TAYLOR-MADE BIOMATERIALS FROM COLLAGENIC WASTES: FEASIBLE LINK BETWEEN TANNING INDUSTRY AND TISSUE ENGINEERING

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Abstract: The basic aim of the present work is the conversion of non-tanned solid wastes from the tanning industry (splits) into high added value biopolymers for its application in tissue engineering within fields such as cosmetics, medicine or veterinary.
From all the previous experience of our team, a specific technology for this kind of waste has been developed. Those tannery wastes have, as an essential feature, a high content of collagen, whose properties and characteristics make this material suitable for a wide range of applications, highlighting their use in tissue engineering.
The first stage of the project was centred on finding, through a factorial experimental design, the most suitable treatment for the isolation of biopolymers with optimum properties. The input of this treatment was the extraction of those materials with a minimum hydrolytic effect on the triple helix of the collagen molecule. The following stage was the purification and separation of the biopolymer by molecular weight fractions, using tangential flow ultra-filtration.

Those biomaterials could take different forms depending on the future application: 1) gel, 2) film, 3) sponge or 4) fibres. The evaporation of the gels resulted in the formation of films; sponges were obtained by lyophilisation of the samples; and two different kinds of fibres, macro-fibres and nano-fibres were formed through extrusion and electro-spinning, respectively. The formation of these fibres, based on the reconstitution of the collagen structure, can be characterised through optical microscopy (macro-fibres) and electronic microscopy (nano-fibres). All the different samples were thoroughly characterised using calorimetric techniques, microscopy, electrophoresis, mechanical properties analysis and stability.
The final stage of the project will be based on the identification of the requirements for the applicability of those new biomaterials. Studies of biocompatibility, toxicity and biodegrability will be carried out for the application of the biopolymers in biomedical fields such as cosmetics, medicine and/or veterinary; more specifically within the tissue engineering in tissue and/or organ regeneration, manufacture of suture fibres, etc.

Those “taylor-made” biopolymers will be designed, with the desired molecular weight and “shape”, according to their specific future applications.

Key words: biopolymer, waste, fibres.

1. Introduction

The environment is one of the most relevant topics nowadays, the ecological conscience and the practice of an environmentally friendly and sustainable policy is increasing day by day all over the world. The concept of “Sustainable Development” transmits the idea of the rational use of the resources, the improvement of life quality and the maintenance of the ecosystems without jeopardising future generations. The improvement of the manufacturing processes, the finding of new types of renewable energies, the application of “clean” technologies in the processing, the installation of water treatment plants for the effluents, the reduction of waste generation and the finding of new treatments for each type of waste; are essential steps to make compatible industrial development, environmental protection and social welfare. Waste treatments, in addition of reduce the volume of industrial waste could increase their value through the

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production of high added value products, entailing a great progress in both, environmental and economical terms.

In terms of waste generation, the production of leather gives rise to significant quantities of solid waste product for which tanneries are responsible for the cost of disposal and since most of this waste ends up in landfill it may be considered an environmental problem. However such waste is not without some potential value since it contains collagen which could be recycled and reused. Collagen is a very versatile and special high-added value protein and the most abundant and ubiquitous in vertebrates[1, 2]. This collagenic nature of the tannery solid waste permits us to think about treatments for obtaining biopolymers of reconstituted collagen, and their use in a wide range of potential applications.

Up to now, "low cost" biomaterials have been obtained. Their main applications have been: as filler, re-tanning agents and finishing agents in the tanning industry itself; and as a binder in the paper industry, partially substituting casein (much more expensive). The objective of the present work is the extraction, characterisation, optimisation and application of new "Taylor-made" smart biopolymers with high-added value, finding a new and feasible link between solid tannery waste and the rising market of tissue engineering.

Tissue engineering can be defined as an interdisciplinary field which applies, for one side, the principles of tissue engineering and, for another side, the sciences of life, with the aim of obtaining "Biological Structures" in order to regenerate and/or improve the tissue function[3]. Although lots of synthetics biodegradable or bio-stable polymers have been employed on these "special structures", the affinity of the grafted cellules is quite low. Biologically derived materials are advantageous in that they contain information that facilitates cell attachment and function, whereas synthetics may not interact with cells in the desired manner[4]. The importance and special appeal of collagen as a biomaterial is based on the fact that collagen is a natural material and therefore it is assimilated by the human/animal body as a normal constituent and not as a foreign material, subjected to rejection, with a minimum of inmunogenicity. A great competitiveness of reconstituted collagen fibres in the field of regenerative medicine (tissues and/or organs) has been found in literature[4].

