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Silk fibroin-polyurethane scaffolds for tissue engineering

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Silk fibroin (SF) is a highly promising protein for its surface and structural properties, associated with a good bio- and hemo-compatibility. However, its mechanical properties and architecture cannot be easily tailored to meet the requirements of specific applications.

In this work, SF was used to modify the surface properties of polyurethanes (PUs), thus obtaining 2D and 3D scaffolds for tissue regeneration. PUs were chosen for their well known advantageous properties and versatility; they can be obtained either as 2D (films) or 3D (foams) substrates. Films of a medical-grade poly-carbonate-urethane were prepared by solvent casting; PU foams were purposely designed and prepared with a morphology (porosity and cell size) adequate for cell growth. PU substrates were coated with fibroin by a dipping technique. To stabilize the coating layer, a conformational change of the protein from the α-form (water soluble) to the β-form (not water soluble) was induced.

Novel methodology in UV spectroscopy were developed for quantitatively analyzing the SF-concentration in dilute solutions. Pure fibroin was used as standard, as an alternative to the commonly used albumin, allowing real concentration values to be obtained.

SF-coatings showed good stability in physiological-like conditions. A treatment with methanol further stabilized the coating.

Preliminary results with human fibroblasts indicated that SF coating promote cell adhesion and growth, suggesting that SF-modified PUs appear to be suitable scaffolds for tissue engineering applications.

Introduction

Silk fibroin (SF), extracted from *Bombix Mori* silk, is a highly promising protein for its structural properties, associated with a good bio- and hemo-compatibility [1, 2]. Its peculiar structural characteristics have been making this protein a good substrate for cell growth [3]. However, its mechanical properties and architecture cannot be easily tailored to meet the requirements of specific applications.

In this work, SF was used to modify the surface properties of polyurethanes (PUs), thus obtaining 2D and 3D scaffolds for tissue regeneration. PUs were chosen for their well known advantageous properties and versatility [4]; they can be obtained either as 2D (films) or 3D (foams) substrates. Films of a medical-grade poly-carbonate-urethane were prepared by solvent casting; PU foams were purposely designed and prepared with a morphology (porosity and cell size) adequate for cell growth.

PU substrates were coated with fibroin by a dipping technique. For the preparation of SF-PU coated scaffolds, the water soluble α-form of SF was used; then a conformational change of the protein from the α-form to the β-form (not water soluble) was induced to stabilize the protein coating.

A crucial point is the extraction of fibroin from degummed silk, as minor changes in the environmental conditions may alter the secondary conformation of the protein and modify its behavior. Moreover, the protein can be degraded during the purification process.

For low concentrations quantitative analysis of fibroin in solution, UV spectroscopy was used. Different colorimetric techniques, based on color change of a dye in response to the various concentration of the protein, are commonly used for protein microassay. Bradford dye-binding procedure [5] is primarily based on the interaction of the dye, Coomassie blue, with basic aminoacid residues (mainly arginine) and aromatic aminoacids (tyrosine). As relative standards, bovine gamma globulin (IgG) and bovine serum albumine (BSA) are commonly used and results are expressed in equivalent of IgG or BSA.

In our work, silk fibroin itself was used as standard. Standard curves were also drawn avoiding the use of any dye, on the basis of the UV spectrum of fibroin.

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Materials and methods

Regeneration of silk fibroin (SF)

Bombix Mori silk twill 2/2 fabric (Ratti SpA, Como, Italy) was treated in 9.3 M LiBr at 60 °C for 30 min [3]. This suspension was filtered and extensively dialyzed against distilled water using SpectraPor membrane tubing (BIO-RAD, MWCO 3500). The concentrations of the resulting solutions were obtained from the dry weight of 1 g of SF solution.

Films of SF were cast from water solution at 25 °C. β-form crystallization was induced by immersion in absolute methanol (MeOH) at 25 °C.

