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Immobilized Enzymes and Cells in Poly(*N*-Vinyl Caprolactam)-Based Hydrogels

Preparation, Properties, and Applications in Biotechnology and Medicine

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Abstract

A one-step mild method for entrapping animal cells and enzymes in macroporous composite poly(*N*-vinyl caprolactam)-calcium alginate (PVCL-CaAlg) hydrogels is described. Some properties of immobilized enzymes, such as thermal and storage stabilities and stability in water/organic media were investigated. Composite PVCL-CaAlg gels were successfully applied to immobilize a number of proteases, namely, trypsin, α-chymotrypsin, carboxypeptidase B, and thrombin. Thermal stability of the immobilized preparations obtained by entrapment in hydrogel beads allowed us to use them at 65–80°C, while the native enzymes were completely inactivated at 50–55°C. Various applications of enzymes and cells immobilized in beads were demonstrated. Immobilized trypsin and carboxypeptidase B were applied to prepare human insulin from recombinant

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proinsulin. The hydrogel beads with entrapped α-chymotrypsin were used in enantioselective hydrolysis of Shiff's base of D,L-phenylalanine ethyl ester (SBPH) in acetonitrile/water medium. Thrombin immobilized in PVCL-based hydrogel films was shown to be a promising compound for wound treatment. To prepare pure preparations of monoclonal antibodies (MAb) several hybridoma cell lines, including hybridoma cell lines producing MAb to interleukin-2, were successfully cultivated in the hydrogel beads.

Index Entries: Poly(*N*-vinyl caprolactam); composite macroporous hydrogel; gel-entrapped proteases; biocatalysis in water–organic media; enantioselective hydrolysis; thrombin; wound healing; immobilized animal cells.

Introduction

Presently, enzyme and cell immobilization in polymer hydrogels is widely used in biotechnology and biomedical fields. Although natural polymers have many advantages including mild immobilization conditions, synthetic polymers have the benefit that adequate properties can be more precisely and reproducibly designed. The porosity of a gel as well as the ionic and hydrophobic/hydrophilic properties can be adjusted. Although the list of synthetic polymers used for enzyme and microbial cell immobilization is rather large (polyacrylamide, poly[*N*-isopropylacrylamide], various polymethacrylates, polyuretane, poly[vinyl alcohol], and so on), there are only few synthetic materials proposed for animal cell immobilization. The reason is that many synthetic polymers-based techniques include severe thermal- or chemical-treatment of living cells, and, therefore, cannot be applied for sensitive animal cells.

On the other hand, there are some unstable enzymes (carboxypeptidase B, thrombin, etc.) that cannot be successfully immobilized by covalent attachment. It is well known that the reducing of the mobility of enzyme polypeptide chains by their covalent binding to a polymer support often results in a significant decrease in the catalytic activity, especially in the case of unstable enzymes. The gel entrapment method provides a more or less loose attachment of enzyme to a polymer matrix without rigidly fixing of a protein molecule. Therefore, it seems to be the most promising mild technique for stabilization of unstable enzymes like thrombin or carboxypeptidase B.

The present paper describes the simple and mild technique to prepare composite macroporous poly(*N*-vinyl caprolactam)-based beads and films, some properties of immobilized systems, in particular animal cells and proteolytic enzymes, and their various applications in the biotechnology and biomedical fields.

Materials and Methods

Chemicals

PVCL (MW 900,000) was obtained by polymerization of *N*-vinyl caprolactam, as described by Kirsh et al. *(1)*. Sodium alginate was from

