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Biological properties of copolymer of 2-Hydroxyethyl methacrylate with sulfopropyl methacrylate

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Interaction of organism with non-toxic implanted polymers depends on the physicochemical properties of the implant surface, which influence the adsorption of bioactive proteins and subsequently adhesion and growth of cells. The synthetic hydrogels are known as poorly adhesive surfaces. In this study we demonstrated the adsorption of albumin, fibrinogen, fibronectin, basic fibroblast growth factor, heparin-binding epidermal growth factor-like growth factor and epidermal growth factor to poly(2-hydroxyethyl methacrylate) (pHEMA) and copolymer of 2-hydroxyethyl methacrylate (HEMA) and potassium salt of 3-sulfopropyl methacrylate (SPMAK). The adhesion and growth of 3T3 cells and human keratinocytes on surface of these polymers was tested without and with pretreatment of polymers with heparin-binding epidermal growth factor-like growth factor. The adhesion of mixture of human granulocytes and monocytes to these surfaces was also tested. The strips of both polymers were subcutaneously and intracerebrally implanted into the rat and the extent of foreign body reaction and brain biocompatibility was evaluated. The results showed the extensive adsorption of basic fibroblast growth factor and heparin-binding epidermal growth factor-like growth factor to copolymer containing SPMAK. However the adhesion (and growth) of cells to this type of copolymers was very low. Preadsorption of human plasma to pHEMA clearly stimulated the leukocyte adhesion in contrary to copolymer containing SPMAK. The extent of foreign-body reaction was significantly higher against the pHEMA compared to tested copolymer p(HEMA-*co*-SPMAK). In conclusion, the tested copolymer was a poorly adhesive substrate that is only poorly recognized by the non-specific immunity, although the adsorption of basic growth factors to this substrate is highly significant. Both polymers were well tolerated by the brain tissue. The phenotype of surrounding neurons was more close to the control neurons in the brain tissue surrounding the p(HEMA-*co*-SPMAK) implants.

Introduction

Interaction of implanted devices with host tissues and immune system represents a very important aspect of its functionality and biological safety [1, 2]. The biological properties of polymer implants are highly complex and depend on numerous parameters such as physicochemical properties of the surface influencing the adsorption of bioactive proteins (fibrinogen, complement, immunoglobulins and fibronectin). These bioactive molecules are recognized by specific pro-adhesive cellular receptors such as integrins [3–8]. The molecular structure of synthetic polymer precisely controls the bioactive protein adsorption including their 3D-structure and biological activity [9]. The synthetic hydrogels such as pHEMA are generally considered as non-adhesive surfaces because of their very low interfacial-free energy relative to water or physiological environment and low adsorption of bioactive proteins. However, pHEMA implant is extensively colonized by macrophages (MPh) and induces chronic inflammatory response, the foreign-body reaction, after

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subcutaneous implantation into the rat [10]. The presence of carboxylate anions, even at very low concentration (3 wt % of sodium methacrylate) in copolymer with HEMA, significantly reduces the adhesion of inflammatory cells on the copolymer surface *in vitro* [11, 12] and minimizes the extent of foreign-body reaction *in vivo* [10, 13–15].

Sulfate is a chemical group frequently present in biologically important macromolecules such as glycosaminoglycans and proteoglycans. Important members of these families were described in the extracellular matrix of connective tissue or in the basal membranes of epithels [16]. The copolymer of SPMAK (as a carrier of $-SO_3^-$) with HEMA was biologically characterized. The pHEMA was used as a reference polymer. The study includes adsorption of the human albumin (Alb), fibrinogen (Fbg), recombinant heparin-binding epidermal growth factor-like growth factor (HB-EGF) and recombinant basic fibroblast growth factor (BFGF), fibronectin (Fibro) and epidermal growth factor (EGF). The proliferation of non-immune cells was studied using mouse 3T3 fibroblast cell line *in vitro*. The interaction of p(HEMA-*co*-SPMAK) with the immune cells was evaluated in study of the short-term adhesion of suspension of human neutrophil granulocytes with monocytes also *in vitro*. Moreover, the extent of foreign-body reaction was studied by the implantation experiments in the laboratory rat. The brain biocompatibility was evaluated after the polymer stereotactic implantation into the striatum of the rat brain.

