Construction of Sandwich-like cell sheets with natural electrospun gelatin/chitosan nanofibrous delivered plasmid VEGF for accelerated wound healing

Yanxia Zhu

Shenzhen University

Yuqi Liao

Shenzhen University

Yuanyuan Zhang

Shenzhen University

Xinyi Wang

Shenzhen University Health Science Center

Jianhao Zhang

Shenzhen University

Ziyang You

Shenzhen University

Cuihong Lian

Shenzhen University

Qianjun He (✉ nanoflower@126.com)

<https://orcid.org/0000-0003-0689-8838>

Research

Keywords: electrospun nanofibrous, gelatin, chitosan, VEGF, adipose-derived stem cell, cell sheet, wound healing

Posted Date: February 19th, 2020

DOI: <https://doi.org/10.21203/rs.2.23977/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background Development of natural biodegradable electrospun nanofibrous with appropriate physical properties and biocompatibility is highly desirable to support multi-layer cell sheets construction for wound healing.

Results We developed a series of electrospun gelatin/chitosan nanofibrous with different gelatin/chitosan ratios and controlled pore sizes, and impregnated plasmid VEGF into membrane, which as supporting membrane to construct sandwich-like adipose-derived stem cells (ADSCs) cell sheets with a simple and effective technique for accelerated wound healing. We found that the physical properties of the electrospun nanofibrous including water retention, stiffness, strength, elasticity and degradation could be tailored by changing the proportion of gelatin/chitosan. We further observed that the optimized electrospun nanofibrous with the optimal ratio of gelatin to chitosan (7:3) which were soft and elastic could most effectively support cell adhesion, proliferation and migration into the whole nanofibrous membranes. Nanofibrous delivered plasmid VEGF facilitating multi-layer ADSC cell sheets formation and promoting regeneration of cutaneous tissues within two weeks.

Conclusions Such natural biodegradable and biocompatible electrospun gelatin/chitosan nanofibrous with plasmid have the potential to become fully cellularized and support sandwich-like ADSC cell sheets formation, which will make it suitable for widespread applications such as skin substitute or wound dressing.

Background

Regenerative medicine is promoted as a promising treatment for the difficult-to-treat diseases and physically impaired function in patients, which heralds a revolutionary shift from conventional symptomatic treatment to radical treatment [1]. Replacement of injured or lost tissues with appropriate cells or tissues is one of desired mechanisms in regenerative medicine. To maximize the regenerative effects, it is indispensable to transplant amount of cells for a long time. Tissue engineering is a promising strategy to overcome inadequate cell number and cell loss after transplantation [1]. Researchers have developed biodegradable scaffolds for tissue engineering and scaffold-free cell sheet-based tissue engineering. Cell sheet engineering involves many disciplines including medicine, biology, engineering, and pharmacy [2, 3], because the noninvasive cell sheet can be engrafted to the desired transplantation site without suturing the cell sheet. Preclinical and Clinical studies have been conducted by cell sheet transplantation for the regeneration of cornea [4], bone [5], tendon [6], myocardium [7] and peridontium [8]. Transplanted cell sheets can replace injured tissue and compensate impaired function when implanted in the ectopic region.

Although the cell sheet formation is a promising and compelling technology for tissue engineering, it is challenging to harvest cell sheet with excellent matrix stability. Single layer cell sheets are quite fragile and easily crumple when they are removed from the culture surface with forceps or other tools, so cell

sheet formation and transplantation processes, such as the layering of sheets, are delicate and labor-intensive tasks [9]. Moreover, layering multiple cell sheets provides limited space for cell growth and nutrient supply, so it's hard to obtain superior cell viability and differentiation potential. A natural biodegradable and non-toxic nanofibrous membrane to support cell sheets would be of great benefit for cell-sheet engineering. The supporting membrane should have unprecedented porosity, a high surface volume ratio and three dimensional (3D) porous structures for cell growth and nutrient supply. Electrospinning is considered to be a simple and versatile method to fabricate nanofibrillar membrane, and the morphology of electrospun nanofibrous can closely mimic the structure of the native ECM and, thus, facilitate cell adhesion, proliferation and differentiation [10]. Electrospun nanofibrous is well known for its native tissue mimicking properties in tissue engineering application [11]. Thus, incorporating essential components of ECM in electrospun nanofibrous to mimic native microenvironment is necessary and efficient approach to support cellular proliferation and differentiation in tissue regeneration.

As is well known, The primary constituent of the ECM is typically collagen [12], gelatin is a denatured form of collagen, it is completely resorbable in vivo, its physicochemical properties can be suitably modulated due to the existence of many functional groups, because of its biological origin, biocompatibility, biodegradability, commercial availability and has not shown any antigenicity, it has been popularly used in tissue engineered scaffolds [13]. Chitosan is also promising biopolymers for tissue engineering because of its biocompatibility, biodegradability, antibacterial and antifungal activity [14, 15]. Moreover, it can be easily processed and formed into various shapes [16], and could achieve hemostasis and allow the promotion of normal tissue regeneration. Chitosan have been applied to diverse biomedical research and therapy, such as drug-delivery carriers, surgical thread and bone healing materials, especially wound dressing [15, 17].

Thus in this study, by combining chitosan and gelatin, with impregnation of plasmid DNA pCMV6-AC-VEGF, we synthesized a crosslinkable electrospun gelatin/chitosan nanofibrous with plasmid. Modification of gelatin and chitosan proportion further introduced glutaraldehyde crosslinking, endowing the natural electrospun gelatin/chitosan nanofibrous with controllable porosity, hydrophilicity, degradation and mechanical properties, as well as good biocompatibility with ADSC, and nanofibrous with plasmid VEGF also have good biocompatibility with ADSC, which all required for aiding wound healing. By mimicking the structure of the native skin, we constructed ADSCs-gelatin/chitosan nanofibrous with plasmid-ADSCs sandwich-like cell sheets, and proved that the sandwich-like cell sheets with plasmid VEGF can effectively aid wound healing by guiding cellular processes, making them ideal candidates for skin regeneration.

