Multifunctional calcium polyphenol networks reverse the hostile microenvironment of trauma for preventing postoperative peritoneal adhesions

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Methods

Determination of sustained release of gallic acid *in vitro*

In order to create a diluted solution of gallic acid with a concentration of 0.1 mg mL^{-1}, 10 mg of gallic acid was accurately weighed, placed in a 100 mL volumetric flask, dissolved in PBS solution (pH 7.4), added to the scale, and shaken well after constant volume. To create standard solutions with concentrations of 1, 5, 10, 15 and 20 g mL^{-1}, respectively, the 1, 5, 10, 15, and 20 mL GA solutions were transferred into a 100 mL volumetric flask with a pipette, diluted to the scale with PBS solution (pH 7.4), and shaken well. The absorbance values of various GA concentrations were measured using a microplate reader at a wavelength of 260 nm using PBS (pH 7.4) as the blank solvent.

10 mL of PBS solution (pH 7.4) was added to the dialysis bag as the release medium, and 10 mg of the prepared CaPNs powder (M_0) was precisely weighed and dissolved in it. The two ends of the dialysis bag were clamped and placed into a sample bottle containing 10 mL of the same PBS solution. The above solutions were fixed and shaken in a culture shaker (37 ℃, 100 r/min). All the external solution was removed at 1, 3, 6, 24, 48, 72, 96, 120, 144, and 168 h, respectively, and 10 mL of the same PBS release medium was added at the same time. The content of GA (M_{GA}) in the release medium was determined by microplate reader at each time point. The cumulative release rate-time curve was plotted. The cumulative release rate (CR%) was calculated using the following formula: $CR\%=M_0/M_{GA} \times 100\%$.

Swelling experiment

The swelling behavior of the CaPNs powder was investigated using the gravimetric method. Briefly, dried CaPNs powder was immersed in PBS at 37 °C and weighed after 1, 2, 3, 4, 5, 6, and 10 min. Excess water on the surface of the sample was absorbed by filter paper. The wet weight of each sample (W_s) was measured with an electronic balance. The swelling ratio was calculated using the following equation:

Swelling ratio (%) = $(W_s - W_d)/W_d \times 100\%$,

where W_s is the specimen weight at a defined time period during swelling and W_d is its dry weight.

Hemolysis assay

Blood compatibility was determined by incubating diluted blood in PBS with various materials and measuring absorbance at 540 nm with a microplate reader. In 24-well culture plates, different concentrations (0.5, 1 or 2 mg mL^{-1}) of Celox and CaPNs suspensions were gently mixed with 900 L of red blood cell suspension, incubated at 37 °C for 2 hours, centrifuged for 10 minutes, and the absorbance of hemoglobin in the supernatant was measured with a microplate reader at 540 nm. PBS and 0.1% Triton X-100 were employed as negative and positive controls, respectively. Hemolysis rate (%)= (sample - negative control)/(positive control - negative control)×100% was the formula for estimating hemolysis rate.

***In vitro* hemostatic assay**

For the determination of *in vitro* hemostasis time, 10 mg samples (Celox and CaPNs)

were placed in 2 mL plastic tubes and placed in a 37 °C water bath. 1 mL of fresh blood containing anticoagulant was added and maintained at 37 °C. Added 100 μL of 0.1 mol/L $CaCl_2$ aqueous solution to the above fresh blood and started the timing. The tube was tilted every 10 s to observe whether the blood was gel. The time required to record the complete loss of blood fluidity was the hemostasis time. Blood without samples was used as a blank control and each sample was repeated 3 times.

The blood coagulation index (BCI) was measured according to the method reported in the previous literature,[S1,S2] 5 mg samples (Celox and CaPNs) were placed in 2 mL centrifuge tubes, preheated at 37 °C for 5 min, then 10 μL fresh anticoagulant blood was slowly added to different samples, incubated at 37 °C for 5 min, afterwards, 2 mL deionized water was added to dissolve the uncoagulated red blood cells. At the same time, in order to avoid interfering with the already formed blood clots, Further incubated for 5 min. The absorbance of the supernatant containing hemoglobin was measured at 540 nm. The negative control was the absorbance of 10 μL whole blood in 2 mL deionized water. Finally, the BCI of Celox and CaPNs in whole blood was determined using the following formula: BCI (%) = $A_t/A_0 \times 100\%$, A_t and A_0 were the absorbance of the experimental group and the negative control group, respectively.

