FORMULATION AND CHARACTERIZATION OF SILK FIBROIN FILMS AS A SCAFFOLD FOR ADIPOSE-DERIVED STEM CELLS IN SKIN TISSUE ENGINEERING

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Skin substitutes are epidermal, dermal or complete bilayered constructs, composed by natural or synthetic scaffolds and by adherent cells such as fibroblasts, keratinocytes or mesenchymal stem cells. Silk fibroin is a promising polymer to realize scaffolds, since it is biocompatible, biodegradable, and exhibits excellent mechanical properties in terms of tensile strength. Moreover, fibroin can be added of others components in order to modify the biomaterial properties for the purpose. The aim of this work is to prepare silk fibroin films for adipose-derived stem cell (ADSCs) culture as a novel feeder layer for skin tissue engineering. Pectin has been added to promote the protein conformational transition and construct strength, while glycerol as plasticizer, providing biomaterial flexibility. Eighteen formulations were prepared by casting method using fibroin, pectin (range 1-10% w/w), and glycerol (range 0-20% w/w); films were characterized by Fourier transform infrared spectroscopy and differential scanning calorimetry assay, to select the optimal composition. A stable fibroin conformation was obtained using 6% w/w pectin, and the best mechanical properties were obtained using 12% w/w glycerol. Films were sterilized, and human ADSCs were seeded and cultured for 15 days. Cells adhere to the support assuming a fibroblastic-like shape and reaching confluence. The ultrastructural analysis evidences typical active-cell features and adhesion structures that promote cell anchorage to the film, thus developing a multilayered cell structure. This construct could be advantageously employed in cutaneous wound healing or where the use of ADSCs scaffold is indicated either in human or veterinary field.

The development of skin substitutes dates back to 1975, when Rheinwald and Green (1) cultured keratinocytes on a layer of lethally irradiated 3T3 murine fibroblasts. Currently, this method remains the most reliable and widely used for *in vitro* keratinocyte culture, but some concerns are emerging about their safety (2-5). To overcome these limits, Sugiyama et al. (6) proposed the use of human irradiated adipose-derived stem cells (ADSCs) as a feeder cell layer: results showed that the morphology of irradiated stem cells was similar to 3T3, and both cell lines (stem and 3T3 cells) expressed genes promoting keratinocyte proliferation. Other researchers also showed that non-irradiated ADSCs can boost epithelialization, angiogenesis (7), capillary density and granulation thickness of transplanted cell-enriched collagen sponges (8). Altman et al. (9) observed that ADSCs, once seeded on acellular dermal matrix and applied on murine wound, spontaneously differentiated along vascular endothelial, fibroblastic and epidermal epithelial lineages and significantly improved wound healing; moreover, cells localize at implantation site.

Current cutaneous substitutes are epidermal, dermal or

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complete bilayered constructs seeded with keratinocytes, fibroblasts or both, respectively. Skin substitutes are composed by natural or synthetic scaffolds and must lead cells to tissue reconstruction. Commercial skin grafts are principally collagen-based matrices, such as hydrogels, sponges or lattices. Other natural materials (e.g. chitosan, fibrin and hyaluronan) are commercialized, but further clinical data are needed for FDA approval (10). A promising natural polymer is represented by silk fibroin, because of its peculiar biocompatibility (11), biodegradability (12), tensile strength and versatility (13). Besides textile industry, silk fibroin has been employed as suture material for centuries (14) and, more recently, in several biomedical fields as in hydrogels, membranes or scaffolds for regenerative medicine (13, 15-23).

The silk fibroin, usually extracted from the silkworm (*Bombyx mori*) cocoons, can assume three molecular conformations: *Silk II* (β-sheet structure), stable and water-insoluble; *Silk I* (a mixture of random coil, α-helix and β-turn), metastable and water-soluble; *Silk III* (α-helix structure at the water/air interface) (24, 25). The native silk fibroin is purified from sericin by the de-gumming process, and has a predominant *Silk II* structure. De-gummed fibers of silk fibroin can be used as they are or can be regenerated in order to obtain an aqueous fibroin solution. The regenerated fibroin is then processed in different ways to produce adequate scaffolds (13, 20, 26-28). The regenerated silk fibroin, in aqueous solution or as scaffold, mainly presents *Silk I* form. Since the *Silk II* conformation is a fundamental condition for the employ of silk fibroin in regenerative medicine, it is mandatory to promote the conformational transition from *Silk I* to *Silk II* by treating the *Silk I* form with organic solvents (such as methanol or ethanol) (29-31), mechanical stress, temperature change or presence of other polymers, such as alginate, polyethylene glycole, chitosan, pectin (24, 32), and glycerol (33). The pectin, a natural polysaccharide polymer constituting the plant cell wall, has been recently employed for several biomedical applications, including drug and gene delivery, wound healing and tissue engineering; in particular, pectin hydrogels were used for bone tissue regeneration, as prosthetic nucleus pulposus substitutes or as wound healing patches (34). Glycerol is a plasticizer and has been added to fibroin by Lu et al. (33) to obtain insoluble and flexible films, improving their mechanical properties when compared with pure silk fibroin films.