Results

The schematic protocol of electrospun gelatin/chitosan nanofibrous development and sandwich-like ADSC cell sheets construction for wound repairing was summarized in Fig. 1. Electrospun gelatin/chitosan nanofibrous with plasmid pCMV6-AC-VEGF were developed by electrospinning. The cross-linked electrospun gelatin/chitosan (7:3) nanofibrous had appropriate degradation behavior, water

retention, mechanical property, and the best biocompatibility with ADSCs compare to the other membranes. Gelatin/chitosan (7:3) nanofibrous was selected for plasmid loading and cells-membrane-cells sandwich-like cell sheets construction, ADSCs were cultured on temperature responsive surface for cell sheet formation. After transplantation, compare to no transplantation and transplantation of nanofibrous with or without plasmid, sandwich-like cell sheets can promote the wound healing significantly, and the structure of repaired skin healing by cell sheets with plasmid is close to normal skin.

Physical characteristics of the electrospun nanofibrous

SEM was used to characterize morphologies of different electrospun nanofibrous and to image the morphological change after cross-linking. Figure 2A shows SEM images of electrospun nanofibrous (non-cross-linked and cross-linked) with different proportion. A well interconnected and randomly oriented electrospun nanofibrous formed a highly pore network structure. SEM images revealed that the electrospun nanofibrous were fine, smooth and overlapped, forming thick network of electrospun nanofibrous, but after cross-linking the uniformity and smoothness of nanofibrous were lost. When the concentration of chitosan was increased, both the average nanofibrous diameter and the pore size of non-cross-linked electrospun nanofibrous decreased. After cross-linking, both the average nanofibrous diameter and the pore size almost remained unchanged (no significance) (Fig. 2B ,2C).

All electrospun gelatin/chitosan nanofibrous displayed typical stress-strain curves. The increase of the Young's modulus with the increase of chitosan was observed in gelatin/chitosan nanofibrous 9:1, 8:2 and 7:3, however, among nanofibrous 7:3, 6:4, 5:5 and 4:6, the Young's modulus decreased with the increase of chitosan. The Young's modulus of all nanofibrous increased after crosslinking, and the Young's modulus was lowest for crosslinked gelatin/chitosan nanofibrous (5:5) at 29 ± 4 MPa and incrementally increased to 84 ± 5 MPa for 9:1 (Fig. 2D). The Young's modulus of normal skin is about 35 kPa, Gelatin/chitosan nanofibrous 7:3, 6:4, 5:5 and 4:6 appeared more flexible/compliant (with slightly lower Young's modulus) compared to the cases of 9:1 and 8:2.

The degradation fluid had an initial pH value of 7.0 ± 0.1 , it almost remained in this level in 35 days (Fig. 2F). The nanofibrous mass changed significantly during the 35 day degradation. Figure 2E shows the residual mass as a percentage of the original sample mass at day 0. The largest loss of mass was observed in the first 10 days, and electrospun gelatin/chitosan nanofibrous 4:6 have the largest mass loss, all the membranes degraded slowly.

The cross-linked electrospun nanofibrous were further characterized to determine their fluid absorption ability after submersion in the PBS solution. The degree of swelling of the gelatin/chitosan nanofibrous 9:1 and 8:2 was 450%, while that of 4:6 electrospun nanofibrous were 230%. The membrane with more chitosan showed less absorption (Fig. 2G). It supported our results in SEM which showed that nanofibrous pore size was decreased with the increase of chitosan showed less percent swelling than all.

ADSC adhesion, spreading and proliferation on electrospun nanofibrous

Figure 3A showed that the ADSCs were spread on the electrospun gelatin/chitosan nanofibrous after seeded for 1 day, well grown and had spread extensively, adhered on the surfaces of the nanofibrous, and more gelatin seems help more cells adhesion and spreading. After 3 days proliferation, under SEM, we observed that the shape of ADSCs changed from round to elongated and almost covered the electrospun nanofibrous 7:3 (Fig. 3B). The cells almost covered the whole membrane after 1 week proliferation on electrospun gelatin/chitosan nanofibrous 7:3 and 6:4 (Fig. 3C), the shape of ADSCs changed from round to elongate. Nanofibrous with more chitosan (5:5 and 4:6) seems not helpful for ADSCs proliferation.

The proliferation of ADSCs on the electrospun gelatin/chitosan nanofibrous was evaluated by quantitative analysis using the cck-8 test. Cell proliferation on the electrospun gelatin/chitosan 7:3 nanofibrous increased significantly on day 14 compared with the control. Based on the results, it was concluded that the nanofibrous displayed no cytotoxicity in this test, as shown in Fig. 3E. Further analysis using confocal laser scanning microscope showed layered cells on different electrospun nanofibrous, especially on nanofibrous of 7:3 (Fig. 3D), which suggested that the change of electrospun nanofibrous substrate property did not pose any obvious toxicity to ADSCs, and the nanofibrous of 7:3 have the best biocompatibility with ADSCs. Cell migration was quantified using the whole thickness of cell-nanofibrous, starting from the top to the bottom of ADSC-GFP (Fig. 3F). It was found that cells seeded on the electrospun gelatin/chitosan 7:3 nanofibrous migrated deepest compared to the other nanofibrous. After 14 days of culture, the cells seeded onto the electrospun gelatin/chitosan 7:3 nanofibrous migrated to the full depth of 921 μm , whereas those on the nanofibrous 9:1, 8:2, 6:4, 5:5 migrated to approximately 706–780 μm (Fig. 3F). Nanofibrous 4:6 showed the lowest cell migration depth (666 μm) compared to the other nanofibrous.