PU substrate preparation

Films (2D-substrates) were obtained by solvent casting from a 15% THF : Diox (2 : 1) solution of Bionate 80A (PTG, USA). PU foams [6] (3D-substrates) were prepared by reacting a polyol mixture (Elastogran, Italy) with polymeric MDI (B141, BASF). Fe-acetylacetonate as catalyst and water (5% w/w polyol mixture) as expanding agent were used.

2D and 3D silk fibroin/PU scaffold preparation

Both 2D and 3D PU substrates were coated with SF by dipping in freshly prepared fibroin aqueous solution (3–4% w/w). Samples were dried until constant weight at room temperature on polystyrene plates. Some samples were then immersed in methanol and allowed to dry at room temperature. The amount of fibroin on the surface of the samples was evaluated from the dry weight of the scaffold.

Differential scanning calorimetry (DSC)

Analyses were performed on 5–10 mg samples in the temperature range − 150 °C to + 350 °C at a heating rate of 20 °C/min under N_2, with a DSC 6200 SII Seiko Instruments calorimeter, calibrated with indium. A first run was performed from + 25 to + 160 °C at 20 °C/min to eliminate residual moisture.

FT IR spectroscopy

ATR FT-IR spectra were performed on SF films with a FT-IR Magna 560 Nicolet spectrometer equipped with Omnic 4.1 Software. ATR Spectra-Tech attachment, mod. 300, was used.

UV spectroscopy

For quantitative analyses, fibroin, dialyzed against phosphate buffer saline (PBS, pH 7.4), was chosen as standard. For this aim, a set of SF standard solutions with decreasing concentrations, ranging from 460 μg/g to 5 μg/g, were obtained by successive dilutions with PBS. UV spectra of these solutions were recorded with a UNICAM® UV/VIS Spectrometer UV2 in quartz cells.

Three different standard curves were obtained from the absorbance at 216, 223 and 276 nm. The SF solutions were also used as standard in Bradford assay: a standard curve was obtained from the absorbance at 595 nm.

A protein microassay kit (BioRad) based on the Bradford dye-binding procedure, was also used. A standard curve was obtained from the absorbance at 595 nm.

SEM

Specimens, mounted on aluminum stubs, were sputter-coated with gold, and examined with a Leica Cambridge Stereoscan 360 microscope at 3–7 kV acceleration voltage.

SF-coating stability

To test the SF-coating stability, SF/PU scaffolds, both MeOH-treated and not, were immersed in PBS at 37 °C for increasing times (6 h/14 days). Sodium azide (0.01% w/v) was added to prevent bacterial colonization. The PBS extracts were then analyzed by UV and the SF-concentration drawn from the standard curves.

Results and discussion

Silk fibroin in the soluble amorphous and α-form, at concentrations up to 4% w/v, was obtained only with a careful control of all the extraction parameters. Thermal and structural properties of the regenerated silk fibroin were investigated.

DSC curve of a water cast film presents the typical features [7–9] of this morphological heterogeneous protein. The presence of a random coil phase can be deduced by the presence of a Tg at 178 °C and an exothermic transition at 216 °C ($\Delta H = 8.3$ J/g) due to the conformational change from random coil to β-sheet. A large, multiple endothermic peak at 290 °C ($\Delta H = 165.1$ J/g) is attributed to the presence of β-sheet form which is known to degrade at that temperature [7]. Moreover, an endothermic shift of the baseline, whose nature should be further investigated, is observed at 30 °C.

IR spectra of regenerated SF shows a broad peak attributed to the NH stretching of the peptide groups (3270 cm^{-1}). The stretching of the different C–H groups (3084, 2985 and 2933 cm^{-1}) are probably due to the ν_{C-H} aromatic, ν_{C-H} (asymmetrical) and ν_{C-H} (symmetrical). Bands due to C–H vibrations can be observed in the zone 1450–1330 cm^{-1} (Table I, first column, and Fig. 1, trace A). Amide I, amide II, amide III can be

