Supplementary Information for

Fouling Resistant Zwitterionic Polymers for Complete Prevention of Postoperative Adhesion

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This PDF file includes:

Supplementary text (Materials and Methods)

Supplementary figures (Figures S1 to S7)

SI references

Supplementary Text: Materials and Methods

Materials. The carboxybetaine acrylamide (CBAA) monomer was prepared following a previously published method [1]. Ammonium persulfate (APS), fluorescein isothiocyanate (FITC, isomer I suitable for protein labeling, F7250), 2-aminoethyl methacrylate hydrochloride, rat plasma fibronectin (F0635), phosphate buffered saline (PBS, pH 7.4), and paraformaldehyde were purchased from Sigma-Aldrich. Cyanine7 (Cy7)-NHS ester was purchased from Lumiprobe Corporation. Heat-inactivated fetal bovine serum (FBS) was obtained from Hyclone Laboratories. Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) and 0.25% trypsin-EDTA were purchased from Thermo Fisher Scientific. Commercially available Interceed® Absorbable Adhesion Barrier (Johnson & Johnson) was purchased from Medex Supply. The SD rat dermal fibroblasts carrying red fluorescence (TurboFP602 red fluorescent protein) were purchased from Innoprot (Spain).

Preparation and characterization of zwitterionic PCBAA solution. To synthesize zwitterionic PCBAA, an appropriate amount of CBAA monomer and APS initiator (0.5% relative to the total monomer mass) were dissolved in water at a concentration of 10 wt% and then the polymerization was carried out at 37 °C for 24 h. The resulting solution product was dialyzed against sterile deionized water for 72 h using a dialysis bag (MWCO 8000 Da) and then freeze-dried to obtain PCBAA powder. To label PCBAA, 2-aminoethyl methacrylate hydrochloride (5% in molar) was added to form a PCBAA polymer with -NH_2 group. 600 mg of PCBAA with -NH_2 group was dissolved in 10 mL of HEPES buffer (pH 8.2), followed by addition of 10 mg of Cy7-NHS in 2 mL of DMSO and stirring in the dark at room temperature for 48 hours. The resulting solution was dialyzed against sterile deionized water for 72 h (MWCO 3500 Da) to remove unreacted Cy7-NHS and then freeze-dried to obtain PCBAA-cy7.

The molecular weight (Mw) of the prepared PCBAA was characterized using gel permeation chromatography (GPC) (Waters 1525 pump and Waters 2414 differential refractometer) with phosphate-buffered saline (PBS) as eluent at a flow rate of 1.0 mL/min. The monodisperse poly(ethylene glycol) was used as standard. PCBAA solution was reconstituted in sterile PBS (pH 7.4) at the different concentrations (10%, 20%, and 30% by weight). Shear rheology of various polymer solutions was studied using a TA Instruments ARG2 rheometer (TA Instruments Inc., New Castle, DL) equipped with a 20 mm diameter parallel plate at 25 °C. Dissolution rate of PCBAA solution was monitored by measuring the weight loss over time. 1 mL of PCBAA solution was immersed in 10 mL of PBS and incubated in a shaking bath at 37 °C with 125 rpm. At predetermined time points, the supernatant was removed and the remaining sample was lyophilized and weighed.

In vivo protein adsorption study. The fluorescein isothiocyanate-labeled rat plasma fibronectin (FITC-Fn) was prepared as previously described with some modifications [2]. Briefly, 100 uL of FITC solution (0.18 mg/mL) was added to 1 mL Fibronection solution (1 mg/mL) in the carbonate-bicarbonate basic buffer at pH 9.0 and then incubated for 1 h at room temperature in the dark. The unbound FITC was removed by dialyzing in sterile PBS (pH 7.4) for 2 h and then in sterile

deionized water for 4 h (MWCO 3500 Da). After freeze-drying, the lyophilized FITC-Fn powder was reconstituted in sterile PBS at the concentration of 1 mg/mL and stored in the dark before use.

