ORIGINAL RESEARCH PAPER

Health Science

POTENTIAL SKIN SUBSTITUTE OF BIOMIMETIC PROTEINS AND TERPOLYMER WITH PROVEN IMMUNO-COMPATIBLE AND BIODEGRADABLE PROPERTIES

KEY WORDS: Skin Substitute, Electrospinning, Terpolymer PLGC, Fibrin, Hyaluronic Acid, Wound Healing.

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ABSTRACT

Non-immunogenic matrix with desirable biological and mechanical properties could be a valuable graft to treat large area acute wounds and non-healing chronic wounds. In this study, an electrospun biodegradable polymer scaffold composed of a terpolymer PLGC [poly(L-Lactide-co-Glycolide-co-Caprolactone)] incorporated with a fibrin-hyaluronic acid (HA) based biomimetic composite (PLGCFIBHA) was evaluated. An appropriate mechanical properties, fibroblast growth potential, biodegradability and absence of immune response were established. A significantly increased fibroblast attachment and proliferation was observed with optimal physical properties. Intracutaneous (intradermal) reactivity test and guinea pig maximization test was negative as per requirements of ISO-10993-10:2010(E): biological evaluation of medical devices part 10 proving immunocompatible nature. *In vitro* and *in vivo* studies showed suitable degradability for skin tissue engineering purposes. Hence the fabricated scaffold PLGCFIBHA is advocated as a potential candidate for the engineering of dermal tissue which can be used as an off the shelf product.

INTRODUCTION:

Wound healing is often compromised in diabetic, severe burn and aged patients resulting in non-healing ulcers which currently has no effective pharmacologic remedy, and requires skin grafting (Han & Ceilley, 2017; Perez-Favila et al., 2019). Principles of regenerative medicine gives a ray of hope in such conditions which is an emerging technology- based on biomaterials, stem cells or a combination of both (Chen & Liu, 2016). A wide variety of biomaterials are commercially available for wound care which are based on natural materials; mainly xenogenic or allogenic collagen products with or without cells faceing risk of infection, immune rejection, donor site morbidity, and scar formation (Axibal & Brown, 2019). Therefore, potential approach may be adopted to develop a biomimetic scaffold that is mechanically compliant with native skin and having biological cues that can mobilize stem cells, keratinocyte precursors, fibroblasts and vascular cells from neighbouring tissue to promote proliferation and differentiation of cells to bridge the gap, heal the epidermal and dermal wound and produce vascularity and thus bringing out continuity of native tissue with regenerated tissue (Kaur et al., 2019).

Biomaterials have played an increasingly prominent role in the success of biomedical devices and in the development of tissue engineering. Biodegradabile, non-toxic, non-immun ogenic, and non-inflammatory with low risk of disease transmission, easy availability, long-shelf life, and user-friendliness are the major properties considered to make an ideal engineered substitute (Chen & Liu, 2016). Bioabsor bable polyesters comprising glycolide (GA)/lactide (LA) /ε - caprolactone are preferred as they are biocompatible and approved by the U.S. Food and Drug Administration. A nearly similar terpolymer based wound care product available in the international market is Suprathel (Uhlig et al., 2007). This degradable terpolymer scaffold with a different composition has been FDA approved, commercially available and in clinical use (Rashaan et al., 2017). Electrospinning can be employed to generate nano- and micro-architectural scaff olds similar to the fibrous structures of native ECM with controllable parameters like porosity, evaporative water loss, oxygen permeability, and fluid drainage, etc (Law et al., 2017). However, poor cell growth on synthetic polymers could be a limitation, and this may be countered by combining natural polymers such as collagen, chitosan, gelatin, fibronectin, or fibrin (Williams, 2019). Fibrin and hyaluronic acid (HA) forms important constituent of the natural extra cellular matrix (ECM) of skin and its beneficial role in wound healing has been reported (Anilkumar et al., 2011; Chen & Liu, 2016).

