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Simple fabrication of a chitin wound healing membrane from Soft-Shell crab carapace

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ABSTRACT

A chitin wound healing membrane was extracted and minimally processed from soft-shell crab carapace, a fabrication method simpler than the conventional process of chemically modifying chitin into more soluble and bioactive derivatives. We observed an interesting difference between the topography of the membrane's internal and external surface, which correlated with their different abilities to promote cell adhesion and proliferation. The internal surface was dotted with prickles where cells could adhere and elongate, while the smooth external surface demonstrated poor cell adhesion and proliferation. Applying the internal surface of the membrane onto the wound improved the epidermis surface homogeneity and accelerated epidermis-dermis re-attachment. This membrane also showed an excellent tensile strength of 105.7 ± 29.9 MPa and ultimate strain of 6.5 ± 4.2%, higher than those of common wound healing membranes from cellulose, and collagen. These results showed the ability of chitin membranes from soft-shell crab carapaces to support cell attachment, proliferation, and migration and its potential for further wound healing applications.

1. Introduction

Chitin is a linear polymer of N-acetyl-glucosamine monomers, commonly found in crustacean shells. Although chitin is biocompatible, antimicrobial, and thermally stable [1], its insolubility in water limits its biomedical applications, and its deacetylated derivative chitosan which is soluble in weak acids [2] is more commonly used. However, chitin is more favorable than chitosan in many regards. Chitin contains acetamide groups similar to native proteins, making it more biocompatible than chitosan. Chitin's monomeric units N-acetyl-glucosamine are present in hyaluronic acid, a naturally occurring extracellular molecule that promotes dermal repair [3]. Most importantly, deacetylating chitin to chitosan requires intensive energy, generates concentrated alkaline waste, and produces a broad range of soluble and insoluble products that are challenging to processed before being released into the environment [4].

To overcome these limitations, we studied a novel source of chitin—soft-shell crab carapaces, which have large surface areas suitable for wound dressings. Soft-shell crabs are molting crabs, shedding their old carapaces to form bigger shells. These newly formed shells are soft and flexible and have not been previously characterized. Most studies on crab carapace have resulted in chitin powder as the product, but few have directly harvested a chitin membrane for wound dressing applications. Direct extraction eliminates the membrane fabrication step and reduces the time, energy, and environmental impact. This study aims to directly extract a chitin wound healing membrane from the carapace and evaluate its mechanical properties, biocompatibility, and wound healing ability.

2. Experimental

2.1. Chitin membrane preparation and characterization

Soft-shell crabs were obtained from a local market, and their carapaces were extracted with scissors. The shells were deproteinized in 1 M NaOH at 50 °C for 5 h, rinsed three times with

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distilled water, and dried overnight at room temperature. They were then demineralized in 0.5%w/v HCl for 5 h, rinsed three times, and dried overnight at room temperature. The scaffold was characterized with scanning electron microscopy (SEM) (JSM-IT100 (JEOL, Japan), accelerating voltage 10 kV), Fourier-transformed infrared spectroscopy (FT-IR) (Spectrum GX, PerkinElmer, USA), and the Texture Analyser (TA.XTplus, Stable Micro Systems, USA).

2.2. Cell proliferation assay

In a 24-well plate, mouse fibroblasts L929 (5×10^4/well) were seeded onto 1×1 cm membranes submerged in 1 mL Dulbecco's Modified Eagle Media (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA). Culture media were changed every two days. The negative control was culture medium. After 1 h, 1 day and