Biopolymers are polymers generated from renewable resources, often biodegradable and from non-toxic production. They can be produced from biological systems or chemically synthesised from biological raw materials. They are an alternative to the petrol-based polymers. The main problems of biopolymers are bio-compatibility, mechanical properties and adaptability. Collagenic biopolymers present huge possibilities due to the possibility of manufacture, and application, in different ways, forms and shapes, with well determined characteristics. We can talk about "Taylor-made" biopolymers: it is possible to produce easily said biopolymers as gel, film, fibres, tissue and/or sponges, using techniques such as freeze drying/lyophilisation, extrusion, or electro-spinning for nano-fibres formation.

In addition to the technical and scientific benefits obtained from the isolation of biopolymers from solid waste, this research could entail different economical benefits: In the first place, it presents a solution to a problem of dumping/storage of wastes, avoiding taxes for accumulating those wastes. Secondly, whole hides of low quality can be used as raw material, those hides, catalogued as a 4^{th}-5^{th} class, would be used to produce low quality articles of very low price on the market; however, the biopolymer extracted from this hide would have a high-added value. Thirdly, the treatment process is simple and cheap; environmentally and economically much more plausible than other treatments such as incineration, landfilling, etc. Finally, a wide range of potential applications for the produced bioproducts could be taken into consideration; with specific applications on medicine, veterinary and/or cosmetics, expanding field nowadays.
The technology to be used on the development of this research is focused on the production of macro-fibres (extrusion) and nano-fibres (electro-spinning), films, sponges (lyophilisation) and different types of scaffold material for tissue engineering.

The use of mathematical experimental designs permits to study the degree of significance of the different variables and the corresponding interaction between them in the different processes for obtaining collagenic biopolymers. This ensures that the experimentation can be rationalised and the optimum determined, being able to achieve a controlled production of "Taylor-made" biopolymers for each specific application.

2. Experimental

2.1. Materials

Bovine pickled hides, supplied by the Leather Technology School of Igualada, were used as a raw material for the biopolymers extraction. Acetic acid (glacial) was supplied by Carlo Erba and Panreac. Standard marker for SDS-PAGE (from 6.5 to 205 kDa) was supplied by Bio-Rad. Analytical grade chemicals were used for the fibre formation: the phosphate buffer comprised disodium phosphate heptahydrate and monosodium phosphate monohydrate, supplied by Riedel-de Haen and Fluka, respectively. Polyethylene glycol Mw 8000 and sodium chloride were supplied by Sigma and Carlo Erba, respectively.

2.2. Biopolymer extraction

The basis for the preparation of biopolymer-gelatin was the degradation of collagen by hydrolysis. Based on previous studies, bovine hide grinded at 0.25mm size, was mixed in a concentration of 50 g hide per liter of acetic acid 0.5M, by mechanical stirring at 50rpm and 10ºC during 24 hours.

2.3 Film formation

A solution of biopolymer (10 ml) was placed in a small Petri dish and allowed to air dry at a constant temperature (20ºC) and relative humidity (60%).

2.4 Fibre formation (extrusion)

The process for fibre formation was based on previous work[4] with slight modifications. A syringe was loaded with biopolymer gel solution and placed on a syringe pump system supplied by KDScientific. One end of a silicone tube was connected to the syringe and a needle was fitted at the other end and then placed at the bottom of a container. The fibres were extruded into a "Fiber Formation Buffer" (FFB) remaining there for 30 minutes and then transferred into a "Fiber Incubation Buffer" (FIB) for another 10 minutes. Finally the fibres were air-dried under the tension of their own weigt at room temperature. The "Fiber Formation Buffer" comprised 118 mM phosphate buffer and 20% of polyethylene glycol (Mw 8000) at pH 7.55 and 37ºC. The "Fiber Incubation Buffer" comprised 6 mM phosphate buffer and 75 mM sodium chloride at pH 7.10 and 37ºC.

2.5 Sponge formation (Lyophilisation)

Samples of the extracted biopolymer were dried by lyophilisation technique, using a freeze drier supplied by Telstar. Samples were frozen in an acetone/dry ice solution prior to the lyophilisation.

2.6 Characterisation:

2.6.1. *Yield*

The yield of biopolymer was calculated as the percentage of hide material converted to biopolymer and calculated according to the following formula: $Yield(\%) = 100(1-W_{res}/W_{shav})$. Where W_{res} is the residual weight of biopolymer after filtration, and W_{shav} is the initial weight of hide.

2.6.2. *Swelling*

The films were weighed then immersed in a phosphate buffered saline (PBS) solution for different periods of time. Wet samples were blotted with tissue to remove excess liquid and re-

weighed. The percentage of swelling was calculated as follows: Swelling (%) = 100(W_{wet} – W_{dried})/W_{dried}; where W_{wet} is the weight of the film after being immersed in PBS solution for a determined period of time and W_{dried} is the initial weight of the film.

