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**Songhao Liu**

Qinghai University

**Juan Yan**

Qinghai University

**Yuxia Zhang**

Northwest Institute of Plateau Biology

**Lin Rong**

Northwest Institute of Plateau Biology

**Liang Gao**

Northwest Institute of Plateau Biology

**Lixin Wei**

Northwest Institute of Plateau Biology

**Hongxia Yang**

[hxyang@nwipb.cas.cn](mailto:hxyang@nwipb.cas.cn)

Northwest Institute of Plateau Biology

**Mengyu Gao**

Qinghai University

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## Article

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# Preparation and in-vitro screening of yak-skin collagen/PMVE-MA composite biomaterials toward biodegradable vascular stents

Songhao Liu<sup>a, c, 1</sup>, Juan Yan<sup>a, b, 1</sup>, Yuxia Zhang<sup>b, c</sup>, Lin Rong<sup>b, c</sup>, Liang Gao<sup>b, c</sup>, Lixin Wei<sup>b</sup>, Hongxia Yang<sup>b, \*</sup>, Mengyu Gao<sup>a, \*</sup>

<sup>a</sup> Qinghai University, Xining, Qinghai, 810016, China;

<sup>b</sup> Qinghai Key Laboratory of Tibetan Medicine Pharmacology and Safety Evaluation, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining 810008, China;

<sup>c</sup> University of Chinese Academic of Sciences, Beijing 100049, China;

<sup>1</sup> These authors contributed equally to this work and should be considered co-first authors.

**Correspondence:** Hongxia Yang, Qinghai Key Laboratory of Tibetan Medicine Pharmacology and Safety Evaluation, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Chinese Academy of Sciences, Xining 810008, China. Email: hxyang@nwipb.cas.cn

## Abstract

Cardiovascular stents play a decisive role in the treatment of coronary artery disease. Conventional metallic stents suffer from long-term complications including in-stent restenosis and chronic inflammation due to permanent foreign-body retention. Degradable metallic stents are limited by corrosion-derived particle-mediated inflammatory responses. Polymer-collagen composite represents promising alternative candidate for biodegradable stents, yet it remains challenging to simultaneously achieve adequate mechanical stability, anti-swelling property and favorable biological performance. Herein, we extracted type-I collagen from yak skin, which exhibits elevated denaturation temperature compared with common mammalian collagens. A composite biomaterial consisting of yak-derived type-I collagen and FDA-approved PMVE-MA was fabricated via cross-linking and freeze-drying. Box-Behnken response-surface methodology was applied to systematically optimize preparation parameters including mass ratio, stirring temperature and reaction duration. The optimized composite displayed appropriate compressive modulus, controlled degradation profile and satisfactory hemocompatibility. Nevertheless, the material exhibited strong surface hydrophobicity, which severely impeded cell adhesion, spreading and infiltration, despite its acceptable blood compatibility. This work demonstrates an interesting trade-off phenomenon: good hemocompatibility does not guarantee satisfactory cellular compatibility for collagen-PMVE-MA biomaterials. Our findings provide baseline performance data for this composite system, and highlight that surface modification is mandatory to overcome hydrophobicity-associated biological barriers before this material can be translated toward vascular stent applications.

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**Keywords:** Cardiovascular disease, Stent material, Biodegradable

## 1 Introduction

Cardiovascular disease (CVD) remains one of the leading causes of global mortality, among which coronary artery disease contributes enormously to disability and death worldwide[1, 2]. Percutaneous coronary intervention with vascular stent implantation has become the dominant therapeutic strategy for revascularization of stenosed coronary arteries. Conventional permanent stents, including bare-metal stents (BMS) and drug-eluting stents (DES), can mechanically dilate narrowed blood vessels and restore blood perfusion efficiently[3]. Nevertheless, permanent metallic implants bring unavoidable long-term risks: chronic foreign-body inflammatory responses, stent fracture, and impaired natural vasomotor function of vessels[4, 5]. Long-term clinical follow-up has demonstrated that both BMS and DES are associated with non-negligible risks of in-stent restenosis and late-stage stent thrombosis[6]. Moreover, permanently retained stent hardware can complicate potential re-interventions for recurrent vascular lesions, which limits long-term clinical benefits.

To circumvent the drawbacks of non-degradable metallic implants, bioresorbable vascular stents have attracted intensive research interest[3]. Degradable metal systems based on magnesium, zinc and iron alloys are well-established candidates, possessing adequate mechanical strength for vascular support at early implantation stages[7, 8]. However, metallic degradable stents suffer from intrinsic limitations originating from physiological corrosion inside blood vessels[9]. Electrochemical degradation generates metallic ions and insoluble corrosion particles. These particulate debris can be phagocytosed by macrophages, triggering foreign-body giant-cell granuloma, aggravating local inflammation and delaying endothelialization of vessel walls[10-12]. Such particle-provoked inflammatory events severely hamper the expected long-term performance of metal-based bioresorbable stents, driving the demand for alternative non-metallic stent materials.

