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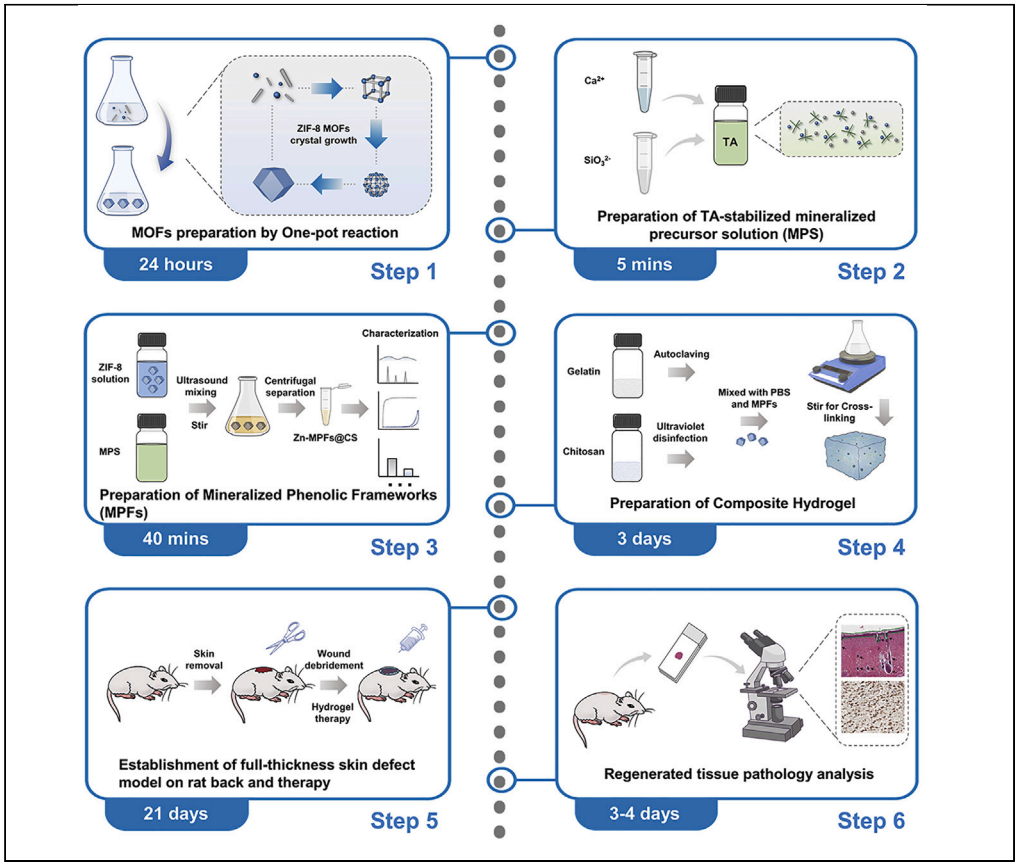
Protocol to assemble metal-phenolic framework nanoparticles based on polyphenol-mediated biomimetic mineralization for wound healing in rats



Here, we present a protocol for controllable biomimetic mineralization at the nano-scale, simulating natural ion-enriched sedimentary mineralization. We describe steps for treatment of metal-organic frameworks with polyphenol-mediated stabilized mineralized precursor solution. We then detail their use as templates to assemble metal-phenolic frameworks (MPFs) with mineralized layers. Furthermore, we demonstrate the therapeutic benefits of MPF delivery by hydrogel to the full-thickness skin defect model in rats.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol to dynamically control the biomimetic mineralization process at nano-scale

Protocol to prepare metal-phenolic framework nanomaterials with bioactivities

Establishment of the full-thickness skin defect model and therapy monitoring

Quality assessment of regenerated skin based on tissue biopsy techniques

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Protocol to assemble metal-phenolic framework nanoparticles based on polyphenol-mediated biomimetic mineralization for wound healing in rats

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SUMMARY

Here, we present a protocol for controllable biomimetic mineralization at the nano-scale, simulating natural ion-enriched sedimentary mineralization. We describe steps for treatment of metal-organic frameworks with polyphenol-mediated stabilized mineralized precursor solution. We then detail their use as templates to assemble metal-phenolic frameworks (MPFs) with mineralized layers. Furthermore, we demonstrate the therapeutic benefits of MPF delivery by hydrogel to the full-thickness skin defect model in rats.

