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ORIGINAL RESEARCH

Engineering of epidermis skin grafts using electrospun nanofibrous gelatin/polycaprolactone membranes

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Abstract: Skin engineering provides a new strategy for treating a wide variety of skin defects. In particular, electrospun nanofibrous membranes have been used as carriers for epidermis engineering. The aim of this study was to investigate the feasibility of a modified gelatin and polycaprolactone (GT/PCL) electrospun membrane for epidermis engineering. The biocompatibility of the membranes was evaluated by seeding HaCaT cells (human keratinocyte cell line) on the membrane and the mechanical properties of the membranes were determined with and without cells after culture. A cell proliferation assay showing that HaCaT cells attached and proliferated well on the membranes demonstrated that the membranes possess good biocompatibility. Mechanical tests showed that the membranes are strong enough to be handled during transplantation. Further in vivo transplantation studies revealed that epidermises engineered with GT/PCL membranes were able to repair skin defects in the nude mouse. These results demonstrate that GT/PCL electrospun membranes could be suitable scaffolds for skin engineering.

Keywords: epidermis engineering, electrospun nanofibrous membrane, gelatin, polycaprolactone

Introduction

The early and permanent coverage of extensive skin injury caused by trauma, burns, or diabetic diseases is usually hampered by insufficient supplies of donor skin. Skin engineering provides a new strategy to treat a wide variety of skin defects.[1] In early studies, cultured keratinocyte (KC) sheets from autologous skin were applied in clinical use;[2] however, the cell sheets are too fragile for engraftment. To improve the mechanical properties of the grafts, various membranes that can support the growth of KCs have been developed.[3–6] Such scaffolds can be classified into naturally occurring or artificial substrates, or combinations of the two.

An ideal scaffold for epidermis engineering should provide suitable mechanical properties that can support the transfer of engineered graft from a culture dish to the wound. It should also have good biocompatibility that can provide a favorable environment for KC growth.[7] In addition, the quality of the scaffolds should be controllable so that stable clinical outcomes can be achieved. Electrospinning technology, which can easily mass-produce thin nanofibrous membranes with good conformability, could offer a solution to the manufacture of scaffolds for epidermis engineering. Electrospun nanofibers resemble the native topographical features of the natural extracellular matrix and may thus promote the cell's natural functions in a biomimetic fashion.[8,9] Several electrospun nanofibrous membranes have been tested in epidermis engineering, including those fabricated from pure natural materials or natural materials combined with

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synthetic polymers.[3,5,10–14] The latter hybrid materials, which combine the merits of the natural and synthetic polymeric components, have shown great advantages. One applicable hybrid scaffold is the gelatin and polycaprolactone (GT/PCL) electrospun membrane. The presence of GT enhances the biodegradability and biocompatibility of the membranes, whereas PCL improves the mechanical properties of the sheets. This hybrid material has been used in both skin and nerve engineering.[12,15]

However, electrospinning of GT and PCL could raise a phase separation problem during electrospinning because of the dissimilarity of the two materials, which might be detrimental to the resultant fiber performance. Fiber inhomogeneities at the ultrastructural level could lead to unfavorable performance (eg, weakened mechanical properties).[3,16–18] To address phase separation related issues, we previously used a tiny amount (<0.3%) of acetic acid to improve miscibility, which clarified the originally turbid solution to be single-phase stable for more than 1 week. The resultant nanofibers appeared to be thinner, smooth, and homogeneous, with enhanced performance in wettability and mechanical properties.[19] Thus, it was of great interest to determine whether this improved membrane could be used for epidermis engineering.

In this study, the mechanical properties and biocompatibility of the improved GT/PCL nanofibrous membranes were investigated in vitro by seeding with human KCs. The potential of the scaffold for skin engineering was further evaluated in vivo by transplantation of engineered epidermis into a wound-healing model in the nude mouse.

Materials and methods

Preparation of GT/PCL membranes

Composite GT/PCL (50:50) nanofibers were fabricated as described previously.[20] The GT/PCL membranes were tailored into round shapes (diameter 15.6 mm), sterilized for 30 minutes under ultraviolet irradiation, and then lyophilized in a vacuum freeze-drier (Virtis Benchtop 6.6; SP Industries, Inc, Gardiner, NY, USA).