TABLE I ATR FT-IR band attribution of regenerated silk fibroin

	Untreated	MeOH-treated
ν_{N-H}	3276	3274
ν_{C-H}(aromatic) + ν_{C-H}(asymmetrical) +	3084	3084
ν_{C-H}(symmetrical)	2985	2978
	2933	2935
amide I	1635	1640 + 1620
amide II	1513	1510
C–H_2 scissoring + δ_{CH3} + $V_{C=C}$ (aromatic)	1449	1447
δ_{CH2}	1409	1409
δ_{CH3}	1380	1381
C–H_2 wagging	1333	1333
amide III	1232	1265 + 1229

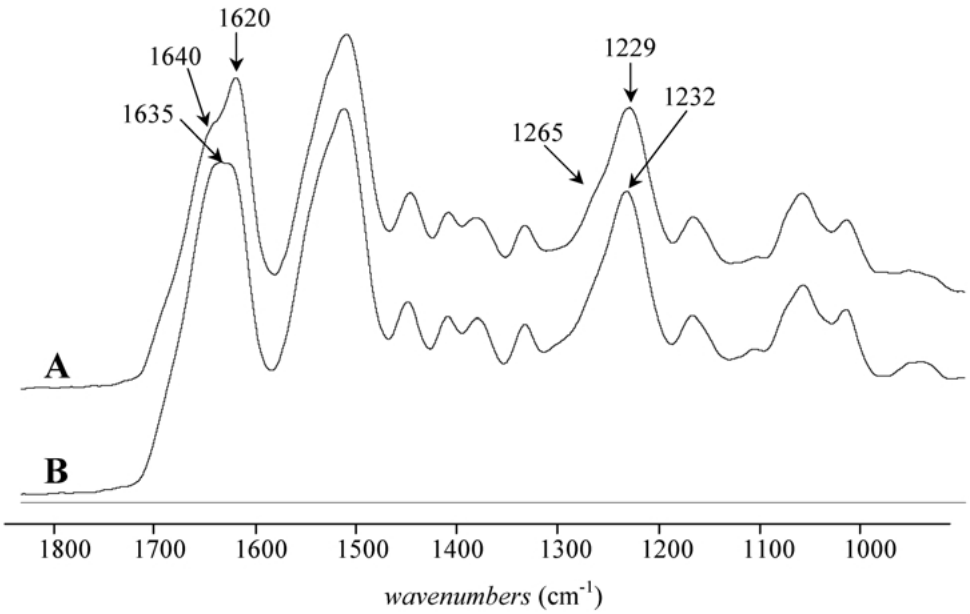

Figure 1 ATR FT-IR of regenerating silk fibroin: (A) film cast from water solution after methanol treatment and (B) the same film before methanol treatment.

observed in the spectrum at frequency typical of the random coil and silk I form, as reported in literature [10–12].

The methanol treatment induces a transition to silk II form, with the appearance of typical bands of the β-sheet form in the amide I band (shoulder at 1620 cm^{-1} and 1695 cm^{-1}), and in the amide III (shoulder at 1265 cm^{-1}) (Table 1 and Fig. 1, trace B). However, after methanol treatment, random coil and α-form bands do not completely disappear.

UV spectra of SF solutions showed an intense electronic transition at λ_{max} 204 nm, with two shoulders at λ_{max} 216 and 223 nm. In concentrate solutions (more than 300 μg/g) these bands were off-scale. A less intense transition was observed at 276 nm, and it could not be detected for solutions with concentrations lower than 20 μg/g. This band results from the overlapping of two electronic transitions and it can be attributed to the contribution of the two aromatic groups in the aminoacids tyrosine and tryptophan [13].

A linear dependence of the absorbance at 216, 223, 276 nm of SF solutions versus concentration was observed, with good R^2 values ($R^2 > 0.99$) (Fig. 2). The standard curve obtained by the Bradford method showed a deviation from linearity at higher concentration, due to the instability of the conformation of this protein in the acidic solution of the dye: at concentrations higher than 250 μg/g, the protein partially precipitated with the addition of the dye.