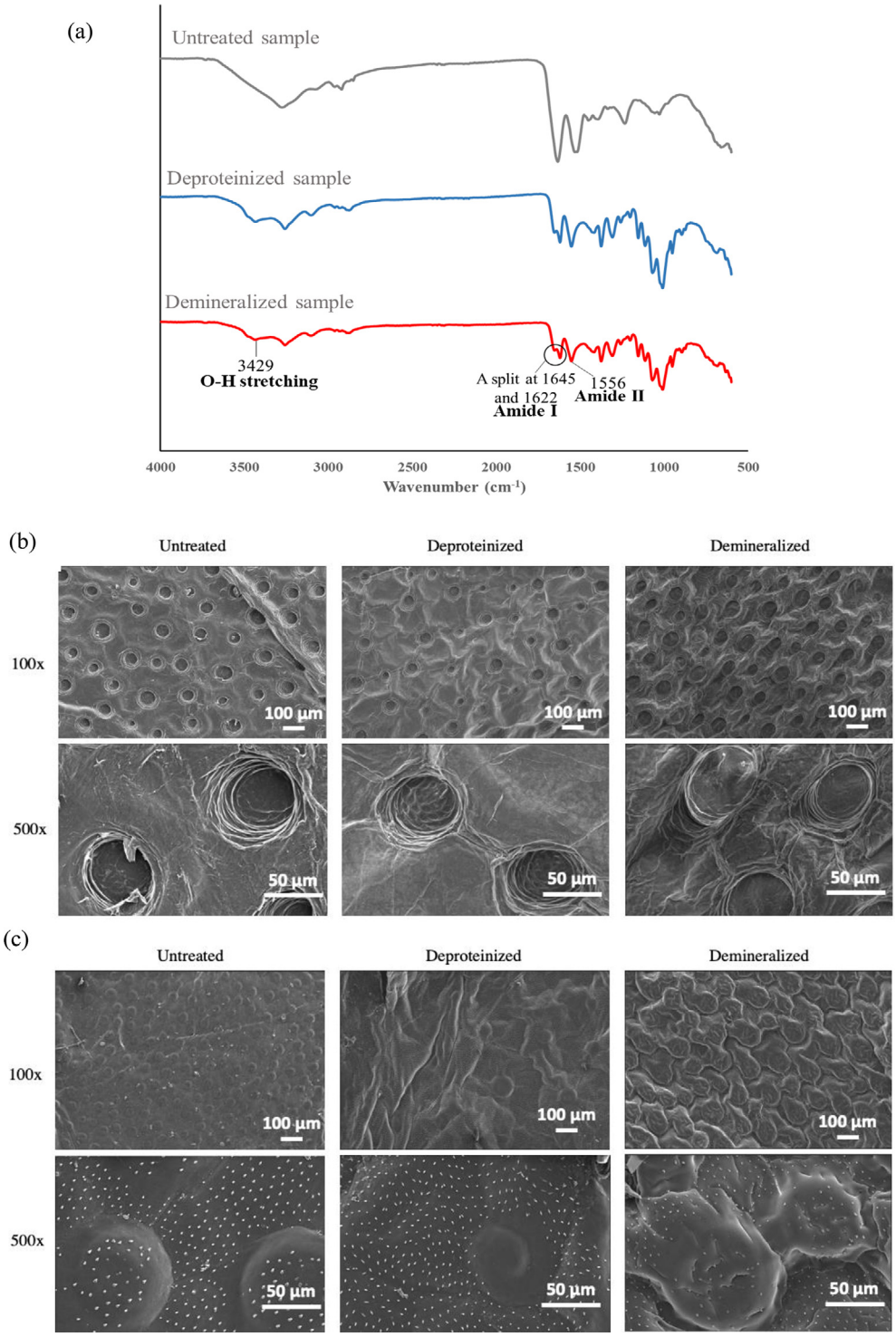

Fig. 1. (a) FT-IR spectrum of the original, demineralized, and deproteinized membranes; SEM micrographs pre- and post-treatment of the (b) external surface and (c) internal surface of the membrane (magnification 100x and 500x).

7 days of incubation at 37 °C, old culture media was replaced with new media containing 0.02 mg/mL resazurin. The samples were incubated for 4 h and read at Ex/Em 530/590 nm using a multimode microplate reader (VariosKan, Thermo Fisher, USA).

2.3. Cell adhesion visualization

After being cultured for 1 h, 1 day and 7 days, samples were washed with phosphate-buffered saline (PBS), fixed in glutaraldehyde 4% (Sigma, USA) for 20 min, dehydrated with graded ethanol (Sigma, USA) 50°, 60°, 70°, 80°, 90°, 95° (3 min each) and 100° (3 times, 10 min each), dried for 12 h, and examined with SEM.

2.4. Animal study

Animal study was approved by the Ethics Review Committee of International University and performed by veterinarians at the University of Agriculture and Forestry (Supporting information, Supplementary method 1). 2 adult male British giant rabbits (2–2.5 kg) were fasted overnight, and a 12 × 14 cm dorsal area on each side of the spine was fully shaved and disinfected with povidone prior to surgery. The rabbits were anesthetized with xylazine (5 mg/kg) and ketamine (80 mg/kg) [5]. On each rabbit, six 1 × 1 cm full-thickness wounds were excised. Three wounds were controls, and three were sutured with membranes rinsed in PBS and soaked in 5% antibiotics for 30 min (Fig. S1). All samples were covered with gauze to prevent infection. Images were taken on day 1, 3, 5, 7, 10 and 14.

