ISSN 0003-6838, Applied Biochemistry and Microbiology, 2006, Vol. 42, No. 6, pp. 631–635. © MAIK "Nauka/Interperiodica" (Russia), 2006.
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Preparation of Biodegradable Porous Films for Use as Wound Coverings

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Received Janury 23, 2006

Abstract—We studied the preparation of polymeric films formed from solutions of poly-3-hydroxybutyrate and poly-ε-caprolactone in chloroform and methylene chloride. A morphological study of film chips (electron microscopy) showed that solvent evaporation results in the formation of a heterogeneous structure with interpenetrating pores (1–20 μm). We proposed a new method for introducing the proteolytic enzyme and the aminopolysaccharide chitosan into the composition of polyester films. Composite films possessed necrolytic activity and were characterized by increased hydrophilicity. Properties of enzyme-containing films from a mixture of polymers (proteolytic activity, porous structure, and increased hydrophilicity) account for their use in the preparation of biodegradable wound coverings.

DOI: 10.1134/S0003683806060160

Biodegradable polymers hold much promise for use in medical practice. They serve as a polymeric matrix to prepare prolonged-action medicinal agents, absorbable sutures, and wound coverings. Moreover, these polymers are used in genetic and cellular engineering to obtain bioartificial organs and tissues. Development of new materials from various classes of biodegradable polymers solves a variety of complex problems.

Much recent attention has been paid to biocompatible polyesters. The natural polymer poly-3-hydroxybutyrate, possessing degrading activity in vivo, is of particular interest [1–3]. The requirements for functional properties of a biodegradable material depend on its destination. Targeted regulation of compositional and structural characteristics of a polymeric preparation is of considerable importance in this respect. The preparation of mixed solutions from polymers in the solvent is an efficient approach to regulate the supramolecular and porous structure of fibers and films. Composite materials from these solutions may form various structural types, which determines the biodegradation time, the adsorption capacity, and the kinetics of the release of medicinal compounds from polymeric matrices.

Our previous studies [4] showed that the polymeric composition of polyhydroxybutyrate (PHB) and synthetic polyester poly-ε-caprolactone (PCL), capable of inducing hydrolytic degradation *in vivo*, is one of the most promising biodegradable materials. These compounds have a similar chemical composition, but differ in the supramolecular structure of crystallizing polymers. The films prepared from various solutions of PHB and PCL were structurally heterogeneous. Some films had a porous structure. These properties are important for developing new products for replacement surgery. Spontaneous formation of this structure results from phase separation during solvent evaporation unaccompanied by extraction of one of the polymers. Therefore, these films may be used as therapeutic agents to promote healing of tissue damage.

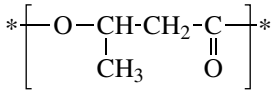
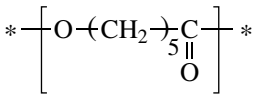
A wound covering to treat the first stage of the wound process should include proteolytic enzymes. During local application, proteolytic enzymes cleave dead tissue, decrease the viscosity of wound exudate, and promote its elimination. The conditions for propagation of new tissue (fibroblasts and epithelium) should be provided at the second stage of wound healing.

This work was designed to develop an efficient biodegradable wound covering. We prepared and studied the properties of polyester films from solutions in organic solvents containing the proteolytic enzyme trypsin.

MATERIALS AND METHODS

Polyesters, poly-3-hydroxybutyrate (Bach Institute of Biochemistry, Russian Academy of Sciences), and poly-ε-caprolactone (Sigma–Aldrich, United States) served as film-forming polymers. Table 1 illustrates characteristics of these polymers. Experiments were performed with chitosan (molecular weight, 180 kDa; deacetylation degree, 0.87; All-Russia Institute of Fish Industry and Oceanography, Rus-

Table 1. Characteristics and properties of biodegradable polyesters

Polymer	Repeated element	Polymerization degree	Molecular weight of polymer, kDa	Density, g/cm^3	T_{mlt}, °C	Degree of crystallization, %
PHB		3500	300	1.2	175	71
PCL		500	42	1.1	60	68

sia), trypsin (EC 3.4.21.4; active site concentration, 68%; Merck, Germany), *N*-benzoyl-*L*-arginine methyl ester (BAME; Sigma–Aldrich, United States), and 25% aqueous solution of glutaraldehyde (Merck, Germany).

