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Sponge-Like Dressings Based on the Association of Chitosan and Sericin for the Treatment of Chronic Skin Ulcers. I. Design of Experiments–Assisted Development

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ABSTRACT

The aim of the present work was the development of sponge-like dressings based on chitosan glutamate (high molecular weight) and sericin for the treatment of chronic skin ulcers. Dressings were prepared by freeze-drying and glycine was added as cryoprotectant agent. Dressing development was assisted by design of experiments, using a simplex centroid design. Mechanical resistance, hydration propensity, viscous, and viscoelasticity properties of dressings were considered as response variables. The superimposition of the contour plots, calculated by the best fit model for each response variable, permitted to individuate a region of the factor space where the dressing of optimized quantitative composition was chosen. Such a dressing was able to absorb high amount of phosphate-buffered saline forming a gel characterized by rheological properties enabling both a lubricant and a protective effect. The optimized formulation was characterized by optimal mechanical properties and by cell proliferation and antioxidant activity on human fibroblast cell line.

Introduction

In the last decade, the recognition of the disabling conditions derived from painful chronic wounds and the lack of fully efficacious treatments have prompted further research on this topic, which led to a progressive change in the therapeutic approaches devised for wound treatment. “Inert” dressings, uniquely aiming at isolating and protecting wound bed from infections, have been substituted by “bioactive” medications, capable to promote healing. The bioactive properties of such dressings derive from the presence of the so-called enabling excipients, which are biopolymers able of interacting with tissue components, taking part in the healing process.[1,2]

Recently, it has been demonstrated that some natural polymers are promising excipients for wound dressings. Among these, chitosan (CS), a polysaccharide obtained by chitin deacetylation and composed of N-acetyl glucosamine and glucosamine residues, has been recognized as a biopolymer able to promote tissue reparation.[3-10] CS is characterized by unique biological properties, such as biocompatibility, biodegradability, nontoxicity, antimicrobial, and hemostatic properties, that make it an optimal candidate for wound healing. Nowadays, several CS-based dressings and bandages are commercially available.[4]

Different mechanisms are reported in literature to explain CS activity on wound healing: it acts as chemoattractant for neutrophils, possesses a stimulatory effect on macrophages, stimulates granulation tissue formation or reepithelization, promotes dermal regeneration, and inhibits metalloproteinases, which are enzymes excessively expressed in chronic wounds. Moreover, chitooligomers, produced by enzymatic degradation of CS in the wound bed, participate to the synthesis of hyaluronan, which, in turn, promotes cell motility, adhesion, and proliferation and plays important roles in wound repair.[4] In addition, CS is characterized by antimicrobial properties that are beneficial for the healing of infected wounds.[7,11-13] CS has also been proved to protect cells from oxidative damage due to free radicals in the wound bed, promoting healing process.[12,14]

Sericin (Ser), a silk protein derived from *Bombyx mori* cocoons, has been demonstrated to play a role in wound healing[15] and to possess antioxidant activity,[16] which is functional to healing. The antioxidant action is related to the high number of hydroxyl groups in Ser structure, which is rich in polar aminoacids, such as treonin

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and serin.[17] *In vitro* studies have demonstrated that Ser is able to promote the recovery of corneal ulcers and to improve the adhesive properties of skin human fibroblasts and, consequently, their proliferation.[18] Ser is also an ideal support for mammalian cells because it is able to promote cell growing without being toxic when added to the culture medium.[18] Recently, some authors have developed CS-Ser nanofibers intended for wound healing.[20,21] In these articles, concentrations of CS and Ser solutions have been chosen with the aim to facilitate nanofiber preparation, whereas no attention has been paid to optimizing either technological or bioactive properties of the finished products.

Among various bioactive dressings, sponge-like medications are interesting products, being capable of absorbing excess wound exudate, thus, behaving like a sponge and producing, on liquid absorption, a viscoelastic gel that protects a lesion area.

Given these premises, the aim of the present work was the development of sponge-like dressings intended for the treatment of chronic skin ulcers. Formulation development was not carried out following a trial and error approach, but, rather, to achieve the desired technological and bioactive properties, dressing composition was optimized by means of design of experiments (DoE).[22-24]

Dressings, prepared by freeze-drying, were based on the association of CS glutamate (high molecular weight [MW]) (CSG hMW) and Ser with the addition of glycine (Gly) as cryoprotectant agent.

Optimized dressings should be characterized by mechanical resistance appropriate to withstand stresses occurring during manufacturing, packaging, and administration as well as by capability of absorbing wound exudate, thus, forming a viscoelastic gel.

Three stock aqueous solutions having different CSG hMW, Ser, and Gly concentrations were considered. These solutions were used as such or mixed in predetermined wt/wt ratios to obtain the significant points (input variables) of a simplex centroid design. The response variables considered were: fracture force, amount of phosphate-buffered saline (PBS) (buffer mimicking wound exudate) absorbed after a prefixed hydration time, and rheological (viscosity and viscoelasticity) properties of hydrated dressings. To confirm the prediction power of the best fit model correlating dressing composition with each response variable, the optimized formulation was subjected to the same characterization used for the dressings of the simplex centroid design and the experimental results were compared with those predicted by the model.