Bellco. Aromatic polyamide POLAR (MW 25,000) was obtained by copolymerization of isophthalic acid and 4,4'-diaminodiphenyl-2,2'-disulfonic acid *(2)*. Carboxypeptidase B (EC 3.4.17.2), 100 U/mg, and bovine trypsin (EC 3.4.23.1), 22 U/mg, were from Biolar, Latvia. α-Chymotrypsin (EC 3.4.21.1) from bovine pancreas, lyophilized powder, was obtained from Fluka, Switzerland. *N*-Acetyl-L-tyrosine ethyl ester (ATEE), *N*-benzoyl-L-tyrosine-*p*-nitroanilide (BTNA), and calcium chloride were from Sigma. *N*-Benzoyl-L-arginine ethyl ester (BAEE) was from Reanal, Hungary, and hippuryl-L-arginine (Hipp-Arg), *N*-*p*-tosyl-Gly-Pro-Arg *p*-nitroanilide (Chromozym TH) were from Boeringer Mannheim, Germany. Bovine thrombin (EC 3.4.21.5), 2500 NIH U/mg, was obtained by purification of the commercial preparation (Kaunas, Lithuania) by the method described in ref. *3*. Acetonitrile (MeCN) and *N*,*N*-dimethylformamide (DMF) were from Sigma. Recombinant proinsulin was obtained at the Shemyakin-Ovchinnokov Institute of Bioorganic Chemistry. Preparation of SBPH was accomplished according to the method in ref. *4*.

Entrapment of Enzymes in PVCL-CaAlg Beads

Enzymes (trypsin, α-chymotrypsin, carboxypeptidase B) were entrapped as described previously *(5)*. Briefly, the enzyme solution (1.0 mL) was mixed with 10% (w/v) PVCL solution (2.0 mL) and 1% POLAR solution (0.5 mL), then 5.0 mL of 2% (w/v) sodium alginate solution was added. The mixture was dropped into 100 mL of 2% (w/v) $CaCl_2$ solution at 37–40°C, and 0.4–0.6 mm size beads were obtained using a special sprayer *(6)*.

Entrapment of Thrombin in Composite PVCL-Ca Alg Films

To immobilize thrombin in hydrogel film, 8% (w/v) PVCL solution (1.2 mL) was mixed with 20–230 μL of thrombin solution (5–50 NIH U or 334–3236 mU, respectively) and 1% (w/v) POLAR solution (0.1 mL). After the addition of 3 mL of 2% (w/v) sodium alginate, the mixture was transferred to the Petri dish. Then 2% (w/v) $CaCl_2$ solution, previously heated to 40°C, was added to provide gelation. The prepared films were allowed to stay at 37°C for 30 min, then washed three times with 0.9% NaCl. The films were stored either in physiological solution containing 0.025% (w/v) of sodium azide and 1% of $CaCl_2$, or in a dry state at 37°C or room temperature. To prepare the films for model rat experiments, all solutions were previously sterilized. The initial total thrombin activity in the polymer mixture was 33 NIH U/film.

Study of the Effect of Immobilized Thrombin in a Rat Model

The effect of immobilized thrombin on tissue repair was tested in rats. All experiments were carried out under standardized conditions with regard to anesthesia and surgery. The films with immobilized thrombin were put on back wounds (2×3 cm^2) of experimental rats just after wounding. Control rats either had no films on the wound at all (open wound) or

had blank polymer dressings (without thrombin). The granulation tissue samples were studied by light microscopic radioautography on d 3 and 7 after wounding as described in ref. *7*. Briefly, tissue patterns (1 mm^3) were incubated in medium 199 containing [^{3}H]thymidine (20 μCi/mL) at 37°C for 90 min. Then the samples were washed with 0.1 *M* cold Na-phosphate buffer (pH 7.4), fixed with 2.5% (w/v) glutaraldehyde solution and 1% (w/v) OsO_4, after that embedded in araldite resin. Semi-thin sections were examined using a light microscope (Leitz, Germany). The total and proliferating fibroblast number (TFN and PFN), as well as the total number of microvessels (TVN) and a number of vessels with proliferating cells ([^{3}H]thymidine-labeled vessels, VPC), were counted in 10 fields of the slide. The field size was defined through the microscope optical system (1000×).

