S6

Bombyx mori Silk Fibroin Films: Preparation, Characterization of Physical and Chemical Properties, Use as Biomaterial.

Giuliano Freddi

Stazione Sperimentale per la Seta, Milano, Italy

Abstract

The silk fibre produced by the larvae of the lepidopter of the *Bombyx mori* species is no doubt one of the most precious raw materials employed in manufacturing textile products. The present report, however, deals with silk not as a traditional textile fibre, but as a starting material for biomedical applications. In recent years, the unique chemical and mechanical properties of silk have made this protein polymer highly attractive for innovative applications, which mainly focus on the development of devices for biomedical uses. The most recent achievements in the study of silk as biomaterial will be reviewed.

Introduction

The use of silk for non-absorbable surgical sutures has been known for years [1]. They feature considerable resistance, elasticity, and excellent knotting, though their use is diminishing compared to the past owing to competition by a wide range of similar products manufactured with synthetic polymers. However, other biomedical applications of silk fibres and films are being developed. Tissue engineering is an emerging field of study focusing on the development of synthetic substitutes of damaged biological tissues and organs. The need of polymer matrices with different chemical, mechanical, and biological properties, versatile enough to meet various application requirements, has urged scientists to address more attention to natural fibrous polymers, such as silk. Silk fibres have been considered as starting material for the preparation of various kinds of medical devices, such as silk-hydroxyapatite composite prostheses for bone regeneration [2], protective gauzes for the treatment of skin burns [3], wire-ropes for the substitution of the anterior cruciate ligament [4], and bioactive textiles with antibacterial activity [5]. The observation that fibroin films are permeable to oxygen and water vapour [6], and able to effectively support cell adhesion and growth has led to suggest the development of silk-based devices that can assist repairing of damaged human tissues [7].

Owing to the increasing interest on silk fibroin films as biomaterial, the research work has recently focused on key subjects, such as: determination of structure-property relationships [8], study of the biocompatibility [9] and of the degree of biostability of silk films [10]. These studies will be reviewed in the present report.

Results and Discussion

Structure-property relationships

It is well known that the functional performance of silk fibroin films, as well as the interaction with living cells, depends on their chemical, physical, and structural properties, which may change as a function of

the preparation conditions. Some preparation steps are quite crucial and their careful control is required. Dissolution of silk fibres is needed to prepare silk films. For a dissolution process to be effective, swelling of the ordered fibrous structure and breaking of the hydrogen bonding network are required. An ideal solvent should be capable of penetrating into the fibre and dissolving the individual fibroin chains without inducing adverse reactions, such as depolymerization. The most common solvents for silk are concentrated salt solutions. Although salts are considered safe for the chemical and structural integrity of silk, undesired negative effects can be induced if the dissolution conditions are not carefully controlled. The effect of LiBr on the degree of polymerization (DP) of silk fibroin was investigated as a function of the dissolution time. The values of DP decreased gradually with increasing the treatment time with LiBr at 60°C. However, the changes in molecular weight were confined within a narrow range. These results show that the intrinsic physico-chemical properties of silk are are not severely affected if the parameters of the dissolution process, such as time and temperature, are kept under a strict control.

Silk films cast from dilute aqueous solutions are essentially amorphous, showing characteristic IR and Raman bands typical of the random-coil conformation. As the concentration of fibroin in the aqueous solution increases, the metastable Silk I crystalline modification is formed. Convertion from Silk I to Silk II, which corresponds to the well-established β-sheet crystalline structure existing in the fibres, is needed because amorphous/Silk I films are still water soluble. This structural transformation can be easily achieved by applying simple thermal and mechanical treatments, or by annealing with various dehydrating solvents, such as methanol. The latter system is the most commonly used for inducing crystallization. The FT-Raman spectra of silk films annealed with 80% v/v aqueous methanol for different times showed that complete β-sheet crystallization was achieved after 1 hr, while for shorter annealing times the bulk material was still amophous. This effect can be attributed to the fact that methanol is a poor solvent for silk and its diffusion into the bulk film is time dependent, being driven by the water present in the system. For treatment times shorter than 1 hr it is reasonable to expect structural differences between the outer and inner parts of the film. FT-IR spectra measured with the ATR techniques confirmed this hypothesis. In fact, the film surface crystallized as soon as it was immersed into the annealing solution, while the bulk material was still prevalently amorphous (Figure 1).