For complete details on the use and execution of this protocol, please refer to Zhan et al. (2022).[1]

BEFORE YOU BEGIN

Mineralization is a natural process featuring the sophisticated balance of physicochemical interactions at diverse interfaces.[2] However, the bulk mineralization of inorganic materials in solution is difficult to avoid the classical crystallization, e.g., the rapid formation of phosphate precipitates by simply mixing Ca^{2+} with PO_4^{3-}, which limits its application at the nano-scale interface. Inspired by the natural product polyphenols, which can coordinate with various metal ions attributed to the abundant galloyl groups of its structure.[3–5] The protocol shows that tannic acid (TA) can play a role as a capping agent to insulate Ca^{2+} from other mineralization-related ions (e.g., SiO_3^{2-} and PO_4^{3-}) in the mineralized precursor solution (MPS). Here, ZIF-8 (2-Methylimidazole zinc salt), a kind of typical metal-organic frameworks (MOFs), is presented as a template example to mix with TA-mediated stabilized MPS containing calcium silicate (CS) precursors. Then well-assembled metal-phenolic frameworks (MPFs) nanoparticles, Zn-MPFs@CS could be easily obtained with a Zn-based framework structure and CS mineralized layer. In addition, this customizable protocol is also feasible when we change the MOFs template to ZIF-67 (zeolitic imidazolate framework-67) and HKUST-1 (Hong Kong University of Science and Technology-1), and change the CS precursors to calcium phosphate (CaP) precursors.[1]

The plug-and-play property of this protocol allows us to assemble various bioactive ingredients into the "all-in-one" therapy agent for better wound healing.[6,7] We deliver Zn-MPFs@CS to the

full-thickness skin defect model via gelatin chitosan hydrogel. Here, these heuristic composite nanomaterials preparation, wound modeling, and pathology analysis processes are detailed in this protocol for potential investigators to better conduct more interesting studies and wound therapy-related experiments.

Institutional permissions

All animal experiments were carried out with the approval of the Ethical Committee of the South China University of Technology in compliance with Chinese laws and policies (AEC: #2020060).

Note: Before you begin, you should prepare a statement identifying the committee approving the experiments and confirming that all experiments conform to the relevant regulatory standards.

Preparation of non-sterile solution

Timing: 30 min

1. Prepare the metal and ligand stock solution for synthesis of ZIF-8.
   a. Dissolve 8.96 g (40.82 mmol) of $Zn(OAc)_2{\cdot}2H_2O$ in 40 mL of DI water, as the stock metal solution for the synthesis of ZIF-8 (1.02 M).
   b. Dissolve 2.40 g (29.23 mmol) of 2-MIM in 40 mL of DI water, as the stock ligand solution for the synthesis of ZIF-8 (0.73 M).
2. Prepare TA stock solution: Dissolve 400 mg of TA in 60 mL of DI water, as the TA stock solution (3.92 mM).

⚠ CRITICAL: The metal and ligand stock solution can be stored at 25°C up to 2–3 weeks. The polyphenol aqueous solution is easy to oxidize after a long time, so the TA stock solution needs to be used for the following experiments as soon as possible.

Preparation of sterile agent

Timing: 4 h

3. Sterilize 0.027 g of gelatin by high-pressure (100 kPa) steam for 120 min.
4. Sterilize 0.054 g of chitosan hydrochloride by UV irradiation for 30 min.
5. Sterilize sufficient 1× PBS by high-pressure steam and used as the stock PBS solution (10 mM) for the subsequent experiments.

Note: These materials are prepared to be used in step 12 of the "step-by-step method details" section.

KEY RESOURCES TABLE

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Monoclonal Interleukin-6 (IL-6) antibody (working concentration: 10 μg/mL)	Novus	Cat# NBP2-89149
Monoclonal CD31/PECAM-1 antibody (working concentration: 10 μg/mL)	Novus	Cat# NB100-64796SS
Anti-mouse IgG Alexa Fluor 594 conjugated secondary antibody (working concentration: 1 μg/mL)	CST	Cat# 8890S

(*Continued on next page*)