The aim of this work is to prepare silk fibroin films for ADSCs culture as a novel feeder layer for skin tissue engineering. Pectin has been added to promote the protein conformational transition and construct strength, while glycerol as plasticizer, providing biomaterial flexibility.

MATERIALS AND METHODS

Preparation and sterilization of silk fibroin films

The fibroin solubilization was performed as reported by Chlapanidas et al. (35): briefly, cocoons of *Bombyx mori* were degummed in autoclave, dried at room temperature and treated with a solution of $Ca(NO_3)_2$ (75% w/v) in methanol. After filtering under vacuum, the solution was dialyzed, and the final concentration of aqueous silk fibroin solution was 5% w/v.

Pectin powder (Pectine Industrie, Italy) was solubilized in deionized water under stirring at 80°C, while glycerol (Fluka, Germany) was diluted 1:1 in water; silk fibroin, pectin and glycerol were mixed under soft stirring, cast into molds (8.5 cm diameter) to guarantee solvent evaporation (60°C, 6 hours) (32). Eighteen films were obtained using different fibroin-pectin-glycerol ratios reported in Table 1: the first group of films (A-F films, Group 1, see Table 1) is composed by fibroin and pectin only, with a pectin concentration between 1 and 10% w/w. In the second and the third group (G-N and O-T films, Group 2 and Group 3, respectively, see Table 1) glycerol is added at concentration 1-10% and 2-20% w/w respectively, and for each formulation the ratio pectin:glycerol is 1:1 (for Group 2) or 1:2 (for Group 3). Two film formulations were selected and sterilized by three methods: moist heat (autoclave, 121°C for 20 minutes), dry heat (160°C for 20 minutes) and gamma irradiation (at 20 and 60 kGy). A microbial test was performed after sterilization processes: films were incubated for 7 days in DMEM medium (Euroclone, Italy) at 37°C, 5% CO_2: after this period, 5mL of the supernatant were inserted into adequate flask and analyzed using BacT/ALERT 3D system to detect bacteria and fungi.

Fourier Transform Infrared Spectroscopy and Differential Scanning Calorimetry analysis

Before and after sterilization, films were analyzed by Fourier Transform Infrared Spectroscopy (FTIR) on a Bruker Alpha-E spectrometer equipped with a MIRacle™ attenuated total reflection Diamond crystal cell in reflection mode. Background measurements were taken twice with an empty cell and subtracted from the sample readings. The FTIR spectra in the absorbance mode were obtained in the spectral regions of 500–3500cm^{-1}. Each spectrum of the samples was acquired by accumulation of 32 scans with a resolution of 4 cm^{-1}.

Samples were also analyzed by Differential Scanning Calorimetry (DSC) using a Mettler TA30 differential calorimeter. Thermal scanning was carried out on 3mg of each sample under nitrogen atmosphere (temperature range: 10°C–500°C, rate: 10°C/min).

Cell culture

A sample of adipose tissue (about 50 mL), collected from one informed subject, was suspended in phosphate buffered saline (PBS) (Euroclone) with penicillin/streptomycin 1% (Euroclone), put into a sterile box, and forwarded to the laboratory at a temperature of 4°C. Adipose tissue was digested as reported by Faustini et al. (36), using a collagenase concentration of 0.02% w/v (Sigma, USA) for one hour; cells were then centrifuged and washed twice with PBS. Stromal vascular fraction was plated on plastic surface (10.000 cell/cm^2) in DMEM/F12 (Euroclone),

10% fetal bovine serum (ThermoFisher, USA), 1% penicillin/streptomycin and 1% Amphotericin B (Euroclone); adherent stem cells were expanded till the 3rd passage. Films were cut in 1cm^2-squares and cells were then cultured on the prepared films (20,000 cells/cm^2 of film) for 15 days at 37°C, 5% CO_2.