The above results demonstrated that the degradation behavior, water retention, and mechanical strength of g electrospun elatin/chitosan nanofibrous could be changed upon variation of the ratio of gelatin and chitosan. To increase the amount of chitosan in the cross-linked electrospun gelatin/chitosan nanofibrous resulted in decreased water retention, strength and elasticity but increased degradation, and the cross-linked electrospun gelatin/chitosan (7:3) nanofibrous had the best biocompatibility with ADSCs compare to the other nanofibrous. Gelatin/chitosan (7:3) nanofibrous was selected for plasmid loading and in vivo experiments because it was more comparable with natural skin in terms of the capability to retain water, elasticity, strength and good biocompatibility with ADSCs.

Sustained release of plasmid VEGF by electrospun gelatin/chitosan nanofibrous

The structure of nanofibrous-plasmid complex was observed under SEM. The electrospun nanofibrous of gelatin/chitosan were fine and smooth, however, there were some beads, which are complexes of gelatin, chitosan and plasmid DNA on the nanofibrous, and the nanofibrous with beads overlapped layer by layer, and the diameter of nanofibrous seems thinner than the nanofibrous without plasmid (Fig. 4A).

Interestingly, the elastic strength of both nanofibrous has no significant difference (Fig. 4C). The in vitro release profile of plasmid DNA from nanofibrous showed that plasmid DNA was released from

nanofibrous quickly in the first 8 days, nearly 75% plasmid was released. After two weeks, plasmid began to release slowly, and nearly 90% plasmid was released from electrospun nanofibrous at that time (Fig. 4B). ADSCs can spread and grow very well on both electrospun gelatin/chitosan nanofibrous and nanofibrous with plasmid, and VEGF proteins (green fluorescence) in ADSCs were shown on nanofibrous with plasmid (Fig. 4D), after 3 days proliferation, it seems that plasmid pCMV6-AC-VEGF has no obvious effect on ADSC growing on nanofibrous (Fig. 4E, 4F).

Wound closure was affected by cell sheets constructed by nanofibrous with plasmid

Wound closure was assessed by macroscopic observation along the time of implantation and by planimetric analysis of the wound area. There was no significant difference in wound closures between the nanofibrous with or without plasmid groups and the control group (Fig. 5). However, a significant difference in wound area ($p \leq 0.05$) was observed from day 3 post-transplantation in both cell sheets groups, compared to the control and nanofibrous groups (Fig. 6A). It seemed that the presence of the electrospun nanofibrous did not affect wound closure significantly, and the plasmid in the nanofibrous seems has little help to wound healing compare to control group, while the nanofibrous with or without plasmid together with ADSCs synergized to promote wound closure. At day 13, wound transplanted cell sheets with or without plasmid were both healed, while after transplanted 7:3 gelatin/chitosan nanofibrous or nanofibrous with plasmid, the wound was closed at 17 days and 16 days separately, and the wound without transplantation was closed till day 19.

The mechanical property of neo-skin in the cell sheets and cell sheets with plasmid group were similar to that of normal skin, while that of the neo-skin in the control group was significantly lower compared to normal skin (Fig. 6B). To examine the wound healing effect, the thickness of neo-skins at the day of healing was determined considering both the epidermal and dermal layers in H&E histological sections (Fig. 6C, 6D). Masson's trichrome staining carried out at the same time shows the nascent collagen (blue staining) in the regenerating skin. Quantitative analysis demonstrated that the overall thickness of neo-skin in the cell sheets and cell sheets with plasmid group was significantly thicker than that in the other groups (Fig., 6C), and the matrix density has increased significantly with amount of collagen (Fig. 6E). Interestingly, the presence of nanofibrous with or without plasmid slightly increased the thickness and collagen deposition of the neo-skin, compared to the control, and the plasmid in the nanofibrous alone or in the cell sheets can increase the skin thickness and collagen deposition, then improve the wound healing.

Discussion

In this study, we demonstrated a simple and effective technique to construct multi-layer cell sheets using a natural electrospun gelatin/chitosan nanofibrous and ADSCs for accelerating wound healing. The architecture of electrospun nanofibrous made from natural biodegradable and biocompatible material (gelatin and chitosan) was similar to real tissue, which can satisfy physiological requirements. However,

the strength properties of electro spun chitosan fibers are weak [19], and gelatin is sensitive to degradation [20, 21]. In this work, the blending of chitosan with gelatin can improve the mechanical properties of chitosan fibers, what's more, chitosan is a hard electrospinning biomaterial, and gelatin can increase the spinnability of chitosan. The blend electro spun method also creates a new component without the need for synthesizing a new co-polymer. Therefore, we combined gelatin with different ratios of chitosan to fabricate electrospun gelatin/chitosan nanofibrous.

The advantages of the electrospinning technique include the production of very thin nanofibrous membranes with large specific growth surface, which allows for cell proliferation and functionalization. It is reported that both fiber diameter and pore size of electrospun nanofibrous membrane affect cell proliferation and differentiation [22]. The morphology of gelatin/chitosan nanofibrous clearly demonstrates that gelatin concentration was very important in obtaining fine and large gelatin/chitosan fibers, more gelatin in membrane exhibited much higher mechanical strength than the composite containing more chitosan. The mechanical property of skin is important to its structure, appearance and functionality [23], so the mechanical strength of electrospun nanofibrous is also important for the cell sheets construction and wound healing. However, electrospun nanofibrous of natural biomaterials usually possess inferior mechanical properties and fast degradation rates, crosslinking is a common approach for improving the mechanical and degradation properties of natural biomaterials for tissue engineering applications [24]. A crosslinking treatment of gelatin/chitosan nanofibrous with glutaraldehyde effectively optimizes mechanical properties and modifies biodegradation rate. Our crosslinked gelatin/chitosan (7:3) electrospun nanofibrous showed the highest mechanical strength because this fiber was uniform and had medium-size in diameter, both of which are important factors dictating the mechanical properties of electro spinning [25].

