PROTEASES ENTRAPPED IN POLYMER COMPOSITE HYDROGELS: PREPARATION METHODS AND APPLICATIONS

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Methods for entrapment of proteases in magnetic polymer beads have been developed. Entrapped trypsin and carboxypeptidase B (CPB) were shown to retain 80 and 65% of their initial activities, respectively. As a polymer matrix synthetic temperature-sensitive polymer, namely poly(N-vinyl caprolactam), and various natural and modified polymers (alginate, chitosan, chitosan sulfate) were used. On the base of these polymers dressings (hydrogel films) with entrapped thrombin and peptides that mimic thrombin and trypsin action were prepared. Film-entrapped thrombin and the peptides (thrombin receptor agonist peptide TRAP-6 and trypsin receptor agonist peptide Ag-PAR-2) were found to markedly accelerate healing of skin incisional wounds in a mouse model. This was confirmed by a marked decrease of wound sizes in experimental animal groups (mice having films with entrapped thrombin or peptides) compared to a control animal group (films without thrombin or peptides). Accelerating fibroblast proliferation and neovascularization in granulation tissue samples also gave evidence of stimulating effect of film-entrapped thrombin and peptides on wound healing.

Gel entrapment method is attractive because is very simple, can be carried out under soft conditions (physiological pH and temperature) and also allowed to vary polymer matrix composition and structure. This method is often the only one which can be used for entrapment of such unstable enzymes as CPB and thrombin are. Providing magnetic properties to polymer carriers with entrapped enzymes (by introducing magnetic materials into a polymer matrix) opens new technological advantages for their use in various fields.

Presently magnetic carriers are widely employed in biotechnology and medicine, in particular in immunoenzyme analysis [1]; for separation of cells [2]; as affinity sorbents [3]; for target drug delivery [4]; in particular for tumor treatment [5]; in solid phase organic synthesis [6], for cultivation of microorganisms [7] and surface-dependent animal cells [8]. Various synthetic polymers, such as polyacrylamide [9], polyacrolein [10], polyvinyl alcohol [11], co-polymers N-isopropylacrylamide and methacrylic acid [12], poly(ethylene glycol) derivatives [5] as well as natural polymers, such as starch [13], alginate [11], chitin, chitosan and derivatives [14, 15, 16], cellulose [17] are proposed as polymer matrix of magnetic carriers. There are also several reports on preparation of magnetic liposomes with entrapped enzymes [18]. Magnetic carriers are used to immobilize various enzymes, including chymotrypsin, acid and alkaline phosphatases, beta-galactosidase, beta-D-fructofuranosidase [16], glucoseoxidase and glucoamylase [9, 10, 16], trypsin [12, 14, 15, 16], cytochrome C-oxidase [18].

In the current research we would like to present recent results on entrapment of trypsin and carboxypeptidase B in PVCL-based composite polymer hydrogel beads containing magnetic filler as well as thrombin (and its peptide analogues) in composite polymer hydrogel films. All entrapped enzymes were shown to retain rather high enzymatic activity independently on the bead preparation method (an extrusion technique using water solution or an emulsification method involving hydrophobic phase). They can be used in biocatalysis in both water and organic media. Film-entrapped thrombin and its peptide analogues could be promising drugs for wound therapy.

Methods

Polymers. In the current study poly(N-vinyl caprolactam) (M_r 900 000) and aromatic polyamide POLAR-2 (M_r 25 000) were kindly provided by Prof. Yu. E. Kirsh (Karpov Physico-Chemical Institute, Moscow). Sodium alginate from *Macrocystis pyrifera* (medium viscosity) was from Sigma. Chitosan (M_r 330 000, minimum 85% deacetylated) was pursed at Sevryba (Russia); chitosan sulfate (M_r 80 000) were kindly provided by Prof. G. A. Vikhoreva (Textile Academy, Moscow).

Enzymes. Bovine trypsin (EC 3.4.23.1), 22 U/mg and carboxypeptidase B (EC 3.4.17.2) were from Biolar (Latvia). Bovine thrombin (EC 3.4.21.5, 2500 NIH U/mg) was obtained by purification of the commercial preparation (Kaunas, Lithuania) [19].

Peptides. TPAP-6 (Ser-Phe-Leu-Leu-Arg-Asn-OH) and Ag-PAR-2 (Ser-Leu-Ile-Gly-Lys-Val-OH) were synthesized at Institute of Molecular Biotechnology (Jena, Germany) and kindly provided by Prof. E. Glusa.

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Abbreviations: PVCL poly(N-vinyl caprolactam); Ca-alginate calcium alginate, CPB carboxypeptidase B; BAEE N-Benzoyl-L-arginine ethyl ester; Hipp-Arg Hippuryl-L-arginine; TRAP-6 thrombin receptor agonist peptide; Ag-PAR-2 trypsin receptor agonist peptide.