Characterization of cell culture

After cell culture, silk fibroin cell seeded films were treated with 2% glutaraldehyde (Sigma) for 30 min at room temperature, then 2% glutaraldehyde and cacodylate buffer (Sigma) 0.1M for 30 min at 4°C, and finally washed with cacodylate buffer 0.1M. Samples were dehydrated using a graded ethanol series (from 30% to 100%) (Carlo Erba, Italy), freeze-dried, critical point dried, sputter coated with gold and analyzed using a scanning electron microscope (JEOL JSM-6380LV) operating at low vacuum degree, 20 kV, retrodiffused electron signal.

Moreover, samples were first fixed with 2.5% glutaraldehyde in cacodylate buffer 0.1M ph 7.4, then with osmium tetroxide (Sigma) 0.1% and finally dehydrated with alcohol scale. Films were included in Epon 812/Araldite resin (Sigma) before being sectioned using Ultracut S Ultramicrotome. The thin sections were treated with Toluidine Blue staining (Sigma) and observed under an optical microscope, while the ultrathin sections were observed for transmission electron microscopy with a JEOL JEM 1200 EX instrument.

RESULTS

The eighteen different films had different characteristics according to pectin and glycerol content. Films composed by fibroin and pectin only resulted rigid, fragile and they broke during mold removal; the presence of glycerol improved film elasticity and resistance. FTIR and DSC analysis were carried out to evaluate the fibroin molecular conformation. Considering the Amide I peak obtained from FTIR spectra, a wavenumber of ~1650 cm^{-1} was related to *Silk II*, while a wavenumber of ~1625 cm- 1 corresponded to *Silk I* form. Among A-F groups, A and B films presented the *Silk I* conformation (Table 1); increasing pectin concentration, the Amide I peak shifted to lower frequencies and then remained unchanged in the case of films containing 6%, 8% and 10% pectin (D-F films, respectively) (Table 1).

These results indicated that the fibroin conformational transition was obtained using 6% of pectin, and the same behavior was observed for the second and third group of films (Table 1). The FTIR spectrum of Q film (4% pectin) presented the characteristic absorption bands of both

Table 1. *Weight/weight percentage composition of silk fibroin films and wavenumber of Amide I peak.*

	Film	Fibroin	Pectin	Glycerol	Amide I peak (cm^{-1})
Group 1	A	99	1	0	1648.93
	B	98	2	0	1648.53
	C	96	4	0	1628.79
	D	94	6	0	1625.51
	E	92	8	0	1625.58
	F	90	10	0	1625.83
Group 2	G	98	1	1	1647.99
	H	96	2	2	1647.44
	I	92	4	4	1630.68
	L	88	6	6	1627.86
	M	84	8	8	1627.33
	N	80	10	10	1626.45
Group 3	O	97	1	2	1648.00
	P	94	2	4	1647.80
	Q	88	4	8	1644.30 and 1627.00
	R	82	6	12	1626.29
	S	76	8	16	1626.02
	T	70	10	20	1625.69