In the current study, a biomimetic fibrin-HA matrix immob ilized on the electrospun terpolymer PLGC [poly(L- Lactide-co-Glycolide-co-Caprolactone)] (PLGCFIBHA) was developed as an off-the-shelf skin substitute with enhanced wound healing properties. The objective was to evaluate its physical and biological properties and its immunological response and biodegradability for using as an off-the-shelf skin substitute.

MATERIALS AND METHODS:

FABRICATION OF STERILE SKIN SUBSTITUTE PLGCFIBHA:

The skin substitute PLGCFIBHA was prepared aseptically using electrospun terpolymer poly (L-Lactide-co-Glycolide-co-Caprolactone) / PLGC, clinical grade Fibrin Sealant (prepared in-house) and umbilical cord tissue derived pure HA prepared using in-house standardized method as described previously (Anilkumar et al., 2011).

Briefly, the electrospun and characterized terpolymer PLGC fiber matrices prepared as described earlier was cut into patches of the required size (Ramakrishnan et al., 2019). The lyophilized constituents of fibrin sealant kit was dissolved in the respective solvents as per the instructions for use for clinics and HA in sterile water. The solutions were then diluted to get final concentrations of fibrinogen concentrate (10 mg/mL), thrombin (10 IU/mL) and HA (200 µg/mL). The electrospun PLGC was cut into pieces according to the size of the mold and 200 µL / cm^2 fibrin-HA composite was delivered to the mold on the PLGC and allowed to clot for 30 min at 37° C, frozen at - 80° C, lyophilized (Edwards, Modulyo 4K) and sterilized using plasma sterilization (Sterrad, USA) and stored

sealed at 4° C until use. The final product in the form of lyophilised wafer was used for its evaluation (Figure 1 a-c).

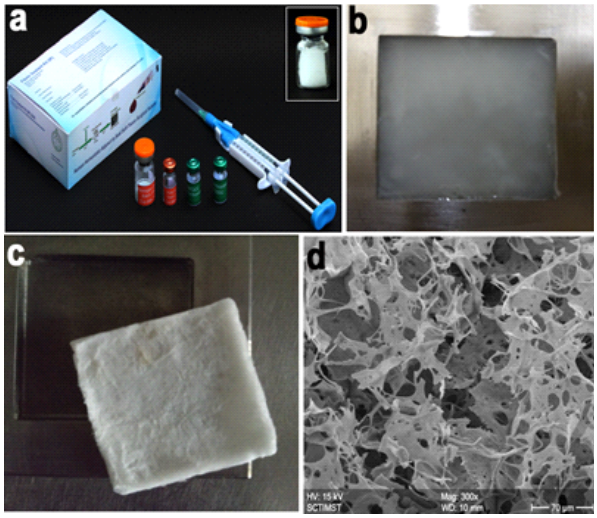

Figure 1. Representative images of a) fibrin sealant kit and dual syringe applicator (inset –hyaluronic acid), b & c) final product PLGCFIBHA before and after lyophilisation and d) ESEM image of PLGCFIBHA.

PHYSIOCHEMICAL EVALUATION OF THE PLGCFIBHA SKIN SUBSTITUTE:

The surface topography was analysed using environmental scanning electron microscope (ESEM, FEI Quanta 200, Netherlands). The surface wettability was determined by sessile drop method using a video-assisted contact angle measuring goniometer (Data Physics OCA15 plus, Germany) and imaging software (SCA20 software, Germany). Percentage swelling studies were carried out in simulated body fluid (SBF; pH = 7.4) and phosphate buffered saline (PBS; pH = 7.4) at 37° C for 24 h. Water vapour transmission rate (WVT°C) studies were carried out according to the ASTM Standard Test Method E96/E96M 14 (E96-00 procedure D) at a temperature of 37° C and 50 % humidity and monitored for 3 d.