Continued

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Tannic acid (TA)	Macklin (Shanghai, China)	Cat# 1401-55-4
Calcium chloride anhydrous ($CaCl_2$)	Macklin (Shanghai, China)	Cat# 10043-52-4
Sodium silicate ($Na_2O \cdot 3SiO_2$)	Macklin (Shanghai, China)	Cat# 1344-09-8
2-Methylimidazole (2-MIM)	Aladdin (Shanghai, China)	Cat# 693-98-1
Zinc acetate dihydrate $[Zn(OAc)_2 \cdot 2H_2O]$	Aladdin (Shanghai, China)	Cat# 5970-45-6
Phosphate-buffered saline (PBS)	Procell	Cat# PB180327
Goat serum	BOSTER	Cat# AR0009
Trypan blue	Thermo Fisher	Cat# 15250061
Cold water fish skin gelatin	Sigma	Cat# G7041
Chitosan hydrochloride	Zhejiang Golden shell Pharmaceutical Co., Ltd, China	Cat# M-YYHK-2008001
Distilled water	N/A	N/A
Transglutaminase	Biobomei Biotechnology Co., Ltd, China	Cat# BC5582
4% Tissue fixer	RARBIO	Cat# RA5601
Tissue-Tek O.C.T.	Sakura	Cat# 4583
Tween 20	Biotopped	Cat# 9005-64-5
Immunohistochemical antigen repair buffer	Biolynx	Cat# I30031A
Triton-X 100	MilliporeSigma	Cat# X-100
Xylene	Macklin (Shanghai, China)	Cat# 1330-20-7
4′,6-diamidino-2-phenylindole (DAPI)	Invitrogen-Thermo Fisher Scientific	Cat# D1306
Masson's Trichrome Stain Kit	Solarbio	Cat# G1340
Eosin Y solution	Solarbio	Cat# G1100
Mayer' Hematoxylin solution	Solarbio	Cat# G1080
Neutral balsam	Solarbio	Cat# G8590
DAB Substrate	Sigma-Aldrich	Cat# 11718096001
HRP Goat Anti-Rat IgG (Ready to use)	Vector Laboratories	Cat# MP-7404
Hydrogen peroxide (H_2O_2, 30%)	Chengdu Cologne Chemicals Co., Ltd. (China)	Cat# 7722-84-1
Methanol	Energy Chemical	Cat# 67-56-1
Isoflurane	RWD Life Science Co., Ltd.	Cat# R510-22-10
Paraffin	Sigma-Aldrich	Cat# 145686-99-3
Experimental models: Organisms/strains		
Sprague Dawley rats: 7 weeks, female	Hunan SJA laboratory animal Co., Ltd (Changsha, China)	N/A
Other		
Magnetic stirrer	RH digital, IKA	N/A
Transmission electron microscope	Thermo Scientific, USA	Cat# Talos F200x
X-ray diffractometer	Rigaku HomeLab, Japan	N/A
N_2 adsorption-desorption measurement instrument	BSD Instruments, China	Cat# BSD-PM1
Dynamic light scattering (DLS)	Zetasizer Nano ZS, Malvern	N/A
DMi8 microscope	Leica, Germany	N/A
Digital Pathology Scanner	Leica, Germany	Cat# Aperio CS2
Automatic rotary microtome	Leica, Germany	N/A
Water bath	Grant Instruments	Cat# SUB36
Ultraviolet (UV) crosslinker	UVITEC Cambridge	Cat# BLX-254
Anesthesia box	RWD Life Science Co., Ltd.	Cat# R500IE
Cover slip	N/A	N/A

MATERIALS AND EQUIPMENT

PBST washing buffer (Prepare Fresh)		
Reagent	Final concentration	Amount
PBS	1×	999 mL
Tween 20	0.1%	1 mL
Total		1,000 mL

Antigen repair buffer (Prepare Fresh)		
Reagent	Final concentration	Amount
Immunohistochemical antigen repairing buffer (100×)	1×	1 mL
Distilled water	N/A	99 mL
Total		100 mL

0.3% H_2O_2 (Prepare Fresh)		
Reagent	Final concentration	Amount
30% H_2O_2	0.3%	1 mL
Phosphate buffer saline	1×	99 mL
Total		100 mL

⚠ CRITICAL: Hydrogen peroxide (H_2O_2) can cause damage to the human body, including skin erosion, and serious eye damage. Make sure you prepare the storage solution while wearing gloves and goggles.

STEP-BY-STEP METHOD DETAILS

Preparation of metal-organic frameworks ZIF-8 templates

Timing: 25 h

This section describes the standard procedure for preparing Metal-Organic Frameworks ZIF-8 templates, the method for removing unreacted monomers by washing, and the storage method.

1. Mix 40 mL of 1.02 mol/L $Zn(OAc)_2{\cdot}2H_2O$ and 40 mL of 0.73 mol/L 2-MIM stock solution under stirring with a magnetic bar.
2. Stop stirring after completely mixing the component solutions.
3. Let the mixture be at 25°C for 24 h to allow the ZIF-8 nanocrystal growth.
4. Collect the formed ZIF-8 nanoparticles by centrifuging (12,850 × *g*), and wash three times with deionized water (DI water) and methanol, respectively.
5. Store the synthesized ZIF-8 NPs in methanol before using, and measure their concentration.

Note: Please ensure that the ambient temperature during each synthesis is the same, which is related to the size of the final nanoparticles. In addition, please adequately wash the nanoparticles under ultrasonic conditions until they are completely dispersed.

Preparation of TA-stabilized mineralized precursor solution (MPS)

Timing: 5 min

This section describes the preparation process of TA-stabilized mineralized precursor solution, focusing on the proportion of various reagents.