Cell Immobilization and Cultivation in PVCL-CaAlg Beads

Mouse–mouse hybridoma cell line producing IgG1 MAb to interleukin-2 was LNKB-2 *(8)*. The cells were entrapped in CaAlg beads by mixing 4 mL of cells (1–2 × 10^6 cells/mL), 2 mL of a sodium alginate solution (4% [w/v]), and adding the mixture to a 2% (w/v) $CaCl_2$ solution heated to 37–40°C. Immobilization in PVCL-CaAlg beads was performed as described above but by mixing 2 mL of a sodium alginate solution (4% w/v), 1 mL of a PVCL solution (7%, w/v), and 3 mL of cell suspension. To prevent the release of cells from beads in the medium during long cultivation, after incubation at room temperature for 5 min, the beads (0.4–0.6 mm in diameter) were washed twice with saline and treated with 0.02% (w/v) poly-L-lysine in saline heated to 37–40°C for 5 min. The cells were cultivated in DMEM supplemented with 10% fetal calf serum (DMEM/FCS), and DMEM/Ham's F-12 mixture (1/1 [v/v]), Gibco, containing 1% (v/v) SPITE, Sigma, (DMEM/SPITE) as described earlier *(9)*. To prepare pictures of immobilized cells the beads were fixed in 2.5% glutaraldeghyde in 100 m*M* Na-cacodylate solution (pH 7.2) overnight at 4°C. After fixation with osmic acid for 6 h at 4°C, the samples were dehydrated in a graded ethanol series, substituted with propylene oxide, and embedded in Epon 812 (TAAB, Berkshire, UK). Thin sections were stained with uranyl acetate and lead citrate and then observed with an Opton light microscope.

Measurement of Enzyme Activities

Spectrophotometric Method

The activities of trypsin, carboxypeptidase B, and amidase thrombin activity were determined using BAEE, Hipp-Arg, and Chromozyme TH as substrates, respectively, according to ref. *10*. The clotting activity of thrombin was assayed using fibrinogen as described earlier *(11)*.

To determine the amidase activity of α-chymotrypsin, BTNA was used as a substrate. The final concentrations of BTNA and the enzyme were 1 × 10^{-4} *M* and 1.2 × 10^{-6} in Tris-HCl buffer (pH 7.5).

Titrimetric Method

Activity of native and entrapped α-chymotrypsin (bead aliquots, 100 μL) was calculated from the initial rate of ATEE (initial concentration 5×10^{-3} *M*) hydrolysis using a TTT60 Radiometer pH-stat (Radiometer, Denmark) at pH 7.5 and 20°C in the presence of 0.1 *M* $CaCl_2$. ATEE and soluble α-chymotrypsin concentrations in the mixture were 1×10^{-3} *M* and 2×10^{-7} *M*, respectively.

Stability of the Immobilized α-Chymotrypsin in Acetonitrile/Water Medium

To estimate the stability of the immobilized enzyme in MeCN, wet beads (1 g) were equilibrated with MeCN (1 mL) and filtered, and then a fresh portion of the solvent (3 mL) was added. The bead suspension was incubated for 90, 180, and 270 h with stirring (25°C). Water content in the mixture was 12.3%. The bead aliquots (100 μL) were taken periodically to determine the enzyme activity as described above. Water content in MeCN was monitored by Karl Fisher's procedure. To get the water contents of 9.0 and 0.5%, the above described procedure was used with volumes of MeCN solutions equal to 3 and 9 mL instead of 1 and 3 mL, respectively.

Enantiomeric Analysis

Gas chromatographic analysis was performed with a chiral glass capillary column (l = 41 m, id = 0.21 mm) of a diamide polysiloxane phase type Chirasyl-Val (synthesized in the authors' institution) and a FID detector. The carrier gas was helium, with a temperature of 147°C, the flow rate was 1 mL/min, and the inlet pressure was 1.6 bar. L-Phe and D-Phe were analyzed as *N*-trifluoroacetyl derivatives of their isopropyl esters.

Enzymatic Hydrolysis Using Immobilyzed α-Chymotrypsin

SBPH dissolved in acetonitrile/water mixtures (20% H_2O) was added to a suspension of the immobilized enzyme in the same solvent, and the reaction mixture was stirred at room temperature for a stipulated period of time. Dry acetonitrile (5 mL) was added at the end of the process. The sediment containing the precipitated L-Phe and the beads with entrapped α-chymotrypsin was filtered and washed twice with MeCN, then twice with 1% aqueous ammonia and filtered. The filtrate was evaporated *in vacuo*, and a residue was purified by the ion-exchange technique on DOWEX–50W(H^+) resin. L-Phe was eluted from the resin with 5% ammonia, the solution was evaporated, and the residue was analyzed. For D-Phe solution, MeCN combined extract was evaporated *in vacuo,* the residue was hydrolyzed with 6 *N* HCl at 20°C for 10 min. The solution obtained was extracted with toluene to remove *p*-chlorobenzaldehyde. The water layer was refluxed for 5 h and evaporated. The residue containing D-Phe was purified on a DOWEX–50W (H^+) column and analyzed. The chemical purity of the Phe enantiomers prepared was examined with ^1H NMR spectroscopy and TLC.