Reagents. N-Benzoyl-L-arginine ethyl ester (BAEE), Sigma, and Hippuryl-L-arginine (Hipp-Arg), Serva, were used. Calcium chloride, sodium chloride, calcium phosphate, ferrum chlorides ($FeCl_2 \cdot 4H_2O$ $FeCl_3 \cdot 6H_2O$), poly(vinyl alcohol) (M_r 11 000), glyceryl monostearate were pushed from DIA-M (Russia).

Preparation of magnetic filler: Two kinds of magnetic filler were used in this study, namely magnetite (Fe_3O_4) and polystyrene magnetic latex beads (1 μm). Magnetite was prepared by addition of 25 ml of concentrated ammonia solution to iron salt solutions (50 g $FeCl_2 \cdot 4H_2O$ and 10 g $FeCl_3 \cdot 6H_2O$ in 50 ml H_2O) at stirring using mechanical agitator (1000 rpm). The obtained Fe_3O_4 precipitate was washed with 1% ammonia solution (100 ml) twice and then with water (200 ml) using a permanent magnet. Polystyrene magnetic latex beads were synthesized by the method of microsuspension polymerization using magnetic fluid on a styrene base as a hydrophobic phase [20].

Entrapment of trypsin and carboxypeptidase B in PVCL–calcium alginate beads. Enzyme solution (trypsin, 0.1 ml; 10 mg/ml; CPB, 4 mg/ml) was added to the mixture of polymer soluitions (1 ml 10%(w/v) PVCL, 0.1 ml 1% (w/v) POLAR-2 and 2.5 ml 2% (w/v) of a sodium alginate), which contained a freshly precipitated and washed magnetite. The mixture was carefully agitated and then added to 1% (w/v) calcium chloride solution at 40 °C as described earlier [21].

Entrapment of thrypsin and carboxypeptidase B in composite PVCL–chitosan–chitosan sulfate beads. Trypsin (0.3 ml, 10 mg/ml) or CPB (0.3 ml, 4 mg/ml), was added to the mixture of polymer solutions (5 ml of 10% (w/v) PVCL solution; 0.5 ml of 1% (w/v) POLAR-2 solution), then 2 g of freshly prepared $Ca_3(PO_4)_2$ and a 2% (w/v) chitosan solution was added to get a final mixture volume of 20 ml. The obtained mixture was emulsified in 50 ml of oil phase containing 0.1% glyceryl monostearate for 20 min. To solidify the drops emulsion "water-in-oil" was heated at 40 °C for 10 min, then 50 ml of 3% (w/v) poly(vinyl alcohol) solution was added. After bead precipitation a sulfate chitosan solution (equal molar quantity) was added dropwise to the mixture. The beads were agitated for 30 min at 20 °C, and then separated by centrifugation (30 min, 450 g). To prepare magnetic beads freshly precipitated magnetite was added to polymer mixture and the beads were separated from the oil phase using a permanet magnet (3–4 kE) without centrifugation.

Measurement of enzyme activity. The activities of soluble and entrapped CPB, trypsin and thrombin were determined by spectrophotometry using substrates Hipp-Arg, BAEE and Chromozyme TH, respectively [22].

Entrapment of thrombin in composite polymer films. The entrapment was carried out as described earlier [23], but instead of adding a 2% (w/v) calcium chloride solution in Petri dish we used 2 cellulose filters which were impregnated by this solution. The polymer mixture containing thrombin or peptide was placed between these filters. The quantity of thrombin in the polymer mixture which was used for preparing one film was 3.5 or 10 NIH U; and peptide content was 1 mM.

Study of the effect of thrombin and peptides-agonists entrapped in composite hydrogel films on wound healing in a mouse model. The influence of the film-entrapped thrombin and peptides was studied as described earlier for a rat model [23]. However, the current research was carried out in a mouse model, and a size of incisional back wounds was diminished (1x1 cm^2).

Results and Discussion

1. Entrapment of trypsin and carboxypeptidase B in magnetic polymer beads

Magnetite (Fe_3O_4) is one of widely used magnetic materials which is used as a magnetic filler for preparation of magnetically susceptible polymer carriers. Usually magnetite is prepared by co-precipitation of Fe^{2+} Fe^{3+} salts with a base or a concentrated ammonia solution [15]. There are several methods for preparation of magnetic supports, such as follows: (1) introducing magnetite (as a freshly precipitated suspension or magnetic fluid) in a polymer solution and then preparation of magnetic polymer beads [11]; (2) adsorption of magnetite in pores of previously prepared polymer beads [14]; (3) co-precipitation of iron salts from a polymer solution with a base during the bead preparation process [15].