HaCaT cell culture on GT/PCL membranes

The human KC cell line, HaCaT, was purchased from Fuxiang Biological Technology Co, Ltd (Shanghai, People's Republic of China) and maintained in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan City, UT, USA) with 10% fetal bovine serum (FBS; Hyclone). Cells at 90% confluency were trypsinized, resuspended in medium, and counted using a hemocytometer. Cells were seeded onto GT/PCL membranes at 2.5×10^4 cells/cm^2 and maintained with medium changes every 2 days. Cell proliferation and biomechanical properties were measured after 7 days of culture.

Confocal microscopic analyses

GT/PCL membranes seeded with HaCaT cells were rinsed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 15 minutes at room temperature, and then counterstained by 4′,6-diamidino-2-phenylindole (DAPI 1:1000; Life Technologies, Carlsbad, CA, USA). Samples were sealed and examined using a confocal microscope (Leica Microsystems, Wetzlar, Germany).

Scanning electron microscopy (SEM) analyses

GT/PCL membranes, with or without cells, were rinsed using PBS, and fixed overnight in 0.05% glutaraldehyde at 4°C. After dehydration through a graded ethanol series, samples were critical-point dried and then examined using a scanning electron microscope (SEM) (JEOL-6380 LV; JEOL, Tokyo, Japan). The pore size of the membrane was measured in SEM by Image J software (National Institutes of Health, Bethesda, MD, USA),[21] and the thickness was determined with the aid of a micrometer.[12]

Cell proliferation assay

The biocompatibility of the scaffold was evaluated by monitoring the cell proliferation of HaCaT cells on the membranes. Briefly, HaCaT cells were seeded on GT/PCL membranes that were commensurate with the size of 24 well plates, at 5×10^4 cells/well in 500 μL of DMEM with 10% FBS. The same amount of culture medium was added into a well with a GT/PCL membrane acting as a control. Cell proliferation was assessed using a Cell Count-8 kit (CCK-8; Dojindo Molecular Technologies, Inc, Kumamoto, Japan) following the manufacturer's protocol. The plates were then measured at 450 nm wavelength on an enzyme-linked immunosorbent assay instrument (Thermo Fisher Scientific, Waltham, MA, USA). Optical density values were calculated from triplicates of each group, and the experiment was then repeated three times.

Biomechanical test

Mechanical properties of the electrospun fibrous membranes with or without cells were determined using a tabletop uniaxial material testing machine (Instron-3343; Instron, Norwood, MA, USA) equipped with a 50 N load cell.

Rectangular shaped specimens (50 mm × 10 mm × 0.10 mm ~ 0.20 mm) were stretched at a constant cross-head speed of 10 mm/minute. Five samples in each group were tested. The load–elongation behaviors of the scaffolds and failure modes were recorded. The structural properties of the scaffolds were represented by typical stress–strain curves, Young's modulus (MPa), breaking strength (MPa), and strain at break (%). The original data were transformed into stress–strain values by

$$\text{Load}/(\text{Width} \times \text{Thickness}) \quad (1)$$

and Change in length/original length using Origin 8.5.1 software (OriginLab, Hampton, MA, USA). For each scaffold, the greatest slope in the linear region of the stress–strain curve corresponding to strain between 0%–20% was used to calculate the tensile modulus.

Transplantation of engineered epidermis in a mouse wound-healing model

Two types of cells were used for epidermis engineering: the HaCaT and human KCs. Human KCs were isolated from foreskin specimens, which were obtained from five donors aged from 6 years to 12 years who underwent a routine circumcision procedure at the Shanghai 9th Hospital with informed consent. The study was performed with approval from the local ethics committee (Shanghai Jiao Tong University School of Medicine, People's Republic of China). KCs were isolated as previously described.[22] Primary KCs were suspended in KC-serum free medium (Gibco®; Life Technologies, Carlsbad, CA, USA) and seeded onto tissue culture plates (BD Pharmingen, Franklin Lakes, NJ, USA) at 3.5×10^4 cells/cm^2 in 10 mL of complete medium and incubated at 37°C with 5% CO_2. Culture medium was changed every other day. Upon reaching 70%–80% confluency, KCs were trypsinized and replated at a 1:3 split ratio. Cells at passage 3 were used for epidermis engineering.