The property of nanofibrous biodegradation is beneficial for skin regeneration as it can support and regulate skin regeneration [20]. Gelatin absorbed an amount of water and made the nanofibrous more hydrophilic and sensitive to degradation, while more chitosan exhibited slower degradation, which may inhibit wound healing [26]. So the crosslinked electrospun gelatin/chitosan (7:3) nanofibrous with appropriate biodegradation may be more suitable for skin regeneration. Additionally, the degradation products of gelatin and chitosan are relatively non-toxic small molecules, which can easily be excreted directly or after entry and exit from various metabolic pathways [20, 27].

Natural biomaterials usually possess better biocompatibility and biofunctionality, we indicated that electrospun gelatin/chitosan nanofibrous had good biocompatibility with adipose-derived stem cells (ADSCs). ADSC is a promising adult stem cell for clinical therapy, because it can be easily isolated from adipose tissue, and has strong proliferation and differentiation ability [28]. The electrospun gelatin/chitosan nanofibrous (7:3) with appropriate mechanical strength and biodegradation can accelerate more ADSC adhesion and proliferation. Studies indicated that culture surface with higher stiffness favored cell adhesion and spreading [29, 30], and hydrophilic surfaces favored BMSC spreading [31], which are consistent with our observation. The electrospun gelatin/chitosan nanofibrous was shown to not be toxic at all compositions.

The electrospun gelatin/chitosan nanofibrous are positively charged, which can be a promising nonviral vector for gene transfection. Negatively charged molecules can be ionically bound to positively charged free amino groups of chitosan, which can protect molecules from the destruction caused by enzymes or other harmful factors [18]. Some researchers combined cationized gelatin with plasmid DNA and impregnated the complex into a collagen/polyglycolic acid scaffold to enhance the formation of engineered bone tissue [32]. Endogenous angiogenic factors, vascular endothelial growth factor (VEGF), are naturally produced in response to tissue hypoxia and during wound healing [33]. Because of inadequate secretion of VEGF, this restorative process is insufficient to prevent tissue ischemia and necrosis, exogenous delivery of VEGF is necessary for wound healing. After impregnation of plasmid DNA VEGF into electrospun gelatin/chitosan nanofibrous (7:3), the positively charged gelatin and chitosan firmly immobilize negatively charged plasmid DNA, the release of plasmid DNA was unlikely to be simple diffusion through the surface of nanofibrous, it will be completely released only when the nanofibrous is degraded to generate water-soluble fragments. The electrostatic interaction between the negatively charged cell membrane and the positively charged polymer attaches the gelatin/chitosan-plasmid complex to ADSCs, which promote the long-term expression of VEGF.

The electrospun gelatin/chitosan nanofibrous (7:3) with plasmid VEGF can promote ADSC proliferation and VEGF expression, so it could be a good supporting nanofibrous membrane for sandwich-like cell sheets construction. Cell sheet was established with a confluence layer of cell populations on a temperature responsive surface [10, 34]. Our previous study suggested that at least 5 days of culture was necessary for sufficient matrix deposition, and then the thick cell sheet was easily to be peeled off from culture surface with PVDF membrane [35]. Even so, it was hard to transplant intact cell sheets without PVDF membrane because they were too thin and fragile. Using biodegradable electrospun gelatin/chitosan nanofibrous as supporting membrane to replace of PVDF membrane, it was much easier to operate and form ADSC- nanofibrous -ADSC sandwich-like cell sheets, and transplant for wound healing.

A mouse dorsal wound healing model was used to investigate the skin regeneration potential of sandwich-like cell sheets. The whole wound healing process included cell adhesion, proliferation as well as migration, and eventually leads to skin regeneration [36]. Our in vivo skin defect models demonstrated that complete wound closure and re-epithelialization was only observed after implanting sandwich-like cell sheets, and the time of wound healing was faster than the case of no transplantation. In contrast, the treatment with electrospun gelatin/chitosan nanofibrous only had no obvious beneficial effect compared to the no-transplantation group with respect to wound closure, and both of them lack of a complete epithelial layer. It may be reasoned that the ADSC adding to electrospun gelatin/chitosan nanofibrous promoted its biomechanical features, and provided a better wound healing environment. So both ADSCs and nanofibrous were the key elements for wound healing and skin regeneration. Although wound closed almost at the same time after transplantation of cell sheets and cell sheets with plasmid VEGF, the wound skin transplanting of cell sheets with plasmid had thicker and complete epithelial layer than that of cell sheets only, which indicate that VEGF in the nanofibrous can promote wound healing and skin

regeneration. Of course, it will have better repairing effects if we differentiated ADSCs into fibroblasts and epidermal cells before seeding on the nanofibrous, it will be our next work.

Conclusions

In this study, we successfully fabricated electrospun gelatin/chitosan nanofibrous with different proportions. The gelatin/chitosan (7:3) nanofibrous has best biodegradation and mechanical strength, as well as good biocompatibility with ADSCs. After impregnation of plasmid DNA VEGF into gelatin/chitosan (7:3) nanofibrous, we used it as supporting membrane to construct ADSCs- nanofibrous -ADSCs sandwich-like cell sheets, after transplantation, cell sheets with plasmid can obviously promote wound closure and skin regeneration. Here we developed a class of new and easy operating sandwich-like cell sheets combined with ADSC cell sheets, gelatin/chitosan nanofibrous and plasmid DNA VEGF, and demonstrated that it holds promise for use for tissue regeneration and other biomedical applications.

Methods

Preparation of electrospun gelatin/chitosan nanofibrous

15% gelatin and 1% chitosan were suspended in hexafluoroisopropanol (HFIP) separately to form solutions. To this stirred suspension chitosan (1% w/v) and 15% (w/v) of gelatin solution were added with different proportion (15% gelatin : 1% chitosan, 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1), The mixture was stirred at room temperature overnight. Electrospinning was done at a voltage of 8 kV to create micro/nanofibers with a needle having an inner diameter of 20 gauge and a 0.6 mL/h feeding rate of solution using a syringe pump. The cellophane collector plate was placed 12 cm away from the tip of the needle. Upon the completion of the electrospinning, the electrospun nanofibrous were removed from the collector and cross-linked with glutaraldehyde for 1 hour.

