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To cite this article: Mazaher Gholipourmalekabadi et al 2018 Biomed. Mater. 13 035003

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Silk fibroin/amniotic membrane 3D bi-layered artificial skin

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Keywords: scaffold, skin, regenerative medicine, human amniotic membrane, silk fibroin

Supplementary material for this article is available online

Abstract

Burn injuries have been reported to be an important cause of morbidity and mortality and they are still considered as unmet clinical need. Although there is a myriad of effective stem cells that have been suggested for skin regeneration, there is no one ideal scaffold. The aim of this study was to develop a three-dimensional (3D) bi-layer scaffold made of biological decellularized human amniotic membrane (AM) with viscoelastic electrospun nanofibrous silk fibroin (ESF) spun on top. The fabricated 3D bi-layer AM/ESF scaffold was submerged in ethanol to induce β-sheet transformation as well as to get a tightly coated and inseparable bi-layer. The biomechanical and biological properties of the 3D bi-layer AM/ESF scaffold were investigated. The results indicate significantly improved mechanical properties of the AM/ESF compared with the AM alone. Both the AM and AM/ESF possess a variety of suitable adhesion cells without detectable cytotoxicity against adipose tissue-derived mesenchymal stem cells (AT-MSCs). The AT-MSCs show increased expression of two main pro-angiogenesis factors, vascular endothelial growth factor and basic fibroblast growth factor, when cultured on the AM/ESF for 7 days, when comparing with AM alone. The results suggest that the AM/ESF scaffold with autologous AT-MSCs has excellent cell adhesion and proliferation along with production of growth factors which serves as a possible application in a clinical setting for skin regeneration.

1. Introduction

Full-thickness skin wounds, especially caused by third degree burns more than 10 mm in diameter, require skin grafting to fully heal and prevent related complications [1, 2]. Many attempts have been made to develop an ideal skin substitute that would accelerate wound healing and prevent infections. An ideal skin substitute should have mechanical properties similar to normal skin, facilitate cell attachment, proliferation, migration and differentiation, degrade at a comparable rate to the formation of new skin, and prevent infections [1, 3–5]. To date, there is no such product; however there is a number of scaffold materials, such
as decellularized bovine collagen based membrane (Intergra™), silicon-based sheets and decellularized human amniotic membrane (AM) which can serve as the next best substitutes. Unfortunately, these materials are quite expensive, hence skin regeneration is considered as an unmet clinical need [1]. For instance, AM is used in the management of burn wounds today [6].

AM is a natural bio-scaffold and has many favorable characteristics for use as a skin substitute including high elasticity, structural integrity, an extracellular matrix (ECM) composition similar to human skin, an anti-scarring effect, supportive effects on cell migration, proliferation, differentiation and anti-bacterial activity. These properties make this membrane a promising and optimal skin substitute [6–10]. It is reported that epithelial and mesenchymal layers of the AM have anti-angiogenic and angiogenic properties. Despite all the unique characteristics of AM as a wound dressing, its weak mechanical properties and fast degradation remain a challenge. Wet AM is hardly handled during the grafting and degrades before the wounds fully heal [11]. Table S1, available online at stacks.iop.org/BMM/13/035003/mmedia, shows the recent research work on skin regeneration for wound healing applications.

In this work, we hypothesize overcoming the disadvantages associated with AM grafting by multilayer coating it with natural and optimal biomaterial such as nanofibrous silk protein. Silk has favorable characteristics such as aqueous preparation, superior mechanical properties, suitable elasticity, adequate angiogenicity and slow biodegradability. Silk fibroin from the mulberry silkworm Bombyx mori is cyto-biocompatible and extensively used in tissue engineering applications. [12–18]. A number of silk containing products are used in plastic surgery applications, which are approved by the US Food and Drug Administration [19].

In this paper, we report the fabrication and characterization of bi-layer membranes made from decellularized human AM and electrospun nanofibrous silk fibroin (ESF). Our bi-layer artificial skin substitute showed an improved angiogenesis in in vitro evaluation by increased expression of angiogenic factors in adipose tissue-derived mesenchymal stem cells (AT-MSCs) when compared to AM. Our improved bi-layer artificial skin scaffold presents an excellent option as a skin substitute and mesenchymal stem cells carrier for skin tissue engineering applications.