Natural collagen-based biomaterials are highly appealing candidates for biodegradable implantable devices owing to their intrinsic bio-origin, favorable biocompatibility and tunable degradability. Type-I collagen is the primary structural protein of extracellular matrix and has been widely exploited for wound dressing, tissue engineering stents and bio-medical carrier systems[13]. Even so, native collagen materials face critical bottlenecks for vascular stent applications. Un-cross-linked collagen hydrogels or sponges usually exhibit poor

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mechanical performance, excessive swelling and rapid disintegration under aqueous physiological environments, failing to maintain structural integrity required for vascular support. PMVE-MA (poly(methyl vinyl ether-alt-maleic acid)) is a FDA-approved synthetic copolymer with low intrinsic cytotoxicity, which has been adopted in multiple biomedical scenarios[14]. Combining collagen with PMVE-MA is expected to compensate for the mechanical weakness of pure collagen while retaining biodegradable properties. Notably, yak-skin-derived type-I collagen exhibits higher denaturation temperature compared with collagen from pig, fish or bovine sources, implying superior thermal and structural stability, which is potentially beneficial for implant material fabrication (Fig 1 C and Fig S1).

In this work, we aim to establish and characterize a composite biomaterial based on yak-skin-extracted type-I collagen and PMVE-MA for preliminary screening of biodegradable vascular-stent-relevant performance. First, type-I collagen was isolated and purified from yak skin, and its intrinsic molecular, thermal and structural features were characterized. Secondly, Box-Behnken response-surface methodology was used to simultaneously optimize the fabrication parameters including mass ratio of collagen/PMVE-MA, stirring temperature and stirring duration, with comprehensive desirability index integrating hemolysis rate, swelling degree, compressive modulus and degradation rate. After obtaining optimized composite material, comprehensive characterizations covering microstructure, wettability, physicochemical properties, hemocompatibility and in-vitro cellular behaviors were carried out. Special attention was paid to analyzing the potential trade-off between hemocompatibility and cytocompatibility of this composite system. This study provides baseline experimental datasets for yak-collagen/PMVE-MA biomaterials, and identifies key material bottlenecks that need to be resolved before further translation toward vascular stent applications.

## **2 Materials and methods**

### **2.1 Materials**

The yak skins were purchased from Qinghai Yutai Livestock Products Co., Ltd. PMVE-MA and hexamethylene diisocyanate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphate-buffered saline (PBS) and Purinomycetes were purchased from Beijing Soleibao Technology Co., Ltd (Beijing, China). Fetal bovine serum and minimum essential medium (MEM) basal medium were purchased from Gibco (Life Technologies, California, USA). Penicillin–streptomycin solution was purchased from Wuhan Prosei Biotechnology

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Co., Ltd (Wuhan, China). Cell Counting Kit-8 (CCK-8) was purchased from Elabscience Biotechnology Co., Ltd (Wuhan, China). HepG2 cells were purchased from Pu Nuosai Biotechnology Co., Ltd (Wuhan, China). Ten SD rats, with a body weight ranging from 180 to 220 g and an equal number of males and females, were purchased from the Research and Experimental Center of Gansu University of Traditional Chinese Medicine.

## **2.2 Isolation and purification of type-I collagen from yak skin**

Yak skin was thoroughly washed and cut into pieces of approximately 5 mm × 5 mm. Hair removal was performed by immersion in 2 wt% NaOH aqueous solution for 48 h, followed by repeated rinsing with ultrapure water until the pH of the eluate reached neutral. For lipid removal, skin fragments were soaked in 6 wt% Na<sub>2</sub>CO<sub>3</sub> solution (solid-liquid ratio 1:15, w/v) for 12 h. After rinsing, samples were treated with 5 wt% NaCl solution under continuous shaking for 18 h to remove salt-soluble non-collagenous proteins. The pretreated yak skin tissue was homogenized into fibrous fragments [15-17].

The fibrous tissue was dispersed in 0.5 mol/L acetic acid solution containing pepsin (enzyme-to-substrate ratio 3000 U/g), and digested at 30 °C with constant agitation for 8 h. The mixture was subjected to negative-pressure filtration. Solid NaCl was added into filtrate to achieve final concentration of 4 mol/L for collagen salting-out, and left undisturbed at 4 °C overnight. The precipitate was collected by centrifugation and redissolved in 0.5 mol/L acetic acid. The resulting collagen solution was transferred into dialysis tubing (molecular weight cutoff 3500 Da), dialyzed against ultrapure water for 48 h. Dialysis termination was confirmed by silver nitrate test when no chloride ion precipitate could be observed. The dialyzed collagen solution was pre-cooled and lyophilized to obtain purified yak-skin-derived type-I collagen.

## **2.3 Characterizations of pristine yak-skin type-I collagen**

Atomic force microscopy (AFM, Dimension Icon, Bruker) was applied to observe surface topography of collagen samples within 5 μm × 5 μm scanning area, and surface roughness parameters were statistically analyzed. Fourier-transform infrared spectroscopy (FT-IR) was recorded over wavenumber range 4000–400 cm<sup>-1</sup> to identify characteristic amide-A, amide-I and amide-B absorption bands of collagen secondary structure. Differential scanning calorimetry (DSC) was used to determine thermal denaturation temperature. Amino-acid composition analysis was performed and homology modeling of full-length yak-skin type-I collagen was conducted via Modeller, SWISS-MODEL and

## 2.4 Fabrication and Box-Behnken response-surface optimization of collagen-PMVE-MA composite material

Purified yak-skin collagen and PMVE-MA were separately dissolved in 0.5 mol/L acetic acid. According to experimental design, two solutions were blended at predefined mass ratios, magnetically stirred under set temperature and duration. Three independent variables were selected: (A) mass ratio collagen: PMVE-MA, (B) stirring temperature (°C), (C) stirring time (h). Factor levels were set as A (4:1, 8:1, 16:1), B (37, 45, 55 °C), C (2, 3, 4 h).