The dipping technique to prepare 2D and 3D SF/PU

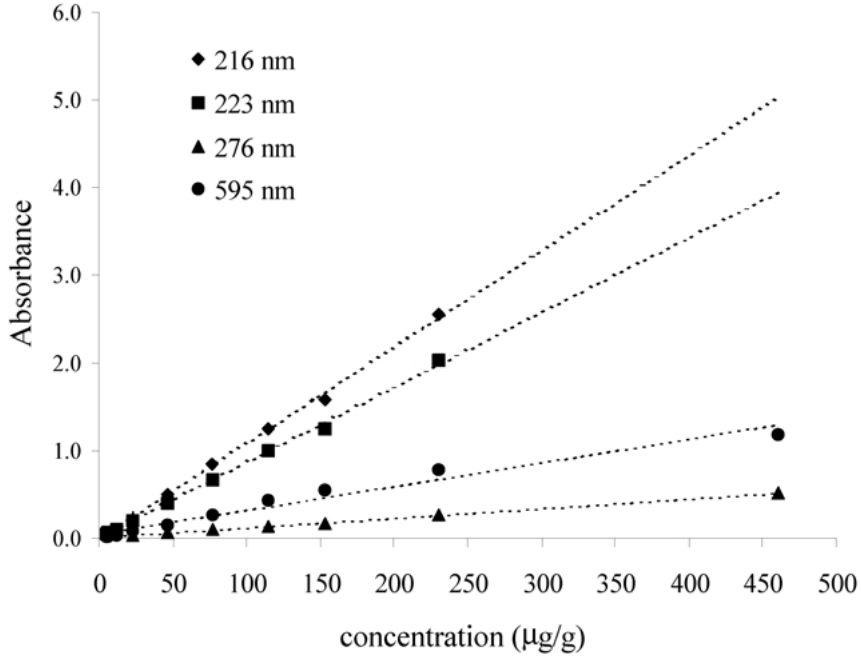

Figure 2 Standard curves for SF quantitive analysis in solution, obtained from the UV absorbance at 216, 223 and 276 nm and standard curve obtained from Bradford assay (absorbance at 595 nm).

scaffolds allowed to obtain a homogeneous coating as can be observed by SEM (Figs 3 and 4). The presence of the coating (thickness about 200–600 nm) is revealed by cross section images (Fig. 3B and Fig. 4B,C) and in the presence of defects of the coating deriving from sample preparation (Fig. 3C). ATR FT-IR of SF/PU 2D scaffold showed the presence of bands attributed to SF and small contribution of the polyurethane substrate [14] at 2935 cm^{-1} (ν_{CH}), 1732 cm^{-1} ($\nu_{C=O}$), at 1409 cm^{-1} (ν_{C-C}, aromatic), 1308 cm^{-1} (wagging CH_2), 1108 cm^{-1} (ν_{C-O-C}) (Fig. 5).

The release of SF from the substrates, both 2D and 3D, was lower than 5% (w/coating w) (Fig. 6), with the exception of the samples SF/PU 2D at 6 h and 14 days. In these cases, some fragments of the SF coating were detached from the PU substrate, mainly caused by the mismatch of the mechanical properties of the polyurethane substrate (elastomeric) and the protein coating.

The crystallization of SF coating into the β-form stabilized the coating, resulting in a lower release of SF (Fig. 6B) and a slower kinetic release. It can be hypothesized that low molecular weight protein chains,