For epidermis engineering, cells (HaCaT or human KCs) were seeded onto GT/PCL membranes (round shape, 1.56 cm in diameter) at 1×10^6 cells/cm^2 in DMEM with 10% FBS. Cell cultures were maintained for 7 days with the medium changed every other day. The engineered epidermises were then used for transplantation.

Twelve male BALB/c nude mice were purchased from the Shanghai Laboratory Animal Center National Rodent Laboratory Animal Resources (Shanghai, People's Republic of China). Mice were anesthetized with intraperitoneal injections of pentobarbital sodium (20 mg/kg body weight). One full-thickness circular wound, 1 cm in diameter, was created on the back of each mouse. The wound was covered by a GT/PCL membrane or engineered epidermis (with HaCaT or human KCs; n = 4 for each group), fixed by 50 nylon sutures at the corners, followed by covering with a Vaseline (Unilever House, London, UK) gauze and adhesive bandages. Animal behavior and bandage integrity were monitored throughout the experiment. Wound healings were evaluated at days 0, 4, 7, 11, and 14 postoperation. Images were recorded with a digital camera (Panasonic Corporation, Osaka, Japan). The wound area was measured by tracing the wound margin using Image-Pro Plus Software (version 5.0; Media Cybernetics LP). The person taking measurements was blinded to the groups and to treatment. Wound-healing rates were calculated as:

$$(\text{Original wound area} - \text{Actual wound area})/\text{Original wound area} \times 100\%. \quad (2)$$

Histological analyses and immunofluorescence staining

For histological analyses and immunofluorescence staining, skin grafts were harvested at day 14, embedded in Tissue-Tek® OCT™ compound (Sakura Finetek, Torrance, CA, USA), followed by snap-freezing and sectioning into 5 μm sections. Cells of donor origin were detected by antihuman human leukocyte antigen (HLA)-ABC staining. Briefly, sections were labeled with a mouse antihuman HLA-ABC monoclonal antibody (1:200; Abcam plc, Cambridge, MA, USA), followed by a secondary Alexa–Fluor 488-labeled goat antimouse immunoglobulin G (1:1000; Life Technologies). The slides were then counterstained by DAPI (1:1000; Life Technologies) and observed under a confocal microscope. After immunofluorescence observation, the slides were further stained with hematoxylin and eosin for histological structure analyses.

Statistical analysis

Data were expressed as the mean ± standard deviation. A two-way analysis of variance was used to determine the statistical significance between groups, and a value of $P < 0.05$ was considered statistically significant.

Results

Preparation of the electrospun GT/PCL membranes

The electrospun GT/PCL membranes were first tailored into a round shape, 1.56 cm in diameter, which was commensurate with the size of 24-well plates (Figure 1A). SEM analysis

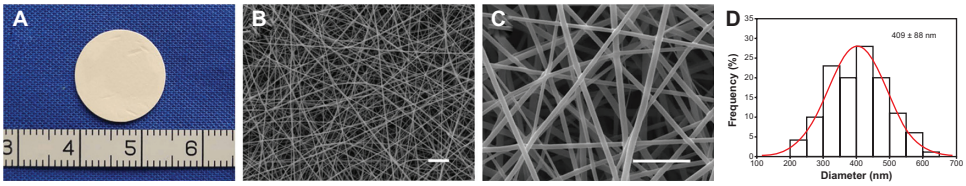

Figure 1 Characterization of electrospun GT/PCL membranes. (**A**) Gross view of a round membrane; (**B** and **C**) Microscopic structure of the membrane by SEM analysis.
Note: Scale bars: 5 μm.
Abbreviations: GT/PCL, gelatin and polycaprolactone; SEM, scanning electron microscopy; D, diameter of fibers.

was performed to evaluate the microscopic structure. As shown in Figures 1B and C, the GT/PCL (50:50) fibers were smooth, uniform, and fine, with an average fiber diameter of 409 nm ± 88 nm (Figure 1D). The average pore size of the membrane was about 7.2 μm ± 1.5 μm and the thickness was 25 μm ± 4 μm (Table 1).

