genus level. Data are presented as mean ± SD (n = 5 biologically independent samples for (**c-f, i,** and **j**)). P > 0.05 (n.s.), *p < 0.05, **p < 0.01, ***p < 0.001, statistical analysis was performed using one-way ANOVA.




**Supplementary Fig. 9. a** The adhesion forces of PPy/PFD@Inulin gel and PPy nanozymes. **b** The photo of the lap-shear test to measure the adhesive energy.

**Q4.** Figure 9d: Showing 1 replicate per condition is not very helpful. It is better to show fewer conditions with more replicates. Please also include the original Western blots for quantification in Figure 9e in the Supplement.

**Response:** Thank you very much for your kind suggestion. We have added the original Western blot for quantitative analysis to Supplementary Fig. 29.




**Supplementary Fig. 29.** To verify the protein imprinting image represented by mouse fibrosis pathway.

Minor:

**Q1.** Discussion of the scalability of the hydrogel synthesis process and any potential manufacturing and regulatory challenges would be beneficial to understanding the path to clinical application.

**Response:** Thank you very much for your kind and valuable suggestions. We have incorporated additional discussion in the manuscript, outlining future prospects of the hydrogel, thereby enriching the content of the revised manuscript.

**Q2.** The manuscript could benefit from professional editing by a native speaker.

**Response:** Thanks for your kind suggestion. We have asked a native speaker to polish the language in the revised manuscript.

# REVIEWER COMMENTS

**Reviewer #1 (Remarks to the Author):**

The authors have addressed my concerns.

**Reviewer #2 (Remarks to the Author):**

The authors have well addressed my concerns. I recommend the acceptance of the manuscript.

**Reviewer #3 (Remarks to the Author):**

The authors have addressed most of my comments and present a signficantly improved manuscript. However, as already mentioned in my previous review, I would kindly ask the authors to improve the presented western blots. For quantification, I highly recommend running all biological replicates on the same gel instead of quantifying several independent gels with each containing only one replicate. This can lead to the wrong conclusions.

In case there are too many conditions, I would rather split the conditions to different gels each containing the meaningful groups and control groups.

The same applies to the new western blots of ZO1 and occluding, which only contain one replicate per condition. Please improve these western blots as well.

Please also upload the raw pictures of the western blot membranes that were used for quantification to the supporting information.

**Responses to referees**

**Reviewer #1 (Remarks to the Author):**

The authors have addressed my concerns.

**Response:** We are proud to have addressed the reviewer's concerns. We thank the reviewer for the positive and constructive comments regarding our paper.

**Reviewer #2 (Remarks to the Author):**

The authors have well addressed my concerns. I recommend the acceptance of the manuscript.

**Response:** We sincerely thank you for your feedback which would help to improve the quality of our manuscript.

**Reviewer #3 (Remarks to the Author):**

The authors have addressed most of my comments and present a significantly improved manuscript.

However, as already mentioned in my previous review, I would kindly ask the authors to improve the presented western blots. For quantification, I highly recommend running all biological replicates on the same gel instead of quantifying several independent gels with each containing only one replicate. This can lead to the wrong conclusions.

In case there are too many conditions, I would rather split the conditions to different gels each containing the meaningful groups and control groups.

The same applies to the new western blots of ZO1 and occluding, which only contain one replicate per condition. Please improve these western blots as well.

Please also upload the raw pictures of the western blot membranes that were used for quantification to the supporting information.

**Response:** We would like to thank you for your professional review work, constructive comments, and valuable suggestions our manuscript. We have re-run all biological repeats on the same gel. Western blot experiment of intestinal barrier proteins ZO-1 and Occludin in mice and western blot experiment of TGF-β1 and α-SMA in fibrosis

mechanism were updated. Finally, all the data have been arranged in the supporting information.

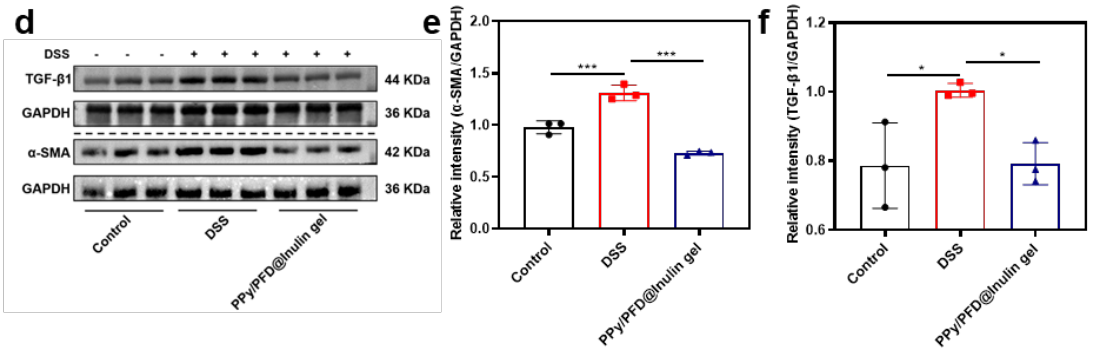
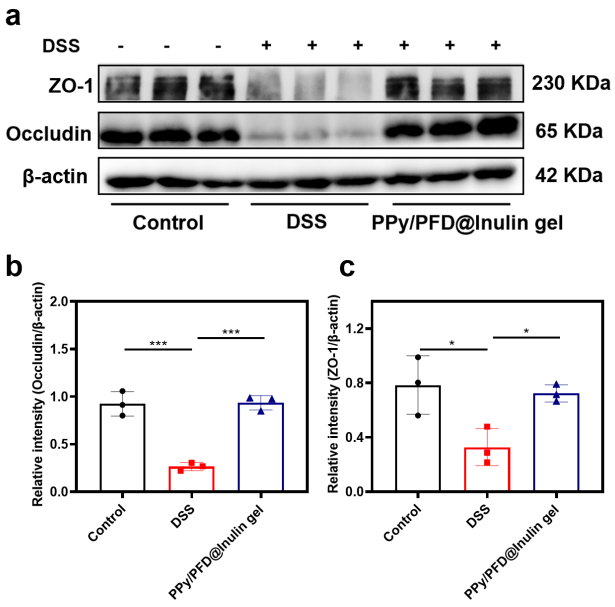



**Fig. 11 | The mechanism of PPy/PFD@Inulin gel for inhibiting intestinal fibrosis.** **d** Representative Western blot images. **e, f** Quantitative Western blot analyses were performed.




**Supplementary Fig. 20. a** Western blotting analysis of Occludin and ZO-1 expression of protein extracts from colon tissue. **b, c** Quantitative Western blot analyses were performed.

# REVIEWERS' COMMENTS

**Reviewer #3 (Remarks to the Author):**

The authors have improved the western blots and I am happy to now recommend the manuscript for publication