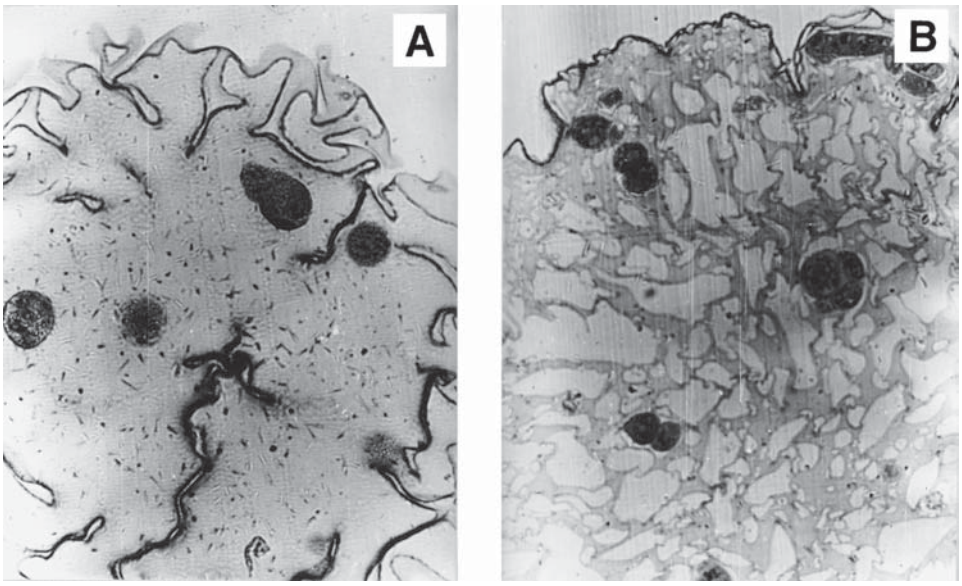

Fig. 1. Photomicrographs of LNKB-2 cells entrapped in Ca-alginate **(A)** and in macroporous composite PVCL-Ca-alginate **(B)** beads (thin sections). Cultivation medium: DMEM supplemented with 10% FCS. Day 3 after immobilization. (Magnification 200×.)

Results and Discussion

Poly(*N*-vinyl caprolactam) is a water-soluble synthetic, biocompatible, temperature-sensitive polymer with a low critical solution temperature. PVCL can form hydrogels by raising the temperature from 20 to 37°C. To obtain hydrogel beads/films with entrapped biocatalyst one can mix cells/enzyme with polymer solution at room temperature, and then introduce the mixture into another solution heated up to 37–40°C. Thus, the entrapment procedure is very simple and mild for both cells and enzymes. Developed earlier by us, the method for preparation of composite PVCL-CaAlg beads *(5)* has at least three intrinsic merits. First, the entrapment procedure is carried out at physiological pH and temperature (37–40°C) allowing a minimal decrease in the enzyme activity (or cell viability) during immobilization. Second, PVCL is able to form enzyme–polymer complexes providing retention of the entrapped enzyme inside the polymer network without covalent binding. Finally, addition of PVCL to the polymer Ca-alginate network allows the macroporous structure of the carrier to develop, which decreases diffusional limitations, and is especially of great value in the case of immobilized cells (Fig. 1).