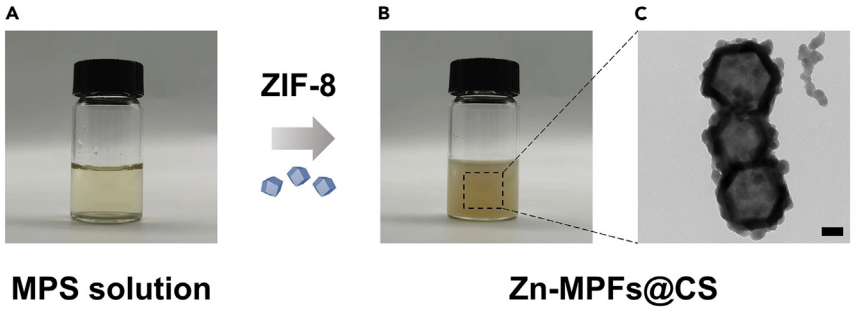

Figure 1. Etching and biomimetic mineralization process of ZIF-8 in MPS solution

(A) Photograph of MPS solution.

(B) Photograph of Zn-MPFs@CS solution.

(C) TEM image of Zn-MPFs@CS nanoparticles. Scale bar, 100nm.

6. Transfer 6 mL of TA aqueous solution to a 20 mL synthetic flask, in which a magnetic bar was added, and place the flask on a running magnetic stirrer.
7. Add 1 mL $CaCl_2$ of solution and 1 mL of $Na_2O{\cdot}3SiO_2$ solution successively to the flask and stir thoroughly, the final molar ratio of TA: $CaCl_2$: $Na_2O{\cdot}3SiO_2$ in the precursor solution is 1:1:1 (problem 1).

⚠ CRITICAL: TA is easy to oxidize after a long time, so the MPS solution needs to be used for the following experiments as soon as possible.

Preparation of mineralized metal-phenolic frameworks (MPFs)

Timing: 40 min

This section describes the preparation process of mineralized Metal-Phenolic Frameworks, including the pre-treatment ZIF-8 nanoparticles, the reaction process, the subsequent washing process and the storage condition of mineralized MPFs.

8. Remove the methanol solution containing 8 mg of ZIF-8 from the previously synthesized ZIF-8 methanol solution and place it in a centrifugal tube. After centrifugation (12,850 × *g*), collect the ZIF-8 nanoparticles and further clean them with DI water.
9. Collect the ZIF-8 nanoparticles by centrifugation (12,850 × *g*), and disperse them into 8 mL of MPS solution under ultrasound.
10. Keep the mixture stirred for 10 min and then centrifuge (12,850 × *g*) it to collect the MPFs nanoparticles Zn-MPFs@CS, which should be washed with DI water and methanol three times, respectively.
11. Store Zn-MPFs@CS nanoparticles in methanol for the following characterization and experiments (problem 2).

Note: When ZIF-8 is mixed with the MPS solution, the mixture solution quickly turns a cloudy brown color (Figure 1), which is a typical phenomenon indicating that ZIF-8 has been etched by TA. In addition, this customizable protocol is also feasible when the MOFs templates are replaced with ZIF-67 and HKUST-1, as well as change the CS precursors to calcium phosphate (CaP) precursors. The optimal molar ratio of TA:$CaCl_2$:K_3PO_4 in CaP precursor solution is 3:3:2.

Preparation of composite hydrogel

Timing: 3 days

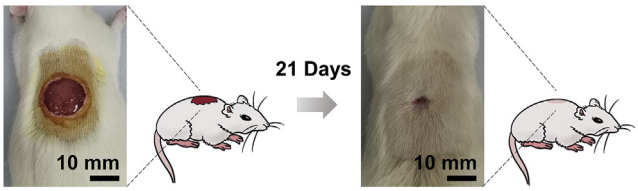

Figure 2. Example of the Wound healing process

This section describes the preparation process of gelatin-chitosan hydrogel loaded with Zn-MPFs@CS, which will be used as a functional hydrogel for wound therapy.

12. Dissolve 0.027g gelatin and 0.054g chitosan hydrochloride (the mass ratio is 1:2) in 1 mL of 1× PBS (these mentioned agents should be sterilized as described in steps 3–5 of the "before you begin" section), and the obtained solution keep stirred at 300 rpm for 36 h.
13. Dissolve 0.1 g transglutaminase (1,500 U/g) in 500 μL of 1× PBS, keep stirred at 150 rpm for 24 h, and filtered with a 0.22 μm filter.
14. Add 100 μL of sterile transglutaminase to the dissolved gelatin-chitosan solution which contains 100 μg of Zn-MPFs@CS for crosslinking for 45 min at 37°C.

Note: Perform these procedures in Class II biological hood with standard aseptic technique.

Establishment of full-thickness skin defect model on rat back and therapy

Timing: 21 days

This section describes how to establish a full-thickness skin defect model on the back of rats to evaluate the effect of wound healing and apply the composite hydrogel to wound therapy.