Biocompatibility

It is generally accepted that biocompatibility is a prerequisite for designing tissue engineering scaffolds able to ensure optimum biological integration. Some concerns exist about the biocompatibility of silk. In fact, several cases of strong inflammatory response attributed to the use of silk sutures have been reported in the medical literature. However, the scientific data available on this subject seem to confirm that sericin, the silk gum, is mostly responsible for the adverse biological response of silk. To address these important items, the inflammatory potential of silk fibroin films was evaluated and compared to that of two model polymers with completely different physico-chemical properties, poly-styrene and poly-(2-hydroxyethyl methacrylate). Fibroin bound lower levels of fibrinogen than did the two synthetic polymers, while the same amounts of adsorbed human plasma proteins were detected. Studies of the binding strength of the complement fragment C3 to fibroin indicated the occurrence of strong hydrophobic interactions at the interface. The activation of the mononuclear cells by fibroin was lower than that of the reference

polymers. Adhesion experiments showed the ability of the macrophages to adhere to fibroin by filopodia without a complete spreading of the cells. The results achieved demonstrate that the interactions of fibroin with the humoral components of the inflammatory system were comparable with those of the two model surfaces, while the degree of activation and adhesion of the immunocompetent cells appeared more limited. The restrained fibroin-induced stimulation of mediators and the adhesion of leukocytes, combined with its previously reported ability to support fibroblast adhesion, re-launches this natural polymer as a promising biomaterial for those applications in which tissue regeneration is required.

Biostability

A key factor that must be considered when silk-based biomedical devices are developed, is the degree of biostability of the polymeric substrate, which must match the functional needs and ensure optimum mechanical and physiological integration of the device. In this context, it is of chief importance to characterize the kinetics and mechanism of the biological degradation of silk films potentially exploitable for the preparation of polymeric scaffolds for tissue engineering. The biostability of silk films "*in vitro*" was studied as a model for investigating their functional properties and interaction with the biological environment "*in vivo*".

Silk films incubated with different proteolytic enzymes (collagenase, □ -chymotrypsin, protease) for different times were analyzed to determine changes of weight loss, molecular weight, amino acid composition, crystalline structure, and morphology. The weight of silk films decreased with increasing the incubation time. The kinetics of weight loss followed different trends depending on the enzyme used. The values of weight average molecular weight of enzyme-degraded films decreased gradually. The inspection of the chromatographic profiles suggests that the enzymatic attack proceeded with different mechanisms. For example, with collagenase and □ -chymotrypsin the molecular weight distribution tended to become narrower and shifted to lower molecular weight values. On the other hand, with protease the pattern was characterized by the presence of polypeptide fractions with medium-to-high and low molecular weight. The amino acid composition of enzyme-degraded films showed systematic changes. The concentration of the three amino acids typical of silk, glycine, alanine, and serine increased gradually, while other amino acids, especially those with bulky and polar side chains, decreased. This suggests that the polypeptides sequences forming the amorphous regions were preferentially attacked by the enzyme, while the crystalline regions remained almost unchanged because of limited accessibility. As a result, the enzymatic attack enhanced the crystalline character of silk films. This observation was confirmed by infrared spectroscopy measurements. The FT-IR crystallinity index increased linearly with the weight loss. The higher the weight loss, the higher the crystallinity of the residual material. The morphological characterization of degraded silk films showed the presence of zones of extensive degradation, indicating how severe the hydrolytic attack was (Figure 2).

Although "*in vivo*". degradation of biopolymers is a very complex process, involving various synergistic pathways of chemical, biochemical, physical, and mechanical origin, the study of the enzymatic degradation of silk fibroin "*in vitro*" has contributed new findings that may allow to elucidate the mechanism by which the material interacts with the biological environment.

Acknowledgements

The author wishes to express his thanks to: M. Tsukada and T. Arai for biodegradation studies; M. Cannas, M. Santin and A. Motta for biocompatibility studies; P. Monti and R. Innocenti for the spectroscopic studies.