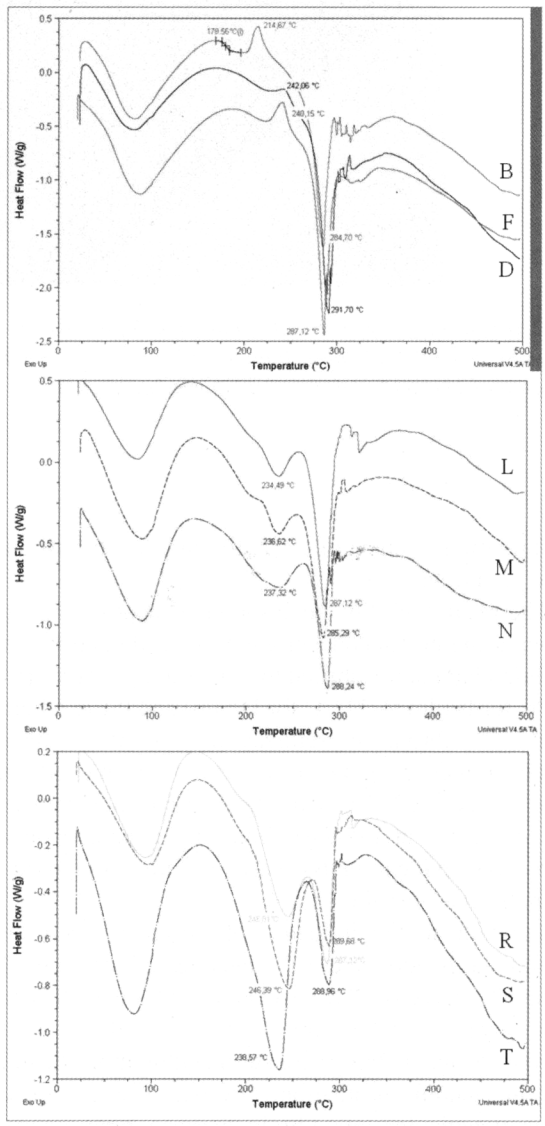

Fig. 1. *Differential scanning calorimetry (DSC) patterns of different representative silk fibroin films.*

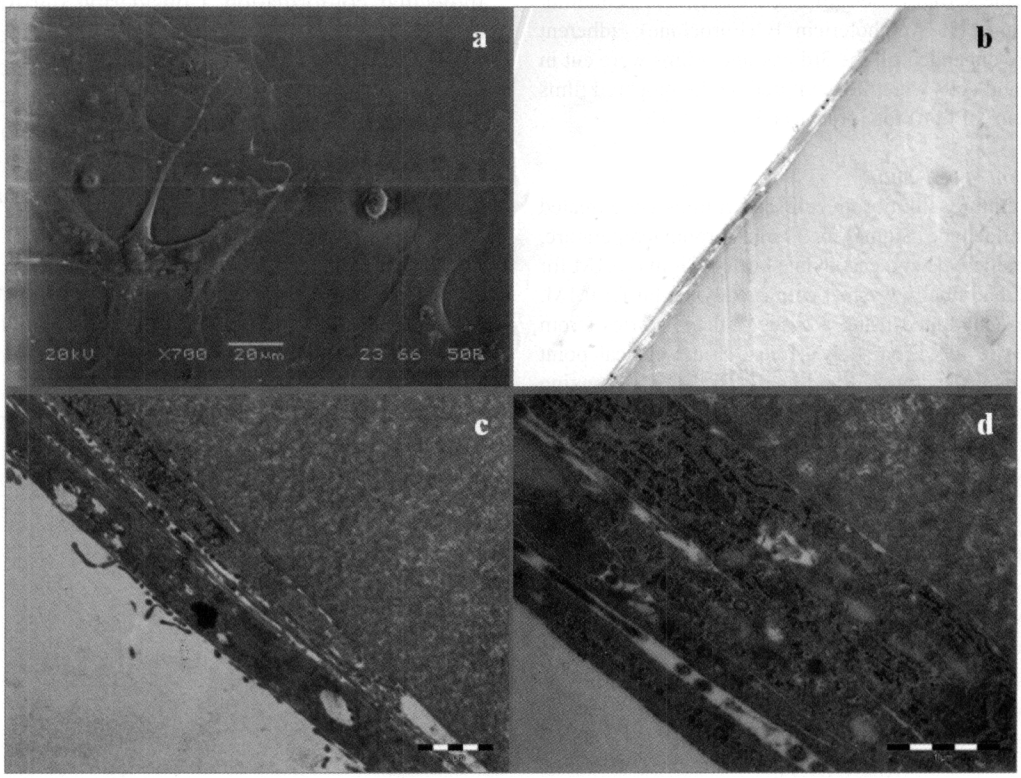

Fig. 2. *Adipose-derived stem cells cultured on R fibroin film: a) SEM image; b) light microscopy microphotograph of a film transversal section showing the cell layer, Toluidine Blue Staining, magnification 20x; c) and d) TEM images of a film transversal section showing the cell attachment organized in multilayer, bar in c): 2 µm; bar in d): 1 µm.*

conformations, index of a conformational transition in place (Table 1).

The results of DSC analysis were reported in Figure 1: increasing pectin concentration, the endothermic event of fibrin degradation shifted to higher temperatures (from 285°C for B film to 292°C for D film) and the exothermic event of fibroin crystallization (215°C) vanished; moreover, increasing pectin concentration, an exothermic event occured at 242°C. Adding glycerol, an endothermic peak compared at 235-240°C and this phenomenon was even more evident in the case of films composed by pectin:glycerol in ratio 1:2.