Male Sprague-Dawley (SD) rats (250 ± 20 g) were purchased from Charles River Laboratory. The animal experiments were approved by the Institutional Animal Care and Use Committee at Wayne State University and performed in compliance with the relevant laws and institutional guidelines. All the animals were housed in a climate-controlled room under 12 h/12 h light/dark cycle with food and water ad libitum. Rats were given buprenorphine SR (0.4 mg/kg, SQ) prior to surgery. Surgery anesthesia was conducted by isoflurane inhalation (inducted in a chamber and maintained via a nosecone). Appropriate anesthesia levels were monitored by observing respiration rate, eye response, and response to front toe pinch. After the abdominal skin was shaved and prepped with three alternating scrubs of betadine and 70% alcohol, a single 4-5 cm long incision was opened along the linea alba on the abdominal wall with surgical scissors. A 1.5 × 2 cm defect (including the parietal peritoneum and ~1 mm of muscle) on the right lateral abdominal wall was created using a scalpel. For the PCBAA group, 500 uL of PCBAA solution was used to cover the sidewall defect. Then 250 uL of prepared FITC-Fn solution (1 mg/mL) was applied on the PCBAA protected defect. For the control group, the FITC-Fn solution was directly applied onto the sidewall defect. Finally, the peritoneum was closed with 3-0 PDS suture (Ethicon), and the skin was closed with 4-0 Nylon suture (Ethicon), respectively. At 2 h and 24 h after the surgery, the rats were euthanized by CO_2 and the FITC-Fn treated abdominal walls were harvested and lightly washed three times with PBS and then photographed with the Carestream In Vivo Xtreme Imaging System (Bruker, 480 nm excitation, 535 nm emission).

In vivo fibroblasts adhesion study. The rat fibroblasts carrying red fluorescence were cultured in DMEM/F-12 medium containing 10% inactivated FBS at 37 °C under the 5% CO_2 atmosphere. Before the in vivo study, fibroblasts were seeded on the coverslip for 48 h in vitro culture and observed by a EVOS FL fluorescence microscope (AMG, 530 nm excitation, 593 nm emission) and the Carestream In Vivo Xtreme Imaging System (Bruker, 550 nm excitation, 600 nm emission) to confirm the red fluorescence.

For the in vivo study, similar to the typical surgery described above, rats were prepped with abdomen opened. After the cecum was identified, the serosal surface was gently abraded with dry sterile surgical gauze (about 50 strokes) until visible hemorrhaging was developed. A corresponding 1.5 × 2 cm peritoneal defect on the right lateral abdominal wall was created using a scalpel. Then the injured cecum was sutured to close the injured abdominal wall by puncturing the surrounding mesentery rather than cecum with 3-0 PDS suture. For the PCBAA group, 1 mL of PCBAA was applied on the injured sites (the damaged cecum surface and the abdominal wall defect) and then 250 uL of fibroblasts carrying red fluorescence (2×10^5 cell/mL) were seeded on the PCBAA protected defects. Likewise, fibroblasts were directly seeded onto the untreated defects as the control. The peritoneum and the skin were closed as described above. At 2 h, 1 d and 4 d after the surgery, the rats were euthanized and the injured abdominal walls were

harvested for fluorescence photography with the Carestream In Vivo Xtreme Imaging System (Bruker, 550 nm excitation, 600 nm emission).

In vivo anti-adhesion evaluation in a rat abdominal wall and cecum defect model. The sidewall defect-cecum abrasion model was established as described in the fibroblasts adhesion study, except that fibroblasts were not applied. For the PCBAA group, 1 mL of PCBAA solution was injected onto the injured abdominal wall and damaged cecum. As a positive control, the defects were also covered by a commercialized 2 × 2 cm Interceed® film. For negative control, no anti-adhesion material was employed on the injured area. 1 week and 2 weeks after the surgery, 6 rats for each group were euthanized by CO_2 asphyxiation. The peritoneum was opened and the extent of adhesion was evaluated and scored based on the standard adhesion scoring system as follows [3]: score 0, no adhesion; score 1, mild, easily separable intestinal adhesion; score 2, moderate intestinal adhesion, separable by blunt dissection; and score 3, severe intestinal adhesion requiring sharp dissection to separate. Tissues collected from different groups were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with Hematoxylin and Eosin (H&E) and Masson trichrome, and then imaged using an EVOS XL Core microscope (Thermo Fisher Scientific).