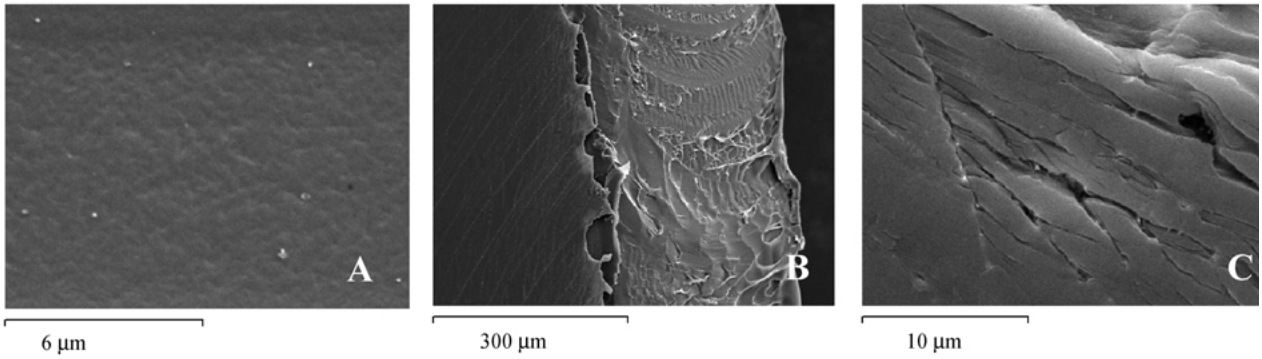

Figure 3 SE micrographs of SF/PU 2D scaffolds, (A) homogeneus surface (10 000×), (B) a cross section (200×), (C) surface cracking of the coating due to sample preparation showing the presence of the Sf layer deposit (5000×).

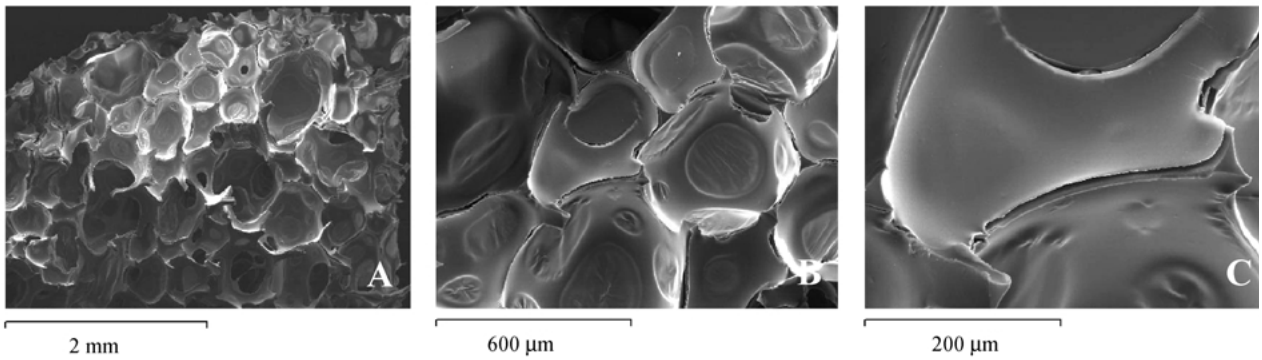

Figure 4 SE micrographs of SF/PU 3D scaffolds, (A) 30×, (B) 100×, (C) 300×.

