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DOI: 10.1557/adv.2019.61

Hierarchical Decoration of Eggshell Membrane with Polycaprolactone Nanofibers to fabricate a Bilayered Scaffold for Skin Tissue Engineering

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Abstract

Eggshell Membrane (ESM) is a naturally occurring proteinaceous microfibrous scaffold capable of mimicking the extracellular matrix (ECM). The extraction methodology deployed for its extraction process impedes its extensive application as a biomaterial in regenerative medicine. Herein, a unique route was deployed to decorate the surface of ESM with electrospun polycaprolactone (PCL) nanofiber in order to ameliorate the above problems and also fabricate a novel ECM mimicking bilayered scaffold for skin tissue engineering applications. Microstructural and surface topographic analysis confirms the formation of bilayered structure with smooth electrospun PCL nanofibers decorated on ESM. Carbodiimide chemistry was utilized to crosslink the two layers. Cytocompatibility evaluation of scaffolds was carried out with Human dermal fibroblast (HDF) cells. The biomimetic architecture and protein rich composition of as fabricated bilayered construct facilitated extensive cell adhesion, proliferation and migration in contrast the bare natural tissue led to impeded cell adhesion.

INTRODUCTION:

The ability of scaffolds to mimick the in vivo microenvironment facilitates its application in varying tissue engineering applications [1,2]. In case of skin tissue engineering, it's of paramount importance to fabricate a scaffold resembling the extracellular matrix (ECM) so that it can facilitate adhesion and proliferation of Human fibroblast and keratinocyte cells [2]. A synergistic selection of polymer and fabrication technology used for fabricating the scaffold plays a vital role in designing an ideal matrix [3]. Further, it's equally essential to maintain suitable parameters during fabrication process to obtain perfect surface topography which can facilitate cell anchorage,

proliferation and cross-talk among cell lines in order to regenerate the extracellular matrix [4]. It was also previously reported that scaffolds bearing a nano/micro fibrous architecture possessed better cell adhesion, proliferation and migration owing to its ability to mimic the ECM [5].

Eggshell membrane (ESM) is a naturally occurring versatile material possessing a microfibrous architecture capable of mimicking the ECM. ESM microfibers are mainly composed of proteins, which includes collagen (Type I, V, X) and other proteins along with glycoproteins [6,7]. Each microfiber is made up of glycoprotein-rich cortex and collagen-rich core [8]. The ESM microfibers are also believe to contain keratin [7].

In this study, we intend to fabricate a unique bilayered scaffold by deploying electrospun nanofibers of PCL on top of ESM followed by crosslinking the same with NHS/ EDC. The as fabricated scaffolds were exposed to various aqueous environment to test for its stability. The microstructure and surface topography of as prepared scaffolds were examined and compared with the natural ESM. The molecular footprint of the scaffold was also studied using FTIR spectroscopy. Finally, the effect of nanofiber decoration on cell viability along with cell adhesion and proliferation was also evaluated using Human dermal fibroblast cells.

EXPERIMENTAL

Herein, the extraction and surface modification of ESM was performed using acetic acid (ESM AA) as demonstrated in our previous report [9]. In brief, yolk was first separated and eggshell containing membrane was washed in DI water followed exposure to 2 % acetic acid (Merck) for 15 mins. Post incubation in acetic acid, the system was rinsed with sufficient amount of DI water in order to remove any unreacted or remaining acid moiety from the surface. The membranes were extracted, washed and dried under room temperature and hereafter termed as ESM AA. The hierarchical decoration of ESM microfibers was performed using electrospun nanofibers of polycaprolactone (PCL). In order to do so, 15 wt% PCL (Sigma) was dissolved in chloroform and was kept under continuous stirring overnight. The above prepared PCL solution was homogenized and carefully loaded onto a 5 ml syringe fitted to a 26-gauge needle tip. The solution loaded syringe was mounted on syringe pump (KD Scientific, Switzerland) and the flow rate was kept constant at 1 ml/ h. Chemically modified ESM AA samples were portioned into appropriate shapes and loaded onto the grounded stationary target while the distance between collector and needle was maintained at of 10 cm. The electrospinning process was carried out under a stable voltage supply of 20 KV (Glass Mann, Japan) for 2 h in order to get a thick sheet. Further, the nonwoven nanofibrous sheets of PCL were crosslinked onto the surface of ESM AA by using NHS/ EDC crosslinking. Briefly the uncrosslinked nonwoven bilayered composites were exposed to 0.2 M EDC/ 0.05 M NHS solution in DI water followed by inactivation by 1M Na_2HPO_4. After deactivation, samples were thoroughly washed in DI water and dried under room temperature on teflon sheets to obtain a bilayered composite hereafter termed as E-PCL prior to other application. The fabricated bilayered scaffolds were further characterized to study its microstructural topography, biochemical composition and biocompatibility.