2. Materials and methods

2.1. Fabrication of bi-layer decellularized human AM and ESF

2.1.1. Human AM

The AM samples were obtained and decellularized by a simple and cost-effective method as previously described [11]. Briefly, the human placentas were obtained from consenting mothers upon cesarean sections under sterile conditions. The samples were screened to be negative for the following agents: the human immunodeficiency virus type II, syphilis, gonorrhea, human hepatitis virus types B and C, toxoplasmosis and cytomegalovirus. The AM was separated from chorion and then treated with 0.2% EDTA and 0.5 M NaOH and scraped to remove the cells. The sample collection and experiments were performed in accordance with the World Medical Association Declaration Of Helsinki [20]. The decellularization of the membrane was confirmed by immunohistochemistry (IHC) and haemotoxylin and eosin (H&E) staining. The decellularized membrane was dried under vacuum and sterilized by gamma irradiation.

2.1.2. Preparation of nanofibrous silk protein fibroin by electrospinning

2.1.2.1. Extraction and purification

The extraction and purification of silk fibroin from cocoons of the mulberry silkworm Bombyx mori were carried out as described previously [21]. Briefly, the cocoons were boiled at 100 °C in an aqueous solution of 0.02 M sodium bicarbonate (Na2CO3) and the fibers were thoroughly rinsed with deionized water to remove the glue-like silk protein sericin. The degummed silk fibers were dissolved in 9.3 M lithium bromide (LiBr) solution at 60 °C for 4 h and samples were dialyzed (molecular weight cut-offs of 12 KDa) against ultrapure water with changes every 2 h. The dialyzed fibroin solution was lyophilized and stored at room temperature until future use.

2.1.2.2. Nuclear magnetic resonance (NMR) analysis

The extracted and dried SF powder was characterized by a high resolution 13C-NMR spectra (FT-NMR (600 MHz) AVANCE 600, Bruker, Germany). The SF powder was dissolved in formic acid (FA) (10% w/v) and subjected to NMR analysis as described previously [22].

2.1.2.3. Fourier transform infrared (FTIR) spectroscopy

Methanol and ethanol are the most common solvents for induction of water insoluble β-sheet conformational transition [23–25]. The SF was treated with 70% ethanol for 1 h. The molecular conformation of the SF was determined by FTIR spectroscopy (55 FTIR EQUINOX spectrophotometer, BrukerOptik GmbH, Germany). For this purpose, before and after ethanol treatment, the dried SF powder was mixed with potassium bromide (KBr) and analyzed in the spectral region of 4000–400 cm⁻¹ with a resolution of 4 cm⁻¹ [26].

2.1.3. Fabrication of AM/ESF bi-layered membrane

10% silk fibroin (SF) solution in FA was prepared as described in our previously published study [21]. For
electrospinning, an AM membrane \((20 \times 3 \text{ cm})\) was fixed to a drum covered with an aluminum foil. The SF/FA solution in a 3 ml syringe was electrospun on the dried AM. The electrospinning was performed under the following conditions: a voltage of 18 kV cm\(^{-1}\), 15 cm distance between the syringe and the collector and 0.3 ml H\(^{-1}\) constant flow rate. In this study, the SF solution was electrospun for 20 min (this confirmed the attachment of ESF to AM) and 3 h (this finalized AM/ESF bi-layer membrane). AM/ESF was treated with 70% ethanol for 1 h and subsequently dried under vacuum. Ethanol treatment was also done on AM alone following same procedure. The synthesized bi-layer membrane was kept at 4°C until further use. The schematic of bi-layer skin scaffold preparation is illustrated in figure 1.

### 2.2. Biological and cellular characterization

2.2.1. Surface characterization using SEM

After 20 min and 3 h of electrospinning the AM and AM/ESF with gold, their morphologies were observed under a scanning electron microscope (SEM, Philips XL30, Netherlands) at an acceleration voltage of 15 kV. The average diameter of the spun nano-fibers \((n = 10)\) before and after ethanol treatment were determined by SEM software and analyzed.

2.2.2. Biomechanical behavior

A universal tensile strength testing machine at a crosshead speed of 10 mm min\(^{-1}\) with a specified sample size (length = 20 mm and width = 10 mm) was used to evaluate the biomechanical behavior of the AM and AM/ESF \((n = 5)\). Maximum load value (N), suture retention strength (mN) (5–0 nylon suture) and strain deflection at break (mm) of the AM/ESF were determined by a protocol previously described and compared to those of AM alone [11]. A Mitutoyo 547-400S Digimatic IDC thickness gauge was used for a measurement of average thickness.