To prepare magnetic beads with entrapped enzymes we used 2 kinds of magnetic filler: (1) a suspension of freshly precipitated magnetite; (2) magnetite particles, encapsulated in polystyrene latex beads (1 μm). The quantity of the magnetic filler in a polymer matrix was varied within a range of 0–40%. Magnetite was obtained either by co-precipitation of Fe^{2+} and Fe^{3+} salts with a base solution, or in a sodium alginate solution which was a good stabilizer for magnetite particles preventing them from formation of big aggregates. As was shown by analysis of size distribution of magnetite particles, that were prepareted in water and in a sodium alginate solution, only 14% of all aggregates had a size within 0.1–1 μm range. The relative number of these particles increased up to 39% in the case of the sodium alginate solution usage. The magnetite preparation in the alginate solution was accompanied by adsorption of polymer molecules on the surface of forming monodomain magnetite particles, thus preventing them from magnetic aggregation. These particles had superparamagnetic properties independently on the size of the obtained aggregates. Thus, the prepared composite PVCL–Ca–alginate and PVCL–chitosan–chitosan sulfate beads with magnetite particles proportionally distributed in the volume of the polymer matrix (Fig. 1), also did not posses residual magnetizatrion, e. g. it was possible to repeatedly manupulate them using a permanent magnet.

To prepare a polymer matrix a synthetic temperature-sensitive polymer PVCL [25] as well as natural and modified polysaccharides, such as alginate, chitosan and chitosan sulfate were used. Earlier we developed a method for entrapment of proteases in non-magnetic carriers based on PVCL [22]. Magnetic polymer beads were prepared by dispersion of polymer solutions containing both the enzyme and magnetite in a water phase (an extrusion method)

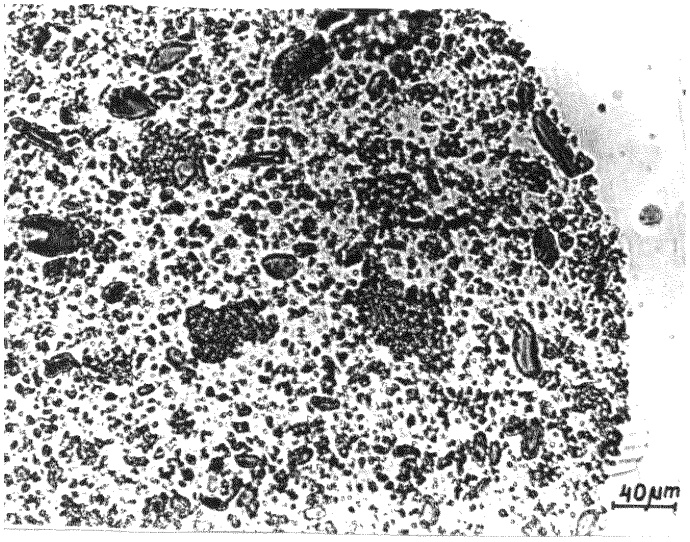

Fig. 1. The PVCL–Ca alginate bead with entrapped enzyme (a semi-thin section, light microscopy). The initial enzyme activity (before entrapment) was taken as 100%.

or in hydrophobic phase (an emulsification method). The latter involved in emulsification of a water phase (PVCL, the enzyme, magnetite and cationic polymer chitosan) into an oil phase with surfactant and then precipitation of the formed microdrops in a water subphase that contained anionic polyelectrolyte, namely chitosan sulfate. The magnetic particle size depended upon the used preparation method and was 0.5–1 mm and 10–100 μm for the emulsification and the extrusion techniques, respectively.

By determing the activities of the bead-entrapped enzymes we observed their desrease compared to initial values of native proteases. This difference can be explained by partial enzyme release from the beads in the supernatant and by the lost of some beads with entrapped enzyme during entrapment process. The relative trypsin and CPB activities in magnetic PVCL–Ca alginate and PVCL–chitosan–chitosan sulfate beads were 80 and 70% (the extrusion method) and 70 and 75% (the emulsification method) of initial values, respectively (Table. 1). It should be noted that the use of the permanent magnet (3–4 kE) for preparation of magnetic beads allowed us to diminish the lost of trypsin activity from 32 to 17% by the extrusion method and from 38 to 38% by the emulsification method. It should be also noted that introducing magnetite in the polymer matrix permited to strongly simplify the emulsification method itself using bead precipitation from the hydrophobic phase on the permanent magnet without time consuming centrifugation.