In vivo anti-adhesion evaluation in a rat repeated-injury adhesion model. The repeated-injury adhesion model was established by creating a first abdominal wall and cecum injury with the peritoneum closed without any anti-adhesion material treatment as untreated control described in the sidewall defect-cecum abrasion model. One week later, a second laparotomy was performed to create a repeated injury. After reopening the incision, the adhesion site resulted from the first surgery was separated by an appropriate dissection as needed, and then the separated abdominal wall and cecum were abraded monodirectionally with a sterilized brush until bleeding surfaces were produced. For treatment groups, the repeated-injured sites were covered either by 1 mL of PCBAA solution or 2 × 2 cm Interceed® film before the final closure. For the untreated control group, no anti-adhesion material was employed on the repeated-injured sites. At 7 and 14 days after the second surgery, 6 rats for each group were euthanized by CO_2 asphyxiation. The peritoneum was opened, and the extent of adhesion was evaluated and scored. The tissues were collected for histological examination.

In vivo anti-adhesion evaluation in a rat 70 % hepatectomy-induced adhesion model. Similar to the typical surgery described above, rats were prepped with abdomen opened. Hepatectomy was performed by ligating the pedicle of the median lobe using 3-0 silk sutures, then cutting the parenchyma of median lobe using surgical scissors near the base of the lobe. Similarly, the left lateral lobe was ligated and resected. After lavaging the peritoneal cavity with 15 mL of sterile saline, the cut surfaces of the excisional liver parenchyma were completely covered with 2 mL of injected PCBAA. For the film treated group, the 2 × 2 cm Interceed® film was applied to cover the cut surfaces and the surface of remnant liver lobes. For the untreated control group, the peritoneal cavity was closed without any anti-adhesion materials applied. After surgery, 20 % glucose water was provided instead of regular water for 3 days. Day 7, 14, and 30 after the

hepatectomy, 6 rats in each group were euthanized and the abdomen was opened to evaluate and score the adhesions. The presented adhesions at the cut surface, diaphragm, hepatic hilum, and remnant liver surface were scored respectively since the locations of adhesion formation were uncertain in this model.

Supplementary Figures

Figure S1. Photos of injectable cream-like zwitterionic PCBAA solution (20 wt%).