After mixing, homogeneous solution was transferred into mould, frozen at −80 °C for 24 h and lyophilized. The obtained porous sponge was cross-linked in 10 vol% HMDI isopropanol solution, subsequently washed three times with pure isopropanol to remove residual cross-linker. After natural volatilization of isopropanol, material was rinsed repeatedly with ultrapure water.

Four response indexes including hemolysis rate, swelling degree, compressive modulus and degradation rate were normalized. Hassan comprehensive desirability method was adopted to calculate overall desirability value (OD)[18].

$$d_{min} = (Y_{max} - Y_i)/(Y_{max} - Y_{min}), \quad (1)$$

$$d_{max} = (Y_i - Y_{min})/(Y_{max} - Y_{min}), \quad (2)$$

$$OD = (d_1 d_2 d_3 \dots d_n)^{1/n}, \quad (3)$$

Where the  $d$ ,  $n$ ,  $Y$ ,  $i$ ,  $Y_{max}$ ,  $Y_{min}$ , are the normalized value of each index, index number, index value, number for experiment, and maximum minimum value as index respectively. For the indicators with small values, Eq. 1 was used to calculate the normalized values while Eq. 2 for large values and Eq. 3 was used to calculate the population normalized values. Design-Expert 8.0.6 software was used to establish quadratic regression model and analyze variance. Validation experiments were carried out under predicted optimal parameters.

## 2.5 Characterizations of composite s

### 2.5.1 Microstructure and wettability

Composite material was sputter-coated with platinum, and surface cross-sectional morphology was observed by scanning electron microscopy (SEM, SU8010, Hitachi, Japan). Static water contact angle measurement (OCA25, Dataphysics, Germany) was performed using a contact-angle goniometer; at least six parallel samples were tested.

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### 2.5.2 Swelling degree

Dry composite material was weighed ( $W_d$ ). Samples were immersed in PBS at 37 °C. At predetermined time points (2, 3, 5, 10, 12, 24 h), samples were taken out, surface free water was gently blotted with filter paper, and wet weight ( $W_w$ ) was immediately recorded. The swelling degree was calculated using Eq. 4.

$$\text{Swelling degree} = \left( \frac{W_w - W_d}{W_d} \right) * 100\% \quad (4)$$

### 2.5.3 Compressive mechanical test

Compressive performance was measured following Chinese national standard GB/T 1448-2005 on universal electronic material testing machine. Specimens were cut into ~10 mm × 10 mm blocks matching fixture dimension. Compressive modulus was derived from stress-strain curves.

### 2.5.4 In-vitro degradation assay

Initial dry weight of composite material was recorded as ( $W_d$ ). Samples were immersed in PBS solution maintained at 37 °C, and PBS medium was refreshed every day. After continuous 14-day incubation, specimens were collected, rinsed with deionized water, lyophilized and weighed as terminal dry weight ( $W_t$ ). The degradation degree was calculated using Eq. 5.

$$\text{Degradation rate} = \left( \frac{W_d - W_t}{W_d} \right) * 100\% \quad (5)$$

### 2.5.5 Hemolysis assay

Rats were anesthetized via intra-peritonea injection of pentobarbital sodium at a dose of 50 mg/kg body weight. Adequate anesthetic depth was confirmed by complete loss of the pedal withdrawal reflex and corneal reflex. After reaching a stable surgical anesthesia state, the rat was secured in a supine position on a sterile surgical platform. A rapid mid-line thoracotomy was performed to fully expose the heart. A 5 mL sterile syringe equipped with an 18-gauge or 20-gauge needle was inserted into the apex of the left ventricle. Blood was aspirated slowly and continuously with minimal negative pressure to prevent hemolysis. Following blood collection, the rat was euthanized by complete exsanguination. Their bodies were all handed over to the Xining Hua Shui Environmental Resources Development Co., Ltd. for disposal.

Rat whole-blood samples were centrifuged to collect erythrocyte pellet, which was washed and diluted to 2 vol% suspension with normal saline. Composite material samples were pre-incubated in normal saline at 37 °C for 30 min, then incubated with 2 vol% erythrocyte suspension for 1 h. After centrifugation, supernatant absorbance was detected by

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spectrophotometer [19]. Normal saline served as negative control ( $A_{ns}$ ), distilled water as positive control ( $A_{ps}$ ), ( $A_{ts}$ ) for test-group sample. The hemolysis rate was calculated using the following Eq. 6:

$$\text{Hemolysis rate} = \left( \frac{A_{ts} - A_{ns}}{A_{ps} - A_{ns}} \right) * 100\%, \quad (6)$$

#### **2.5.6 Platelet adhesion assay**

After centrifuging the upper plasma obtained from part 2.5.5 Hemolysis assay, perform another centrifugation and discard most of the upper platelet-poor plasma and only retain a small amount of liquid. Gently vortex to resuspend the platelet precipitate at the bottom, and this will result in platelet-rich plasma. Platelet-rich plasma was incubated with composite material at 37 °C for 30 min, 60 min and 120 min. After rinsing, adherent platelets were lysed and absorbance at 340 nm was measured to quantify relative platelet adhesion amount.