2.5. Haematoxylin and eosin (H&E) staining

After 7 and 14 days, the regenerated skin was extracted, fixed overnight with 10% paraformaldehyde, and soaked in 30% sucrose. They were cryo-sectioned (3 μm), stained with H&E, and visualized under a light microscope (Nikon Eclipse, Ti-U series, Japan) [6].

3. Results and discussion

The FT-IR spectrum of the untreated sample showed characteristic peaks of β-chitin—an undivided amide I band at 1640 cm^{-1} due to the hydrogen bonds between the chitin chains and an amide II stretch at 1550 cm^{-1} (Fig. 1a). However, after treatment, the

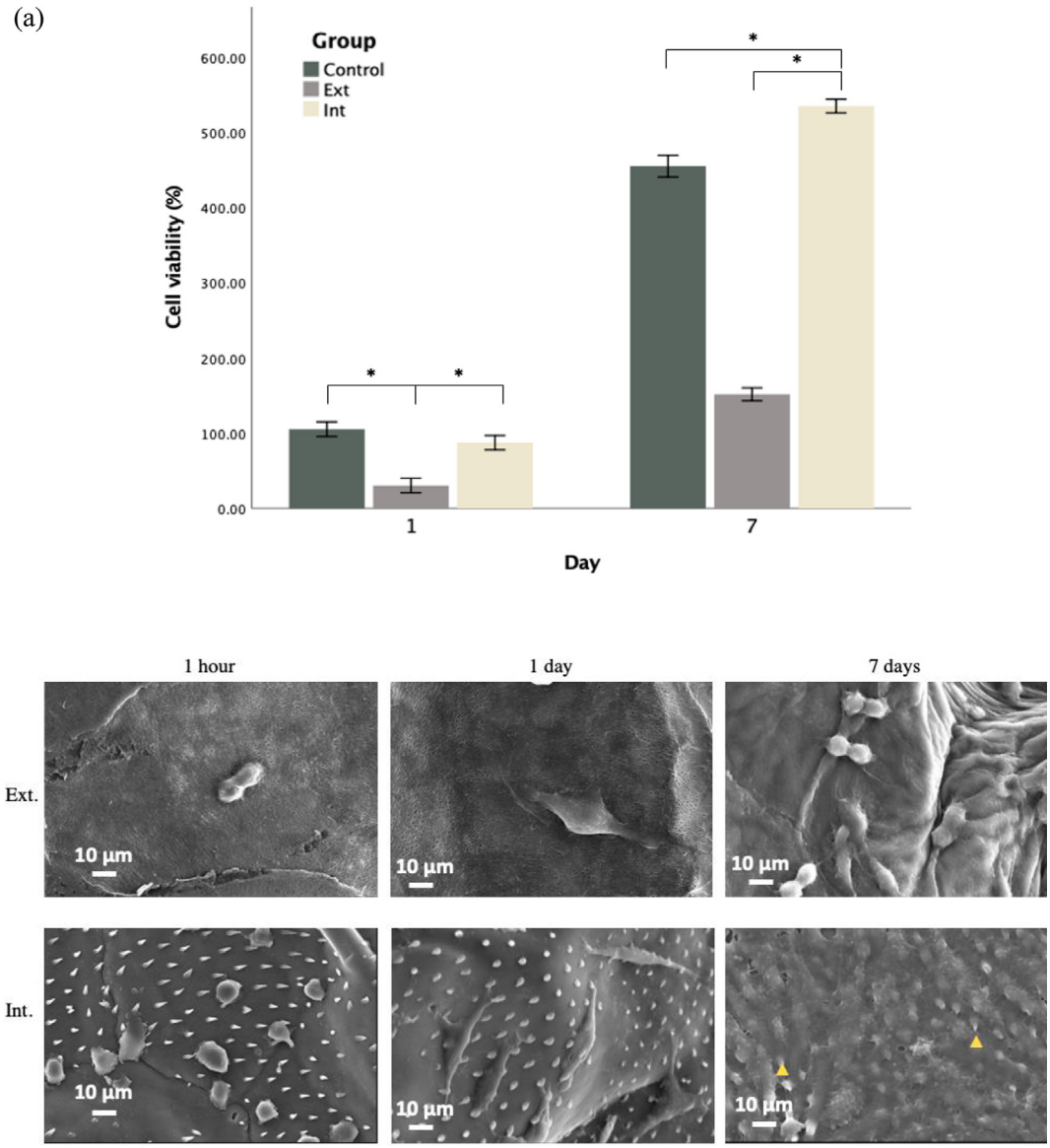

Fig. 2. (a) Cell viability after without the membrane, on the external, and on the internal membrane surface after 1 and 7 days (*: $p < 0.05$) (b) SEM micrographs (1000x) of the membrane after 1 h, 1 day, and 7 days (yellow arrows: prickles).

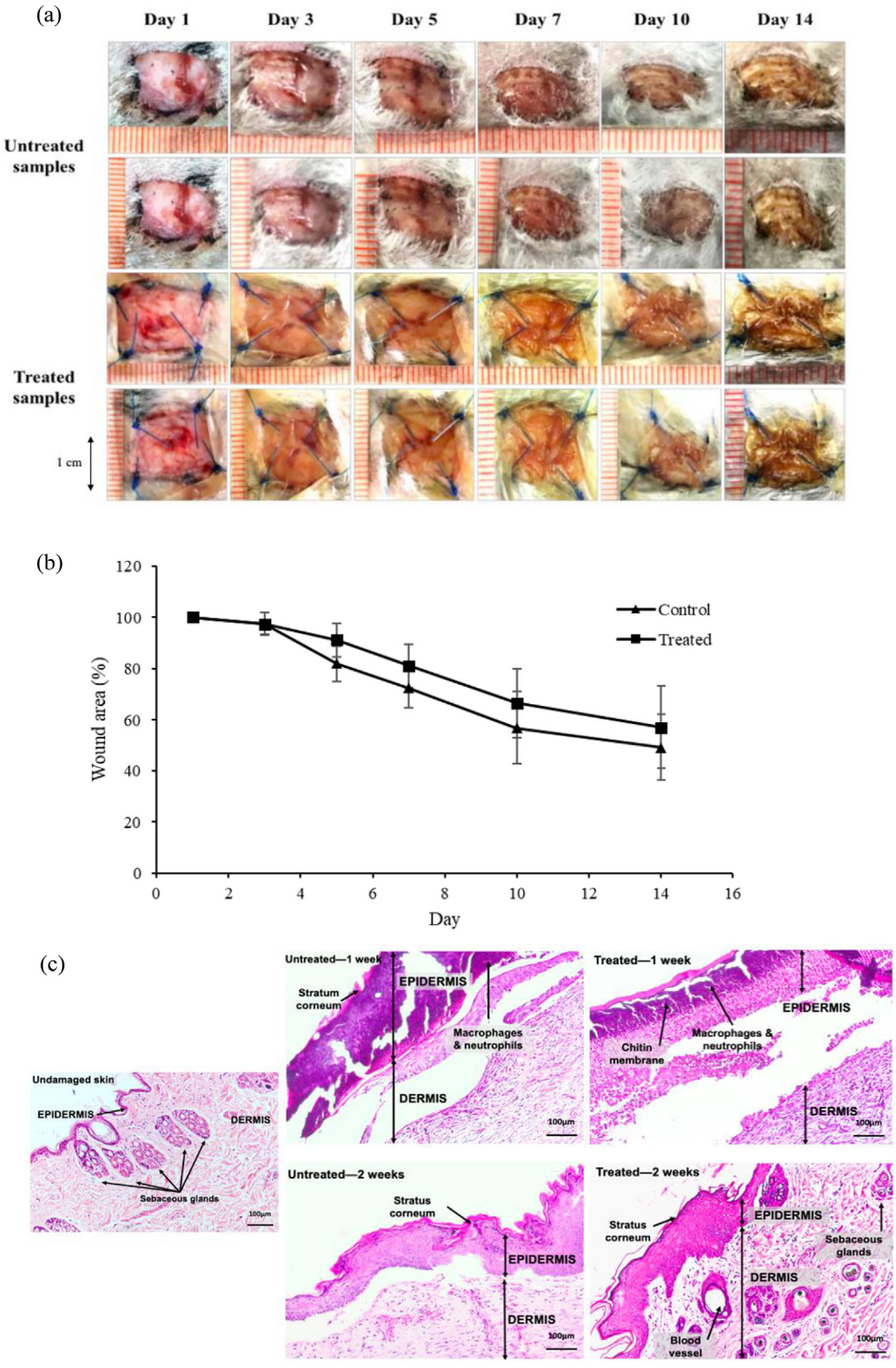

Fig. 3. (a) Wound areas of the treated and untreated samples over 14 days; (b) Average wound closure rate over 14 days (n = 3); (c) H&E staining of the undamaged, treated, and untreated tissue (10x) after 1 and 2 weeks.