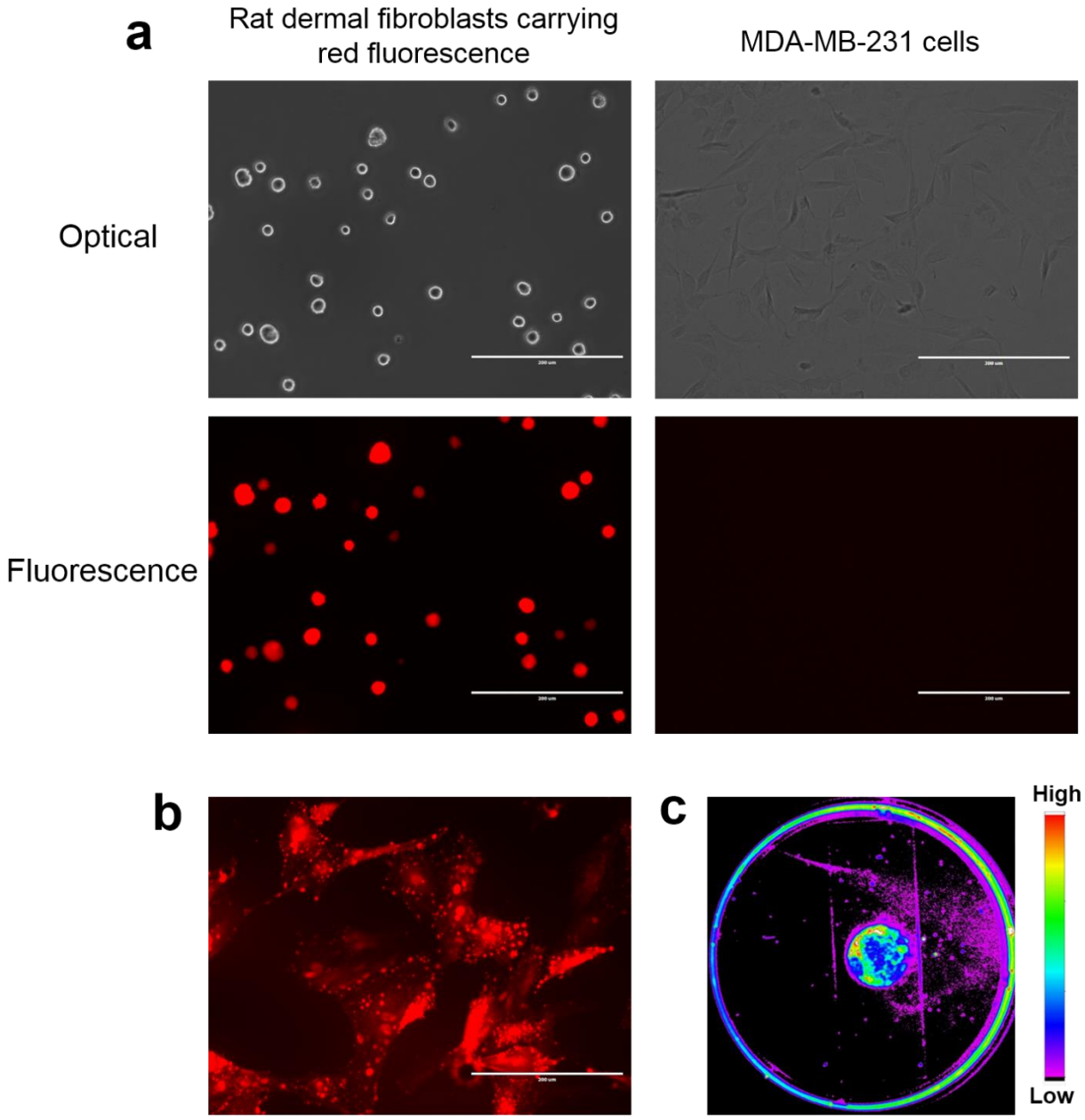

Figure S2. Representative fluorescence images of fibroblasts carrying red fluorescence. a, The optical and fluorescence images of thawed fibroblasts carrying red fluorescence observed by a EVOS FL fluorescence microscope (530 nm excitation, 593 nm emission). The MDA-MB-231 breast cancer cells were used as a control. b, c, The fluorescence images of fibroblasts carrying red fluorescence cultured on coverslip for 4 days observed by (b) EVOS FL fluorescence microscope (530 nm excitation, 593 nm emission) and (c) the Carestream In Vivo Xtreme Imaging System (Bruker, 550 nm excitation, 600 nm emission). Scale bar, 400 µm.