To test CaPNs and Celox's erythrocyte adhesion ability, 10 mg of the material was deposited in a 1.5 mL centrifuge tube, and 300 μL of blood diluted with PBS was dropped on the surface of the different materials, well mixed, and incubated at 37 °C for 30 minutes. The supernatant was then collected, and PBS was added to the supernatant removal tube to remove the red blood cells that did not cling to the various

components. By combining the two solutions (supernatant and PBS) and measuring the absorbance at 540 nm with a microplate reader, the number of free red blood cells was determined.

Platelet adhesion was assessed by lactate dehydrogenase (LDH) assays.[S3] The platelet-rich plasma (PRP) extracted from SD rats was diluted to 2×10^8/mL with PBS. 10 mg Celox, CaPNs or Gauze were incubated with 100 μL PRP suspension in a centrifuge tube at 37 ℃ for 30 min. After that, non-adherent platelets were removed by washing three times with PBS buffer. Adherent platelets were incubated with Triton X-100 to cleave adherent platelets. According to the LDH kit (Shanghai Biyuntime Biotechnology Co., LTD., Shanghai, China), the absorbance was recorded at 340 nm to determine the LDH activity in the lysate. By the known number of platelets and LDH activity built calibration curve was used to quantitatively adhesion of platelets in the material surface.

ELISA analysis

Blood samples were collected from the rat heart and dropped into an anticoagulant tube, left at room temperature for 1 h, centrifuged at 3000 rpm for 20 min at 4 °C, and the supernatant was removed and stored in a -20 °C refrigerator for subsequent use. Enzyme-linked immunosorbent assay (ELISA) was performed according to the protocol of each kit to detect cytokines included IL-6 (Cat#:ERC003), TNF-α (Cat#:ERC102a) and IL-1β (Cat#:ERC007). The ELISA kits used in the experiment were provided by Neobioscience Biotechnology Co., LTD.

RT-qPCR analysis of rat cecum and abdominal wall injury tissues

Using a QIA-shredder and RNeasy mini-kits (QIACEN), total RNA was extracted from freshly harvested rat tissues. Complementary DNA (cDNA) was created using the BioRad Laboratories' iScript cDNA Synthesis Kit. SYBR Green Supermix (Bio-Rad) was used in a StepOnePlus Real-Time PCR machine (Thermo Scientific) for the quantitative measurement of total RNA expression. The housekeeping gene -Actin, which is present in rat tissues, and genes of interest were both examined in cDNA samples. In rat tissues, the target genes included NF-κB, IL-6, and STAT3. The relative transcript levels of each target gene were determined using the comparative Ct ($2^{-\Delta\Delta Ct}$) technique.

Western blotting analysis of rat cecum and abdominal wall injury tissues

Tissue protein extraction was carried out according to the instructions of the RIPA Protein Extraction kit (Thermo Fisher Scientific, USA). The isolated proteins were electrophoresed in 10% sodium dodecyl sulfate polyacrylamide gels and transferred to PVDF membranes. Anti-NF-κB p65 (CST, 8242, 1:1000), anti-Phospho-NF-κB p65 (Abmart, TA2006, 1:1500), anti-STAT3 (CST, 9139, 1:1000), anti-Phospho-STAT3 (CST, 9145, 1:2000), and anti-β-Actin (BioTNT, A20120A0702, 1:1000) were the primary antibodies used. After treatment, PVDF membranes were washed and treated for 1 hour at room temperature with secondary antibodies (Jackson, 111-035-003, 1:20000). A chemiluminescence detection system (Millipore, Billerica, MA) was then used to assess protein expression.