References

1. H.K. Song, K.R. Keuyon, Ophtalmology, 91, 1984, 479
2. Y. Tamada, T. Furuzono, T. Taguchi, A. Kishida, M. Akashi, J. Biomat. Sci. Polym. Ed., 10, 1999, 787
3. T. Furuzono, K. Ishihara, N. Nakabayashi, Y. Tamada, Biomaterials, 2, 2000, 327
4. G.H. Altman, R.L. Horan, H.H. Lu, J. Moreau, I. Martin, J.C. Richmond, D.L. Kaplan, Biomaterials 23, 2002, 4131
5. T. Arai, G. Freddi, G.M. Colonna, E. Scotti, A. Boschi, R. Murakami, M. Tsukada, J. Appl. Polym. Sci., 80, 2001, 297
6. N. Minoura, S. Aiba, M. Higuchi, Y. Gotoh, M. Tsukada, Y. Imai, Biochem. Biophys. Res. Comm., 208, 1995, 511
7. S. Sofia, M.B. McCarthy, G. Gronowicz, D.L. Kaplan, J. Biomed. Mater. Res., 54, 2001, 139
8. M. Tsukada, Y. Gotoh, M. Nagura, N. Minoura, N. Kasai, G. Freddi, J. Polym. Sci., Polym. Phys. Ed., 32, 1994, 961
9. M. Santin, A. Motta, G. Freddi, M. Cannas, J. Biomed. Mater. Res., 46, 1999, 382
10. T. Arai, G. Freddi, R. Innocenti, M. Tsukada, J. Appl. Polym. Sci., to appear

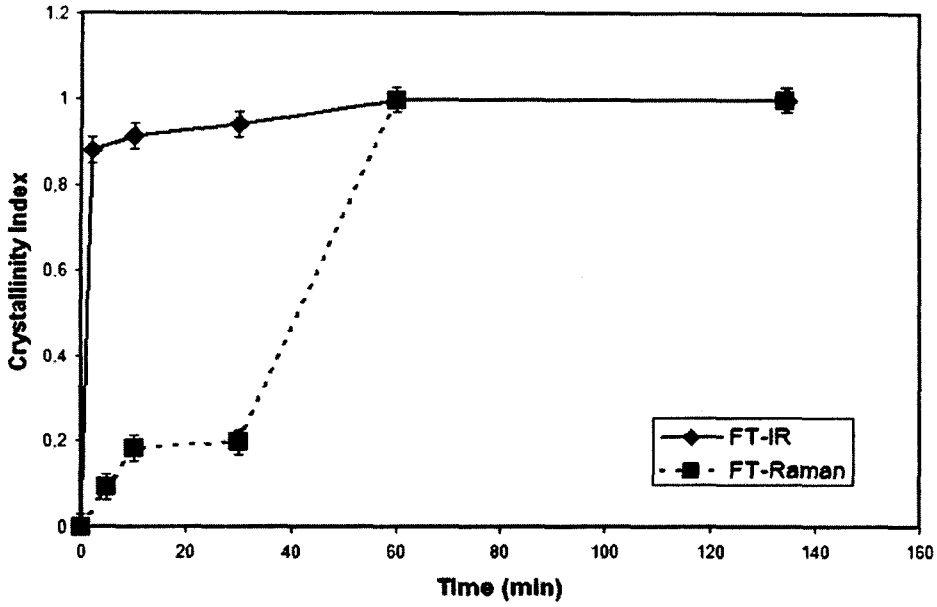

Figure 1 – Behaviour of FT-IR and FT-Raman crystallinity indexes of silk films as a function of the annealing time with water-methanol solution.

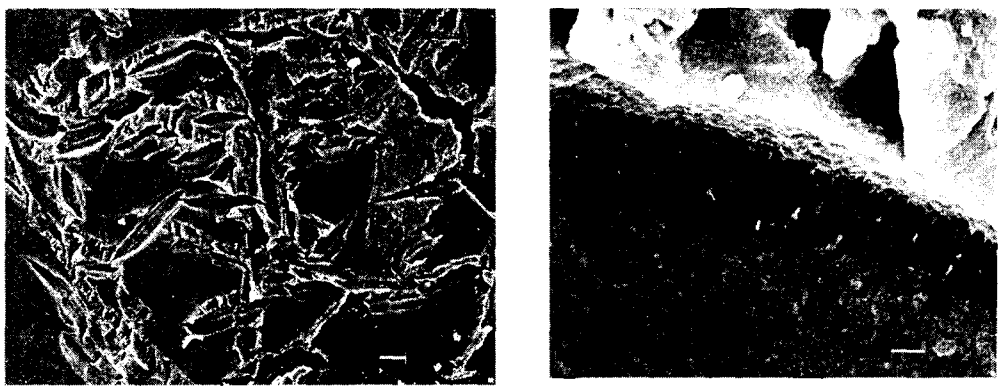

Figure 2 - SEM photographs of the surface of silk films incubated with protease (*Streptomices griseus*) for 17 days (left); detail at higher magnification (right).