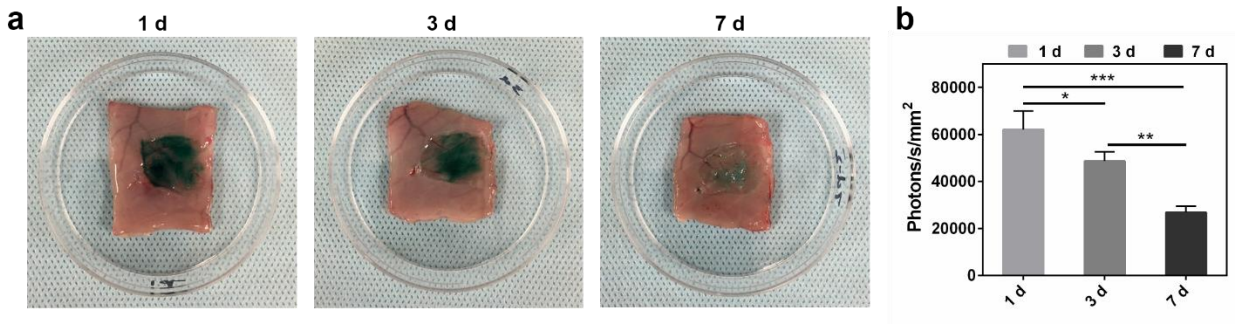

Figure S3. a, Visual examination of retained PCBAA-cy7 polymer at the application site at 1 d, 3 d and 7 d after treatment in the rat sidewall defect-cecum abrasion adhesion model. b, Quantification of Cy7 fluorescent intensity in region of abdominal wall defects. The results are presented as mean ± SD (n = 3). A one-way ANOVA with Tukey multi-comparison was used for statistical analysis. *P < 0.05; **P < 0.01; ***P < 0.001.

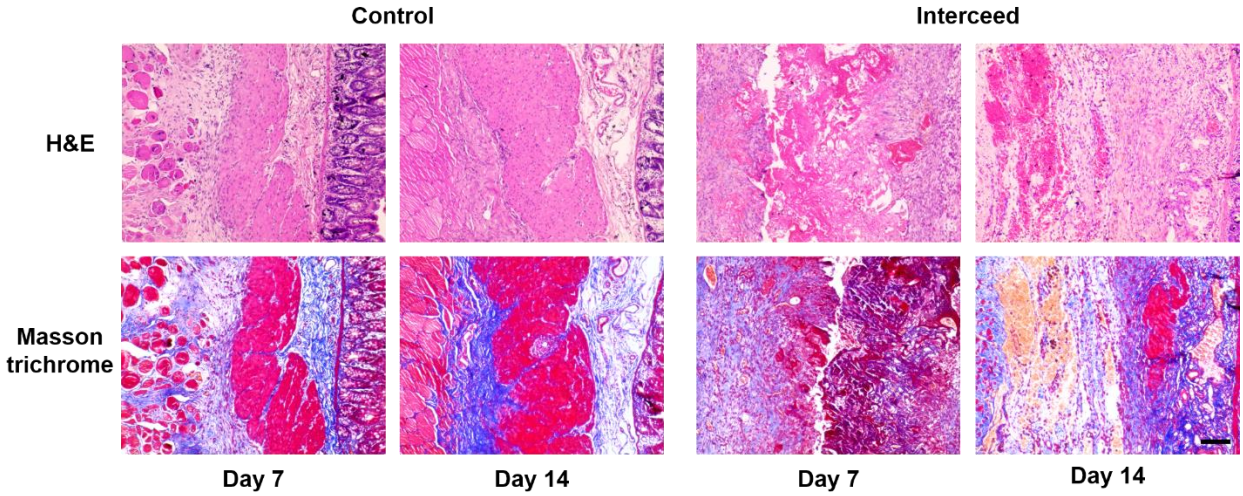

Figure S4. Histological images (H&E and Masson trichrome staining) of adhesion tissues from Interceed® film-treated and untreated control group on day 7 and 14 after surgery in a rat sidewall defect-cecum abrasion model. Scale bar, 100 µm.