RESULTS & DISCUSSION

The present study narrates a route for hierarchical decoration of ESM AA in order to fabricate a bilayered scaffold for skin tissue engineering. The various extraction procedure utilized for extracting ESM have rendered it with rough fibers and uneven morphology that was witnessed to impede cell adhesion and proliferation [10].

Therefore, it was important to design a route to decorate or modify the surface of ESM without affecting its fibrous architecture. The presence of PCL nanofibers not only aided in fabricating a bilayered scaffold but also decreased the surface roughness of the natural tissue. Inclusion of PCL also moderately increased the hydrophobicity of the scaffold thus facilitating cell adhesion. Moreover, the addition of PCL nanofibers decreased the pore size and inter-fiber spacing thus aiding in cell adhesion and proliferation. Microstructural Evaluation: FESEM (SEM, EVO 60/ Zeiss, Germany) micrographs revealed a highly crosslinked network of intact microfibers for ESM AA samples with diameters in the range of 0.5 – 3.5 µm while thickness varied between 50-60 µm (Figure 1a). It was further noted that average inter-fiber distance for ESM AA samples varied

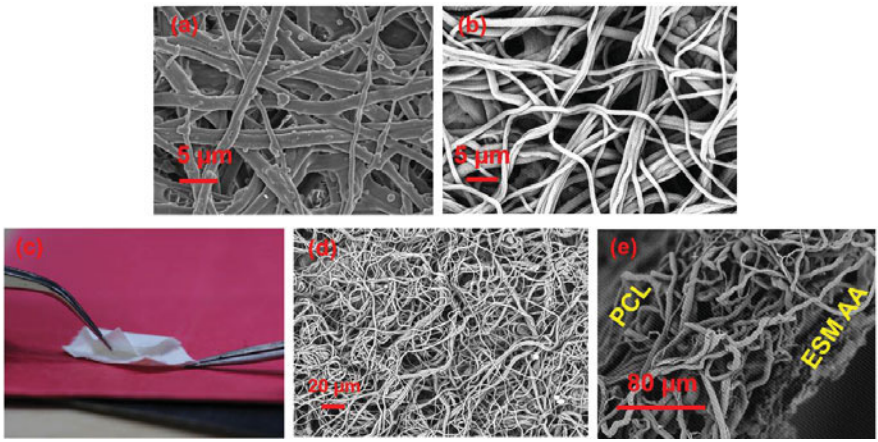

Figure 1. Microstructural assessment of ESM AA and E-PCL samples. FESEM micrographs of (a) ESM AA & (b) E-PCL samples clearly depicts the micro/nano architecture of the as prepared samples. (c) Digital photograph of as prepared samples demonstrating flexibility and the double layer architecture of E-PCL matrix. (d) Lower Magnification image of E-PCL representing the overall structure and crosslinking between microfibers and nanofibers. (e) FESEM micrograph of vertical cross – section of the prepared scaffold thus representing the bi-layered architecture of the construct.

between 10-20 µm. The large gap between fibers may led to cell penetration. Further, no distortion in fibrous architecture of ESM AA was observed but the fiber surface