15. Anesthetize Sprague Dawley rats at 7 weeks old (problem 3).
    a. Place the rats in an animal anesthesia box.
    b. Place cotton adsorbed with isoflurane or another suitable anesthetic in an anesthesia box until the rats are anesthetized before being moved to a clean operating table.
16. Remove the back fur using a razor.
17. Use 75% ethanol(v/v) to disinfect the expected location of the wound.
18. Create a full-thickness skin wound model (diameter: ~15 mm) on each rat's back with sterilized scissors and forceps, and take photos for archiving.
19. Spread the compound hydrogel over the wound surface using a syringe.
20. House each rat individually in a single cage after surgery (problem 4).
21. Follow up on wound healing progress, record the wound size regularly and take photos for archiving (Figure 2).

Note: Please ensure that the distance between the camera and the operating table is the same when the size of the wound is being recorded, which is conducive to the statistical analysis of the size of the wound in the photos. It is necessary to classify and label the records and photographs collected at different points in time.

Regenerated tissue pathology analysis

Timing: 3–4 days

This section describes how to prepare tissue biopsy sections and analyze the healing quality of regenerated skin tissue by different staining methods.

Table 1. Tissue processor

Steps	Reagent	Time (min)	Cycles	Temperature
Dehydration	70% Ethanol	45	N/A	20°C
	85% Ethanol	45	N/A	
	95% Ethanol	45	N/A	
	100% Ethanol	45	3	
Clearing	Xylene	45	3	
Wax infiltration	Paraffin	45	3	60°C

22. Tissue processing.
    a. Collect regenerated wound tissue from each rat with sterilized scissors and forceps, and cut the tissue into two strips.
    b. Place the tissues in the pre-filled 10% neutral buffered formalin container (problem 5).
    c. After being fixed for 48 h, embed the tissues with paraffin and Tissue-Tek O.C.T. (Optimum Cutting Temperature Compound), respectively.
    d. Process the tissues overnight with different reagents following the tissue processor (Table 1).
    e. Choose an appropriate size base mold and add some liquid paraffin into it.
    f. Position the tissues onto the paraffin base mold for subsequent longitudinal sectioning of the tissue.
    g. Place the base mold with the tissues on the cold plate at −10°C to solidify the paraffin, making sure that the tissues are pressed down and fully immersed.
    h. Place the pre-labeled cassette on top of the base mold containing the tissue and fill the mold with additional liquid paraffin.
    i. Let the paraffin completely solidify before removing the paraffin block from the mold.
    j. Trim excess paraffin from the sides of the cassette with the Para Trimmer. The blocks are now ready for sectioning and can be stored at 20°C.
23. Paraffin block sectioning.
    a. Prewarm a 37°C water bath.
    b. Mount the paraffin block on the microtome.
    c. Cut 6 μm thin sections throughout the block to collect different layers of the tissues.
    d. Pick up the sections using a small brush and transfer them gently into the water bath.
    e. Collect the tissue sections by dipping a glass slide into the water bath and allowing the tissue sections to adhere to the surface of the slide.
    f. Exsiccate the slides in a slide box temporarily, then place the slides in a 45°C incubator for 1 h.
    g. Select the proper slide box for the classification, storage, and labeling of slides with tissues, which will be used for different staining and observation.
24. Hematoxylin and Eosin staining for formalin fixed paraffin embedded tissues.
    a. Place the glass slides with paraffin sections in staining racks. Clear the paraffin from the samples in three changes of xylene for 10 min per change.
    b. Stain the samples with different reagents following the H&E staining processor (Table 2).
    c. Add a cover slip over the tissue on each slide.
    d. View the slides using a microscope.
25. Masson's Trichrome staining for formalin fixed paraffin embedded tissues.
    a. Place the glass slides with paraffin sections in staining racks. Clear the paraffin from the samples in three changes of xylene for 10 min per change.
    b. Stain the samples with different reagents following Masson's Trichrome staining processor (Table 3).
    c. View the slides using a microscope.

Note: To save experimental supplies, the executors are encouraged to fix two tissues on a single slide. This will not affect the quality of fixation and subsequent observation under correct operation (Figure 3).

Table 2. H&E staining processor

Steps	Reagent	Time (min)	Cycles	Temperature
Hydration	100% Ethanol	10	3	20°C
	95% Ethanol	8	N/A	
	90% Ethanol	5	N/A	
	80% Ethanol	3	N/A	
Rinse the slide in running tap water	N/A	2	N/A	
Stain	Hematoxylin solution	3	N/A	
Rinse the slide in running tap water	N/A	5	N/A	
Stain	Eosin Y solution	2	N/A	
Dehydration	95% ethanol	2	2	
	100% ethanol	2	3	
Cover the slide	Neutral balsam	1	1	

26. IL-6 immunohistochemical staining for formalin fixed paraffin embedded tissues.
    a. Place the glass slides with paraffin sections in staining racks. Clear the paraffin from the samples in three changes of xylene for 10 min per change.
    b. Stain the samples with different reagents following the IL-6 immunohistochemical staining processor (Table 4).
    c. View the slides using a microscope.