The films for electron microscopic studies and thermomechanical analysis were formed from 5% solutions of PHB and PCL or their mixture in chloroform by means of petri-dish coating and solvent evaporation for 12 h. Trypsin was incorporated into the structure of films. Trypsin was dissolved in 0.1 M phosphate buffer (pH 7.8) and emulsified in the solution of film-forming polymers in a water-insoluble organic solvent (chloroform or methylene chloride). The emulsions were maintained for 30 min to remove air bubbles.

The biocatalytic activity of films was studied after solvent evaporation from a thin layer of the forming composition. It was prepared using Teflon dies with slots of different size. Segments of similar thickness were cut off from the films. Measurements were performed on a micrometer with an accuracy of ±5 μm. We recorded changes in the enzyme activity during immobilization and kinetic characteristics of trypsin release.

Trypsin activity was measured spectrophotometrically by the rate of BAME hydrolysis. The amount of the substrate hydrolyzed at 25°C and pH 7.8 in 1 min was taken to be equal to one unit of trypsin activity. Immobilized enzyme activity was expressed in units per gram of film (U/g) and as percentages of the activity of the native enzyme. The kinetics of trypsin release in 0.95% solution of NaCl was estimated by changes in trypsin activity in the solution under static conditions (a polymeric material/isotonic solution ratio of 1 : 40).

Thermomechanical analysis was performed on a DMA/SDTA 861 thermal analyzer (Mettler, Switzerland; heating rate, 5 K/min; maximum load, 5 N; load frequency, 1 Hz).

Morphological study of film chips by electron microscopy involved a JSM-35 scanning microscope (JEOL, Japan). The chips were prepared in liquid nitrogen. The surfaces of the samples were sprayed with gold.

RESULTS AND DISCUSSION

Table 1 shows that PHB and PCL have a similar chemical structure. Previous studies showed the same solubility for PHB and PCL. However, these crystallizing polymers are rarely compatible with each other. Their intersolubility may be realized only at the level of amorphous regions. Mixed solutions of PHB and PCL are transparent and monophasic. However, solvent evaporation is accompanied by phase separation of the solution and the formation of heterogeneous structures.

Morphological characteristics of film chips prepared from chloroform solutions of PHB and PCL and equiconcentrated mixed solutions of these compounds were studied by scanning electron microscopy. It should be emphasized that both polyesters (PHB and PCL) have a crystallization degree of 70%. The films prepared from solutions of individual polymers are nontransparent. Hence, the turbidity of films from mixed solutions of PHB and PCL does not reflect the incompatibility of these polymers. Morphological characteristics of the film surface and chip for PHB differ from those for PCL (Figs. 1a–b) in the smaller size of structural elements and the absence of orientation along the film surface. The roughness of the chip and the heterogeneity of the film surface are associated with the presence of crystal elements.

The mixture of PHB and PCL has a heterogeneous structure. The films of PHB and PCL (50 : 50) have a system of interpenetrating pores. The size of the pores varies from 1 to 20 μm (Fig. 1c). These pores penetrate the material and appear on the surface. The continuity and the interpenetration of the phases are signs of a spinodal mechanism of phase separation [5]. Under these conditions of phase separation, the matrix is formed by a more concentrated solution of polymers. In