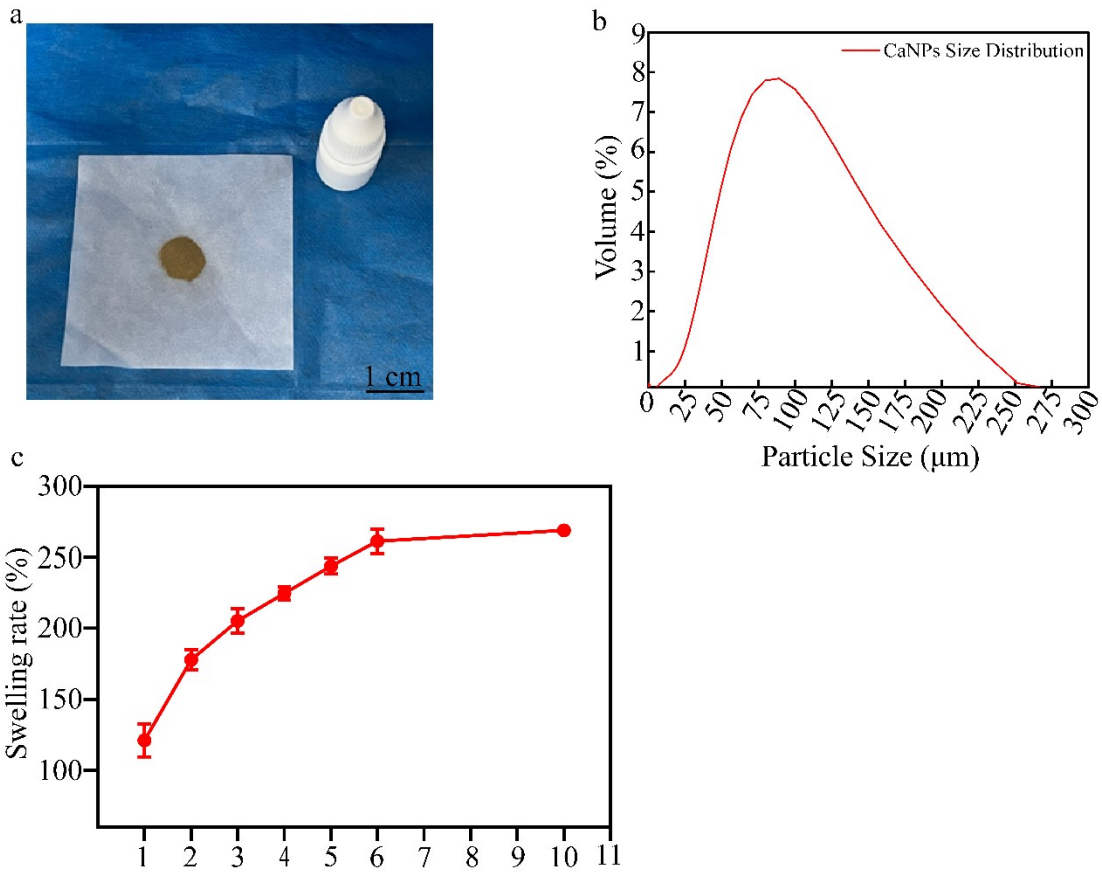

Fig. S1 Characterization of CaPNs. (a) Gross view images of the resultant CaPNs. (b) Size distribution of CaPNs microspheres by Particle Size Analyzer. (c) The swelling rate of CaPNs powder.

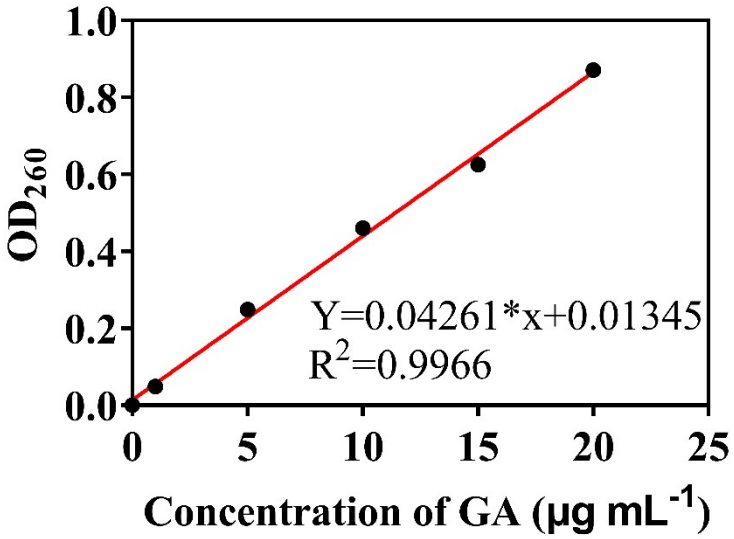

Fig. S2 The standard curve of GA.