#### **2.5.7 Cell proliferation assay (CCK-8)**

Composite material extracts were prepared by incubating sterilized composite material in complete MEM medium at 37 °C for 24 h, supernatant was filtered through 0.22 µm filter. Extracts were diluted to three testing concentrations: 12.5 mg/mL, 25 mg/mL, 50 mg/mL. Complete MEM medium without extract was set as control group.

HepG2 cells were seeded in 96-well plates and cultured with corresponding extracts. On days 2, 4, 6, and 8 after seeding, CCK-8 reagent was added and incubated. Optical density values were recorded, and relative growth rate (RGR) was calculated according to the following Eq. 7 (n=8):

$$\text{RGR (\%)} = (\text{OD value of experimental group} / \text{OD value of control group}) * 100\% \quad (7)$$

#### **2.5.8 Cell adhesion and infiltration measurement**

GFP-expressing HepG2 cells were seeded onto sterilized composite material in 24-well plates with seeding density of 25 000 cells/mL (0.5 mL per well). Incubation condition: 37 °C, humidified 5 % CO<sub>2</sub> atmosphere. At 2 d, 4 d, 6 d, 8 d, triplicate samples for each time-point were collected. Confocal laser scanning microscopy Z-stack scanning was performed with 2 µm interval step size, to visualize cell distribution and quantitatively calculate cell infiltration depth inside composite material [20].

### **2.6 Statistical analysis**

All quantitative data were expressed as mean ± standard deviation (mean ± SD). Excel 2007 and Design-Expert 8.0.6 were adopted for data processing and regression modeling. A t-test was used for statistical comparison.  $p < 0.05$  was regarded as statistically significant difference.

### 3 Results

#### 3.1 Intrinsic physicochemical characteristics of purified yak-skin-derived type-I collagen

Purified yak-skin type-I collagen was obtained via alkaline treatment, degreasing, salt-out, pepsin-assisted enzymatic hydrolysis, dialysis and lyophilization (Fig 1 A). Macroscopic observation showed that uncross-linked lyophilized yak-skin collagen presented fibrous morphology, but dissolved rapidly once immersed in aqueous environment, indicating poor water-resistant performance of pristine collagen. AFM tapping-mode imaging revealed heterogeneous nanoscale surface topography for lyophilized collagen, with an average surface roughness of  $18.50 \pm 0.82$  nm over a  $5 \mu\text{m} \times 5 \mu\text{m}$  scanning region (Fig 1 B).

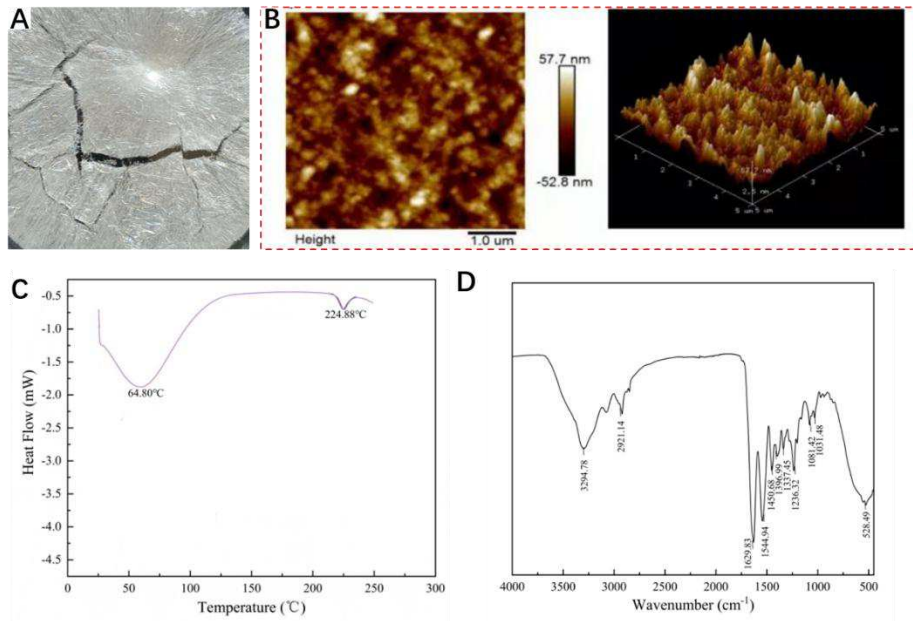


Fig 1 A: The purified type I collagen from yak skin, B: The AFM of the surface of type I collagen, C: DSC of type I collagen from yak skin, D: FT-IR of type I collagen from yak skin

DSC thermal analysis demonstrated that yak-skin collagen possessed a higher thermal denaturation temperature compared with porcine, fish-skin and bovine Achilles-tendon collagen, suggesting enhanced thermal stability (Fig 1 C and Fig S1). Amino-acid composition differences among various collagen sources were summarized in Table S1 and indicated higher content of proline and hydroxyproline. Homology-modeled three-dimensional protein structures of yak-skin collagen chains were shown in Fig. S2 and

Fig. S3.