Stabilization of Proteases by Entrapment in PVCL-CaAlg Hydrogel Beads

The direct interaction of enzyme molecules with an organic solvent changes the catalytically active conformation *(12)*. Gel entrapment is con-

sidered to be one of the most promising methods to maintain enzyme activity in low-water systems *(13)*. The proteases (trypsin, carboxypeptidase B) entrapped in PVCL-based gel can be protected against various denaturing organic solvents, such as acetonitrile or dimethylformamide, as was reported earlier *(10)*. The retained activity of gel-entrapped trypsin in DMF or acetonitrile/water systems (90:10 [v/v]) was approx 30 and 70%, respectively. Native trypsin completely lost its activity at DMF concentrations over 50% and retained only 20% of its initial activity in 90% acetonitrile. The thermal stabilization achieved was also quite impressive: the activity of entrapped trypsin retained up to 65°C and comprised approx 20% at 85°C, while native trypsin was completely inactivated at 50°C. Entrapped carboxypeptidase B retained 60% activity even at 80°C.

Enzymes entrapped in PVCL-based gels can be successfully applied in water and low-water media. Thus, in water medium, high operational stabilities of immobilized trypsin and carboxypeptidase B were demonstrated by their repeated usage in production of human insulin from recombinant proinsulin *(5)*. The immobilized enzymes retained their activity after 20 cycles of proinsulin cleavage.

Application of Gel-Entrapped α-Chymotrypsin for Enantioselective Hydrolysis of a Shiff's Base of D,L-Phenylalanine Ethyl Ester

Chemico-enzymatic methods for preparation of enantiomerically pure substances or their precursors in low-water systems are widely used in the pharmaceutical and food industries. The enantioselective hydrolysis of a Schiff's base of D,L-Phe ethyl ester (SBPH) was chosen to demonstrate the possibility of repeated application of gel-entrapped α-chymotrypsin in acetonitrile/water medium. To estimate the stability of immobilized biocatalyst in a cyclic process, several model experiments were carried out. The beads with entrapped α-chymotrypsin were transferred to a flask with an acetonitrile/water mixture (water content 12.3%), and then stirred at room temperature for an appropriate time. Even after 270 h incubation in acetonitrile/water mixture (Fig. 2), the relative activities of the immobilized enzyme were approx 30 and 47% in the case of ATEE and BTNA substrates, respectively. Immobilized α-chymotrypsin retained its activity for 4 mo storage at the room temperature.

The results on enantioselective hydrolysis of SBPH by gel-entrapped α-chymotrypsin are demonstrated in Table 1. Maximum enantiomeric purity of L-Phe achieved was 87.8%. The most probable reason for the e.e.'s relatively low value could be the side reaction of spontaneous non-enantioselective hydrolysis of the ester substrate, which might be catalyzed by the presence of Ca^{2+}-ions in the beads of composite PVCL-CaAlg gel. On the one hand, the enantiomeric purity of L-Phe is well known to depend on the water concentration in the reaction media, and the lower the water content is in the system, the higher is the enantiomeric purity of L-Phe. On the other hand, the activity of immobilized α-chymotrypsin decreased with a decrease water content in acetonitrile/water mixture

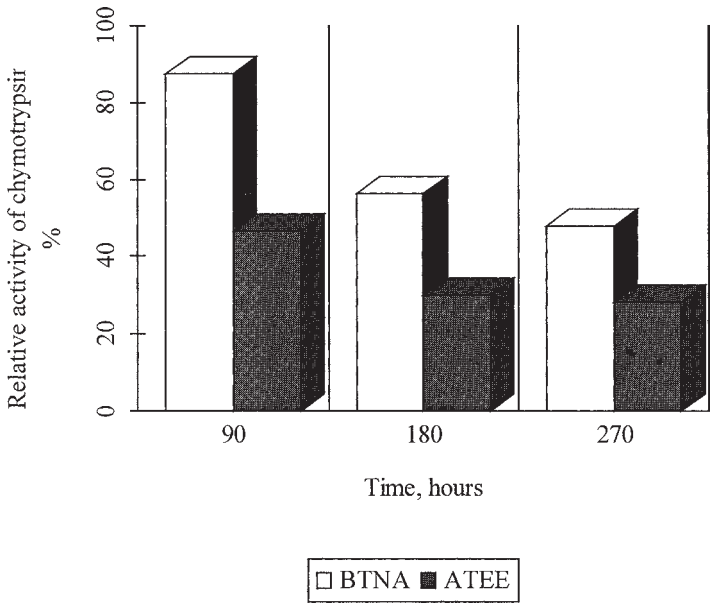

Fig. 2. Stability of immobilized into PVCL-based gels α-chymotrypsin in acetonitrile/water mixture. The activity of α-chymotrypsin in water was taken as 100%. The water content in MeCN/water mixture was 12.3%.