Moreover, the bioactive potential of the optimized formulation was investigated. In particular, antioxidant properties of Ser at the concentration used in the optimized dressing were evaluated on human fibroblast cell line. Cell proliferation properties of the optimized dressing were studied on the same cell line; to this purpose a novel procedure that enables to test dressings on human fibroblasts without any dilution in cell medium was set up.

The optimized formulation was also subjected to a preliminary stability test to investigate the maintenance of its physical properties.

Materials and Methods

Materials

The following materials were used: chitosan high MW (CS hMW) (DD: 91%) (1568, Giusto Faravelli, Milan, Italy); glutammic acid (G) (Sigma-Aldrich, Milan, Italy); Gly (Sigma-Aldrich); *Bombyxmori* cocoons, supplied by Nembri Industrie Tessili S.r.l., Capriolo (BG), Italy.

Micronized Ser Preparation and Characterization

Ser Preparation

B mori cocoons were degummed in an autoclave at 120°C for 1 h (40 mL water/g per cocoon) to obtain Ser solution that was subsequently spray dried with Mini Spray Dryer (Büchi B-290, BÜCHI Labortechnik AG, Flawil, CH) under the following experimental conditions: pump flow rate: 6 mL/min; inlet temperature: 120°C; outlet temperature: 80°C; air pressure: 3 bar.

Ser Characterization

Spray-dried Ser was subjected to granulometric analysis by means of laser light scattering (Beckman Coulter LS230, Beckman Coulter, Inc., Brea, CA) under the following experimental conditions: small cell volume, 120 mL; obscuration 5%; Ser powder suspended in ethanol was sonicated for 4 min, transferred to the measurement cell, and examined in 5 replicates lasting 90 s each. The refractive index was set at 1.359 for ethanol.

Spray-dried Ser cytotoxicity was investigated using normal human dermal fibroblasts (NHDF) (human juvenile fibroblasts from foreskin) (Promocell GmbH, Heidelberg, Germany) from 6th to 16th passage.

Cells were cultured in a polystyrene flask (Greiner bio-one, PBI International, Milan, Italy) with 13-15 mL of complete medium (CM), consisting of Dulbecco's Modified Eagles Medium with 4.5 g/L glucose and L-glutamine (Lonza, BioWhittaker, Verviers, Belgium) supplemented with 1% (vol/vol) penicillin streptomycin 100×, 1% (vol/vol) amphoteric 100× (Sigma-Aldrich), and 10% (vol/vol) inactivated fetal calf bovine serum (Euroclone, Milan, Italy). Cells were maintained at 37°C in 95% air and 5% CO_2 atmosphere (Shellab® Sheldon® Manufacturing Inc., Cornelius, OR). All the operations required for cell culture were carried out in a vertical laminar air flow hood (Ergosafe Space2, PBI International). After reaching 80%-90% confluence in the flask (one week), cells were trypsinized: at first the monolayer was washed with Dulbecco's PBS (Sigma-Aldrich) to remove bivalent cations that could inactivate trypsin, then 3 mL of 0.25% (vol/vol) trypsin-EDTA solution (Sigma-Aldrich) were left in contact with the monolayer for 5 min in the incubator. Afterward, the cell layer was harvested with 7 mL of CM to stop the proteolitic activity of trypsin and to facilitate cell detachment. Subsequently, cell suspension was centrifuged (Sorvall® TC6, Sorvall Products, Newtown, CT) at 112 × *g* for 10 min. The supernatant was eliminated and cells were resuspended in 6-mL medium without serum (M w/s). The amount of cells in suspension was determined in a counting chamber (Hycor Biomedical, Garden Grove, CA), using a 0.5% (wt/vol) Trypan blue solution (Biological Industries, Beit Haemek, Israel) to visualize and count viable cells. For counting, 100 μL of cell suspension were diluted 1:1 vol/vol with the Trypan blue solution and placed in the counting chamber for microscopic analysis.

To assess Ser cytotoxicity 5×10^4 cells/cm^2 were seeded into a 96-well plate (Greiner bio-one, PBI International) in the presence of CM and let to grow overnight. Then, the medium was removed and cells were put in contact for 24 h with a Ser solution prepared in either CM or M w/s. Different Ser concentrations were considered: 0.01, 0.05, and 0.1% wt/wt. CM and medium without serum (M w/s) 10% were used as positive controls. A 10% w/w Tryton (Sigma Aldrich) aqueous solution was used as negative control. After 24 h, an 8-μM MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution in Hank's Balanced Salt Solution (pH 7.4) was added to each well and incubated for 3 h. After shaking for 60 s, absorbance was determined at a wavelength of 570 nm, with a 690 -nm reference wavelength, by means of an iMark® Microplate reader (Bio-Rad Laboratories S.r.l.). Results were expressed as % viability by normalizing the absorbance measured after contact with Ser solutions with that determined after contact with CM or M w/s.

Experimental Design

The choice of the optimal dressing composition was carried out by a suitable experimental design (DoE), the simplex centroid