The FT-IR spectrum exhibited characteristic bands of type-I collagen: the amide-A peak at  $3294.78\text{ cm}^{-1}$  originating from N-H stretching coupled with hydrogen-bonding interaction; amide-I (C=O stretching) located at  $1629.83\text{ cm}^{-1}$ ; and amide-B band at  $2921.14\text{ cm}^{-1}$  corresponding to  $\text{CH}_2$  asymmetric stretching vibration (Fig 1 D). The position of amide-I band indicated dominant  $\alpha$ -helical secondary-structure content within the extracted collagen[15, 21].

### 3.2 Response-surface-based optimization of collagen/PMVE-MA composite material

As shown in Table 1, Box-Behnken experimental design was implemented to optimize three key preparation variables: mass ratio of collagen to PMVE-MA (A), stirring temperature (B), stirring time (C). Hemolysis rate, swelling degree, compressive modulus and 14-day degradation rate were normalized, and Hassan overall desirability value (OD) was calculated to evaluate comprehensive material performance.

Table 1. The results of the single-factor experiment

| Number | A    | B  | C | Hemolysis<br>rate /% | Swelling<br>degree /% | Compression<br>Modulus /MPa | Degradation<br>rate /% | OD    |
|--------|------|----|---|----------------------|-----------------------|-----------------------------|------------------------|-------|
| 1      | 8:1  | 45 | 3 | 0.725                | 152.116               | 0.124                       | 41.722                 | 0.388 |
| 2      | 8:1  | 55 | 4 | 3.352                | 194.485               | 0.145                       | 46.979                 | 0.000 |
| 3      | 16:1 | 37 | 3 | 0.73                 | 105.597               | 0.178                       | 59.277                 | 0.506 |
| 4      | 8:1  | 45 | 3 | 0.726                | 154.348               | 0.183                       | 41.272                 | 0.593 |
| 5      | 8:1  | 45 | 3 | 0.846                | 152.865               | 0.173                       | 44.362                 | 0.551 |
| 6      | 4:1  | 45 | 2 | 0.723                | 217.977               | 0.264                       | 10.432                 | 0.598 |
| 7      | 8:1  | 55 | 2 | 1.805                | 150.881               | 0.117                       | 36.958                 | 0.292 |
| 8      | 4:1  | 37 | 3 | 1.933                | 237.157               | 0.197                       | 11.879                 | 0.000 |
| 9      | 16:1 | 45 | 2 | 0.727                | 163.187               | 0.126                       | 51.841                 | 0.346 |
| 10     | 16:1 | 45 | 4 | 1.825                | 129.754               | 0.13                        | 49.900                 | 0.362 |
| 11     | 8:1  | 37 | 2 | 0.361                | 230.496               | 0.128                       | 14.773                 | 0.269 |
| 12     | 8:1  | 45 | 3 | 0.362                | 160.659               | 0.121                       | 44.678                 | 0.355 |
| 13     | 4:1  | 55 | 3 | 2.721                | 202.388               | 0.179                       | 17.547                 | 0.384 |
| 14     | 8:1  | 37 | 4 | 0.362                | 225.581               | 0.151                       | 24.377                 | 0.364 |

|    |      |    |   |       |         |       |        |       |
|----|------|----|---|-------|---------|-------|--------|-------|
| 15 | 8:1  | 45 | 3 | 1.269 | 151.909 | 0.152 | 37.315 | 0.506 |
| 16 | 4:1  | 45 | 4 | 1.446 | 224.725 | 0.187 | 26.950 | 0.383 |
| 17 | 16:1 | 55 | 3 | 0.723 | 154.697 | 0.111 | 69.361 | 0.000 |

Quadratic regression and analysis-of-variance results were obtained by Design-Expert 8.0.6 software (Table S2). The established regression model was statistically significant ( $p = 0.0179$ ). The interaction terms AB and quadratic term  $B^2$  exerted remarkable influences on overall desirability. According to model prediction, the optimal preparation condition was collagen-PMVE-MA mass ratio of 4:1, stirring temperature of 45 °C, stirring duration of 2 h.

Triplicate validation experiments were carried out under this predicted optimum condition (Table 2). Measured comprehensive desirability value reached 0.617. Key performance indexes of optimized composites were listed: hemolysis rate 0.948–1.288 %, swelling degree 142.960–155.872 %, 14-day degradation rate 13.812–15.550 %, compressive modulus 0.149–0.168 MPa.

Table 2. Properties of composite materials (under optimized conditions)

| Parallel<br>experiment/<br>Material<br>properties | Hemolysis<br>rate (%) | Swelling<br>degree (%) | Degradation<br>rate (%) | Compression<br>modulus<br>(MPa) | OD<br>value |
|---------------------------------------------------|-----------------------|------------------------|-------------------------|---------------------------------|-------------|
| 1                                                 | 1.288                 | 142.960                | 14.331                  | 0.156                           | 0.617       |
| 2                                                 | 0.953                 | 155.872                | 13.812                  | 0.168                           |             |
| 3                                                 | 0.948                 | 144.915                | 15.550                  | 0.149                           |             |

Under 37 °C PBS incubation, optimized composite displayed moderate swelling behavior and controlled mass loss within 14 days of degradation test. The material underwent surface-erosion-dominated degradation mode without obvious fragment shedding. In contrast, the type I collagen composite derived from other organisms, under the same conditions, were basically all dissolved (Fig 2). In addition, the hemolysis rates of optimized samples were far below the 5 % threshold specified for implant biomaterials, satisfying the hemocompatibility requirement for biomaterial preliminary screening.

