

Applications of fungal chitosans in biomaterials

Milos Beran^{a,*}, Petr Hanak^a, Petr Molik^a, Marian Urban^a, Lubos Adamek^a, and Milan Krajicek^b

M.Beran@vupp.cz

^aFood Research Institute Prague, Radiova 7, Cz 10231 Praha, Czech Republic;

^bMedical Sciences s.r.o., Svermova 268, Cz 46010 Liberec, Czech Republic

INTRODUCTION

Chitosan and some of its complexes have been studied for use in biomedical applications such as wound dressing, drug delivery systems and space filling implants. Chitosan also has been reported to be a promising candidate as a scaffold material for engineered human tissue such as skin, cartilage and bone due to its biocompatibility and resorbability.

Fungal mycelial wastes of the antibiotic, citric acid and other industry can become free and rich alternative sources of chitin - chitosan materials, beside the traditional industrial source - shellfish waste materials. Moreover, the fungal chitosans can have unique properties compared with those derived from Crustacea.

Fungal chitosans has been extracted from *Aspergillus niger* and *Penicillium oxalicum* industrial biomass, purified and bleached.

Several applications of the fungal chitosans in biomaterials have been tested:

- 1) composite fungal chitosan - collagen sponge material for wound healing or scaffolds for tissue engineering
- 2) nonwoven fabric from nanofibers composed of fungal chitosan and polyvinyl alcohol with potential medical utilization
- 3) tissue adhesive material for medical usage

EXPERIMENTAL

Chitosan extraction and purification

Aspergillus niger mycelium was obtained as a by-product of submerge production of citric acid from AKTIVA (Kaznějov, Czech Republic). *Penicillium oxalicum* mycelium was obtained from ASCOLOR - BIOTECH (Ústí nad Labem, Czech Republic) as a by-product of production of a red anthraquinone dye. Chitosan was extracted from the mycelial wastes by successive alkali and acidic extraction using a method described formerly and characterized by physico - chemical methods [1].

Physico-chemical characterization of the fungal chitosans:

MW distribution: 5 - 50 000
Degree of acetylation (DA): 4 - 5%

Preparation of composite fungal chitosan - collagen sponge materials

Solutions containing 1% (w/v) of the fungal chitosan, commercial chitosan Primex TM326 (Primex Ingredients ASA, Norway), or commercial bovine collagen (M6, Medical, Czech republic) in 0.5% acetic acid were prepared. After adjustment of pH of the prepared solutions to 6.5 with ammonia, each of the chitosan solutions was mixed with the collagen solution in 1 : 1 ratio (v/v). The suspensions obtained as described above were whisked in a high speed mixer, poured into dishes, frozen immediately and freeze dried to obtain a three-dimensional porous structure. Before biocompatibility testing the sponges were sterilized by gamma-irradiation.

Biocompatibility testing of the chitosan - collagen sponge materials

Biocompatibility of the sponge material was evaluated after subcutaneous implantation of 10 x 10 mm pieces of the tested material to laboratory rats and rabbits. The resorption of the implant was observed macroscopically and by optical and electron microscopy in combination with immunohistochemical methods.

At day 3,7,14, 21 and 29 post-implantation, the implants with surrounding tissue were dissected, examined macroscopically, fixed in 4% (v/v) formalin and examined histologically.

Preparation of nonwoven fabric containing fungal chitosan

The nonwoven fabric was prepared from nanofibres composed from fungal chitosan *A. niger* (10%) and polyvinylalcohol (90%) using a special patented electrospinning technology Manospider at the Department of Nonwovens, Faculty of Textile Engineering, Technical University of Liberec, Czech Republic.

Preparation of test tissue adhesives

Principle of the proposed tissue adhesive is crosslinking gelatine, collagen, or other proper protein with a commercial microbial enzyme transglutaminase to form strong and irreversible gels. This acyl-transfer enzyme catalyzes transamidation reactions that lead to the formation of N-(γ -glutamyl) lysine crosslinks in proteins [2].

Gelatine solutions (5 or 10 w/v %) were prepared by dissolving 5 or 10 g of gelatine from porcine skin (Type A, 300 bloom, Sigma) into 100 ml of demi-water at temperature 45°C, pH was then adjusted to 6.0 with NaOH. A chitosan solution (3.2 w/v %) was prepared by dissolving the fungal chitosan *A. niger* in demi-water after adjusting pH of the solution with HCl to 2 - 3. Finally the pH was increased by NaOH addition to 6.0. Gel formation was initiated by adding 0.25 - 1% (w/v) of transglutaminase ACTIVA WM (Ajinomoto Co., Inc, Tokyo, Japan) to the solutions containing gelatine, or blends containing gelatine and chitosan. Transglutaminase activity: 81 - 135 U/g (Hydroxamate method).