2.6.3. Gel Strength

Gel strength was measured, using 100 ml of gelatin, by Bloom determination which was carried out according to the International Standard (ISO 9665). A Materials Tester designed by Instron, with a 0.5 inch radius cylinder probe (P/0.5R) was used.

2.6.4. *SDS-PAGE*

Aliquots of 50 mg of gelatin were dissolved in 1 ml of sample buffer. The samples then were denatured at 90ºC for 5 minutes, and loaded in appropriate volumes onto a vertical acrylamide gel (4% (v/v) stacking gel, 7.5% (v/v) resolving gel). A standard marker, from 6.5 to 205 kDa was loaded with the samples. The gels were run at 0.01 mA/gel, stained overnight with Coomassie Brilliant Blue solution, and then destained prior to analysis.

3. Results and discussion

- **Biopolymer extraction (lab scale):**

Results of yield, gel strength, percentage of swelling and capacity of fibre and film formation of the extracted biopolymer are shown in Table 1. The percentage of water absorbed by the films versus time and the molecular weight analysis by SDS-PAGE are represented in Figure 1 and 2, respectively.

Tab.1 Characterisation of the extracted biopolymer.

Yield (%)	Gel Strength (g Bloom)	% Swelling @90min	Fibre formation	Film formation
100	252	300	YES	YES

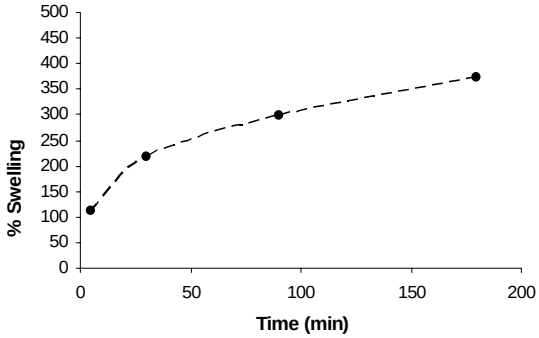

Fig.1 Percentage of swelling vs. time for the extracted biopolymer.

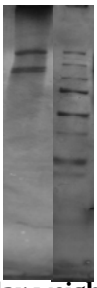

Fig. 2 Molecular weight analysis of the extracted biopolymer using SDS-PAGE.

Collagen isolated from various tissues has a molecular weight of about 300kDa[2]. For collagen derivatives, the molecular weight usually ranges within limits of 15-50kDa for hydrolysates[5] and 50-200 kDa for gelatin[6]. Collagenic biopolymers usually show a wide molecular weight distribution on the low molecular weight area due to the process of extraction, which leads into a material consisting of different molecular weight polypeptides[7]; however, the results of the electrophoresis of the biopolymer extracted according to our methodology show (Figure 2) two distinct bands in the high molecular weight area.

The versatile properties of collagen have made collagenic biomaterials one of the most useful materials for tissue engineering. Those biomaterials can be in the form of and shape of natural tissue, porous scaffolds/sponges, fibres and gels. Reconstituted collagen fibres, and fibre networs have been shown to be a competitive biomaterial for soft and hard tissue replacement

due to their advantageous properties. Those fibres have been used as well as a suture material. It has been postulated that such fibres could be knitted or woven into fabrics, although the production is difficult due to the large amount of material required[8]. The traditional process for formation of collagen fibres involved the extrusion of collagen dispersions into a fibre formation buffer (Figure 3) and subsequent solvent dehydration, air-drying and crosslinking.

Fig. 3 Image of the extrusion system for collagen fibre formation.

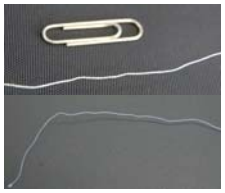

Fig. 4 Collagenic biopolymer extruded fibre.

Fig. 5 Collagenic biopolymer extruded fibre, by optical microscopy (diameter 300μm)

Collagen gels are very attractive for tissue engineering applications because they can retain cells and carry bioactive molecules such as growth factors[2]. Collagen gels have been used in bone, ligament and heart valves tissue engineering as well as in treatment of burns or chronic wounds.

Collagen sponges are generally formed by freeze-drying an aqueous collagen solution. The freeze-drying process includes freezing a collagen gel solution at a low temperature and subsequent sublimation of the ice crystals by vacuum at low temperature. The freezing temperature and freezing rate will have some effect on the porous structure of the resulting sponge[2].

Fig. 6 Collagenic biopolymer gel.

Fig. 7 Collagenic biopolymer film.

Fig. 8 Collagenic biopolymer sponge.