Materials and methods

A. Preparation of polymers

Homopolymers of HEMA and copolymers p(HEMA-*co*-SPMAK) were prepared in both soluble (non-crosslinked) and insoluble (crosslinked) forms.

Non-crosslinked samples were prepared in nitrogen inert medium by 4 h solution polymerization in ethanol at 70 °C, initiated with AIBN. Reaction products were precipitated in petroleum ether, dissolved in ethanol, reprecipitated in water or PBS solution and finally dried. Copolymers p(HEMA-*co*-SPMAK) were prepared in 3 M ratios of monomers with 0.5, 1 and 3 mol % of SPMAK in polymerization feed. For the *in vitro* experiments, 5 wt % solutions of samples in methyl cellosolve were prepared, spincasted on the glass coverslips or poured into 26 multiwell plates in amount of 200 µl/well, dried by solvent evaporation at the clean box and finally vacuum dried.

Crosslinked pHEMA and p(HEMA-*co*-SPMAK) with 3 mol % of SPMAK in polymerization mixture were prepared by 16 h radical polymerization at 70 °C provided between two parallel plates (distance 0.5 mm), by using 0.1% AIBN as an initiator and 0.3% (in both cases relative to the weight of monomers) of ethylene glycol dimethacrylate as a crosslinking agent. By HEMA polymerization, glycerol was added in amount of 10% relative to the monomer weight, by copolymerization of HEMA and SPMAK, methyl cellosolve had to be added in ten-fold weight amount of SPMAK for its dissolution. The function of both additives was to increase the free volume in the block (co)polymer and hence the distance between the chains in crosslinked matrix; they have been washed out from products during the rinsing procedure.

B. Characterization of polymers

In order to obtain a first view about the diffusivity of polymers used, all samples were tested by swelling in water and PBS solution. Real contents of monomeric units in p(HEMA-*co*-SPMAK) copolymers were determined by nuclear magnetic resonance measurement in the solution of deuterated methanol or in the solid state, respectively. The wettability of studied samples was evaluated by means of contact angle apparatus (Krüss, Hamburg, Germany) using the Wilhemy plate method. For this purpose, the soluble non-crosslinked samples were coated on the glass coverslips from 5 wt% ethanolic solution and dried, whereas the insoluble crosslinked samples were used in the form of rectangular plates. As the adhesivity of polymers on the glass in hydrophilic milieu had been found very poor, the contact angle measurement was possible only from the dry state of the samples, being not water-equilibrated in advance. In the case of the crosslinked samples, the contact angles were measured also after 2 h equilibration in water. The advancing (θ_A) and receding (θ_R) contact angles were measured at 25 °C in distilled water, the surface tension of which was determined by platin plate method.

The results of all measurements on studied samples are shown in Table I.

TABLE I Characteristics of polymers

(Co)polymer	Form	Monomer ratio in the feed [mol/mol]	Swelling in		Comonomer content (NMR) [mole]	Contact angle	
			Water [wt %]	PBS [wt %]		θ_A [°]	θ_R [°]
HEMA	Soluble	100	nd[a]	38.2	0	65.4	28.8
HEMA-SPMAK	Soluble	99.5:0.5	nd[a]	44.5		62.9	29.8
HEMA-SPMAK	Soluble	99:1	nd[a]	50.0		61.0	29.3
HEMA-SPMAK	Soluble	97:3	nd[a]	nd[a]		57.0	33.5
HEMA	Insoluble	100	40.9	38.6	0	66.6	34.1
						64.2[b]	31.3[b]
HEMA-SPMAK	Insoluble	97:3	81.9	64.6		57.5	37.9
						56.0[b]	36.5[b]

The swelling is expressed as a weight percentage of water or PBS solution in swollen samples, the wetting as advancing (θ_A) and receding (θ_A) angles, nd = not detected.

[a]Poor adhesion of polymer layer.

[b]Measured after 2 h of equilibration in water.