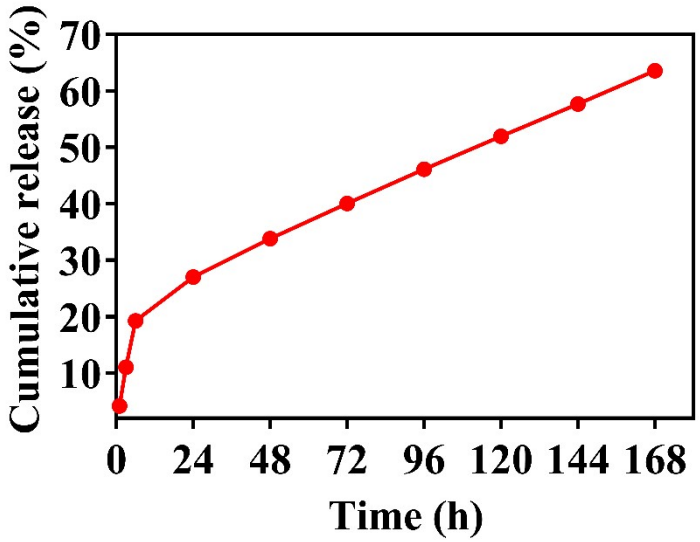

Fig. S3 The curve of accumulative release of GA.

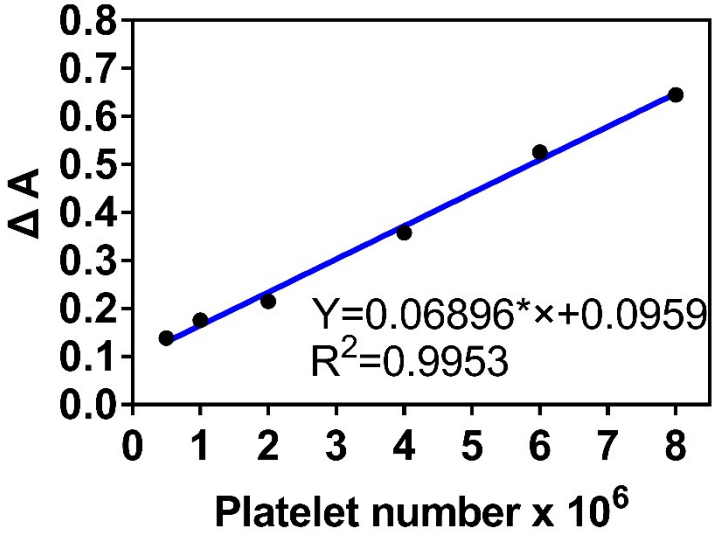

Fig. S4 The standard curve of LDH activity absorbency and the numbers of platelets.