Viscosimetric measurements of gelation time of the tissue adhesives

Viscosimetric measurements of gel formation were performed at constant rotation speed and temperature 37°C using viscosimeter RHEOTEST 21 (MLW Prüfgeräte, Werke Medingen, GDR). Following the addition of transglutaminase into each test blend, polymerization of a gelatine hydrogel is distinguished by the occurrence of gelation at some point in the crosslinking. At this point, the adhesive loses fluidity or its viscosity increases abruptly.

Bonding strength measurement

Bonding strength of the proposed tissue adhesive (gelatine - transglutaminase (TGA) - chitosan) was compared with bonding strengths of gelatine - TGA mixture, pure gelatine, and a commercially used mixture gelatine - resorcinol crosslinked with formaldehyde (GRF glue).

Composition of the test adhesives:

Sample 1: 3.3% (w/v) of porcine gelatine (Type A, 300 bloom, Sigma), 2% (w/v) of TGA (ACTIVA WM), and 1% (w/v) of a fungal chitosan (*P. oxalicum*) in demi-water, pH 5.5

Sample 2: 5% (w/v) of the porcine gelatine, and 3% (w/v) of TGA in demi-water, pH 5.5

Sample 3: 3.3% (w/v) of the porcine gelatine, 6.7% (w/v) of resorcinol (Sigma), and 1.2% (w/v) of formaldehyde (Sigma) in demi-water, pH 5.5

Sample 4: 10% (w/v) of the porcine gelatine in demi-water, pH 5.5

The adhesive characteristics of each of the test adhesives were determined by measuring its bonding strength between two thin muscle slices (8 x 2.5 cm) from a fresh porcine heart. Each of the test adhesives in the amount of 0.4 ml was first applied to 2.5 x 2.5 cm areas of two these slices, which were adhered together. After 800 g.cm⁻² loading for 60 min at 37°C, the bonding strength of each test adhesive was measured by a texture analyzer TA-XT 2 (Stable Micro System, UK) at a constant speed of 48 mm.min⁻¹ until these two muscle slices were separated.

RESULTS and DISCUSSION

Preparation of composite fungal chitosan - collagen sponge material

Prepared chitosan - collagen sponges have uniform porous structure, good mechanical strength and stability, and water absorption capacity, in connection with haemostatic and wound healing properties. Fig. 1 shows a sample of the sponge containing the fungal chitosan.

Biocompatibility testing of the chitosan - collagen sponge material

Macroscopic examination:

The implanted samples of the sponges were significantly decomposed after 3 days and fully degraded after 21 days without any macroscopic inflammatory response in all the experimental animals.

Microscopic examination:

Collagen - chitosan Primex sponge:

Rats

The course of the resorption at microscopic level is recorded on photographs in the Fig. 2. Day 3 and 7: the implant is surrounded by a mixture endemic liquid with a small amount of suppurative infiltrate.

Day 14 - 21: gradual disappearing of the suppurative infiltrate and collagen fibroproduction in the external layer.

Day 21 - 29: multiplication of giant multinuclear cells in the place of the implant

Day 29: no sign of edema or suppuration, predominantly fibrillar and reticulated material, partly granular.

Rabbits

Day 3 and 7: macrophag necrotising reaction.

Day 14 - 21: replacing the necrotising changes by infiltrate containing reticular and monocyte cells.

Day 21 - 29: disappearing of the infiltrate and the implant material and collagen encapsulation.

Collagen - fungal chitosan sponge:

Rats

The course of the resorption at microscopic level is recorded on photographs in the Fig. 2. Day 3 and 7: suppurative infiltrate with polynuclear cells, gradually disappearing by necrosis with fibrin exudation

Day 14 - 21: histiocytar and lymphocytar encapsulation

Day 21 - 29: multiplication of giant multinuclear cells in the place of the implant

Day 29: no sign of edema or suppuration, predominantly fibrillar and reticulated material, partly granular.

Rabbits

The healing process almost identical as in the case of the collagen - chitosan Primex sponge.

Summary of the biocompatibility testing

Results of the biocompatibility testing show that the fungal chitosan is perspective biomaterial however its further purification is necessary. Fragmentation and gradual disappearing of the sponge and the presence of macrophages and foreign body giant cells indicated the sponge was biodegraded in vivo.

Preparation of nonwoven fabric containing fungal chitosan

A sample of the nonwoven fabric containing fungal chitosan is shown in the Fig. 3.

Possible areas of medical applications of the nonwoven fabric include artificial organs, tissue engineering, artificial blood vessels, targeted drug delivery, wound dressing or mouth-screens.