2.2.3. Biodegradation

The degradation properties of the AM/ESF were determined by enzymatic reaction, namely 0.1 wt% solution of lysozyme in Dulbecco’s modified Eagle’s medium (DMEM) culture with the addition of phosphate-buffered saline (PBS) solutions in vitro, as described previously with few modifications [11].

Briefly, the samples were weighed \((m_0)\) and embedded into 10 ml of degradation solution (PBS or enzyme). All the samples were incubated at 37°C for 14 days. Three samples were removed after each predetermined incubation time, washed with distilled water and dried at room temperature for 72 h. The samples were weighed \((m_d)\) and percentage of weight loss was...
2.2.4. AT-MSC responses to AM/ESF

2.2.4.1. The AT-MSC isolation and expansion
The AT-MSCs were isolated from inguinal fat pads of BALB/c mice according to procedures approved by the Ethics Committee of the Medical Sciences. The isolation protocol is described in detail elsewhere [8, 27]. Briefly, 10 g of the adipose tissue was collected and mixed with 0.2% collagenase II (Sigma, St. Louis, MO, USA) for 30 min at 37 °C under sterile conditions. The samples were centrifuged at 2500 rpm for 10 min. The supernatant was removed and the pellet was resuspended in DMEM supplemented with 10% fetal bovine serum, 1% pen/strep, nystatin and amphotericin B, 2 mM Glutamax, 1 mM L-glutamine and 1% non-essential amino acids (all from Gibco, Carlsbad, CA, USA). The cells were harvested in two 75 cm² flasks. The number of viable cells was assessed based on 0.4% trypan blue exclusion (Sigma-Aldrich, USA) method.

2.2.4.2. Characterization of AT-MSCs
The AT-MSCs were characterized by flow cytometry. The cells were examined for the expression of CD34, CD44, CD45, CD73, CD90 and CD105. The AT-MSCs with density of 3 × 10⁵/100 µl were incubated with antibodies in a dark chamber for 30 min at 37 °C. The cells were then washed and analyzed by flow cytometry as described previously [28]. The AT-MSCs were further characterized for their differentiation capacity towards osteogenic and adipogenic lineages [29, 30]. First the cells were treated with osteogenic differentiation media for three weeks and then stained with Alizarin-red (Sigma-Aldrich, USA) to visualize mineralized matrix. Next, the cells were treated with adipogenic differentiation media for three weeks and then stained with Oil-red (Sigma-Aldrich, USA). The stained cells were viewed and images taken under light microscope.

2.2.4.3. Cell adhesion and distribution
For cell adhesion and distribution studies, the cells were seeded on the membranes (1 × 1 cm) and incubated for 72 h. The morphology of the cells cultured on the samples was observed under SEM [11]. The cells were also stained with 4,6-diamidino-2-phenylindole (DAPI) [31] to analyze the cell density along the scaffold. For quantitative analysis, the DAPI stained samples were viewed under fluorescent microscope and the cells were counted from ten different areas (100 × magnification). The test was repeated three times and the average of cell count was reported as cell density.

2.2.4.4. Long-term cell viability and cytotoxicity
The AT-MSC viability and cytotoxicity were evaluated by MTT and lactate dehydrogenase (LDH) specific activity, as described previously [8]. Briefly, the cells (1 × 10⁴ cells/well) were cultured on the membranes (1 × 1 cm) for 1, 5, 10 and 15 days in a cell culture incubator (n = 3). After each time interval, the media were collected and subjected to LDH specific activity assay using an LDH kit (Zist Shimi kits, Tehran, Iran) [8]. For the cell viability assay, the cells after were treated with 10% MTT solution for 1 h and then embedded in dimethyl sulfoxide for 20 min. The optical density (OD) of the samples was measured using an enzyme-linked immunosorbent assay (ELISA) reader at a wavelength of 590 nm. The viability of the AT-MSCs cultured on plastic surface of cell culture plate was considered as positive control (100% cell viability). Sterile cell culture media with no cells or membrane served as a negative control (ODnc). The cell viability was determined by the following formula (equation (2)):

\[
\text{absorbance value} = \text{ODs} - \text{ODnc}. \tag{2}
\]

2.3. Angiogenesis
To determine whether the AM/ESF may accelerate post-injury neovascularization, the effects of AM/ESF/AT-MSCs cross-talk on secretion of the pro-angiogenic factors such as vascular endothelial growth factor (VEGFa) and basic fibroblast growth factor (bFGF) from these cells were evaluated in vitro. For this, the AT-MSCs were seeded on the decellularized AM and AM/ESF membrane and incubated at 5% CO₂ and 95% air for 7 days. The cells cultured on the tissue culture plate served as a control. After specified time, the cells were cultured on the tissue culture plate served as a control. After specified time, the cells were evaluated for their expression of VEGFa and bFGF in vitro by real-time polymerase chain reaction (PCR) and immunofluorescence staining [32].