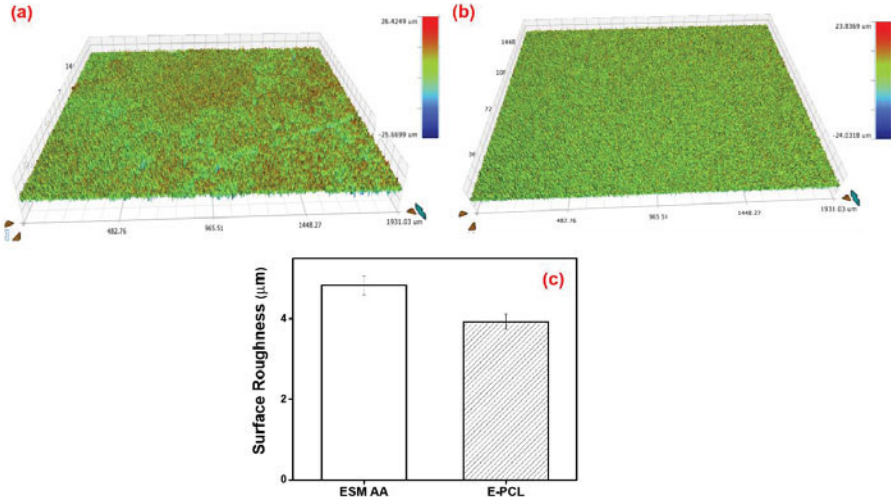

Figure 2. 3D Optical Surface Profilometry (OSP) of (a) ESM AA (b) E-PCL. (c) represents the average surface roughness of the scaffolds

portrayed a rough topography which may again impede cell adhesion. In contrast the surface of E-PCL samples presented smooth electrospun nanofibers possessing an average fiber diameter between 700 nm – 1.2 µm (Figure 1b). Moreover, the inter-fiber distance was also reduced to 6 – 12 µm thus creating an ambient microenvironment for cell adhesion and proliferation. The presence of the nanofibers also decreased the overall porosity of scaffolds from 93% in ESM AA samples to 86% for E-PCL matrices which may facilitate its application in skin tissue engineering. The wider view of the E-PCL scaffold as demonstrated in figure 1 d, also conveniently reveals effective crosslinking between microfibrous ESM AA and nanofibrous PCL sheet thus ratifying bilayered architecture of the fabricated scaffold.

Further, 3D Optical Surface Profilometry (Bruker Contour GT 3D optical microscope) of above samples were carried out to investigate the effect of nanofiber decoration on surface topography of the bilayered composite. It was clear from figure 2 that the inclusion of PCL nanofibers imparted smoothness to the surface while in contrast the ESM AA surface displayed inhomogeneous and rough topography. Moreover, it was also calculated from OSP results that average surface roughness (Ra) of ESM AA was 4.823 µm which eventually decreased to 3.924 µm from E-PCL scaffolds. The above observation from OSP results were concurrent with the FESEM micrographs thus concluding successful decoration of ESM AA scaffold to form a bilayered matrix.

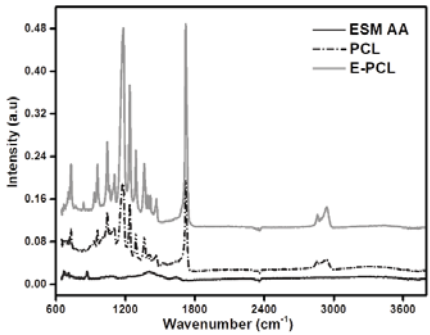

Figure 3. ATR - FTIR Spectra of ESM AA, PCL, E-PCL

Biochemical Composition of Scaffolds: The biochemical composition of scaffolds was further evaluated using ATR - FTIR spectroscopy and upper side of the scaffold was scanned where presence of PCL nanofibers was suspected as shown in Figure 3. It's clear from the spectra that peaks of PCL were dominating the footprints of ESM AA in E-PCL sample and the noted observation was obvious considering the architecture of E-PCL scaffold. However, the PCL spectra consisted of its common band comprising of asymmetric and symmetric -CH2- stretching stationed at 2940 cm^{-1} and 2866 cm^{-1} respectively. The carbonyl (C=O) stretching for PCL was also centered at 1724 cm^{-1}. Importantly, symmetric and asymmetric –C-O-C- bond stretching for PCL was observed at 1174 cm^{-1} and 1238 cm^{-1} respectively while the peak for C–O stretching was positioned at 1295 cm^{-1}. Common peaks for ESM AA spectra was found around 1648 cm^{-1} and 3400 cm^{-1} representing carbonyl stretching and $–NH_2$ in amide stretching respectively.