- **Industry implementation:**

Figure 7 represents the diagram of the industrial plant for the extraction and purification of collagenic biopolymers of different molecular weight fractions from tannery solid wastes. The basic steps of the process are as follows:

***- 1st Step _Grinding up Bovine Hides*:** Dried pickle bovine hides are ground up (grinder: J-110) into defibered small size (0.25 mm) and therefore giving a more homogeneous collagenic material with a greater surface, and consequently saving chemicals and shorten down the reaction time. Final product is impulsed through a pneumatic conveyor to the reactor.

- 2nd Step _ Production of collagenic biopolymers: The biopolymer extraction, the most important part of the whole process, is carried out in a stainless steel jacketed stirred reactor (R-120). The grounded hide is submitted to a mild controlled hydrolysis reaction; then, by means of a pump (P-121) is driven to filter unit (S-125), where the suspension particles are separated from the viscous collagenic solution.

***- 3rd Step _ Ultrafiltration*:** The ultrafiltration is carried out by a combined sequentially connected set of three membrane-based tangential flow filtration spiral-wound modules of different cut-off ranges: 1kDa (U-141); 50kDa (U-142) and 100kDa (U-143). In each one of these subunits, the retentate fraction feeds the next subunit and the correspondent permeate fraction is kept apart. At the end of this 3rd step, four molecular weight collagenic biopolymer fractions were isolated: 1kDa; 1–50 kDa; 50–100kDa and over 100kDa.

***- 4th Step _ Freeze-Drying*:** Each one of the four ultrafiltration fractions must be freeze-dried (lyophilisation) in order to keep their original structural and chemical properties. As shown on Figure 9, each unit of lyophilisation equipment was composed by the following parts: freezer (D-); condenser (E-); vacuum pump (V-) and storage tanks (T-).

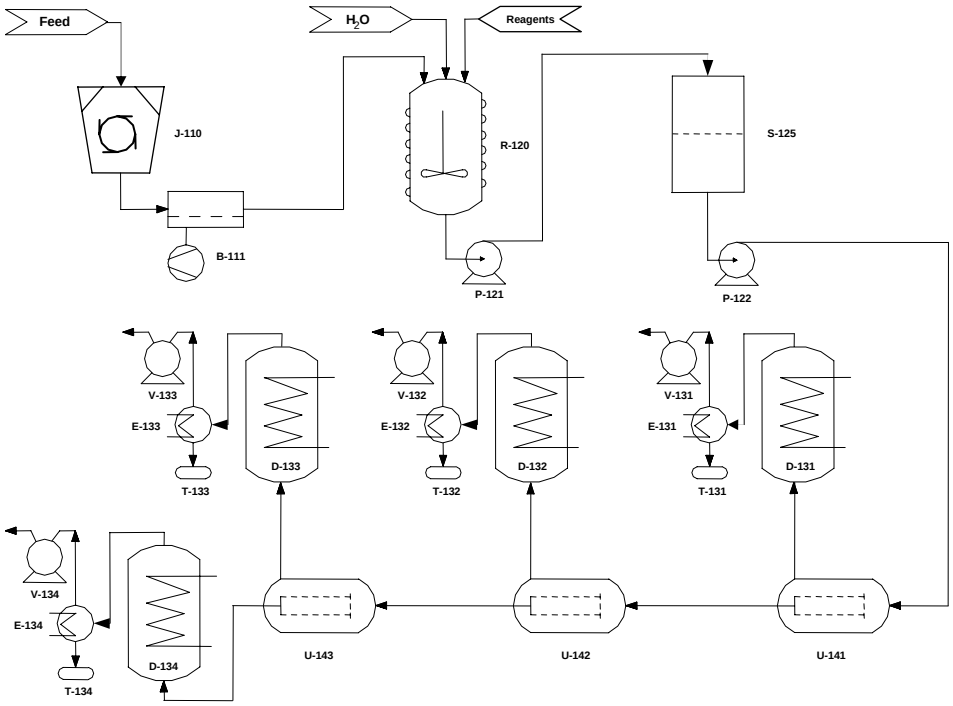

Fig. 9 Scheme of the industrial plant for the processing of biopolymer extraction and purification.

4. Conclusions

A complete methodology for the extraction of new biopolymers from tannery solid waste with special mention to include low quality bovine pickle hides has been developed. It has been demonstrated that those biopolymers can be produced in form of gels, films, fibres and/or sponges. Reconstituted collagen fibres have been shown to be a competitive biomaterial.

In addition to the traditional extrusion procedure for obtaining collagen fibres, for future studies electro-spinning shows to be a promising technique to manufacture in vitro fibrous scaffold for tissue engineering applications. The incorporation of crosslinking can be used to further tailor and control the material properties of the fibres, sponge/scaffold to specific applications.

5. References

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