HEMOCOMPATIBILITY OF THE SKIN SUBSTITUTE PLGCFIBHA:

Hemolysis assay was carried out as per ISO 10993-4:2002 (E) procedure using empty polystyrene dishes as a reference (generating negligible hemolysis < 0.1 %) to analyse the hemocompatibility of the scaffold.

CYTOCOMPATIBILITY OF THE SKIN SUBSTITUTE PLGCFIBHA:

The characterised human ADMSCs derived fibroblasts were used for the validation of PLGCFIBHA scaffold. MTT assay was done to assess cytotoxicity of the scaffold. To evaluate the cell attachment and growth of fibroblasts on PLGCFIBHA scaffold, cells were seeded at a density of 5000 cells/cm^2 and cultured under normal conditions for 7 d and stained for cytoskeletal actin with Texas Red Phalloidin (Molecular probes, USA). Electron micrographs were taken to observe the spreading of cells on the scaffold after 7 d of culture.

BIOCOMPATIBILITY & BIODEGRADABILITY OF THE SKIN SUBSTITUTE PLGCFIBHA:

TOXICOLOGICAL EVALUATION OF THE SCAFFOLD PLGCFIBHA:

Toxicological evaluation of PLGCFIBHA scaffold was done according to the OECD guidelines to meet the requirements of ISO-10993-10:2010(E): biological evaluation of Medical Devices part 10 : test for irritation and skin sensitization test clause 6.4: animal intracutaneous reactivity test & USP 38/NF33:2015 and clause 7.5: guinea pig maximization test (GPMT) respectively. All animal studies were done after obtaining Institutional Animal Ethics Committee (IAEC) approvals, conforming to the CPCSEA, Government of India regulations.

Intracutaneous (intradermal) reactivity test was done in albino rabbits (n=3) using physiological saline (PS) and cotton seed oil extracts from scaffold incubated at 37 ± 1 °C for a time period of 72 ± 2 °C at a agitation of 50 rpm. The extracts were aseptically injected (0.2 mL/site) at five different sites which was compared with injections with control solution (PS alone) with scaffold incubation. The grading of Erethema of test and control sites of all animals at 24, 48 and 72 hours was recorded.

GPMT was evaluated to analyse the skin sensitization potential for the PLGCFIBHA scaffold, intended to use as skin substitute/graft. GPMT was done in Hartley albino guinea pigs (n=15) using PS extract from scaffold incubated at 37 ± 1 °C for a time period of 72 ± 2 °C at a agitation of 50 rpm. The PS extract of the test and control (PS alone) was intradermally injected (0.1 mL/site) and after 7 d it was topically applied. Challenge test was carried out for 14 d on all the animals. The appearance of the challenge skin sites of test and control animals were observed at 24, 48 and 72 hours after removal of dressings and patches. The skin reactions for erythema and oedema were scored and recorded the numerical grading as per guidelines (Savoji et al., 2018).

DEGRADATION PROFILE OF THE PLGCFIBHA SKIN SUBSTITUTE:

The *in vitro* hydrolytic degradation properties of PLGCFIBHA scaffolds were studied as per the procedure stipulated in ISO 10993-13 confirmed by gravimetric analysis. The i*n vivo* degradation of bare PLGC and hybrid scaffold PLGCFIBHA was evaluated by GPC after subcutaneous implantation in New Zealand white rabbits. The implants were recovered after two time periods; 60 d and 120 d, minced finely, the polymer PLGC was extracted using solvent and GPC analysis was done and compared the weight/ number (Mw/Mn) average molecular weights of degraded scaffold with original (0 d) scaffold.

STATISTICAL ANALYSIS:

For all quantitative assays in the above experiments, more than three replicate experiments were carried out and the values were averaged and expressed as the mean ± standard deviation (SD). Statistical analysis using a one-way analysis of variance (ANOVA) and p values showing statistically significant differences were given in the figure legends.