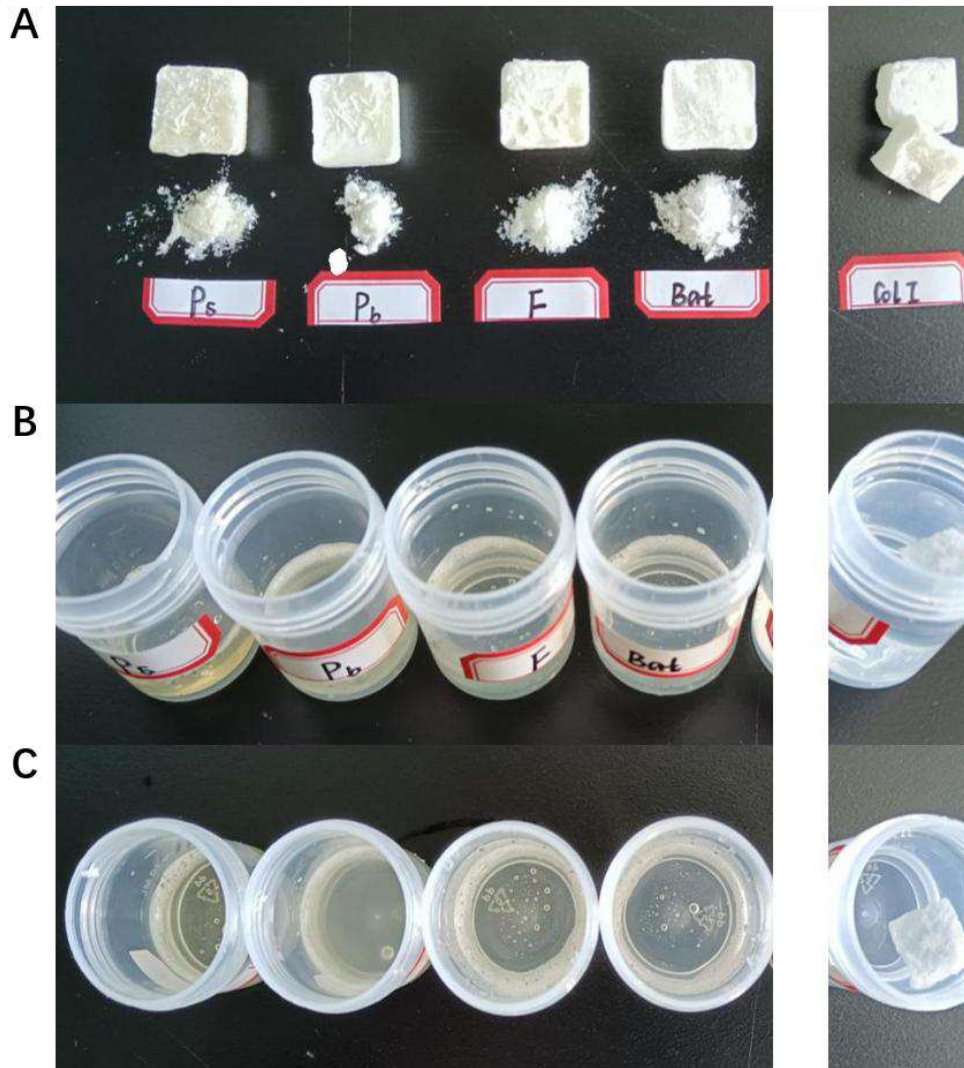


Fig 2 A: Presented macroscopic comparison of composites fabricated from different collagen sources, B: The corresponding composite after being immersed in 37° PBS for 48 hours is shown in the frontal view, C: The corresponding complex after being immersed in 37° PBS for 48 hours is shown in the top view.

### 3.3 Micro-morphology and surface wettability of optimized collagen-PMVE-MA composite

SEM observation demonstrated that cross-linked composite exhibited anisotropic porous architecture originating from ice-crystal growth during freeze-drying (Fig 3 C). Pore structure was lamellar-shaped with limited inter-pore connectivity. Static water-contact-angle test showed that optimized composite possessed prominent hydrophobic surface property, with water contact angle of  $142.3 \pm 2.4$  (n=6). The nanoscale surface roughness observed from micro-scale morphology may have a certain enhancing effect on

its hydrophobicity.

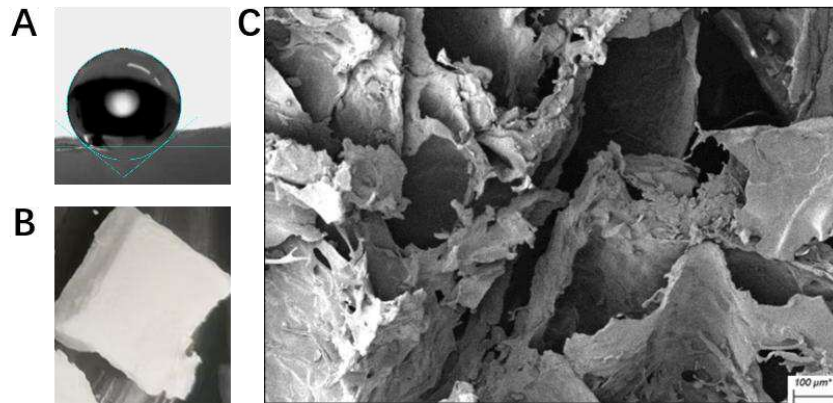


Fig 3 A: The result of the surface wetting property of the complex, B: Macroscopic photo of the composite, C: The SEM image of composite.