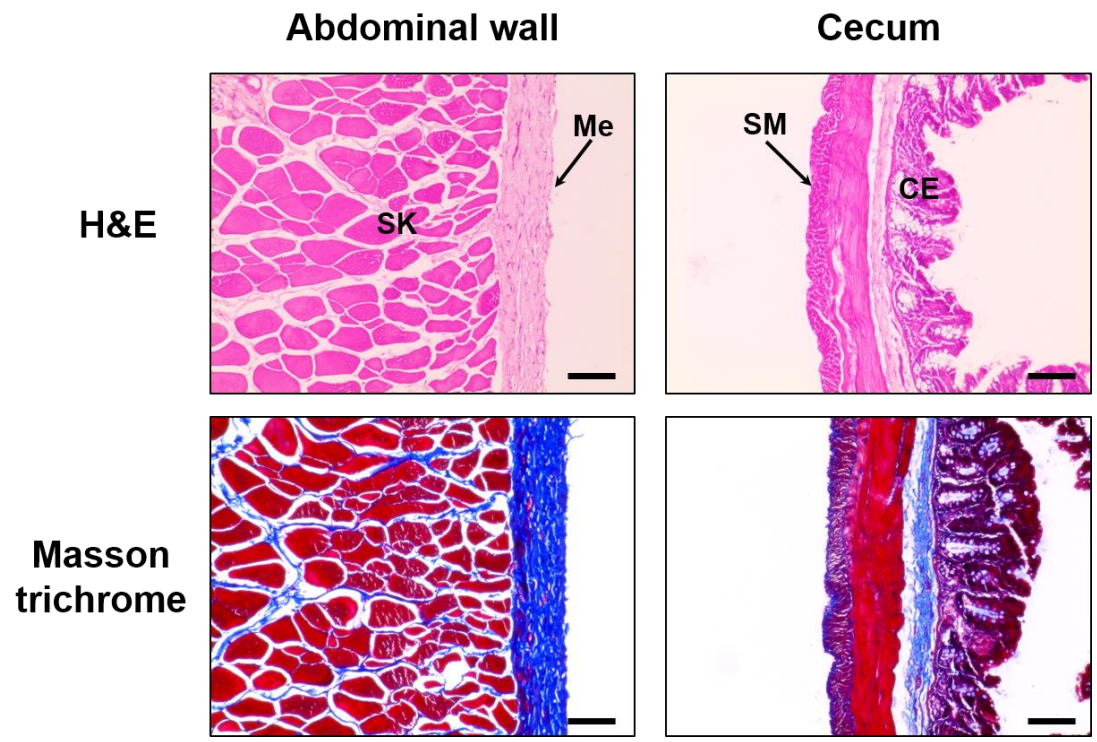

Figure S5. Histology images of the normal abdominal wall and cecum. Me: mesothelial layer; SK: skeletal muscle; CE: cecal mucosa; SM: visceral smooth muscle. Scale bar, 100 µm.

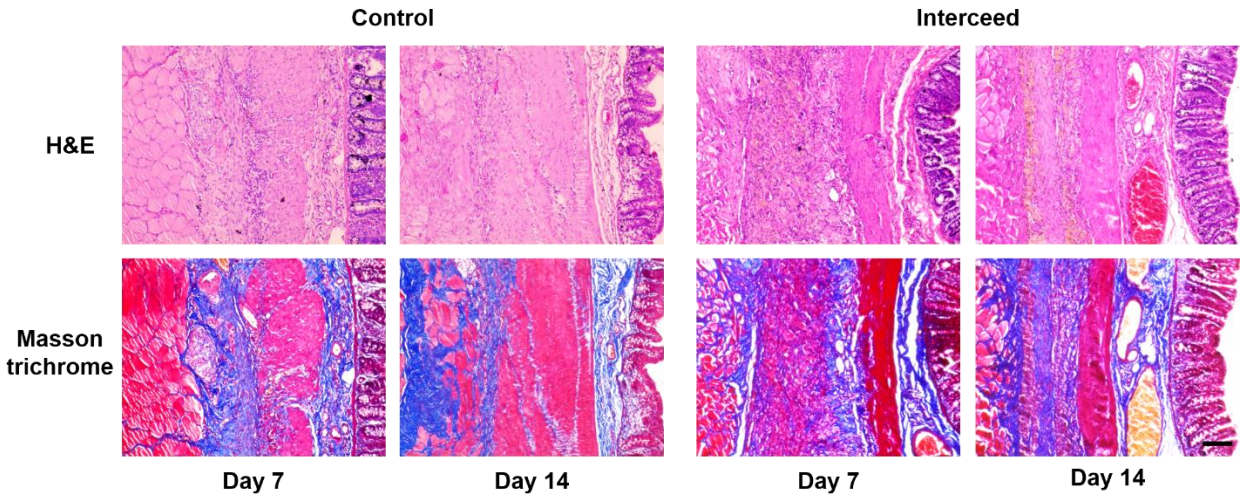

Figure S6. Histological images (H&E and Masson trichrome staining) of adhesion tissues from Interceed® film-treated and untreated control groups on day 7 and 14 after the second surgery in a rat repeated-injury model. Scale bar, 100 µm.