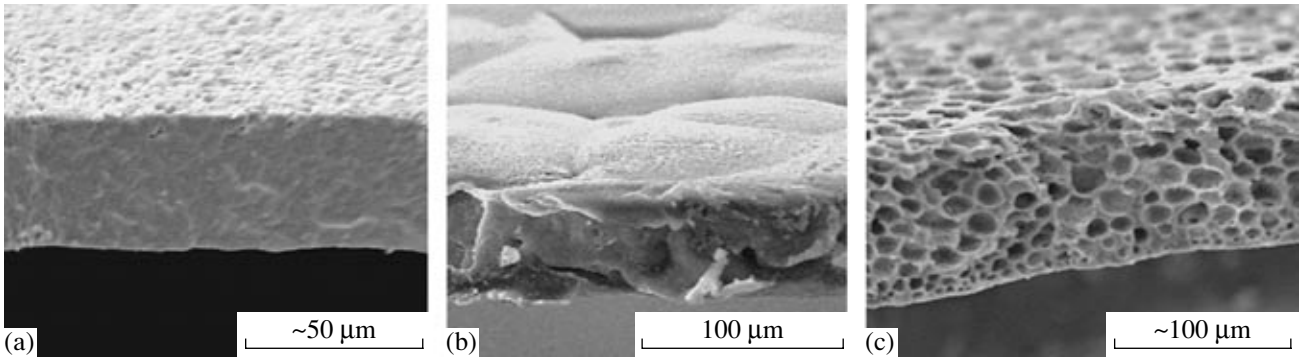


Fig. 1. Microphotographs of film chips: (a) PHB; (b) PCL; and (c) mixture of PHB and PCL (50 : 50).

contrast, the phase is formed by a highly diluted solution. Structural characteristics of the system formed during solvent evaporation provide for regeneration and temporal replacement of damaged tissues over biodegradation of a porous covering.

The film from a mixture of polymers is characterized by one glass transition temperature (Table 2), which reflects the partial compatibility of amorphous regions in PCL and PHB. However, the mechanical properties of the film cannot be described by the additivity rule. The values of the elasticity modulus (E) for a mixture of PHB and PCL (50 : 50) and pure PCL are 220 and 200 MPa, respectively. For a film from PHB, E is 1200 MPa. These features are associated with a decrease in the efficient area of the normal section of the film from a mixture of polymers due to the formation of a highly porous structure.

A wound covering for use at the first stage of the wound process should contain proteolytic enzymes approved for local application. In the present work, emulsions from a solution of PHB and PCL in chloroform or methylene chloride were used as forming compositions to prepare the films. A solution of trypsin served as an aqueous phase of the emulsion. In addition to trypsin, the hydrophilic aminopolysaccharide chitosan was administered to an aqueous phase. This procedure was performed to evaluate the possibility of regulating the kinetics of proteolytic enzyme release from biocompatible film coverings, as well as to increase moisture absorption properties of the material. The choice of chitosan for trypsin modification was related to the presence of primary amino groups in the polysaccharide macromolecule. On the one hand, these groups determine pH-dependent solubility of chitosan in water. On the one hand, they provide for chitosan reactivity with glutaraldehyde. Glutaraldehyde is a bifunctional cross-linking reagent extensively used for protein immobilization. Chitosan and trypsin in the forming composition were cross-linked with glutaraldehyde. An aqueous solution of glutaraldehyde (10%) was added to the emulsion. The glutaraldehyde/NH_2 group ratio was selected taking into account the rate of gel formation in trypsin-containing solutions of chitosan [6].

The ability of inverse emulsions to retain aggregative stability is determined by the interphase surface tension and ratio between the viscosities of the continuous phase and dispersion medium. Given the same concentrations of the surface active protein and the use of the same solvent, the stability of the emulsion depends on the viscosity of the dispersion medium. Stability studies of trypsin-containing emulsions allowed us to determine the lower viscosity limit of the dispersion medium (a solution of polyester in the organic solvent) that ensured the temporal stability of the emulsion. This value was 50 mPa's, which corresponded to 4–6% PHB and 5–7% PCL in chloroform and methylene chloride. Table 3 illustrates the dependence of the activity of immobilized trypsin on the concentration of PCL solution and the nature of the solvent. The activity of immobilized trypsin in films decreases as the concentration of the solution is increased (Table 3, nos. 2 and 3). The decrease in the activity is probably associated with increased influence of pore diffusion limitations on the kinetics of hydrolysis. This is related to the

Table 2. Thermomechanical properties of films from PHB, PCL, and mixture of these compounds

Film composition, %		Glass transition temperature, T_{gt}, °C	Young module (E, 25°C), MPa
PHB	PCL		
100	0	20	1200
0	100	–35	220
50	50	–30	200

Table 3. Characteristics of films* from PCL containing immobilized trypsin

Number of sample	Solvent	Solution concentration, %	Trypsin modification in emulsion	Trypsin activity in film, U/g	Immobilized trypsin activity	
					U/g	% of the initial level
1	Methylene chloride	5	–	20.0	4.4	22.1
2	″	5	+	20.0	4.0	20.0
3	″	7	+	20.0	2.9	14.4
4	Chloroform	5	+	20.0	7.3	36.5
5	″	5	–	20.0	7.0	35.1

* Film thickness 50 ± 5 μm.