Biocompatibility of GT/PCL membranes in vitro

The biocompatibility of the GT/PCL membranes was tested by seeding HaCaT cells on top of the membrane. HaCaT cells adhered to and spread on the membrane 1 day after seeding. Confocal microscope and SEM analyses showed that cells proliferated continuously on the membrane and reached approximately 85% confluency after 7 days of culture (Figures 2A and B). The results were confirmed by CCK-8 analysis. An increase in the optical density value was observed with increasing culture time (Figure 2C). These data proved that GT/PCL membranes possess good biocompatibility.

Mechanical properties of GT/PCL membranes and engineered epidermis

The engineered epidermis should ideally be mechanically strong for handling during transplantation. Thus, the mechanical properties of GT/PCL membranes with or without cells were evaluated after culture. The representative stress–strain behaviors of electrospun GT/PCL 50:50 nanofibers soaked in culture medium for 8 hours, 1 week, 2 weeks, or seeded with HaCaT cells for 1 week are shown in Figure 3A. Young's modulus and breaking strengths were comparable, and no significant difference was observed between groups (Figures 3B and C). The elasticity (elongation) of the nanofibrous membranes decreased with extended immersion time (Figure 3D), which is likely because of the rapid degradation of GT. Interestingly, seeding of the HaCaT cells could improve the elasticity of the membranes ($P < 0.05$, compared with membranes at 1 week without cells). The membranes were strong enough to be handled after 1 week of culture.

Table 1 Diameter, thickness, and pore size of GT/PCL nanofibrous membrane

Diameter (nm)	Thickness (μm)	Pore size (μm)
409 ± 88	25 ± 4	7.2 ± 1.5

Notes: Data are representative of three independent experiments, and all data are recorded as the mean values ± SD.
Abbreviations: GT/PCL, gelatin and polycaprolactone; SD, standard deviation.

Repair of skin defects with engineered epidermis in vivo

The appearances of animals treated with HaCaT-membranes, KC membranes, or membranes alone were digitally recorded on days 0, 4, 7, 11, and 14 postsurgery. Representative views in each group are shown in Figure 4. Wound closure with shrinking of wound size was observed in all groups at day 14. Statistical analyses from four animals in each group showed that wound closure rates in the groups treated with engineered epidermises, made of either HaCaT cells or human KCs, were significantly ($P < 0.05$) higher than the group treated with membranes alone at days 4, 7, and 11. However, no significant difference was observed between the three groups at day 14, and the majority of the defects were closed (94.4% ± 6.8% in the membrane-alone group; 99.7% ± 0.6% in the HaCaT-membrane group; and 99.8% ± 0.3% in the KC membrane group) (Figure 5). These results indicate that the engineered epidermis could accelerate the wound closure progress.

Histological analyses of repaired skin at day 14 showed that in the epidermis-treated groups, multiple layers of epithelial cells covered the wound area. The repaired area could be distinguished by the absence of skin appendages, which only existed in the native mouse skin (Figure 6). To further determine whether HaCaT cells and human KCs contributed to the wound healing, immunofluorescence staining of anti-human HLA-ABC was performed using cell nuclei count stained by Hoechst. As shown in Figure 6, positive staining of HLA-ABC on the epithelial layers was observed in the groups treated with engineered epidermises, but not in the