Note: Please incubate slides in 0.3% H_2O_2 on a gentle rocker and make sure the slides are in a dark container as H_2O_2 is light-sensitive.

27. CD-31 immunofluorescent staining for formalin fixed paraffin embedded tissues.

Table 3. Masson's Trichrome staining processor

Steps	Reagent	Time (min)	Cycles	Temperature
Hydration	100% Ethanol	10	3	20°C
	95% Ethanol	8	N/A	
	90% Ethanol	5	N/A	
	80% Ethanol	3	N/A	
Stain	Bouin's solution	60	N/A	60°C
Rinse the slide in running tap water	N/A	5	N/A	20°C
Stain	Iron hematoxylin	2	N/A	
Rinse the slide in running tap water	N/A	5	N/A	
Rinse the slide in DI water	N/A	1	3	
Stain	Biebrich scarlet-acid fuchsin solution	5	N/A	
Rinse the slide in DI water	N/A	1	3	
Stain	phosphotungstic/phosphomolybdic acid solution	10	N/A	
Drain	N/A	N/A	N/A	
Stain	Aniline blue	5	N/A	
Rinse the slide in DI water	N/A	1	3	
Differentiate the colors of the dyed tissue structures	95% ethanol	2	2	
	1% acetic acid	1	N/A	
Rinse the slide in DI water	N/A	1	3	
Dehydration	95% ethanol	2	2	
	100% ethanol	2	3	
	Xylene	2	N/A	
Mounting	2 drops of Cytoseal XYL mounting medium	N/A	N/A	
Covering the slide with a cover slip	N/A	N/A	N/A	
Dry	N/A	N/A	N/A	

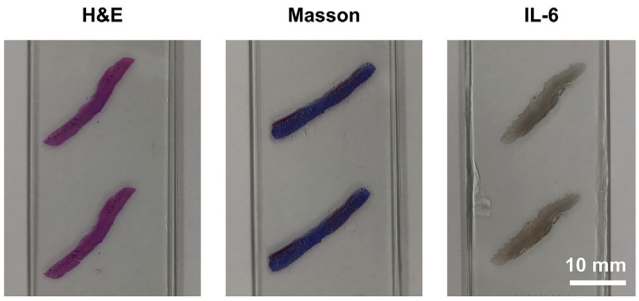

Figure 3. Example of the photograph of different stained tissue slides

a. Embed tissues in OCT and freeze slides (10 μm) using a cryostat.
b. Stain the samples with different reagents following the CD-31 immunofluorescent staining processor (Table 5).
c. View the slides using the DMi8 microscope.

EXPECTED OUTCOMES

This proposal presents the preparation of the "all-in-one" bioactive ingredients delivery system. Under laboratory conditions, we simulated the mineralization process in nature. By controlling the TA-mediated capping and decapping process, the executors of this proposal can achieve the preparation of stable CS MPS, and then realize the enrichment of mineralized ions on the surface of MOFs at the nanoscale when mixing MOFs and CS MPS. Thus, biomimetic mineralization can be completed on the surface of obtained MPFs. To verify the structure and property of Zn-MPFs@CS, we presented several key characteristics for executors to check whether the materials that they obtain are satisfactory (Figure 4). In addition, we have successfully obtained other metal-based MPFs by replacing the type of MOFs templates, e.g., copper-based HKUST-1 and cobalt-based ZIF-67. The mineralized layer can also be changed from CS to CaP by simply replacing the

Table 4. IL-6 Immunohistochemical staining processor

Steps	Reagent	Time (min)	Cycles	Temperature
Hydration	100% Ethanol	10	3	20°C
	95% Ethanol	8	N/A	
	90% Ethanol	5	N/A	
	80% Ethanol	3	N/A	
Repair samples' antigen with microwave	Citrate buffer	5	3	
Cooling	N/A	20–30	N/A	
Washing	PBST solution	1	3	
Clear the endogenous enzymes	0.3% H_2O_2	5	N/A	
Washing	PBST solution	1	3	
Block endogenous non-specific binding	5% goat serum	30	N/A	
Incubate tissues in a dark wet box	50 μL primary antibody (IL-6, diluted at 1:100 in 1× PBS)	Overnight	N/A	4°C
Washing	PBST solution	1	3	20°C
Incubate tissues with secondary antibody	HRP-labeled secondary	60	N/A	
Incubate tissues in DAB with gentle shaking	3,3′-Diaminobenzidine chromogen	2–10	3	
Washing	DI water	1	3	
Staining	Hemotoxylin Solution	10	N/A	
Rinse the slide in running tap water	N/A	5	N/A	
Dehydration	95% ethanol	2	2	
	100% ethanol	2	3	
Covering the slide with a cover slip	N/A	N/A	N/A	