2.3.1. Real-time PCR
The total RNA was extracted by RNX-Trizol reagent according to the manufacturer’s protocol (Cinagen, Tehran, Iran). Transcription of 0.1 µg RNA to cDNA was performed by a High-Capacity cDNA Archive kit using random hexamer primer according to the supplier’s instructions (Applied Biosystems, Foster City, CA, USA). The relative expressions of VEGFa and bFGF were determined by RT-PCR (rotor-gene 6000, Corbett Life Science, Sydney, Australia) using PCR Master Mix (TaKaRa, Dalian, China). The expression level of each target gene was normalized against beta-actin (b-actin) gene. The relative expression was defined based on 2^-ΔΔCt. All the primers used in this study and the product lengths are listed in table 1.

2.3.2. Immunostaining
For immunostaining, the cells were washed with PBS twice and fixed with 4% paraformaldehyde (PFA) for
30 min. After permeabilization of the cells with 0.025% Triton X-100 and washing with PBS, the nonspecific binding was blocked by treatment of the cells with 10% goat serum for 30 min. The cells were then treated with primary antibodies anti-VEGFα and anti-bFGF for 1 h at 37 °C. The fluorescent isothiocyanate (FITC)-conjugated goat polyclonal antibody to rabbit IgG was used as secondary antibody [33]. All the antibodies were purchased from Abcam, Cambridge, MA, USA. The counter stain was performed using DAPI. The positive cells for VEGFα and bFGF were counted under fluorescent microscope (Olympus BX51, Japan) and the results were reported as average cell number per high-power field.

2.3.3. Statistical analysis
The results were analyzed by Student’s t-tests, one-way ANOVA and Tukey’s test. The analysis was performed using SPSS software (Version 16, SPSS Inc., Chicago, IL, USA). Statistical analysis was carried out by applying one-way ANOVA and Tukey’s test. P < 0.05 was set as level of significance.

3. Results and discussion
3.1. Human AM
The decellularization of the AM was described previously [11]. The complete removal of the cells and cellular debris was confirmed using dead and live staining, H&E staining and DNA assay. It was also confirmed that the decellularization did not affect the ECM of the AM using IHC staining as described in our previous studies [8, 11]. It is generally known that the graft rejection limits the use of AM as an allograph. Denudation of the AM may improve cell proliferation along with decreased immunogenicity [11, 34].

3.2. SF characterization
3.2.1. NMR
The results obtained from 13C-NMR spectra of the regenerated SF are illustrated in figures 2(a) and (b). The distinctive peaks appeared at 16.9 ppm (Cβ of Ala), 43.1 (Co of Gly) and 50.2 (Coα of Ala) of the SF/FA solution. The secondary structure of SF molecules in solution can be determined by Cβ (Ala) chemical shift. The random coil (Structure III) and β-sheet conformation (Structure II) display different peaks at 16.9 and 19.5 ppm, respectively [22]. According to the NMR spectra, Cβ (Ala) chemical peak was at 16.9 ppm, indicating random coil structure of SF in FA. This is similar to aqueous SF solution.

3.2.2. FTIR
The induced insoluble β-sheet conformational transition of the SF was confirmed by FTIR spectroscopy [23, 25]. The infrared spectra of the SF before and after ethanol treatment are depicted in figure 2(c). The FTIR spectra results of the ethanol-treated SF showed characteristic peaks of β-sheet conformation at 1636 cm⁻¹, 1516 cm⁻¹, 1235 cm⁻¹ and 965 cm⁻¹ with no changes in α-helix characteristic peaks (621 cm⁻¹) [23, 26].