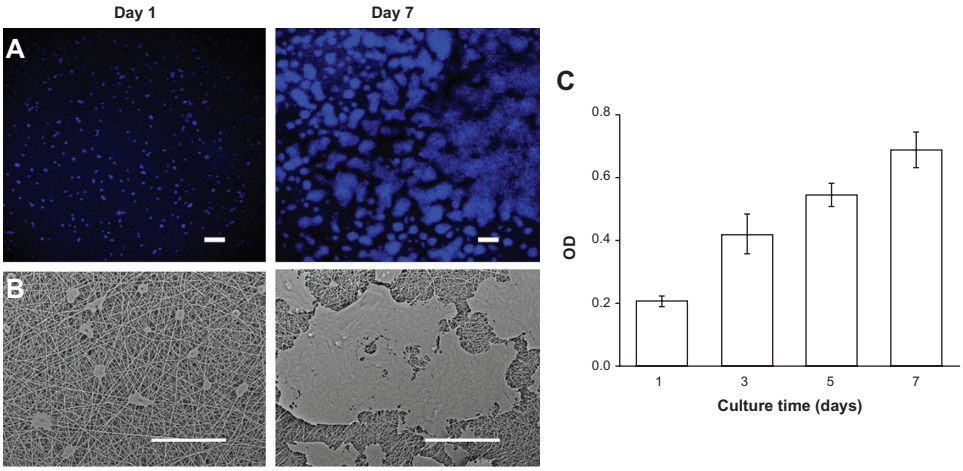

Figure 2 Biocompatibility of GT/PCL membranes. (**A**) SEM images of HaCaT cells on a membrane at day 1 and day 7. Scale bars: 200 μm; (**B**) Confocal microscope images of HaCaT cells on a membrane at day 1 and day 7. Scale bars: 100 μm; (**C**) Proliferation of HaCaT cells on a membrane measured by a CCK-8 kit.
Abbreviations: GT/PCL, gelatin and polycaprolactone; SEM, scanning electron microscopy; CCK-8, Cell Counting Kit-8; OD, optical density.

group treated with the membrane alone, suggesting that donor cells survived and contributed to the wound healing.

Discussion

Good biocompatibility and sufficient mechanical strength are basic requirements for membranes in epidermis engineering. The electrospun nanofibrous membranes of GT/PCL meet the requirements by taking advantage of both material properties. GT is a natural component of the extracellular matrix that can provide a suitable ground for cell adhesion, proliferation, and differentiation, whereas PCL provides good mechanical properties because of its slow degradation rate.[20] Several ratios of GT/PCL combinations were tested in the preliminary studies (unpublished data). An increase in GT content aided in cell adhesion, but decreased the mechanical strength of the membrane. On the contrary, an increase in PCL content

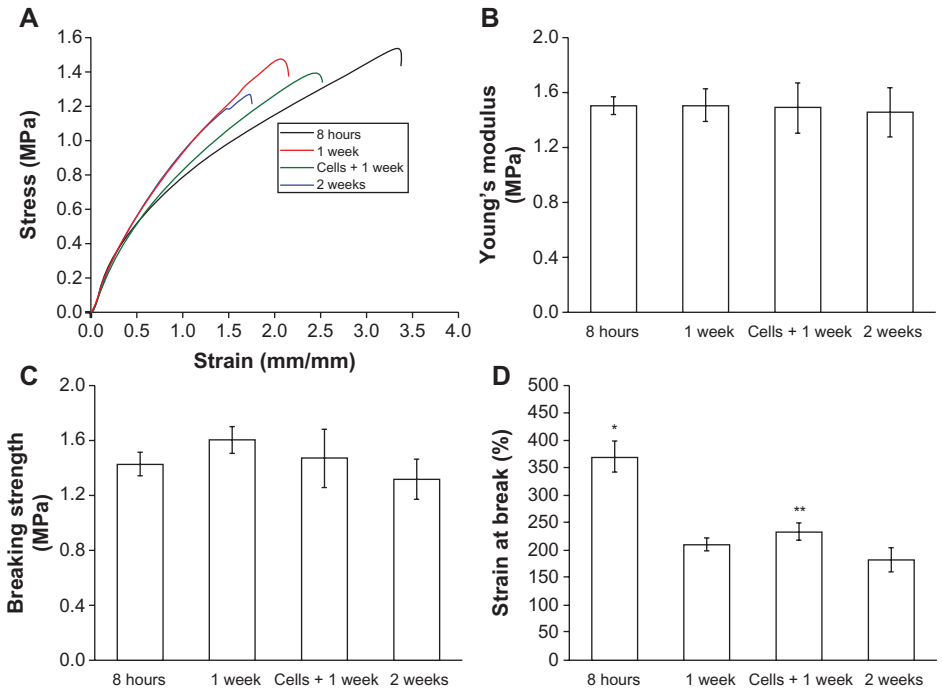

Figure 3 Mechanical properties of the GT/PCL membranes with or without cells. (**A**) Representative stress–strain curves of membranes with or without cells. (**B**) Young's modulus; no significant difference was observed between groups. (**C**) Tensile strength; no significant difference was observed between groups. (**D**) Strain at break; the elasticity of the membranes decreased with extended immersion time.
Abbreviation: GT/PCL, gelatin and polycaprolactone.