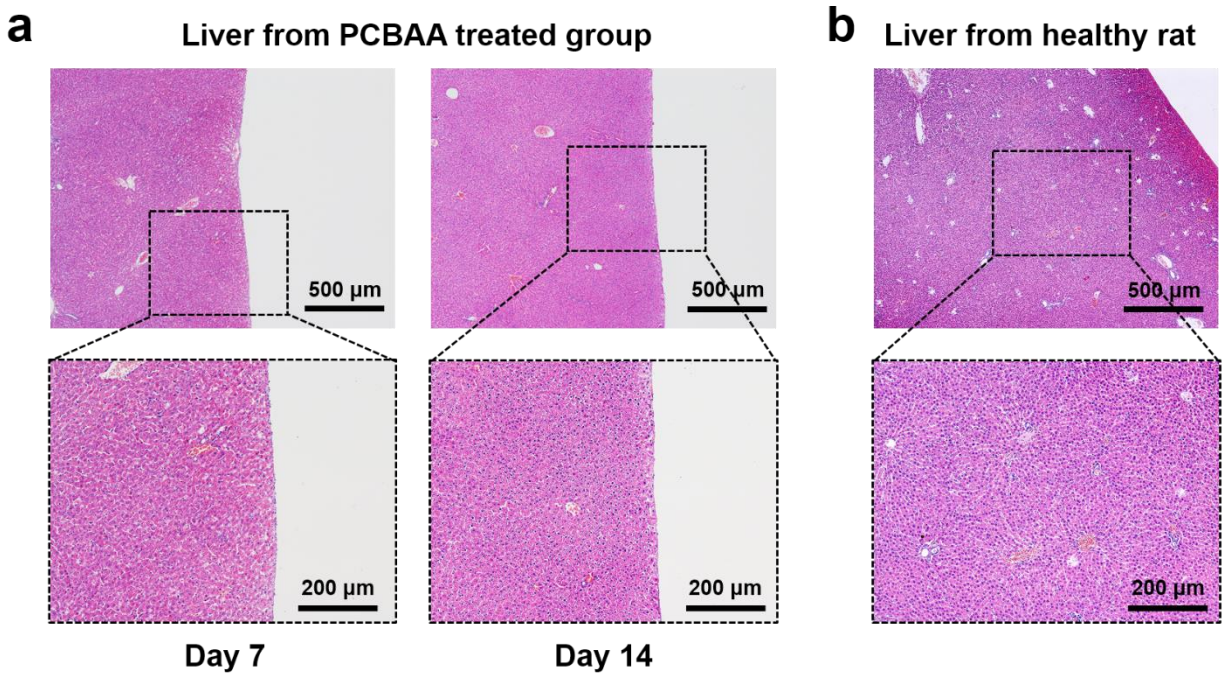

Figure S7. Histological images (H&E staining) of liver from PCBAA treated group on day 7 and 14 in a rat 70 % hepatectomy-induced adhesion model (a) and healthy rat (b).

SI References

1. Zhang, Z., Chao, T., Chen, S. & Jiang, S. Superlow fouling sulfobetaine and carboxybetaine polymers on glass slides. *Langmuir* **22**, 10072-10077 (2006).
2. Hoffmann, C., Leroy-Dudal, J., Patel, S., Gallet, O. & Pauthe, E. Fluorescein isothiocyanate-labeled human plasma fibronectin in extracellular matrix remodeling. *Anal. Biochem.* **372**, 62-71 (2008).
3. Zhang, Z., et al. Biodegradable and thermoreversible PCLA–PEG–PCLA hydrogel as a barrier for prevention of post-operative adhesion. *Biomaterials* **32**, 4725-4736 (2011).