### 3.4 Hemocompatibility and in-vitro cellular performance

Platelet-adhesion assay was performed by measuring absorbance at 340 nm after platelet lysis. At incubation time of 30 min and 60 min, platelet-adhesion level maintained relatively stable (Fig 4. A) Significant increase of adherent platelet amount was detected at 120 min. This phenomenon may be attributed to hydration-driven pore expansion of hydrophobic composite material, which generated extra available surface sites for platelet capture after water infiltration. Collectively, hemolysis and platelet-adhesion results indicated acceptable in-vitro blood compatibility of this composite material.

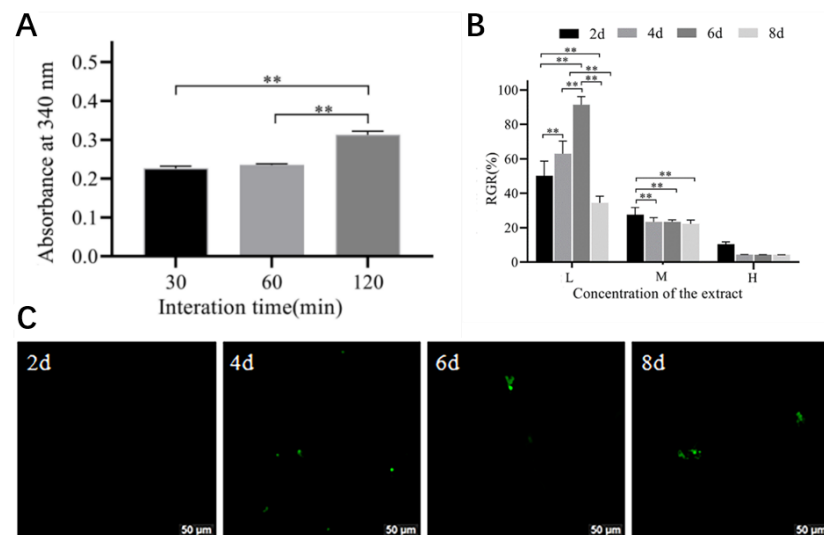


Fig 4 A: Platelet adhesion to the composite material, B: Effect of extracts from composite material on cell proliferation, C: Confocal laser scanning microscopy images of the cells adhering to the composite

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material.

The CCK-8 assay was applied to assess cell proliferation under gradient concentrations of composite material extracts (Fig 4 B). Low-concentration extract group showed partial cell tolerance; relative growth rate (RGR) achieved maximum value of 91.42 % at day 6, followed by subsequent decline. Medium-concentration extracts exerted obvious proliferation-inhibitory effect, keeping RGR consistently below 50 %. High-concentration extracts induced severe cytotoxicity, where RGR remained lower than 20 % for all culture time points. Overall, extracts from collagen-PMVE-MA composite presented concentration-dependent inhibition upon HepG2 cell proliferation.

GFP-labeled HepG2 cells were seeded onto composite material substrates for adhesion and infiltration evaluation via confocal Z-stack scanning (2  $\mu\text{m}$  step interval). Almost no adherent cells could be detected after 2-day incubation (Fig 4 C). Scattered cell clusters appeared on material surface on day 6, and only very limited cell clusters were observed on day 8. Quantified infiltration depth inside composite material was 0  $\mu\text{m}$  (2 d), 10  $\mu\text{m}$  (4 d), 34  $\mu\text{m}$  (6 d), and merely 46  $\mu\text{m}$  at day 8 (Table 3). These results demonstrated that the composite composite cannot support efficient cell attachment, spreading and deep cellular infiltration under current formulation.

Table 3 The depth ( $\mu\text{m}$ ) up to which cells had infiltrated in the composite material

| Incubation time (d) | Infiltration time ( $\mu\text{m}$ ) |
|---------------------|-------------------------------------|
| 2                   | 0                                   |
| 4                   | 10                                  |
| 6                   | 34                                  |
| 8                   | 46                                  |

## 4 Discussion

### 4.1 Merits of yak-skin collagen / PMVE-MA composite system

In this study, a composite material combining yak-skin-derived type-I collagen and FDA-approved PMVE-MA copolymer was successfully fabricated via HMDI cross-linking followed by freeze-drying. Compared with collagens isolated from pig-skin, fish-skin and bovine Achilles tendon, yak-skin collagen exhibited higher thermal denaturation temperature, which originates from its unique amino-acid composition[16]. This intrinsic property confers better thermal stability for the starting biomacromolecule and benefits the structural stability of final composite material.

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Pure un-cross-linked yak-skin collagen sponge dissolves rapidly in aqueous environment, which makes it impossible for implant-related applications[18]. After blending with PMVE-MA and chemical cross-linking, the composite obtained greatly improved water-resistant capacity. Only the yak-collagen-based composite maintained intact morphology after 48 h immersion, whereas other collagen-PMVE-MA groups collapsed. The Box-Behnken response-surface design efficiently screened the interactive effects among mass ratio, stirring temperature and stirring duration [22]. The optimal condition (4:1 mass ratio, 45 °C, 2 h) balanced multiple contradictory indexes including hemolysis, swelling, compression property and degradation rate, rather than merely optimizing single performance metric.