Table 1
Enantioselective Hydrolysis of Shiff's Base of D,L-Phe Ethyl Ester Catalyzed by Immobilized α-Chymotrypsin in PVCL-Based Beads[a–c]

Cycle no.	Amount of substrate, mg	Cycle time, h	Chemical yield of L-Phe, %	Enantiomeric purity of L-Phe, %	Enantiomeric purity of D-Phe, %
I	100	144	40	80.9	40.6
II	75	168	90	87.8	87.3
III	33	192	50	81.5	50.3

[a]0.35 mg of α-chymotrypsin/1 g of PVCL-beads.
[b]The water content in the MeCN solution was 20%.
[c]The same beads recovered from the reaction mixture were repeatedly used in the next cycles.

(Fig. 3), but it was still rather high (37–40% of its initial value) even after 20 h incubation of beads in a low-water medium (0.5% water). Therefore, all the above-mentioned parameters should be taken into consideration in the process optimization.

Application of Hydrogel Film-Entrapped Thrombin for Wound Healing

Thrombin, a multifunctional serine proteinase, is one of growth factors promoting tissue repair. Because thrombin is one of the most expensive and rapidly inactivated enzymes *(14)*, its stabilization is of particular importance. Stabilized thrombin is widely used for controlling heparin

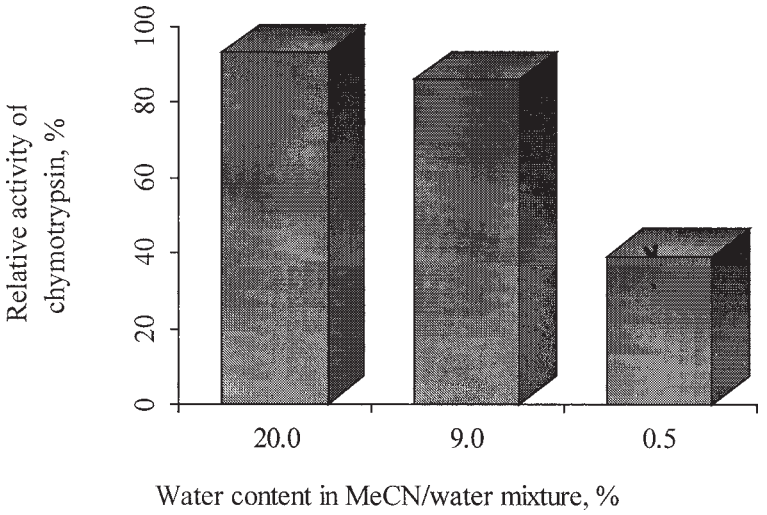

Fig. 3. The effect of the water content in the acetonitrile/water mixture on the esterase activity of α-chymotrypsin entrapped into PVCL-based gels. The chymotrypsin activity in water was taken as 100%. The water content in the MeCN/water mixture was determinated by Fisher's procedure, after incubation of beads with immobilized protease in acetonitrile.

therapy and molecular disorders of fibrinogen in clinics. Stabilization of lyophilized enzyme preparation can be achieved by introduction of certain hydrating additives, for example, bovine serum albumin (BSA). However, protein additives suffer from exogenic microorganisms lowering the quality of the thrombin preparations. Recently, we have demonstrated the stabilization effect of PVCL on amidase thrombin activity *(10)*. The activity of PVCL-containing thrombin preparations increased after lyophilization compared to that of native thrombin or thrombin in the presence of BSA additives. PVCL addition also resulted in enzyme stabilization when previously lyophilized thrombin samples were kept in PVCL solution for 8 d at 4 or 20°C. Native thrombin was completely inactivated under the same conditions. As shown previously for some other growth factors, thrombin introduction should be modulated in order to prevent adverse effects. Some controlled release forms, such as liposomes, hydrogel beads/capsules, or polymer films could be proposed to solve the problem. On the other hand, polymer dressings are considered as a suitable support for cell migration, adhesion, and proliferation in wounds *(15,16)*. Thrombin entrapped in PVCL-CaAlg hydrogel film could be proposed to accelerate wound healing. Wound healing is divided into three phases: inflammation, proliferation, and maturation *(17)*. The proliferation phase is characterized by the formation of granulation tissue in the wound. Fibroblasts are the primary synthetic element in the repair process and produce collagen and other structural components used during tissue reconstruction. Fibroblasts first appear in significant numbers in the wound on d 3 postinjury and achieve