3.3. AM/ESF characterization
3.3.1. Morphology
The morphology of the AM/ESF was observed under SEM after 20 min and 3 h electrospinning (figure 3(a)). The SF (10%) nano-fibers were successfully electrospun and arranged in a specific orientation as displayed by the SEM micrographs. They were homogenous throughout with no beaded fibers. In 20 min electrospun samples, the attachment of the SF nano-fibers to the AM is clearly observed after ethanol treatment. After alcohol treatment, ESF was tightly coated and ensured non separation of the AM. The average diameters of the spun nano-fibers before and after ethanol treatment were 93 ± 25 nm and 157 ± 32 nm, respectively (figure 3(b)). The results are consistent with a previously published report [21]. The tilt view SEM image of the AM/ESF (figure 3(a)) confirmed the coating of the AM by ESF.

3.3.2. Biomechanical behavior
The effects of the SF electrospinning on the mechanical behaviors of the AM/ESF are summarized in table 2. The AM/ESF showed significantly improved mechanical properties including maximum load value, suture retention strength, strain deflection at break and thickness when compared with AM samples. Tissue engineering scaffolds must provide sufficient biomechanical properties for better regeneration of damaged tissues. In our previous study [23], we showed that ethanol treatment and subsequently β-sheet structure formation of ESF significantly increase its mechanical behaviors. Vasconcelos et al [35] fabricated a 3D silk fibroin/elastin wound dressing with excellent chemical

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession number</th>
<th>Sequence</th>
<th>Product size (BP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta-actin</td>
<td>NM_007393.5</td>
<td>F: 5’CTCTTTGGGGATGGAAATCTG R: 5’GTGTTGCGCATAGGTCCTTAC</td>
<td>96</td>
</tr>
<tr>
<td>VEGFa</td>
<td>NM_001110268.1</td>
<td>F: 5’TGCTCTCTCCACTTCTGAGG R: 5’GGCCATTACCAGGCCTCTTC</td>
<td>93</td>
</tr>
<tr>
<td>bFGF</td>
<td>NM_008006.2</td>
<td>F: 5’CCGGTCACGGAAATCTCCA R: 5’CCTCTGTCCAGGTCCCGTT</td>
<td>89</td>
</tr>
</tbody>
</table>
and mechanical properties for biological applications. The biomechanical properties of AM/ESF were comparable with other skin substitutes using waxes and chondroitin sulfate/hyaluronic acid [14, 36]. For example, Kanokpanont et al showed that coating SF fabric with wax increases its biomechanical properties with no cytotoxic effects [14]. Insufficient mechanical properties of AM make it difficult to use for grafting. To address this issue, researchers are attempting to find a way to enhance the biomechanical behavior of AM without affecting its cell adhesion and non-cytotoxic properties. For example, cross-linking of AM with carbodiimide and glutaraldehyde successfully enhanced its biomechanical behavior. Negative effects of such agents on the structure, molecular stability and cytocompatibility of AM were demonstrated previously [37, 38]. The biomechanical testing of this study indicates that coating the AM with ESF significantly increases its mechanical properties and may make it an excellent 3D skin substitute for skin tissue engineering applications.

3.3.3. In vitro degradation of AM/ESF

The degradation rate of scaffolds for tissue engineering purposes is very critical and can profoundly affect healing effectiveness. Quick degradation of the biomaterial causes the scaffold to disintegrate too early before the healing of the damaged tissue is complete. The in vitro degradation rates of the engineered functional materials (AM and AM/ESF) were determined by treatment with PBS (figure 4(a)) and lysozyme (figure 4(b)) solution for 14 days. The degradation rate of the AM/ESF sample showed a significant decrease in weight after 6 and 7 days post-treatment in enzymatic and PBS solution, respectively, when compared to the AM sample alone (independent sample t-test, p < 0.05). The complete degradation of AM occurs during first two weeks [11]. Ma et al [37] reported more than 90% weight loss of AM after 2 weeks in collagenase degradation solution. In addition, it was reported that degradation rate of AM varied and depended on its preparation, decellularization and preservation conditions [11, 39]. The results

![Figure 2](image-url)
of our previous study showed that the denuded AM was degraded faster than the natural AM in both PBS and lysozyme solutions. Based on the biodegradation results in the current study, coating the AM with ESF slowed the degradation rate of the resulted AM/ESF bi-layer membrane. Skin regeneration is an integrated process involving both cells and the supporting scaffold. The AM, due to its biological properties and tissue-like characteristics, makes an excellent base membrane for the cells to grow and proliferate, while ESF, with its viscoelastic nature, ensures the integrity of the product and results in slow destruction of the AM membrane. A suitable skin substitute should stay on the skin wound between 2–3 weeks,

Table 2. Mechanical properties of human amniotic membrane (AM) and the bi-layered AM/electrospun nanofibrous silk fibroin (ESF) scaffold. Freeze dried samples. *Significant difference.