RESULTS AND DISCUSSION:

CHARACTERISTICS OF PLGCFIBHA SKIN SUBSTITUTE:

The lyophilized & plasma sterilised PLGCFIBHA was pliable and easily handled with forceps (Figure 1c). The PLGCFIBHA scaffold showed porous surface topography with electrospun fibres of PLGC completely covered with FIBHA matrix. The deposition and distribution of fibrin network is seen throughout the scaffold. The ESEM images were analysed using image J software and showed an average pore diameter of 45.45 ± 20 μm (Figure 1d). The surface wettability analysis using the contact angle measurement showed hydrophobic nature of the bare PLGC with a value of 123 ± 1° whereas, the hybrid PLGCFIBHA scaffold showed hydrophilic character making it difficult to capture the contact angle. Average swelling percentage of the bare PLGC was found to be 77.3 ± 11.7 and 69.8 ± 20 and for PLGCFIBHA scaffold swelling were 240 ± 38 % and 260 ± 34 % in PBS and SBF respectively. Bare PLGC has no swelling characteristics of its own and the results suggested that bio mimetic matrix deposition enhanced water retaining ability of the PLGCFIBHA scaffold. The WVTR of the bare PLGC and the hybrid PLGFIBHA scaffold ranged from 2639 to 2972 g/m^2/day and 2278 - 2401 g/m^2/day. Statistical analysis showed that WVTR decreased significantly

upon fibrin composite deposition still in the normal range for wound healing applications. The observation suggests that the use of liquid components of fibrin sealant enabled penetration of the components to the pores and formation of fibrin on the internal fiber surfaces.

CYTO- AND HEMOCOMPATIBILITY OF PLGCFIBHA SCAFFOLD:

The MTT assay showed % cell viability of 94 % and 127 % for PLGC and PLGCFIBHA scaffolds respectively. In vimentin-actin co- staining, cell adhesion on the PLGCFIBHA scaffold was found to be visibly higher than that on bare PLGC. The entire surface area in the PLGCFIBHA scaffold was found to be positive for vimentin and cytoskeletal actin of cells when compared to bare PLGC scaffold within 7 d. When counter stained with Hoechst dye, blue coloured nuclei seen in actin stained regions confirmed the specificity of fibroblast vimentin and actin stained cytoskeletons (Figure 2 a - j). The ESEM analysis further confirmed better fibroblast coverage on the scaffold surface of the scaffold indicating that the cells are not only spread on the outer surface; but, also probably penetrated into the pores and proliferated. Fibroblast covered fibre to fibre distance; and the entire scaffold surface was found to be covered with a canopy of fibroblast cells, whereas in the case of bare PLGC only few patches of fibroblast cells were observed (Figure 2 k & l). The increased fibroblast attachment and proliferation may be attributed to biological cues from fibrin-HA components (Pankajakshan et al., 2008).

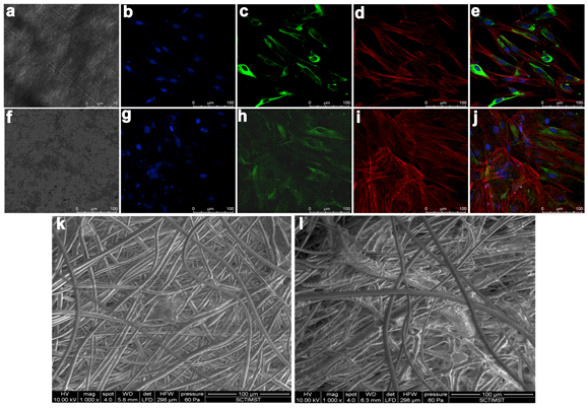

Figure 2. Fluorescent micrographs of vimentin & actin-stained fibroblast on scaffold: a & f) phase contrast images, b & g) DAPI staining, c & h) vimentin staining, d & i) actin staining, e & j) merged images of fibroblast cultured for 7 d on PLGC and PLGCFIBHA scaffolds respectively, scale bar =100μm and k & j) ESEM images of bare PLGC and PLGCFIBHA scaffolds after 7 d of fibroblast culture, respectively.