For the impregnation of plasmid DNA into electrospun nanofibrous, 2 mg plasmid DNA pCMV6-AC-VEGF (500 µg/µL, vector pCMV6-AC-GFP was bought from ORIGENE, Beijing) were gently mixed with gelatin/chitosan electrospun solution, electrospinning was done as previously described. The charge ratio (N:P) that is indicated as the molar ratio of the free amino groups of electrospun gelatin/chitosan nanofibrous to the phosphate molecules of plasmid DNA is 5.0, according to our previous results, to obtain the highest transfection efficiency [18]. The electrospun nanofibrous with plasmid also need to be cross-linked with glutaraldehyde to avoid fast-degrading.

Characterization of electrospun nanofibrous

The morphology of the electrospun nanofibrous was studied by a scanning electron microscope (JCM 6000 JEOL, Japan). The samples were sputter coated, placed on SEM holders and an accelerating voltage of 10 kV was utilized for imaging. Using image processing software (Image J) the average parameter of 100 pores and the diameters of 100 electro spun fibers were measured from the obtained SEM images.

Electrospun nanofibrous were placed in a well of 24 well plate with 2 mL phosphate buffer solution (PBS) and kept at 37°C for degradation time periods of T = 5, 10, 15, 20, 25, 30, and 35 days (n = 5 samples at each time point). An additional time point of T = 0 days refers to nondegraded reference samples. Since the non-cross-linked nanofibrous is fast-degrading(degraded in PBS within 2 h, data didn't show), it is not suitable for the construction of cell sheets, only cross-linked nanofibrous was examined. After degradation, the samples were removed from PBS and dried in a vacuum (YIHENG17, Shanghai, China) at room temperature for 90 min. The remaining weight percentage of nanofibrous was calculated according to the following equation:

$$\text{Residual mass (\%)} = \frac{W_t}{W_0} \times 100\%.$$

Where W_0 is the initial weight of the nanofibrous and W_t is the remaining weight of the nanofibrous at each time point. The pH of the degradation fluid of each sample was measured at each time point (METTLER TOLEDO SevenGo pH meter SG2, Switzerland).

Cross-linked electrospun nanofibrous were submerged in PBS at 37 °C for 24 h. Swelling degree was then measured by the formula: Degree of swelling (%) = $[(M - M_d) / M_d] \times 100$. Where M is the weight of each sample after immersion in the PBS for 24 h, M_d is the weight of the sample in its dry state. Only cross-linked nanofibrous was examined.

The nanofibrous were collected using cellophane, and then the thickness of each sample was measured by SEM. These samples were measured, cut carefully and glued on the frame, and then the cellophane was removed. The paper frame was cut before initiating the tensile strength measurements. The tensile properties of these samples were characterized by a tensile testing machine (UTM150, Advanced Nanomeasurement Solutions, Agilent Technologies). The velocity was 100 $\mu\text{m/s}$. The data were analyzed with linksys32 software. The reported Young's modulus represented average results of the five samples.

Sustained release of plasmid DNA from electrospun nanofibrous was measured by Microplate Reader (Epoch2, Biotech, USA). Phosphate buffer saline (pH 7.4) was used for in vitro plasmid release studies. Three plasmid loaded nanofibrous were cut into small equally sized pieces separately. 1 mg of nanofibrous was analyzed for the release studies at 37 °C and stirring at 100 rpm. 1 μL of aliquots of each plasmid loaded samples were assayed at predetermined regular time intervals: 0, 1, 2, 4, 6, 8, 10, 12, 14 and 16 days, 5 aliquots of each sample were done at each time point. The release profiles were plotted as a function of time in terms of cumulative percentage plasmid released.

Biocompatibility of different nanofibrous with cell

Electrospun nanofibrous 10 mm in diameter were sterilized with 70% ethanol (30 min), then washed with PBS and suspended in culture medium (15 min). Human adipose-derived stem cells were isolated according to our previous method [18] (Human subcutaneous adipose tissue was obtained from subjects undergoing surgery with informed patient consent, and the study was approved by the Medical Ethical

Committee and the Animal Ethical and Welfare Committee of Shenzhen University), and cultured in DMEM with 10% FBS, then ADSCs were transfected with green fluorescent protein (GFP) by adenovirus vector to obtain ADSCs-GFP) ADSCs-GFP were cultured on electrospun nanofibrous. The same cell number was seeded on different nanofibrous and cultured at 37°C in a humidified air atmosphere with 5% CO₂. For the VEGF protein expression, ADSCs without GFP were seeded on the nanofibrous, because the plasmid vector pCMV6-AC-GFP can expression GFP, and VEGF proteins will show green under confocal laser scanning microscope after expression in ADSCs. After 3 days of culture, cellular constructs were harvested and fixed with 2% glutaraldehyde for 15 min and washed with PBS two times (15 min/time). The samples were then dehydrated through a series of graded ethanol solutions (50, 75, 90, 95 and 100%) and 5 min was used for every washing time. The cells were then washed three times in 100% ethanol. All samples were air-dried overnight. Dry cellular constructs were sputtered with gold and imaged by SEM.

After 2 weeks culturing, ADSCs on electrospun nanofibrous were washed once with PBS, and confocal laser scanning microscope (Leica Microsystems) was used to observe cells on nanofibrous. Cell migration was quantified using the whole thickness of cells, defined as the average depth of cell layers detected by confocal laser scanning microscope.

To test cell proliferation on the electrospun gelatin/chitosan nanofibrous, we seeded the cells at the same density and incubated the samples for different time periods. The prepared nanofibrous were cut according to the diameter of the well in a 96 well flat bottom plate. Each of the 96 wells contained nanofibrous adjusted separately at the bottom of each well under aseptic conditions. The electrospun nanofibrous were sterilized with 70% ethanol followed by washing with PBS and culture medium. Culture medium containing cells suspension at a density of 5×10^4 cells/ml was added to each well. Wells devoid of nanofibrous were filled with same amount of cell suspension used as control. After the incubation period, 10 μ l of sterile cell counting kit-8 were added to each well and incubated for 3 h at 37°C. The percentage of viable cells was determined every day for 15 days. The optical density values were examined at a test wavelength of 450 nm and reference wavelength of 630 nm, and at least in triplicate against a reagent blank.