Table 5. CD-31 Immunofluorescent staining processor

Steps	Reagent	Time (min)	Cycles	Temperature
Blocking	5% goat serum	60	N/A	20°C
Washing	Cold PBS solution	5	2	
Incubate tissues with primary antibody	CD 31 diluted at 1:100 in 1× PBS	Overnight	N/A	4°C
Washing	Cold PBS solution	5	2	20°C
Incubate tissues with secondary antibodies in a dark wet box	anti-mouse IgG Alexa Fluor 594 conjugated secondary antibody (diluted at 1:1000)	120	N/A	
Nucleus staining	1× DAPI stock solution	3	N/A	20°C
Washing	Cold PBS solution	5	2	
Drain	N/A	N/A	N/A	
Mounting	One drop of Vectashield® fluorescence mounting medium	N/A	N/A	
Covering the slide with a cover slip	N/A	N/A	N/A	
Dry in a dark container	N/A	240	N/A	

mineralized precursor solution. We believe this proposal could be used for the design of other composite systems, depending on which ingredient the executor wants to integrate.

This proposal takes Zn-MPFs@CS as the typical application example to show the therapy effectiveness of the obtained composite system for wound healing. For executors to better conduct wound therapy experiments, we detail the method to establish a full-thickness skin defect model on the rat's back and evaluate the quality of regenerated tissues by pathology analysis. We also provided expected outcomes of H&E staining, Masson's Trichrome staining, IL-6 immunohistochemical staining, and CD-31 immunofluorescent staining (Figure 5). In H&E staining, epidermal thickness and the regeneration of hair follicles can be observed. In Masson's Trichrome staining, collagen deposition in regenerated skin tissues can be evaluated. As for the regeneration of vessels and the expression of inflammatory cytokines, they can be displayed by IL-6 immunohistochemical staining and CD-31 immunofluorescent staining, respectively.

QUANTIFICATION AND STATISTICAL ANALYSIS

We encourage those who implement this protocol to monitor the progress of wound healing daily and take photographs of it. However, if this is difficult for them, we recommend that they record the changes in wound size from the following time points. On days 0, 3, 7, 14, and 21, the size of the wound can provide valuable information indicating the progress of wound healing. The wound area images recorded by the camera can be calculated with ImageJ, then the relative wound area rate is calculated by the following equation:

$$\textit{Relative Wound Area}\ (\%) = \frac{100 \times \text{Current Wound Area}\ (\text{cm}^2)}{\text{Initial Wound Area}\ (\text{cm}^2)}$$

LIMITATIONS

Although we demonstrated that TA-induced capping could be used for the preparation of stable solutions of CS or CaP precursor solution in this protocol, this is based on the reversible coordination of calcium ions and polyphenols. When transition metal ions like iron coordinate with polyphenols, the interaction force between them is strong and difficult to unravel. Because of this, it is extremely important to select the proper metal ions to prepare the desired mineralized precursor solution. In addition, the state of the mineralized precursor solution also plays a key role in the preparation of MPFs. The reason why we emphasize that mineralized precursor solutions with polyphenols need to be newly prepared and used in each experiment is that the phenol hydroxyl groups of

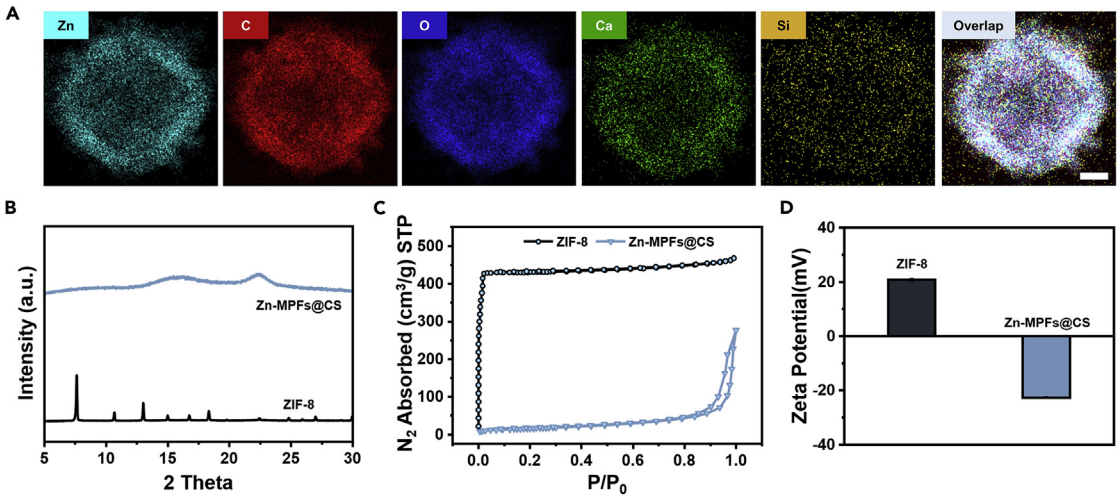

Figure 4. Key characteristics of mineralized metal-phenolic frameworks Zn-MPFs@CS

(A) EDX elemental mapping of Zn-MPFs@CS. Scale bar, 100 nm.