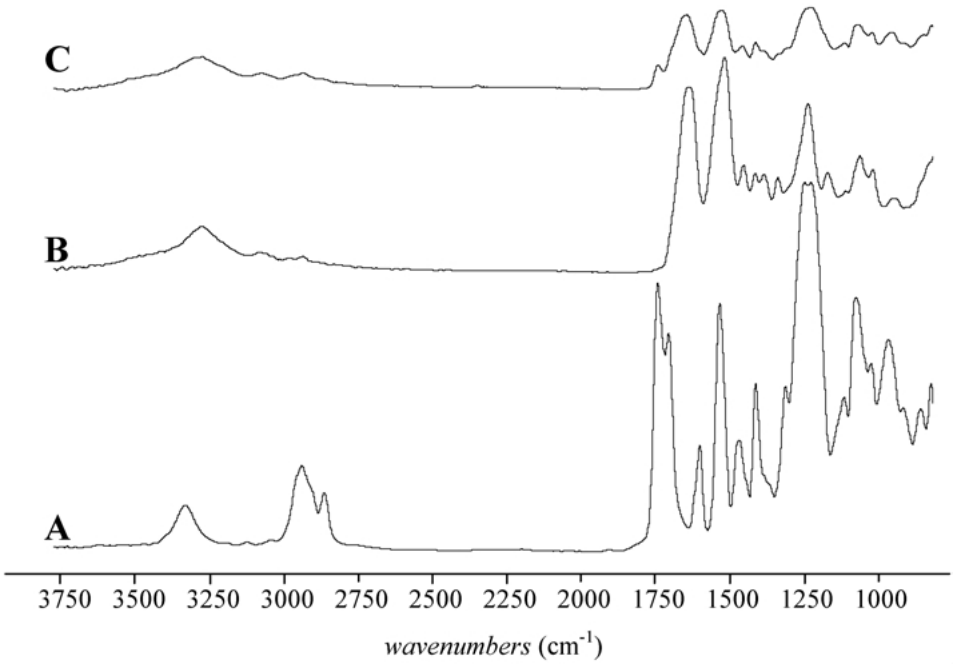

Figure 5 ATR FT-IR spectra of (A) the polyurethane substrate, Bionate 80A, (B) film of regenerated silk fibroin, (C) SF/PU 2D scaffold.

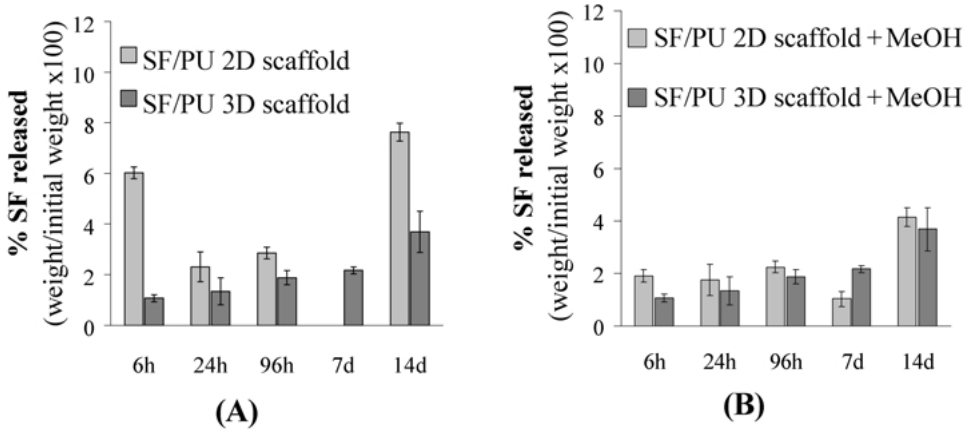

Figure 6 Weight percent of SF released from SF/PU 2D and 3D scaffolds (A), and from the respective MeOH-treated samples (B), after immersion in PBS at 37 °C.

deriving from the low molecular weight cut-off of the dialysis membranes other than the degradation of the protein in the test conditions, are more easily released. They could also impend the crystallization of the protein in the not soluble conformation.

Conclusions

By adequate choosing of the parameters of the extraction process (time, temperature, concentration, dialysis conditions) SF water solution (up to 4% w/w concentration) were obtained.

DSC of water cast SF film was in agreement with the data reported in scientific literature. By FT-IR analysis of these films, typical bands between 4000 cm^{-1} and 600 cm^{-1} were identified and the attribution of other bands due to CH vibrations were proposed. Structural changes of SF after the MeOH-treatment were also detected by FT-IR.

New methods in UV spectroscopy were developed for quantitatively analyzing the SF-concentration in dilute solutions ($< 10\,\mu g/g$). The use of pure fibroin as a standard, as an alternative to the commonly used albumin, allowed real concentration values to be obtained.

The presence of the SF coating was confirmed by ATR FT-IR and its homogeneity was showed by SEM.

SF-coating onto both 2D and 3D substrates showed good stability in physiological-like conditions, with a weigh loss lower than 8% (w/coating w). The methanol treatment further stabilizes the coating, as shown by a lower release of SF in physiological conditions. In addition, the SF release kinetics from MeOH-treated samples was slower.

Preliminary results with human fibroblasts indicate that SF-coated polyurethane scaffolds induce cell adhesion and growth at a major extent than the uncoated materials and polystyrene cell culture plates (Prof U. Armato, University of Verona, Italy). All the obtained results appear to indicate that SF-modified PUs are suitable scaffolds for tissue engineering applications.

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