Percentage hemolysis estimation has been used as a simple and reliable technique for estimating blood compatibility of materials. As per ISO 10993-4:2002 (E), for material to be non-hemolytic, the percentage hemolysis should be less than 0.1 %. All tested samples displayed less than 0.1% hemolysis. The PLGCFIBHA scaffold showed % hemolysis values of 0.03 ± 0.009 indicating hemocompatible nature.

BIOCOMPATIBILITY OF THE PLGCFIBHA SCAFFOLD:

TOXICOLOGICAL EVALUATION OF THE PLGCFIBHA SCAFFOLD:

Intracutaneous (intradermal) reactivity test: The results indicated that PS and cotton seed oil extract of PLGCFIBHA scaffolds induced a total mean score of 0 and 0.89 in PS and cotton seed oil extract respectively following intradermal injection confirming no adverse reaction to the test solutions. In both cases, there was no reduction of body weight was seen.

GPMT: GPMT did not show any adverse skin reaction during the induction or challenge period and confirmed that PS extract of PLGCFIBHA scaffolds are non-irritant at the laboratory conditions simulated. In both cases, there was no reduction of body weight seen.

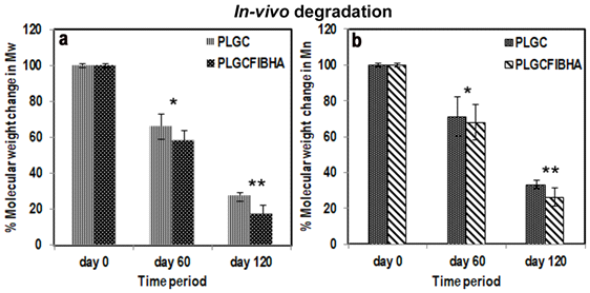

Figure 3. Data on in vivo degradation of PLGCFIBHA hybrid scaffold analysed by GPC: A) number average molecular weight (Mn) of hybrid scaffolds and B,) weight average molecular weight (Mw) of hybrid scaffolds on 0 d, 60 d and 120 d of study respectively. Bars represent means ± SD (n=6), * p value < 0.05, ** p value < 0.01.

***DEGRADATION PROFILE OF PLGCFIBHA SKIN SUBS TITUTE*:**

Biodegradation of the scaffold is a desirable prerequisite in tissue engineering endeavours for facilitating natural tissue formation. *In vitro* hydrolytic degradation showed a gravimetric weight loss of ~ 23 ± 4 % within 30 d. *In vivo* degradation of bare PLGC and hybrid PLGCFIBHA scaffold after 60 d and 120 d suggest that the degradation was slightly higher for the hybrid PLGCFIBHA than bare PLGC scaffold. The number average molecular weight (Mn) of the bare and hybrid PLGCFIBHA scaffold was decreased by 28.8 ± 11 % and 32 ± 10 % within 60 d and 66.7 ± 2.4 % and 73.7 ± 5 % by 120 d, respectively (Figure 3). *In vitro* and *in vivo* degradation of the PLGCFIBHA scaffolds showed biodegradable nature which could be cleared from the wound site with time and pave way for regeneration (Wang et al., 2013).

CONCLUSION:

Biodegrabable and non-immunogenic matrix as skin substitute that promotes cell proliferation *in vitro* could guide wound regeneration posing scope in the wound care product market. This study established design of a suitable combination scaffold, meeting defined primary requirements and comprising biomimetic matrix for improved cell adhesion/migration supported by a mechanically strong, biocompatible scaffold. The fabricated skin substitute was biodegradable and immunocompatible with optimum properties which can be used as an off-the-shelf product.

CONFLICT OF INTEREST DISCLOSURE:

The authors declare no conflict of interest.

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