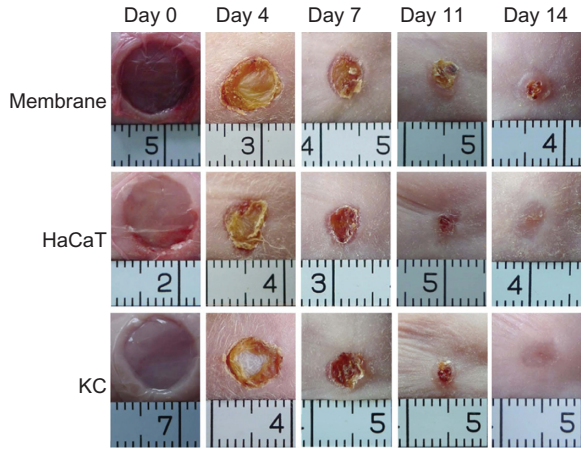

Figure 4 Repair of skin defects with engineered epidermis in the nude mouse.
Notes: Animals were treated with engineered epidermis (HaCaT or KC) or membrane alone, and observed for 14 days.
Abbreviation: KC, keratinocyte.

improved the mechanical strength but reduced cell adhesion. The current GT/PCL ratio (50:50) takes the requirements of both epidermis engineering and further surgical operation into account. The in vitro and in vivo experiments demonstrated that the GT/PCL (50:50) membranes are suitable for epidermis engineering.

Degradation of the GT/PCL membranes is slow. Undegraded membranes could still be observed even after 9 months when used for cartilage engineering (unpublished data). The membrane scaffold in epidermis engineering acted simply as a carrier that supports KC growth. After the KCs covered the wounds and wound healing had progressed, the membranes eventually detached from the skin; therefore, the slow degradation of the membrane is not problematic in practice. Our previous work demonstrated that chitosan–gelatin membranes, which are also slowly degrading materials, could be used for epidermis engineering in animal models as well as in patients.[6,22] Compared with chitosan–gelatin membranes, the current GT/PCL nanofibrous membranes could provide a biomimetic substrate for cell adhesion and proliferation. In addition, the fiber networks create better permeability, which allows for the easy passage of liquid. With this latter advantage, an epidermis engineered with GT/PCL membranes would give a better performance in the repair of wounds with inflammation and exudates. A comparative study of the two membranes in a skin burn model is under investigation.

It is well known that wound repair processes are initiated immediately after injury by various growth factors and cytokine secretions, cellular proliferation, and neovascularization.[23] The improvement of wound healing in the groups treated with engineered epidermis was demonstrated by accelerating the wound closure progress at early time points (Figure 5). The wounds treated with membrane alone were also healed at day 14. This is due to the contraction of the wound in the mouse model. Although a shrinking of wound size was also observed in the experiment groups, HLA-ABC staining confirmed that the wounds were covered by grafted cells, indicating that donor cells played a role in this wound healing process. Studies have shown that engineered epidermis can not only cover the wound, but also secrete growth factors to stimulate skin regeneration.[24–26] Therefore, instead of using epithelial cells, many studies have focused on the modification of membranes with growth factors. Choi et al[27] immobilized recombinant human epidermal growth factor on an electrospun scaffold for diabetic ulcers, whereas Yang et al[28] imbedded human basic fibroblast growth factor into nanofibers to improve the wound recovery rate. Other factors, including granulocyte colony-stimulating factor and platelet-derived growth factor-BB, have also been used for skin tissue engineering.[29,30] Blending of growth factors into the GT/PCL electrospun nanofibers is worthy of investigation in the future.