Viscosimetric measurements of gelation time of the tissue adhesives

Fig. 4 and 5 illustrate the influence of concentrations of TGA and gelatine on gelation time of the tested mixture. The rate of gel formation and the strength of the resulting gels increased markedly with increasing gelatine and transglutaminase concentrations. Fig. 6 illustrates the influence of chitosan presence on gelation time. Chitosan addition increased the rate of gel formation. However, chitosan was not required for transglutaminase - catalyzed reaction. We have no direct evidence to support or reject suggestion that transglutaminase can transfer the protein directly onto chitosan's amino groups.

When gelatine concentration was 10% (w/v) and TGA concentration 1% (w/v), gelation time of the tested mixture was about 15 minutes. This rate of gel formation should be sufficient for most applications of the biological glue.

Bonding strength measurement

The results of the bonding strength measurement are shown in the Fig. 7. The bonding strength of the sample 2 (5 w/v % of gelatine - 3 w/v % of TGA) is comparable with the bonding strength of the sample 3 (3.3 w/v % of gelatine - 6.7 w/v % of resorcinol - 1.2 w/v % of formaldehyde), a commercially used mixture. Taking into consideration lower concentrations of gelatine (3.3 w/v %) and TGA (2 w/v %), chitosan presence didn't influence the bonding strength significantly. Therefore, transglutaminase provide an alternative method for crosslinking collagen, gelatine or other proteins and may offer interesting opportunities for in situ applications. The enzyme can replace the cytotoxic crosslinking agents such as formaldehyde (GRF glue) or glutaraldehyde (GRG) in many cases.

CONCLUSIONS

The chitosan - collagen sponges have proper characteristics for usage as biodegradable wound healing dressing and scaffolds for tissue regeneration.

The composite chitosan containing nanofibers and nonwoven fabric can be tested for applications in tissue engineering, such as tissue scaffolds or artificial bone and vessel materials.

The proposed tissue adhesive material has sufficient bonding strength and is fully biodegradable without any cytotoxic components. All components of the product are available commercially at reasonable prices. Chitosan addition to the tested adhesive increases the rate of gel formation and improves haemostatic and wound healing properties of the product.

The adhesive material can be used as a surgical glue or for other in situ applications. The fungal chitosan is perspective biomaterial however its further purification is necessary.

References

- [1] Adamek, L., Beran, M., Hanek, P., and Molik, P.: Isolation and some applications of fungal chitin - glucan complex and chitosan. 6th Asia Pacific Chitin and Chitosan Symposium, May 23 - 26, 2004. Singapore. <http://www.vupp.cz/cz/vupp/publik/04poster/04Chitosan2004.pdf>
- [2] Greenberg, C.S., Birchtachler, P.J., and Rice, R.H.: Transglutaminases: multifunctional cross-linking enzymes that stabilize tissues. FASEB J 1991, 5: 3071-7.



Fig. 1: A sample of the sponge prepared from collagen and the fungal chitosan (*A. niger*).

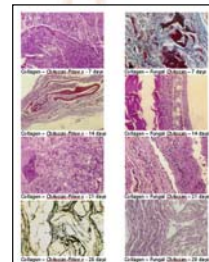


Fig. 2: Course of resorption of the collagen - chitosan sponges at microscopic level



Fig. 3: A sample of a nonwoven fabric prepared from nanofibers composed of fungal chitosan *A. niger* (10%) and polyvinylalcohol (90%)

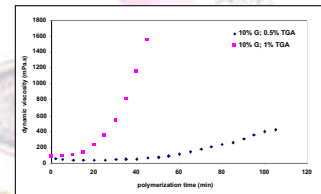


Fig. 4: Influence of TGA concentration on gelation time of 10% (w/v) gelatine (G) solution at 37°C

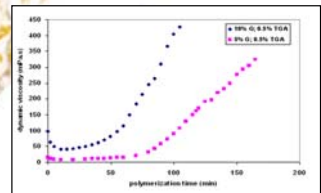


Fig. 5: Influence of gelatine (G) concentration on gelation time in the presence of 0.5% (w/v) TGA at 37°C

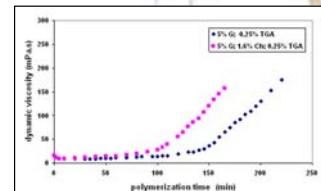


Fig. 6: Influence of chitosan (Ch) presence on gelation time in 5% (w/v) gelatine solution with 0.25% (w/v) of TGA at 37°C

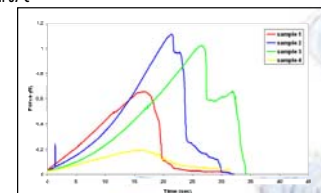


Fig. 7: The results of the bonding strength measurement

- Sample 1: 3.3% (w/v) G; 2% (w/v) TGA, and 1% (w/v) CH
- Sample 2: 5% (w/v) G, and 3% (w/v) TGA
- Sample 3: 3.3% (w/v) G; 6.7% (w/v) R, and 1.2% (w/v) F-GRF glue
- Sample 4: 10% (w/v) G