(B) XRD indicates the amorphization of Zn-MPFs@CS caused by stepwise etching.

(C and D) (C) N_2 adsorption-desorption and (D) ζ-potential difference of Zn-MPFs@CS and ZIF-8.

polyphenols will be oxidized to quinone groups after a long period of standing, and then its capping effect will be greatly reduced, resulting in the subsequent experiments not as expected.

TROUBLESHOOTING

Problem 1

When the calcium silicate mineralized precursor solution is prepared with the optimal molar ratio, but the obtained solution is turbid (step 7).

Potential solution

This may be due to the high concentration of TA, which leads to the formation of nanoparticles with calcium ions. We suggest that the concentration of TA in the precursor solution should not be greater than 10 g/L. Based on that, the executors of this protocol can change the concentration of mineralized ions accordingly.

Problem 2

The substitutability of storage media of MPFs or MOFs nanoparticles (step 11).

Potential solution

In this protocol, we use methanol as an example of a storage medium for MPFs or MOFs nanoparticles, but it can also be replaced with an organic solvent such as ethanol.

Problem 3

The rats die from hyperanesthesia (step 15).

Potential solution

In order to protect animal rights, anesthesia should be used with caution. We recommend the use of commercial anesthesia machines, which have safety valves and air tube for continuous anesthesia. When the limbs of rats are pressed with tweezers, the rats did not respond, which could be regarded as meeting the standard of anesthesia requirements.

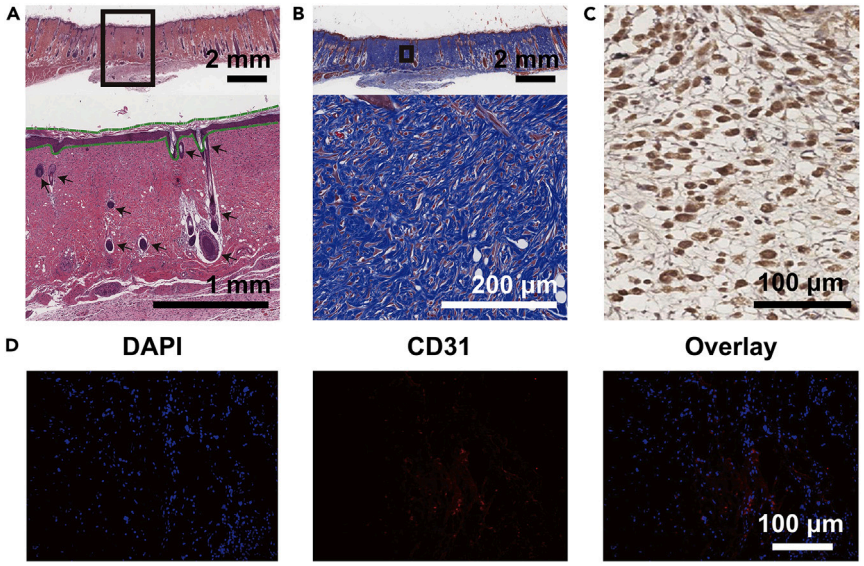

Figure 5. Expected outcomes of pathology analysis

(A) H&E staining.

(B) Masson's Trichrome staining.

(C and D) (C) IL-6 immunohistochemical staining, and (D) CD-31 immunofluorescent staining of regenerated skin tissues.

Problem 4

The composite hydrogel on the wound bed of the rat's back may drop out when the rats move (step 20).

Potential solution

The wound treated with hydrogel could be fixed with gauze. We do not recommend keeping two rats in the same cage as this can cause them to destroy each other's gauze that we have wrapped.

Problem 5

When we fixed the tissues in 10% neutral buffered formalin directly, the tissues may curl and it is difficult to be embedded with paraffin and Tissue-Tek O.C.T (step 22b).

Potential solution

To solve this problem, we can place tissues in the base mold first and it can keep the shape of the tissue normally, then we place the base mold in the pre-filled 10% neutral buffered formalin container to fix tissues.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Wei Zhu (zhuwei86@scut.edu.cn).

Materials availability

This study did not generate new unique materials.

Data and code availability

The data presented in this work are available from the lead contact upon reasonable request.

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AUTHOR CONTRIBUTIONS

W.Z. and H.C. designed the project and discussed the results with all other authors. X.Z. and Z.W. conducted the related materials preparation, structural characterization, and biological experiments. X.C. provided technical support in biological experiments. J.F. assisted in the design and drawing of pictures in the manuscript. K.L. provided photography support during these related experiments. L.X. and P.H. assisted in carrying out animal experiments. X.Z. and Z.W. prepared the first version of the manuscript, and all authors contributed to the final version.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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