<table>
<thead>
<tr>
<th>Properties checked</th>
<th>AM</th>
<th>AM/ESF</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thickness (μm)</td>
<td>97 ± 8.2</td>
<td>127 ± 6.7</td>
<td>P &lt; 0.05*</td>
</tr>
<tr>
<td>Maximum load (N)</td>
<td>1.3 ± 0.17</td>
<td>1.9 ± 0.18</td>
<td>P &lt; 0.05*</td>
</tr>
<tr>
<td>Suture retention strength (mN)</td>
<td>512 ± 63</td>
<td>692 ± 31</td>
<td>P &lt; 0.05*</td>
</tr>
<tr>
<td>Strain deflection at break (mm)</td>
<td>7.3 ± 0.49</td>
<td>8.5 ± 0.33</td>
<td>P &lt; 0.05*</td>
</tr>
</tbody>
</table>

Figure 3. Morphology of the membranes (a) SEM micrographs of the human AM, AM/ESF (20 min and 3 h electrospinning) before and after treatment with ethanol as well as tilt view SEM image of the AM/ESF. Black and white arrows indicate ESF and AM, respectively. EUT: Ethanol untreated. ET: Ethanol treated. (b) Fiber diameter distribution in EUT and ET samples.

Figure 4. In vitro degradation time (% mass loss) of the samples in PBS and lysozyme solutions during 14 days of treatment.
depending on the wound type and severity [40, 41]. AM has shown full degradation during the first week post-grafting [11] which is not optimal for service as a skin substitute. Our results showed an improved effect of ESF on enhancing the degradation time of AM/ESF to 2 weeks.

3.3.4. The AT-MSC responses on AM/ESF

3.3.4.1. Characterization of AT-MSCs

According to the results obtained from the flow cytometry, 69.6%, 96.8%, 76.5% and 94.4% of the cell population were positive for CD44, CD73, CD90 and CD105, respectively (figure 5(a)). Only 2.72% and 3.56% of the cells were positive for CD34 and CD45, respectively. The Alizarin-red and Oil-red staining confirmed that the cells were successfully differentiated into both osteogenic and adipogenic lineages (figure 5(b)) [27, 29, 42].

3.3.4.2. Cell adhesion and distribution

Interaction of cells with the surface of biomaterials profoundly affects various cell behaviors such as adhesion, proliferation and differentiation. These properties strongly depend on the physicochemical characteristics and hydrophilicity of the materials [43]. Therefore, cell adhesion of tissue engineering scaffolds is of critical importance. The morphologies of the AT-MSCs cultured on the AM/ESF before and after ethanol treatment were analyzed by SEM (figure 6(a)). The spindle morphology of the AT-MSCs on all the samples was clearly observed at day 3 post-seeding. The conformation of the AT-MSCs with the surface of the membranes clearly represented the effective cell–substrate attachment. The elongated morphology of the cells as well as
their filopodia-like membrane suggests that all the membranes are suitable for cellular adhesion and proliferation. Moreover, DAPI staining was performed to determine the density and distribution of the AT-MSCs on the membranes (figure 6(c)). No significant difference was observed between the samples \( p > 0.05 \) in the cell density. These results are consistent with earlier investigations [8, 29, 44] which showed a high attachment of both silk fibroin and collagen to L929 cells. Similarly, we previously showed high cell adhesion and increased growth of such materials for bone marrow and adipose tissue-derived mesenchymal stem cells [8, 11, 21, 45]. The presence of proteoglycans, laminin, fibronectin and collagens within the AM matrix endow it with an excellent cell attachment ability [10]. As we reported previously, the decellularization process does not affect the adhesion of mesenchymal stem cells to AM [11]. According to this and our previous studies [8, 11, 21, 23], both AM and AM/ESF offer desirable cell adhesion. Such favorable cell adhesion makes it a promising delivery system in tissue engineering applications.

3.3.4.3. Long-term cell viability and cytotoxicity

The evaluation of the AM/ESF as potential matrix for the viability of the AT-MSCs was carried out based on the survival of the cells in reduced tetrazolium salt solution and formation of the crystals within their mitochondria. Determination of the cell viability (figure 6(b)) revealed that neither AM nor AM/ESF affected the viability of the AT-MSCs after specific incubation intervals. The results were compared to the 100% cell viability (independent sample \( t \)-test, \( p > 0.05 \)).