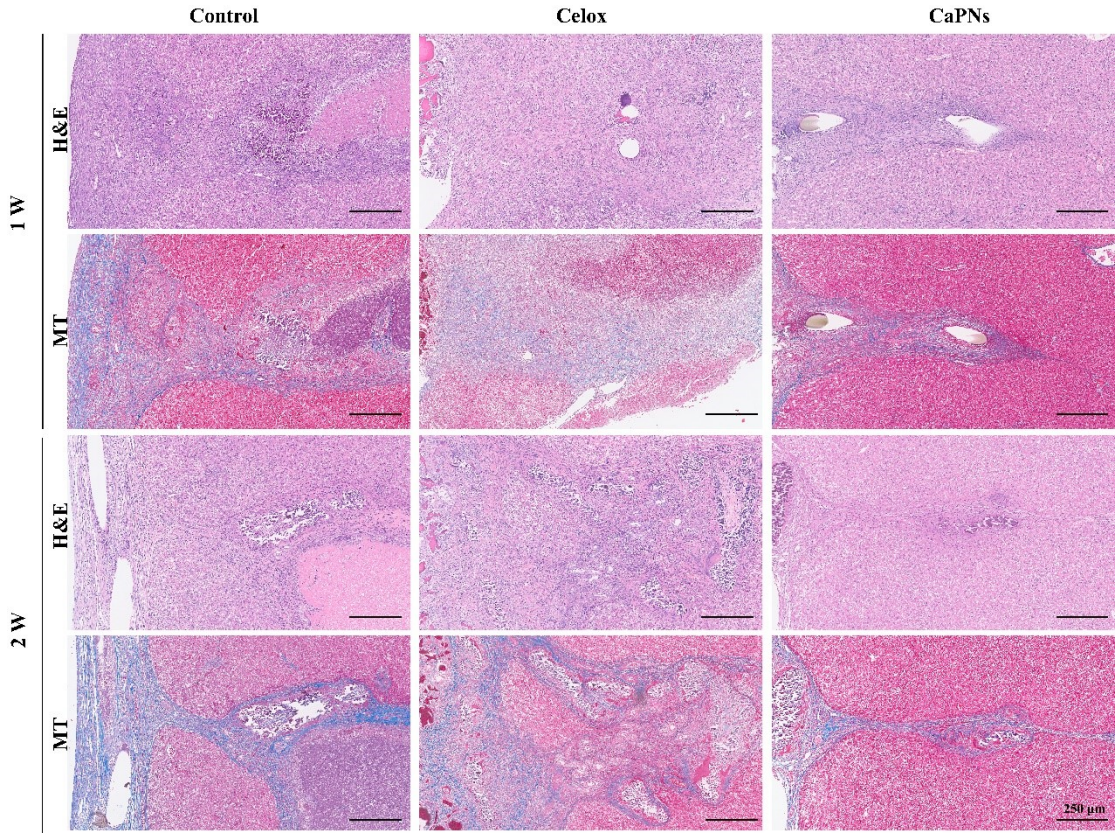

Fig. S5 Histopathological analysis of liver specimens in different groups on day 7 and day 14 postoperation determined by H&E staining and MT staining.

Gene	Primer	Sequence (5'-3')	Tm (°C)
IL-6	Forward	TTCTTGGGACTGATGCTGGTG	60.27
	Reverse	CACAACTCTTTTCTCATTTCCACGA	59.99
iNOS	Forward	GGGCTGTCACGGAGATCAATG	61.08
	Reverse	GCCCGGTACTCATTCTGCATG	61.41
IL-6	Forward	AAT CTG CTC TGG TCT TCT GGA	67.5
	Reverse	ATT GCT CTG AAT GAC TCT GGC	67.9
STAT3	Forward	CCC TGT CTC AGA ACC TTG TGT	67.7
	Reverse	TGG GAA CCA AGC ACA TAG AAT	67.9
NF-κB	Forward	GCT GTT TGG TTT GAG ACA TC	64.2
	Reverse	TCT GCC CTC CTG ACT CTA CT	65.1

Fig. S6 Summary of primer sequences.

Table 1. Quantity score for abdominal adhesion according to the criteria of Nair

Grade	Description of adhesive bands
0	The complete absence of adhesions
1	Only one band of adhesions among the viscera or between one viscera and the abdominal wall
2	Two bands: among viscera's or from viscera to abdominal wall
3	More than two bands: among viscera or from viscera to the abdominal wall or all intestine making a mass without adhesion to the abdominal wall
4	Viscera adhered directly to the abdominal wall, independent of the number and the extension of adhesion bands

Table 2. Quality score for abdominal adhesion according to the criteria of Zuhlke

Grade	Description of adhesive bands
0	No adhesions
1	Filmy (gentle blunt dissection)
2	Mild (aggressive blunt dissection)
3	Moderative (sharp dissection)
4	Severe (not dissectible without damage)

Table. 1 and 2 Nair and Zuhlke grading scoring system.

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2. C. Cao, N. Yang, Y. Zhao, D. Yang, Y. Hu, D. Yang, X. Song, W. Wang and X. Dong, *Nano Today*, 2021, **39**, 101165.
3. C. Liu, W. Yao, M. Tian, J. Wei, Q. Song and W. Qiao, *Biomaterials*, 2018, **179**, 83-95.