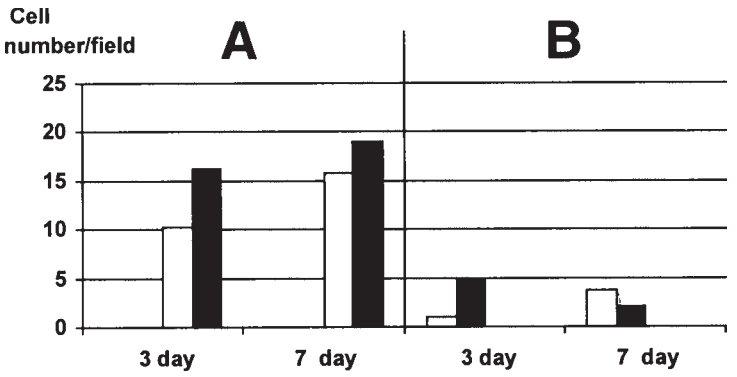

Fig. 4. Effect of immobilized thrombin (■) on total **(A)** and proliferating **(B)** fibroblast number in the incisional wound. The film without thrombin (□).

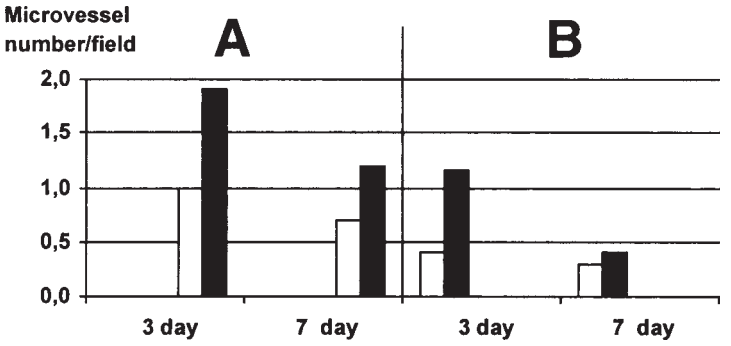

Fig. 5. Effect of immobilized thrombin (■) on total **(A)** and [^{3}H]thymidine labeled **(B)** microvessel number in the incisional wound. The film without thrombin (□).

peak numbers around d 7. Revascularization of the wound proceeds in parallel with the process of fibroblast proliferation and synthetic activity.

All mean parameters for experimental animals markedly exceeded those for control rats on d 3 of healing. Thus, in the case of the open wound as a control, the total fibroblast number (TFN) and the proliferating fibroblast number (PFN) increased 1.7- and 2.6-fold, respectively, compared to the control (open wound). The total microvessel number (TVN) increased 6.2-fold, the number of microvessels with proliferating cells (VPC, [^{3}H]thymidine-labeled vessels) had a 5.5-fold enhancement compared to the control. The increased number of microvessels observed suggested that entrapped thrombin might stimulate healing through an enhanced neovascularization. On d 7, the wound size was reduced approximately twofold for experimental rats, and did not change for the control animals.