Biocompatibility of Scaffolds: The biocompatibility of above fabricated scaffolds was studied with Human Dermal Fibroblast (HDF) cells. The HDF cells were isolated from circumcised sample of human foreskin following a previously reported protocol [5]. The process of isolation was approved by institutional ethical committee (IEC), Indian Institute of Technology, Kharagpur (Ref no. IIT/SRIC/AR/2012). Isolated cells were cultured in DMEM complete medium supplemented with 10% FBS, 1% antibiotic. The cells were cultured until passage 3 to 5 prior to application. Figure 4a demonstrates the mitochondrial activity of HDF cells when exposed to ESM AA and E-PCL samples for 1d, 3d & 7d. Excellent cell viability was witnessed for E-PCL samples with exponential increase in all three time points. The presence of PCL nanofiber not only rendered a better matrix for cell adhesion but also increased the hydrophobicity of the matrix thus leading greater protein binding which directly affected cell growth. On the other, cell

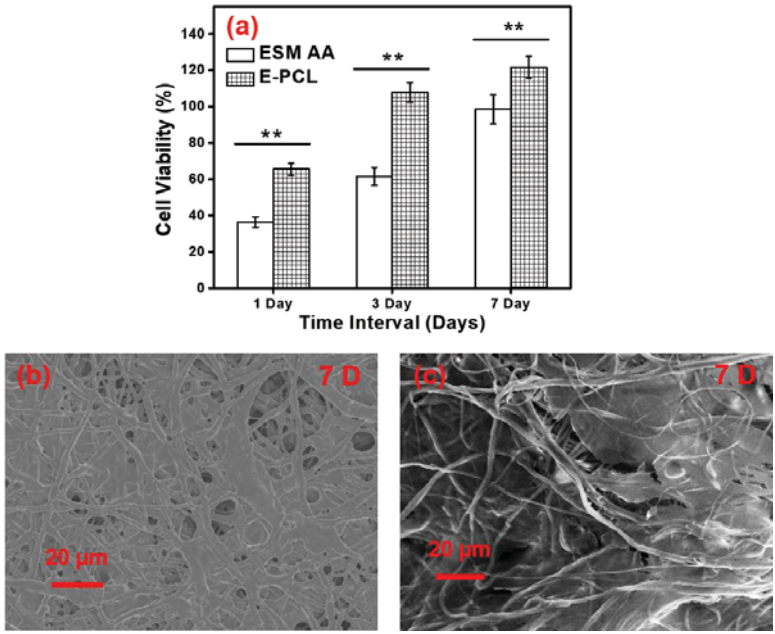

Figure 4. (a) MTT assay demonstrates mitochondrial activity of ESM AA and E-PCL scaffolds when exposed for 1 D, 3 D & 7 D. (b) & (c) HDF cell adhesion and proliferation post 7 D of incubation in (b) ESM AA & (c) E-PCL. Error bars depicts standard deviation ($n \geq 3$) of independent samples per group, double asterisks indicate $P < 0.05$

growth on the natural matrix was slow owing to its rough surface topography and high hydrophilicity. However, a decent growth of HDF cells were observed post 7 D of incubation. The FESEM image represented in figures 4c clearly stated excellent cell adhesion and proliferation for E-PCL samples due to the above stated reasons. The cells were able to form sheets or a layer by layer structure on the E-PCL matrix whereas a retarded cell attachment was apparent for ESM AA samples. The cells were either penetrating inside the scaffold or they were forming lumps by aggregating on top of each other owing to extreme surface topography of the natural matrix. It was ostensible from

the above observation that decoration of nanofibers had an extensive impact on the adhesion and proliferation of HDF cells thus indicative of the fact that it may lead better tissue regeneration when exposed to in vivo conditions.

CONCLUSION

The study demonstrates the fabrication of a novel bilayered scaffold, E-PCL with two bioactive polymers for skin tissue engineering applications. It was observed that the developed scaffolds possessed a nano/ micro architecture with nonwoven nanofibers crosslinked to microfibers of ESM AA. It was certain from the above results that the scaffold not only repaired the drawbacks but also led to an ECM mimicking bilayered structure. E-PCL matrices excellently supported the adhesion and growth of HDF cells thereby reinstating its application in skin tissue engineering. It was apparent from the above observation that the developed scaffold can be explored as a potential alternative in tissue engineering applications.

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