Preparation and transplantation of sandwich-like cell sheets

According to the physical characteristics and biocompatibility with ADSC, we chose electrospun gelatin/chitosan (7:3) nanofibrous with or without plasmid to make cell sheets. After culturing ADSCs on the surface of temperature responsive culture dish to a confluent layer, all media were aspirated and fresh media were added to prevent the cells from drying out. The electrospun membrane nanofibrous with or without plasmid was gently placed on top of the cell layer and incubated at 20°C for 20 min. Forceps were used to grasp under the nanofibrous and cell layer, and carefully withdraw them from the surface. The nanofibrous, with the attached cell layer facing downward, was transferred to another cell layer on the thermoresponsive cell culture surface and incubated at 20°C for 20 min, the detached cells-nanofibrous-cells sandwich-like cell sheets were used for transplantation.

All animals were treated in compliance with the Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8523, revised 1996). The protocol was approved by Animal Ethical and Welfare Committee of Shenzhen University (SYXK: 2018 – 0140). All efforts were made to minimize suffering and numbers of mice used. Specific pathogen free kunming mice, were purchased from Guangdong Medical Laboratory Animal Center, weighting 32 ± 2 g, were anesthetized in a chamber of 2% isoflurane (Jinan Shengqi pharmaceutical Co, China) with 40% oxygen and 60% nitrous oxide. After shaving, one excision of 2 cm diameter round shape was performed in the dorsum of each animal. Wounds were assigned randomly to the following groups (n = 6 wounds for each group): (1) empty wound (control), (2) 7:3 nanofibrous alone (nanofibrous), (3) 7:3 nanofibrous with plasmid (nanofibrous-V), (4) ADSCs-nanofibrous-ADSCs (cell sheets) and (5) ADSCs- nanofibrous with plasmid-ADSCs (cell sheets-V). The nanofibrous or cell sheets were transplanted and covered on the wound, the implanted nanofibrous and cell sheets adsorbed on wounds and wouldn't be dislocated. All wounds were covered and bandaged with surgical dressing. Animals were kept in individuals cages with food and water ad libitum, and observed daily during the total period of the experiment. At the day of wound healing, animals were euthanized by CO₂ inhalation and the regenerated skins were excised out and collected at the established end points, fixed in 4% paraformaldehyde and paraffin embedded. Representative sections were stained for hematoxylin & eosin (H&E) and Masson's trichrome following routine protocols. Sections were analyzed and images acquired with an optical microscope, Olympus BX-41/Q-Color3 digital camera (Olympus, Japan).

Statistical analysis

The results are shown as mean $\pm$ SEM. All experiments were independently repeated for at least 3 times unless otherwise stated. ANOVA with a post hoc Dunn or Bonferroni test was used to analyze the data. $p < 0.05$ was considered to be significant unless otherwise specified.

Abbreviations

VEGF: vascular endothelial growth factor; ADSCs: adipose-derived stem cells; 3D: three dimensional; ECM: extracellular matrix; HFIP: hexafluoroisopropanol; SEM: scanning electron microscope; H&E: hematoxylin & eosin.

Declarations

Ethics approval and consent to participate

All animals were treated in compliance with the Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8523, revised 1996). The protocol was approved by the Medical Ethical Committee and the Animal Ethical and Welfare Committee of Shenzhen University (SYXK: 2018-0140).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request..

Competing interests

The authors declare that they have no competing interests.

Funding

This work was financially supported by the Medical Scientific Research Foundation of Guangdong Province (A2018555), Shenzhen Science and Technology Innovation Commission grants (JCYJ20170818100526471 and KQJSCX20180328095512554) and Natural Science Foundation of SZU (827-000143).

Authors' contributions

YL and YZ2 performed the experiments. JZ and ZY fabricated and examined the electro spun membrane. XW performed the plasmid construction and amplification, and participated the animal experiment. YZ1 and QH wrote the manuscript, YZ1, QH and CL designed the experiments, analyzed and interpreted the data and supervised the working program. All authors read and approved the final manuscript.

Acknowledgments

The authors would like to thank Shihe Li for assistance with confocal laser scanning microscope, and Guolin Yang for helping with animal experiments.