Table 4. Characteristics of trypsin-containing films from emulsions of PHB and PCL in chloroform

Number of sample	Polymer ratio in solution, %		Amount of trypsin in film		Trypsin modification in emulsion	Immobilized trypsin activity	
	PHB	PCL	mg/g	U/g		U/g	% of the initial level
1	100	0	2	20	–	8.5	42.5
2	100	0	2	20	+	6.2	31.0
3	50	50	2	20	–	10.5	52.5

formation of a more compact structure of the film from 7% solution of PCL.

The boiling point of chloroform is higher than that of methylene chloride (61 and 42°C, respectively). Therefore, chloroform exhibits a longer evaporation time than methylene chloride (by 2 h). However, trypsin activity is much higher in films from the solution of chloroform. Titration of trypsin active sites in test samples shows that chloroform has a smaller effect on the protein conformation than methylene chloride. Chloroform was used to prepare enzyme-containing films from a mixture of PHB and PCL.

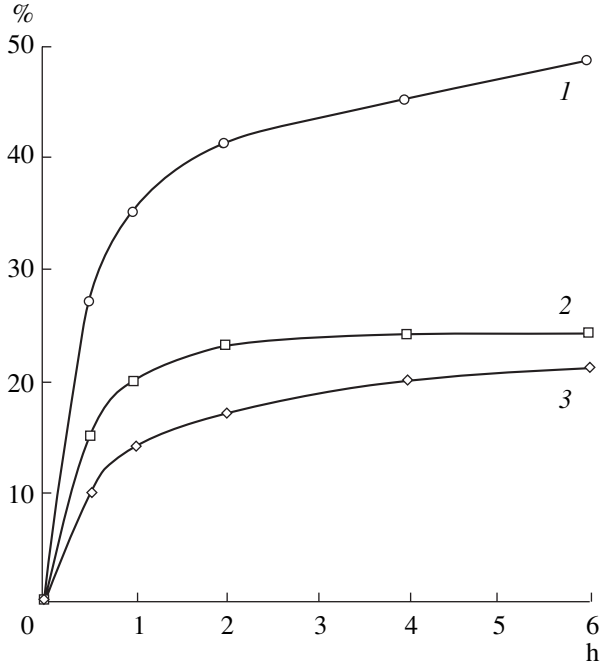


Fig. 2. Kinetics of protein release from films of PHB in 0.9% solution of NaCl (40 ml/g film). The numbers of curves correspond to the numbers of samples in Table 4.

Modification of trypsin with chitosan in the aqueous phase of the emulsion did not result in inactivation of the enzyme (Tables 3 and 4). However, a significant decrease in the rate of protein desorption from the films into physiological saline illustrates the prolonged effect of trypsin (Fig. 2, curves *1*, *2*).

The highly porous structure of the film from PHB and PCL with interpenetrating pores would be expected to provide intensive mass transfer during the interaction with physiological saline. However, this was not the case (Fig. 2, curve *3*). The rate of protein desorption from this film was higher than from others. Taking into account the estimated activity of trypsin, these specific features may be accounted for solely by adsorption of amphiphilic protein molecules from aqueous solutions on a well-developed internal surface of hydrophobic films.

These results indicate that the use of mixed chloroform solutions of biodegradable polyesters PHB and PCL for preparing porous polymeric films with immobilized trypsin makes it possible to regulate their structure and obtain polymeric wound coverings with the desired properties (porosity, biodegradation rate, hydrophilicity, and kinetics of release of biologically active compounds).

ACKNOWLEDGEMENTS

This study was supported by the Complex project no. ZhS-KP.4/002.

Thanks are due Dr. Juergen Schawe (Mettler Company) for his help in thermomechanical analysis.

We are grateful to E. S. Obolonkova (Institute of Synthetic Polymeric Materials, Russian Academy of Sciences) for the preparation of microphotographs.

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