The cytotoxicity of the samples on the AT-MSCs was evaluated by LDH specific activity assay. No cytotoxicity of the AM and AM/ESF membranes on the growth of AT-MSCs was detected (figure 6(b)). These results are consistent with the earlier relevant observations [8, 21, 46–48].

3.4. Angiogenic property of AM/ESF

The AT-MSCs were seeded on the decellularized AM and AM/ESF membranes and incubated in a humidified atmosphere of 5% CO2 and 95% air for 7 days. After a specific time, the cells were evaluated for their expression of VEGFa and bFGF by real-time PCR and immunostaining. The results show that the post-injury neovascularization occurs during 7–14 days of healing depending on the severity of the wounds. IL-1β, FGF, VEGFa and VEGFR2 are reported to be the most important factors involved in angiogenesis. It
is reported that bFGF is secreted during the first 3 days of wound repair, whereas VEGF may have a critical role in angiogenesis 4–7 days post-injury [49, 50]. In this study, we aimed to show whether the AM/ESF membrane can accelerate secretion of these angiogenic cytokines in vitro during first days of healing.

3.4.1. Real-time PCR
The angiogenic property of AM is debatable. It was reported that AM possesses anti-angiogenic properties when it was implanted by its epithelial side [51]. Fast neovascularization during the first hours of wound healing might remarkably decrease local hypoxia and subsequently accelerate the healing process [52–54]. Various biomaterials were investigated for acceleration of the angiogenesis within a damaged area [55, 56]. The angiogenic property of SF was reported by many [36, 57]. AT-MSCs accelerate neovascularization through the secretion of a variety of angiogenesis factors such as VEGFa, bFGF, IL-1β and others [32, 42]. In this study, we showed that the AM/ESF significantly increased the expression levels of VEGFa and bFGF in AT-MSCs after 7 days when compared to AM alone and a control (p ≤ 0.05) (figure 7(a)). Our findings indicate the enhanced the angiogenesis in vitro of the ESF coated over the AM.

3.4.2. Immunostaining
Angiogenesis of the AM/ESF on the AT-MSCs was evaluated by immunostaining of VEGFa and bFGF (figure 7(b)). The cells expressing the angiogenesis factors are reported (figure 7(c)). Immunostaining revealed increased expressions of VEGFa and bFGF in AT-MSCs (figure 7(b)). The results show more than 90% and 70% of the AT-MSCs expressed VEGFa and bFGF, respectively. These angiogenesis data are also confirmed by our real-time PCR results and additional studies [36, 57].

4. Conclusion
The human amniotic membrane (AM) is widely used as a skin substitute in burn injuries, yet it is not fully satisfactory. This stems from the fact that its fast biodegradation rate, low mechanical and insufficient angiogenesis characteristics are not optimal. In this study, we develop and fully characterize a 3D bi-layer artificial skin made from AM and electrospun nanofibrous silk fibroin (ESF). The AM/ESF bi-layer membrane shows improved mechanical properties and slower biodegradation rate when compared to AM alone. The ESF allows for AM to be maintained in its 3D structure by reducing the bio-absorption rate. Our 3D bi-layer scaffold induces the adipose-derived
Mesenchymal stem cells to up-regulate angiogenic factors such as VEGFα and bFGF. These results indicate that the 3D bi-layer AM/ESF scaffold offers an attractive alternative in the skin substitute research area.

Acknowledgments

We express our sincere thanks to Dr Niraman Mosaffa and Dr Mohammad Salehi for providing helpful comments on this work. SC Kundu presently holds an ERA Chair Full Professor position at the 3Bs Research Group, University of Minho, Portugal, supported by the European Union Framework Programme for Research and Innovation Horizon 2020 under grant agreement No 668983—FoReCaST.

Authors’ contributions

MG, AS and AS conceived and designed the study. MG, AS and HG performed the experiments. MG, AS, AMS, MM and MDO interpreted the results. MG, MM, AMS, MM, AMU, JGH, SCK and RLR reviewed the manuscript. MG, AMS and AMU prepared the manuscript. MG, AMS, MM, AMU, JGH performed the experiments.

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The authors declare no conflict of interest both in terms of financial and publication of this work. All authors approved the final version of the manuscript.

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