Figures 4 and 5 demonstrate the results of experiments when a blank polymer film (without thrombin) was used as a control. As could be seen,

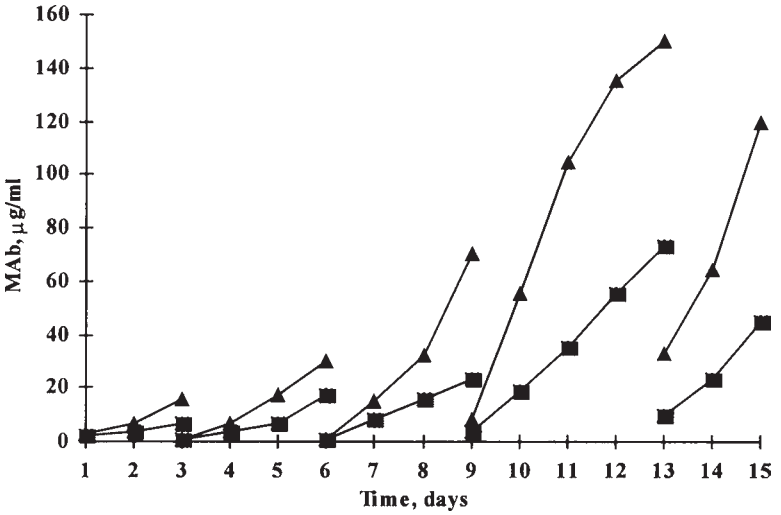

Fig. 6. Monoclonal antibody production by LNKB-2 cells entrapped in PVCL-CaAlg (▲) and CaAlg (■) beads. Cells were cultivated in DMEM/SPITE.

the parameters of healing (TFN, PFN, TVN, VPC) are slightly less than those in the case of control open wound described above. These results suggest the positive effect of the polymer dressings themselves on tissue repair that is in a good agreement with the results of other researches *(15,16)*. It should be noted that the number of proliferating fibroblasts to d 7 decreased for experimental rats, whereas this value still increased for control animals (Fig. 4B). Thus, immobilized thrombin was shown to accelerate healing by shifting the time course forward by up to 3–4 d. This occurs due to stimulation of fibroblast proliferation and enhanced neovascularization causing the increased relative rate of healing. On the other hand, bioencapsulated thrombin may accelerate healing not only due to its activity as a growth factor for the first 3 d, but also as a regulator of angiogenesis.

Preparation of Pure Monoclonal Antibodies to Interleukin-2 by Cultivation of Hybridoma Cells Entrapped in PVCL-CaAlg Hydrogel Beads

Cultivation of entrapped hybridoma cells provides some intrinsic advantages over a common free suspension culture, in particular, cell protection against mechanical stress, higher MAb production due to the higher local and overall cell concentrations (on the order of 10^7 cells/mL), easier cell separation, and MAb purification *(18)*. Moreover, there is a possibility to enhance productivity of specific MAb *(19)*.

MAb productivity strongly depends on cell proliferation inside the polymer beads. Recently, increased attention has been paid to the development of macroporous hydrogels *(20,21)*. The macroporous network of the gel provides good cell distribution within the bead. It minimizes local diffusional limitations expected within dense cell masses in hollow capsules.

According to the microscopic data (Fig. 1), in the case of PVCL-CaAlg beads we have a network in which PVCL precipitates forming polymer coils at 40°C. This increases the number of areas inside the bead that are not crosslinked by calcium ions. Thus, the enhanced number and inner volume of areas may provide better conditions for cell proliferation. In the course of cultivation, cell colonies in composite PVCL-CaAlg beads unified and formed in 2–4 wk spread clusters that filled in approx 90% of the bead volume.

The developed method was used to prepare pure MAbs to interleukin-2 by cultivation of entrapped hybridoma cells in a serum-free medium *(9)*. It is well known that cultivation in a serum-free medium provides high purity MAbs and markedly reduces their cost. However in a serum-free medium, cells suffer from damage during stirring. Immobilization of cells in PVCL-CaAlg beads allow us: (1) to protect cells from stress and (2) to increase cell proliferation and MAb production as compared to "classical" calcium alginate beads (Fig. 6) to obtain pure MAb. Transferrin from SPITE (5% [w/v]) was the only contaminating protein in culture supernatant.

In conclusion, the entrapment of various enzymes and cells in the composite hydrogel PVCL-CaAlg beads and films allows us to stabilize biocatalysts, and provides a higher level of cell proliferation and production of MAb in a serum-free medium due to a macroporous support structure. The method is especially advantageous for application of immobilized unstable enzymes both in biotechnology and biomedical fields.

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