As is well known, sometimes the interaction between magnetite particles and gel-entrapped biologically active compound is undesirable. To prevent from that we proposed an another magnetic filler, namely polystyrene magnetically susceptible latex beads. As can be seen from Fig. 2, the lost of the entrapped CPB activity was 41 and 30–32% in the case of non-magnetic (C) and magnetic (A, B) carriers, respectively. Thus, the relative activity of entrapped CPB practically did not depend upon the type of the magnetic filler, and was 68% and 70% for the beads containing polystyrene magnetic latex or magnetite, respectively. The magnetic filler did not result in the decrease of the entrapped trypsin and CPB activites at storage for 4–5 months.

Thus, novel polymer carriers for entrapment of proteases allowing to simplify markedly the preparation method and to diminish a loss of the enzyme activity.

Table 1

Entrapment of trypsin in magnetic and non-magnetic composite PVCL–Ca alginate and PVCL–chitosan–chitosan sulfate beads

Beads	Magnetite content in the beads, % (w/w)	Relative trypsin activity, %	
		Beads	Lost
PVCL–Ca alginate*	0	68	32
	35	82	15
PVCL–chitosan–chitosan sulfate**	0	62	38
	20	68	32
	40	70	30

Remarks: the initial enzyme activity (before entrapment) was taken as 100%;
* PVCL–Ca alginate beads were prepared by the extrusion method;
** PVCL–chitosan–chitosan sulfate were prepared by the emulsification method.

Table 2

The effect of thrombin and peptides-agonists entrapped in composite PVCL–Ca–alginate films on healing of skin incisional wounds in a mouse model

Films with entrapped	The 3rd day			The 7th day		
	Relative wound size*, %	Vessel number	Fibroblasts/macrophages ratio	Relative wound size*, %	Vessel number	Fibroblasts/macrophages ratio
thrombin (10 NIH)	60.8	22	2.18	34.3	110	3.71
thrombin (3,5 NIH)	74.9	22	1.18	41.8	70	0.65
Ag-PAR-2	97.2	26	0.41	42.9	160	0.72
TRAP-6	96.9	18	0.33	17.1	160	5.79
Control (the film without thrombin/peptides)	*113*	*36*	*1.69*	*63.1*	*90*	*1.03*

* An initial wound size was taken as 100%.

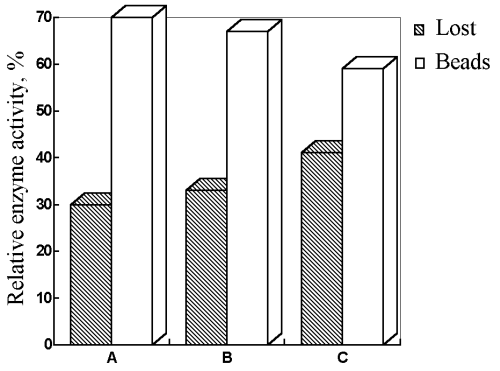

Fig. 2. Entrapment of carboxypeptidase B in composite PVCL–Ca alginate beads: A, with magnetite (35% w/w), B, with magnetic polystyrene latex (35% w/w), C, without magnetic filler. The extrusion method.

2. Entrapment of thrombin and peptides-agonists in composite hydrogel films

Thrombin is a unique serine protease from the trypsin family which regulates blood coagulation and antoicoagulation mechanisms, vascular tone and at the same time is involved in tissue repair process [24]. Being an unstable enzyme thrombin has several effective inhibitors which quickly inactivate it *in vivo*. Some delivery devices such as liposomes, hydrogel beads, capsules as well as films could provide both its activity retention and control release. Earlier we demonstrated thrombin stabilization in PVCL solutions [22]. In the current study a method for entrapment of thrombin as well as two peptides-agonists, namely thrombin receptor agonists peptide (TRAP-6) and trypsin receptor agonist peptide (Ag-PAR-2), in hydrogel PVCL-Ca alginate films is proposed. Both mentioned peptides being receptor agonists activated by proteases (thrombin or trypsin) are strongly involved in tissue repair. The effect of hydrogel film-entrapped thrombin and peptides on wound healing in a mouse model is shown in Table 2. Granulation tissue samples from the wounds were investigated by light microscopy and radioautography on the 3rd and the 7th day after wounding. We tested such parameters, as (1) the ratio fibroblasts/macrophages characterizing dynamics of proliferation and inflammation processes; (2) the number of microvessels that revealed neovascularization in the wound. The obtained results demonstrate that both film-entrapped thrombin and peptides markedly stimulated fibroblast proliferation and promoted neovascularization in an experimental animal group compared to control mouse group (the film without thrombin or peptide). An important parameter of wound healing is the decrease of the wound size. As can be seen from Table 2, the relative wound size under the film with entrapped TRAP-6 was reduced to 17% of its initial value, while it was 63% under the control film (without peptide). Thus, the hydrogel composite PVCL–Ca alginate films with entrapped thrombin or peptides provided acceleration of wound healing and could be considered as promising dressings for wound therapy in clinic.

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