The optimized composite shows suitable compressive modulus and moderate 14-day surface-erosion-dominated degradation without fragment debris shedding. This degradation feature is a meaningful advantage compared with degradable metallic stents. Degradable magnesium- or zinc-based alloys produce insoluble particulate corrosion by-products, which trigger macrophage activation and foreign-body granuloma formation [14]. In contrast, surface erosion mode avoids massive solid particle release and may lower the risk of particle-provoked local inflammation. In-vitro hemolysis test indicated hemolysis rate well below 5 % threshold for implant biomaterials; platelet adhesion test also showed reasonable blood-material interaction behavior [23]. Taken together, from physicochemical and hemocompatibility perspectives, this composite meets primary in-vitro screening requirements for candidate vascular-stent materials.

## **4.2 Key observation: dissociation between hemocompatibility and cytocompatibility**

The most noteworthy finding in present work is the obvious dissociation between satisfactory hemocompatibility and poor cellular-level performance for collagen-PMVE-MA composite. Although hemolysis and platelet-related indexes satisfy biomaterial criteria, the material strongly suppressed cell attachment, spreading, proliferation and infiltration. Even after 8 days incubation, cell infiltration depth only reached 46  $\mu\text{m}$ , and cell clusters were sparse on composite material surface.

The dominant contributing factor is the extreme surface hydrophobicity ( $142.3 \pm 2.4$ ) of cross-linked composite. Despite the lamellar micro-rough porous microstructure revealed by SEM, high surface hydrophobicity inhibits adsorption of cell-adhesive serum proteins such as fibronectin and laminin onto material-liquid interface [24]. Without sufficient adsorbed

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adhesion-mediating protein layer, adherent cells lack effective anchoring sites for focal-adhesion formation, which directly blocks cell attachment and subsequent spreading and inward infiltration.

Two secondary potential influencing factors cannot be completely excluded. Residual trace HMDI cross-linker after washing procedures might introduce mild cytotoxic effects [25]. In addition, surface segregation of PMVE-MA copolymer component during freeze-drying may change surface chemical composition and further worsen cell-material interfacial response[26].

This dissociation phenomenon reminds us that hemocompatibility evaluation cannot replace cytocompatibility assessment. Blood cells (erythrocytes, platelets) and adherent somatic cells rely on totally different interfacial interaction mechanisms when contacting biomaterials. Low hemolysis value only proves that materials do not destroy red-blood-cell membrane; it cannot guarantee favorable conditions for adherent-cell anchoring and growth. Several previous biomaterial literatures have reported similar trade-off effects. Therefore, multi-dimensional evaluation covering both blood-related and cell-related assays is indispensable for screening implantable vascular-stent candidates, rather than relying merely on hemolysis data.

It should also be emphasized that HepG2 tumor cell line was adopted for cytocompatibility screening in this study. HepG2 is not native vascular cell type such as human umbilical-vein endothelial cells (HUVEC) or vascular smooth-muscle cells [27]. Tumor-origin cell line can only serve as preliminary cytotoxicity screening tool, and cannot fully recapitulate real biological response of vascular wall cells *in vivo*.

Based on the above-mentioned mechanistic analysis, several concrete and actionable strategies are suggested for future investigations. Surface modification including plasma treatment, grafting of hydrophilic polymer segments or covalent immobilization of RGD cell-adhesion peptide motifs can be implemented to mitigate surface hydrophobicity and create cell-anchoring sites. Further component-level optimisation, such as readjusting the collagen-PMVE-MA mass ratio and adopting milder cross-linking protocols, is expected to reduce cytotoxic risks originating from cross-linker residues. For biological assessment, primary vascular-relevant cells including HUVECs and vascular smooth-muscle cells should substitute the HepG2 cell line to achieve more physiologically-meaningful *in-vitro* evaluation. If experimental conditions permit, small-animal subcutaneous or intravascular implantation assays are recommended to characterise real-world *in-vivo* degradation profiles, inflammatory cell infiltration and tissue-stent integration.

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## 5 Conclusion

To sum up, the yak-collagen-PMVE-MA composite possesses promising physicochemical and hemocompatible properties, while poor cytocompatibility associated with surface hydrophobicity represents the main bottleneck. This work highlights the non-negligible trade-off between hemocompatibility and cytocompatibility, which deserves attention for future biomaterial screening for vascular implant devices.

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## **Approval for animal experiments**

All the animal procedures were conducted in accordance with the World Health Organization (WHO) Guidance for the Humane Care and Use of Laboratory Animals. All animal procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals produced by the Northwest Institute of Plateau Biology, and were approved by the Animal Ethics Committee of the Northwest Institute of Plateau Biology.

## **Ai Statement**

Deepseek and ChatGPT were used to check for syntax and corrections and did not produce any new content.

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**Author contributions**

S.H.L. analyzed and validated the experimental results, and wrote the manuscript. Y. J. and Y.X.Z. prepared the type I collagen of yak skin, performed and analyzed cytocompatibility of composite materials. L.R. performed the properties of type I collagen. L.G. performed the physical properties of composite materials. Y.J. optimized the manuscript. L.X.W. completed validation. H.X.Y. and M.Y.G. designed the study and reviewed the draft.

**Conflicts of Interest Declaration**

The authors have declared no conflicts of interest for this article.

**Data and code availability**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Supplementary Files

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