References

1. Park K.M, Shin Y.M, Kim K, Shin H. Tissue Engineering and Regenerative Medicine 2017: A Year in Review. *Tissue Eng Part B Rev.* 2018; 24:327-44.
2. Tang Z.L, Okano T. Recent development of temperature responsive surfaces and their application for cell sheet engineering. *Regen Biomater.* 2014; 1: 91-102.
3. Baksh N, Gallant N.D, Toomey R.G. Cell sheet engineering for integrating functional tissue in vivo: Successes and challenges. *Mrs Bulletin.* 2017; 42: 350-5.
4. Kim Y.J, Lee H.J, Ryu J.S, Kim Y.H, Jeon S, Oh J.Y, Choung H.K, Khwarg S.I, Wee W.R, Kim M.K. Prospective Clinical Trial of Corneal Reconstruction With Biomaterial-Free Cultured Oral Mucosal Epithelial Cell Sheets. *Cornea.* 2018; 37: 76-83.
5. Fujii Y, Kawase-Koga Y, Hojo H, Yano F, Sato M, Chung U.I, Ohba S, Chikazu D. Bone regeneration by human dental pulp stem cells using a helioxanthin derivative and cell-sheet technology. *Stem Cell Res Ther.* 2018; 9: 24-36.
6. Zhang C, Zhang E, Yang L, Tu W, Lin J, Yuan C, Bunpetch V, Chen X, Ouyang H. Histone deacetylase inhibitor treated cell sheet from mouse tendon stem/progenitor cells promotes tendon repair. *Biomaterials.* 2018; 172: 66-82.
7. Wang Y, Zhang J, Qin Z, Fan Z, Lu C, Chen B, Zhao J, Li X, Xiao F, Lin X, Wu Z. Preparation of high bioactivity multilayered bone-marrow mesenchymal stem cell sheets for myocardial infarction using a 3D-dynamic system. *Acta Biomater.* 2018; 72: 182-95.
8. Washio K, Tsutsumi Y, Tsumanuma Y, Yano K, Srithanyarat S.S, Takagi R, Ichinose S, Meinzer W, Yamato M, Okano T, Hanawa T, Ishikawa I. In Vivo Periodontium Formation Around Titanium Implants Using Periodontal Ligament Cell Sheet. *Tissue Eng Part A.* 2018; 24: 1273-82.
9. Miyahara Y, Nagaya N, Kataoka M. Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. *Nature Medicine.* 2006; 12: 459-65.
10. Tadakuma K, Tanaka N, Haraguchi Y, Higashimori M, Kaneko M, Shimizu T, Yamato M, Okano T. A device for the rapid transfer/transplantation of living cell sheets with the absence of cell damage. *Biomaterials.* 2013; 34: 9018-25.
11. Mitchell G. R. & Davis F. Electrospinning and tissue engineering. *Computational Methods in Applied Sciences.* 2011; 20: 111-36.
12. Ji W, Yang F, Ma J, Bouma M. J, Boerman O. C, Chen Z, van den Beucken J. J, Jansen J. A. Incorporation of stromal cell-derived factor-1 in PCL/gelatin electrospun membranes for guided bone regeneration. *Biomaterials.* 2013; 34: 735-45.
13. Hwang P. T, Murdock K, Alexander G. C, Salaam A. D, Ng J. I, Lim D.J, Dean D, Jun H.W. Poly(ϵ -caprolactone)/gelatin composite electrospun scaffolds with porous crater-like structures for tissue engineering. *J Biomed Mater Res A.* 2016; 104: 1017-29.
14. Truong Y.B, Glattauer V, Briggs K. L, Zappe S, Ramshaw J. A. Collagen-based layer-by-layer coating on electrospun polymer scaffolds. *Biomaterials.* 2012; 33: 9198-204.
15. Qasim S. B, Zafar M. S, Najeeb S, Khurshid Z, Shah A. H, Husain S, Rehman I. U. Electrospinning of Chitosan-Based Solutions for Tissue Engineering and Regenerative Medicine. *Int J Mol Sci.* 2018; 19:

16. Okamoto M, John B. Synthetic biopolymer nanocomposites for tissue engineering scaffolds. *Progress in Polymer Science*. 2013; 38: 1487-503.
17. Rodríguez-Vázquez M, Vega-Ruiz B, Ramos-Zúñiga R, Saldaña-Koppel D.A, Quiñones-Olvera L.F. Chitosan and Its Potential Use as a Scaffold for Tissue Engineering in Regenerative Medicine. *Biomed Res Int*. 2015; 2015:
18. Zhu Y, Liu T, Ye H, Song K, Ma X, Cui Z. Enhancement of adipose-derived stem cell differentiation in scaffolds with IGF-I gene impregnation under dynamic microenvironment. *Stem Cells Dev*. 2010; 19: 1547-56.
19. El-Tahlawy K, Hudson S, Hebeish A.A. Spinnability of chitosan butyrate/cellulose acetate for obtaining a blend fiber. *Journal of Applied Polymer Science*. 2010; 105: 2801-5.
20. Zhao X, Sun X, Yildirim L, Lang Q, Lin Z.Y.W, Zheng R, Zhang Y, Cui W, Annabi N, Khademhosseini A. Cell infiltrative hydrogel fibrous scaffolds for accelerated wound healing. *Acta Biomater*. 2017; 49: 66-77.
21. Gong M, Chi C, Ye J, Liao M, Xie W, Wu C, Shi R, Zhang L. Icaritin-loaded electrospun PCL/gelatin nanofiber membrane as potential artificial periosteum. *Colloids Surf B Biointerfaces*. 2018; 170: 201-9.
22. Fuller K.P, Gaspar D, Delgado L.M, Pandit A, Zeugolis D.I. Influence of porosity and pore shape on structural, mechanical and biological properties of poly ϵ -caprolactone electro-spun fibrous scaffolds. *Nanomedicine*. 2016; 11: 1031-40.
23. Kruglikov I. L. and Scherer P. E. Skin aging as a mechanical phenomenon: The main weak links. *Nutr Healthy Aging*. 2018; 4: 291-307.
24. Tseng H.J, Tsou T.L, Wang H.J, Hsu S.H. Characterization of chitosan–gelatin scaffolds for dermal tissue engineering. *J Tissue Eng Regen Med*. 2013; 7: 20-31.
25. Cork J, Whittaker A. K, Cooper-White J. J, Grøndahl L. Electrospinning and mechanical properties of P(TMC-co-LLA) elastomers. *Mater. Chem. B*. 2017; 5:2263-72.
26. Tamer T.M, Valachová K, Hassan M.A, Omer A.M, El-Shafeey M, Mohy Eldin M.S, Šoltés L. Chitosan/hyaluronan/edaravone membranes for anti-inflammatory wound dressing: In vitro and in vivo evaluation studies. *Mater Sci Eng C Mater Biol Appl*. 2018; 90: 227-35.
27. Zhao X, Olsen I, Li H, Gellynck K, Buxton P.G, Knowles J.C, Buxton P.G, Knowles J.C, Salih V, Young A.M. Reactive calcium-phosphate-containing poly(ester-co-ether) methacrylate bone adhesives: chemical, mechanical and biological considerations. *Acta Biomater*. 2010; 6: 845-55.
28. Zhu Y, Liu T, Song K, Fan X, Ma X. and Cui Z. Adipose-derived stem cell: a better stem cell than BMSC. *Cell Biochem Funct*. 2009; 26: 664-75.
29. Li D.Y, Brooke B, Davis E.C, Mecham R.P, Sorensen L.K, Boak B.B, Eichwald E, Keating M.T. Elastin is an essential determinant of arterial morphogenesis. *Nature*. 1998; 393: 276-80.
30. Ye K, Cao L, Li S, Yu L, Ding J. Interplay of Matrix Stiffness and Cell-Cell Contact in Regulating Differentiation of Stem Cells. *ACS Appl Mater Interfaces*. 2016; 8: 21903-13.