Electrospun nanofibers have been proven to be a promising scaffold for tissue engineering with limited host response.[31] One of the components, GT, is the nature products of the extracellular matrix that would barely induce an immune response for repairing murine skin trauma,[32] while PCL would not induce inflammatory reactions when used in vivo as a wound dressing.[33] In this study, no significant immune reactivity was observed in the animal study (Figure 6). Whether the GT/PCL membranes possess antiinflammation activity is worthy of investigation in wounds with infection. In addition,

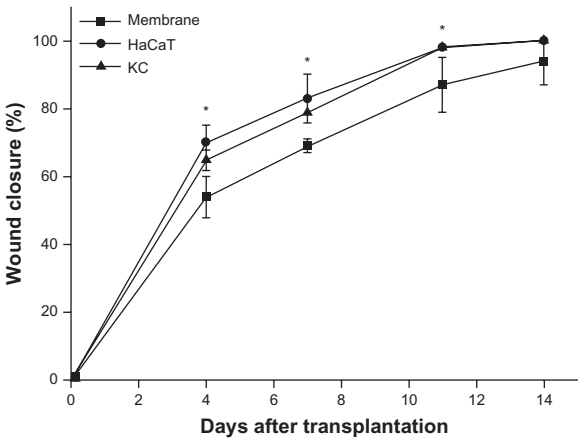

Figure 5 Wound closure rates in each group (n = 4 in each group).
Notes: The groups treated with engineered epidermises were significantly (*$P < 0.05$) higher than the group treated with membranes alone at days 4, 7, and 11, but not at day 14.
Abbreviation: KC, keratinocyte.

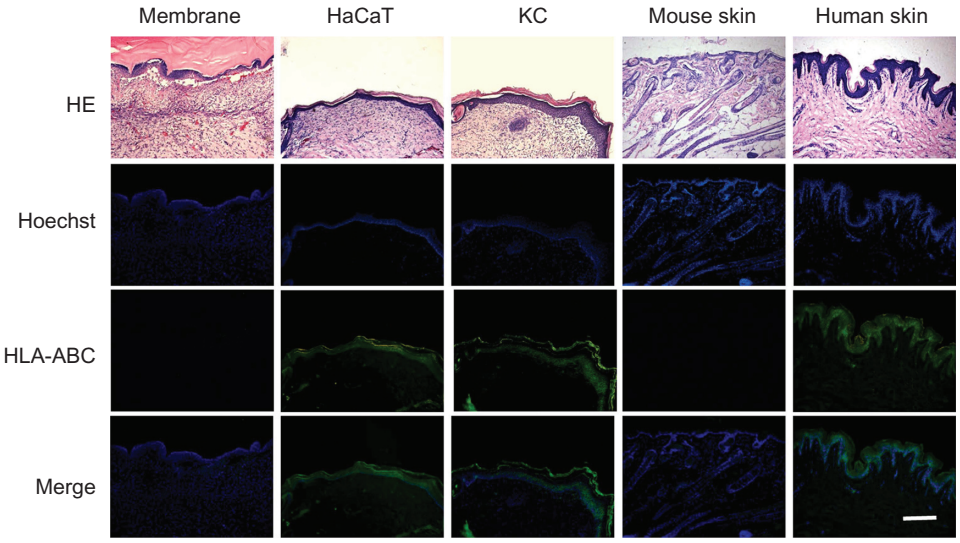

Figure 6 Histology of repaired skin.
Notes: HLA-ABC staining indicates the origin of cells from engineered epidermis. Cell nuclei were count-stained by Hoechst. Scale bar: 200 μm.
Abbreviation: HE, hematoxylin and eosin staining; HLA, human leukocyte antigens; KC, keratinocyte.

nanofibrous membranes embedded with antibiotic drugs are also worthy of being developed.[34]

Conclusion

The current study demonstrated that GT/PCL nanofibrous membranes, improved by the inclusion of acetic acid during the spinning process, possess good biocompatibility and mechanical properties. The membranes may thus be a suitable scaffold for epidermis engineering.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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