amide I band was split into two smaller peaks at 1622 cm^{-1} and 1645 cm^{-1}, indicating the conversion to α-chitin. Additionally, the intensity of the –OH and –NH stretch in the region 3000–3500 cm^{-1} decreased post-treatment, further confirming the change in the intramolecular hydrogen bonds and chitin's conformation [7]. Demineralization and deproteinization made the membrane softer and thinner, but its tensile strength of 105.7 ± 29. 9 MPa (Fig. S2) was more than 30 times higher than previously developed chitin membranes [8]. Cell viability assay demonstrated the membrane's nontoxicity (Fig. S3).

SEM showed significant changes post-treatment and revealed interesting differences between the external and internal membrane surface (Fig. 1b). The internal surface had evenly distributed prickles on 70 μm-diameter dunes not affected by treatments. These prickles were not present on the external surface and have not been previously reported.

These prickles increased the cell-membrane contact area and cell adhesion, shown by *in vitro* assays. After 1 day, cells attached to and spread on both surfaces but adhered more extensively on the internal surface with prickles. The difference in cell proliferation and adhesion between the external and internal surfaces became more pronounced after 7 days (Fig. 2a). SEM observations were consistent and also illuminated the difference in cell morphology. Cells remained spherical on the external surface but elongated on the internal surface. Although after 1 day, cells began to elongate and form filopodia and lamellipodia on both surfaces, after 7 days, cells proliferated more extensively on the internal surface, covering the entire surface and the prickles (Fig. 2b).

Wound healing capacity of the chitin membrane was evaluated on rabbit models. On day 5, a thin scab appeared on the wound of the untreated group, while the membrane started to adhere to the wound. On day 7, the membrane was fully attached to the wound. On day 14, the scab on both groups fell off, with the attached membrane attached on the treated group. No sign of inflammation was observed in either group (Fig. 3a). After 14 days, the wound size of both groups decreased by almost 50%, with no significant difference in wound closure rate between the untreated and treated group (Fig. 3b).

To examine re-epithelialization *in vivo*, we performed histological staining after 1 and 2 weeks of treatment. After 1 week, the epidermis of the untreated wound appeared more damaged and irregular than the treated wound. This could be because the prickles on the internal surface provided adhesion points for proliferating epidermal cells. Furthermore, there was a smaller density of neutrophils and macrophages infiltrated into the treated epidermis, indicating the membrane-treated wound triggered a smaller immune response than the treated wound and the membrane was biocompatible (Fig. 3c). After 2 weeks, the scab and the membrane fell off and were absent in H&E graphs. The difference between the two groups became more pronounced. While there was still epidermis-dermis separation on the untreated wound, these two layers have fully re-attached on the membrane-treated sample. This increase in length of the epithelial migrating tongue could be attributed to unidentified factors in the soft-shell crab carapace, as no growth factors were supplemented during the wound healing process. Future research can determine what this factor is. In addition, the dermis of the treated sample formed sebaceous glands and blood vessels, resembling those of an undamaged dermis, while the untreated dermis did not develop these structures (Fig. 3c).

4. Conclusion

The soft-shell crab carapace demonstrated excellent ability to promote cell adhesion and accelerate epidermis-dermis reattachment, partly due to the prickles suitable for epidermal cells adhesion and unidentified growth factors native to the carapace. This new material requires minimal processing and utilizes soft-shell crabs, an abundant natural chitin source, to reduce the time and energy manufacturing chitin-based wound healing scaffolds.

CRediT authorship contribution statement

Chau Ngoc-Hai Vo: Conceptualization, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Duyen Hong-My Do:** Investigation, Methodology, Visualization, Writing - original draft. **Thang Bach Phan:** Investigation, Methodology. **Quyen Ngoc Tran:** Investigation, Methodology. **Toi Van Vo:** Funding acquisition, Resources. **Thi-Hiep Nguyen:** Funding acquisition, Resources, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.matlet.2021.129995.

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