31. FernándezM, Oberti T.G, Vikingsson L, Ribelles J.L, Cortizo A.M. Biodegradable polyester networks including hydrophilic groups favor BMSCs differentiation and can be eroded by macrophage action. *Polymer Degradation & Stability*. 2016; 130: 38-46.
32. Hosseinkhani H, Inatsugu Y, Hiraoka Y, Inoue S, Shimokawa H. & Tabata. Y. Impregnation of plasmid DNA into three-dimensional scaffolds and medium perfusion enhance in vitro DNA expression of mesenchymal stem cells. *Tissue Eng*. 2005; 11: 1459-75.
33. Rinsch C, Quinodoz P, Pittet B, Alizadeh N, Baetens D, Montandon D, Aebischer P. & Pepper, M. S. Delivery of FGF-2 but not VEGF by encapsulated genetically engineered myoblasts improves survival and vascularization in a model of acute skin flap ischemia. *Gene Therapy*. 2001; 8: 523-33.
34. Shimizu T, Yamato M, Kikuchi A. Cell sheet engineering for myocardial tissue reconstruction. *Biomaterials*. 2003; 24: 2309-16.
35. Wu Z.S, Zhu H.X, Luo Z.Q, Pan W.C, Yang F, Lin X.Y, Zhu Y.X. Combined use of adipose-derived stem cells and electrospun membrane for preparing sandwich-like cardiac cell sheets. *Zhongguo Zuzhi Gongcheng Yanjiu*. 2018; 22: 254-60. (in Chinese)
36. Han G, Ceilley R. Chronic Wound Healing: A Review of Current Management and Treatments. *Adv Ther*. 2017; 34: 599-610.

Figures

Figure 1

Schematics for the fabrication of electrospun gelatin/chitosan nanofibrous with plasmid DNA and construction of sandwich-like cell sheets for its subsequent implantation into the wound. The size of pIRES2-AcGFP1 vector is 5.3 kbp. Human VEGF DNA fragment (699 bp) was inserted into Sgf1 and Mlu1. Electrospun gelatin/chitosan nanofibrous with plasmid was cross-linked with glutaraldehyde, after ADSCs grow to confluence on temperature responsive dishes, the whole cell sheet was peeled off from

the culture surface with gelatin/chitosan nanofibrous with plasmid at 20°C, and the sandwich-like cell sheets were transplanted for wound healing.

Figure 2

Characterization of different electrospun gelatin/chitosan nanofibrous. A. SEM image of different electrospun gelatin/chitosan nanofibrous. Scale bars 20μm. B. Fiber diameter distribution of nanofibrous in SEM image. C. Average pore sizes of nanofibrous in SEM image. D. Young's modulus of different nanofibrous before and after crosslinking. E. Degradation properties of all the nanofibrous. F. pH value of degradation fluid. G. Water absorption ability of all the nanofibrous.

Figure 3

Adhesion and proliferation of ADSCs on different electrospun gelatin/chitosan nanofibrous. A. ADSC-GFP adhered on the nanofibrous after one day cultivation. Scale bars 100μm. B. After 3 days cultivation, under SEM, ADSCs spread on the nanofibrous. Scale bars 20μm. C. After one week culture, ADSC-GFP almost covered the surface of nanofibrous. Scale bars 100μm. D. Morphology of ADSCs on different nanofibrous on day 14 observed by Confocal Laser Scanning Microscope. Scale bars 100μm. E. Cell proliferation on different nanofibrous was tested by CCK-8. F. Cell migration was quantified using the whole thickness of cell-nanofibrous.

Figure 4

Characteristics of the electrospun gelatin/chitosan nanofibrous with plasmid. A. Ultrastructure of electrospun gelatin/chitosan nanofibrous with plasmid observed by SEM. Scale bars 5 μ m B. Release profile of plasmid DNA from nanofibrous. C. Young's modulus of nanofibrous with plasmid. D. VEGF protein expression in ADSCs on nanofibrous. E. ADSCs proliferation on nanofibrous with plasmid observed by Confocal Laser Scanning Microscope. Scale bars 100 μ m. F. Under SEM, ADSCs spread on nanofibrous with plasmid after one week cultivation. Scale bars 20 μ m.

Figure 5

Cell sheets or nanofibrous with plasmid dressing on the mouse wound sites. Extent of wound healing on days 3, 7, 10 and 13. Control: wound without transplantation; Nanofibrous: transplantation of 7:3 nanofibrous alone; Nanofibrous-V: transplantation of 7:3 nanofibrous with plasmid; Cell sheets: transplantation of ADSCs-nanofibrous-ADSCs; Cell sheets-V: transplantation of ADSCs-nanofibrous with plasmid-ADSCs.

Figure 6

Examination of neo-skins at the day of healing. A. Changes in wound size among different group during healing. $P < 0.05$. B. Skin thickness (epidermal and dermal layers) was measured after healing. $P < 0.05$. C. The mechanical property of neo-skin. $P < 0.05$. D. Hematoxylin and Eosin staining images of wounds on day of healing. Scale bars 100μm. E. Masson's trichrome staining for nascent collagen in neo-skin. Control: wound without transplantation; Nanofibrous: transplantation of 7:3 nanofibrous alone;

Nanofibrous-V: transplantation of 7:3 nanofibrous with plasmid; Cell sheets: transplantation of ADSCs-nanofibrous-ADSCs; Cell sheets-V: transplantation of ADSCs-nanofibrous with plasmid-ADSCs.