To evaluate the influence of different composition on film sterilization and cell attachment, R (fibroin 82%, pectin 6%, glycerol 12% w/w) and T (fibroin 70%, pectin 10%, glycerol 20%) films were selected for further analysis. R and T films were sterilized by moist heat, dry heat and gamma irradiation. Dry heat sterilization was not applicable for silk fibroin because films appeared hard, dry and yellowish (data not shown), while the other two methods of sterilization could be exploited. All films stand the sterility test by BacT/ALERT 3D system (data not shown). Moreover, the sterilization, both steam sterilization and gamma rays, did not induce fibroin degradation because the same peaks were appreciated by FTIR analysis (~1622 cm^{-1} after steam sterilization, ~1623 cm^{-1} after 20kGy sterilization and ~1624 cm^{-1} after 60kGy sterilization). However, after gamma radiation a less energy was requested for fibroin degradation: in fact, the DSC analysis shows the endothermic peak at ~288°C after steam steriliazation, ~287°C after 20kGy sterilization and ~288°C after 60kGy sterilization, but the energy was higher after autoclave sterilization than after irradiation with both of the doses.

The results of cell culture on silk fibroin films were reported in Figure 2. Silk fibroin films maintained their structural integrity until the end of cell culture. The morphological investigations showed that cells adhered to the support, with a fibroblast-like shape and reached confluence (Figure 2a). Adhesion of cells occurred without film penetration, since it did not show porosity. Toluidine Blue staining indicated that cells adhered to

both R and T films, but the best adhesion was observed using R film (Figure 2b). The ultrastructural analysis on R film suggested the presence of adhesion molecules that promoted cell anchorage to the film, forming a multilayered cell structure (Figure 2c); moreover, typical active-cell features as nuclei, mitochondria, rough endoplasmic reticulum, lysosomes and vacuoles were observed (Figures 2c and 2d).

DISCUSSION

Silk fibroin films were thecnological characterized and, according to FTIR and DSC analysis, a stable fibroin conformation was obtained using pectin in concentration of 6% w/w. Up to this pectin concentration, silk fibroin conformation is unchanged: probably, a maximum number of sites for pectin-fibroin interaction is saturated at 6% pectin concentration. The best results, in terms of elasticity and resistance, were obtained in presence of glycerol in order to reach a pectin/glycerol ratio of 1:2. Moreover, the DSC analysis has shown an exothermic event at ~240°C associated to pectin degradation and connected to the formation of new links, such as cross-linking or cyclization (37, 38). After adding glycerol, an endothermic peak appeared at ~240°C: this peak is similar to that of pectin degradation, but of opposite nature, and it is probably due to the molecular interation between pectin and glycerol; in fact, this pectin-glycerol interaction phenomenon is even more evident in the case of films composed by pectin:glycerol in ratio 1:2.

The steam sterilization process can be useful for silk fibroin films: several authors have applied moist heat sterilization whithout any appreciable fibroin alteration (16, 17, 39-41). On the other side, Lawrence et al. (25) observed that the sterilization with autoclave increased *Silk II* structure because hydrostatic pressure increases elastic modulus and then crystalline structure content; saturated vapor environment decreases the Tg of silk fibroin, that combined with high temperature increases fibroin chain movement. Moreover, our results indicate that after gamma radiation a less energy was requested for fibroin degradation, probably due to a peptide bond weakening. Kojthung et al. (42) evaluated that gamma radiation reduces silk fibroin tensile strength, molecular weight, and β-sheet content: this effect is more pronounced by increasing radiation intensity. On the other side, George et al. (43) observed that gamma radiation is the most suitable method for the sterilization of silk fibroin films for corneal tissue engineering.

Finally, the presence of pectin could enhance the cell adhesion because it mimics the polysaccharide structure of the extracellular matrix. In fact, pectin improves osteoblasts adhesion and proliferation on porous poly-lactic-co-glycolic acid matrix (44), promotes murine fibroblast and human muscle cell growth (45), improves the biocompatibility of medical devices (46, 47). Munarin et al. (48) obtained an injectable vehicle composed by pectin microspheres suitable for bone tissue regeneration. On the other hand, Lu et al. (34) observed that the glycerol concentration of 30% w/w was necessary to obtain adequate silk fibroin films for fibroblast cell culture. Our results indicate that the concentration of 12% w/w guarantees the adipose-derived stem cell adhesion.

In conclusion, the fibroin casting method with pectin and glycerol confers stability to fibroin structure and elasticity to the films. For these reasons, fibroin films are promising supports for ADSCs culture as feeder layer during the production of bioengineered skin. This construct could be advantageously employed in cutaneous wound healing or where the use of ADSCs scaffold is indicated in both human and veterinary field.

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