C. Protein adsorption

The proteins, human albumin (fraction V), human fibrinogen, human recombinant basic fibroblast growth factor and human recombinant heparin-binding epidermal growth factor-like growth factor, were purchased from Sigma (Prague, Czech Republic). These proteins were biotinylated employing the Biotintag™ Microbiotinylation kit (Sigma, Prague, Czech Republic) as recommended by supplier and frozen until the application. Particles of both crosslinked studied polymers in swelling state, i.e. pHEMA and p(HEMA-*co*-SPMAK) (97/3 mol % monomer ratio in feed) were incubated with biotinylated proteins in concentration of 125 μg/ml of PBS for 1 h at room temperature. After the extensive washing with PBS the ExtrAvidin–TRITC (Sigma, Prague, Czech Republic) was employed for detection of the adsorbed proteins. The reaction with omitted biotinylated proteins was used as a control experiment. The result of reaction was observed using Nikon-Optiphot-2 (Nikon-Cz Optoteam, Prague, Czech Republic) employing the filterblock for TRITC and the H-3 Nikon Photomicrographic Equipment was used for the detection of the emitted light signal by the measurement of the exposition time. The protein adsorption was semiquantitatively estimated according to equation:

$$PA = 10/(ET_m - ET_c) \quad (1)$$

PA, protein adsorption (units); ET_m, exposition time of measured specimen (s); ET_c, exposition time of control specimen (s).

The differences between the pHEMA and p(HEMA-SPMAK) were evaluated statistically employing Student non-paired *t*-test.

D. *In vitro* adhesion of mice 3T3 cells and human keratinocytes

The non-coated 24 multiwell plates (Corning as well as plates coated with pHEMA or p(HEMA-*co*-SPMAK) with 0.5, 1 and 3 mol % of SPMAK were used. Cells were seeded in density 25 000 or 50 000cells/cm^2 in triplets for each tested polymer and cultivated under standard condition. The result of experiments was evaluated at 24, 48 and 96 h. The cells were harvested by trypsinization (Sigma, Prague, Czech Republic, 0.25% solution in PBS) and the cells were counted using by Bürker chamber. To differentiate the living and dead cells Trypan blue assay (Sigma, Prague, Czech Republic) was used. Furthermore, the polystyrene, pHEMA and p(HEMA-*co*-SPMAK) (3 mol %) were preadsorbed with 10 ng/ml of HB-EGF and the growth of 3T3 cells and human keratinocytes at the same density was evaluated in an interval of 24 and 72 h under the same conditions.

E. *In vitro* adhesion of human leukocytes

The leukocytes were harvested from 10 ml of heparinized venous blood of healthy volunteers using Percoll-gradient centrifugation. The cell suspension of 90 % neutrophil granulocytes with 10 % of monocytes was purchased. Pooled serum was added to the cell suspension to obtain a final serum concentration 10 % (v/v) and the cell number were adjusted to 10^7/ml. The adhesion of these cells onto the surface of swollen polymers (and untreated coverslip) was studied without and with the pretreatment with 10 % (v/v) pooled plasma for 90 min. The procedure is described in detail in the previous papers [11, 12]. The cells were stained by Giemsa-Romanowski and calculated. The differences between the polymers under different experimental conditions were evaluated using Student non-paired *t*-test.

F. Foreign body reaction in the rat

The strips 4 × 8 mm of crosslinked pHEMA and p(HEMA-*co*-SPMAK) (3 mol %) were subcutaneously implanted into the Wistar laboratory rats weighing 300 g for 9 days as desribed [17]. Four animals were used for each polymer type. The animals were sacrified by prolonged ether anesthesia, polymer strips were fixed with 4 % paraformaldehyde, stained with hematoxylin and evaluated as whole mount preparation. The percentage of nuclei in foreign-body giant multinucleate cells was calculated according to the equation:

$$PN_{MGC} = Nucl_{MGC}/(Nucl_{MGC} + Nucl_{MPh}) \times 100 \quad (2)$$

PN_{MGC}, percentage of nuclei in foreign-body giant multinucleate cells; $Nucl_{MGC}$, number of nuclei in foreign-body giant multinucleate cells; $Nucl_{MPh}$, number of nuclei in mononuclear macrophages.

G. Intracerebral implantation to the rat

A total of 12 adult Wistar rats of 300 g were used. In 5 rats unilateral stereotaxic implantation of synthetic polymers [2 × pHEMA and 3 × p(HEMA-*co*-SPMAK), 3 mol %] were made into the striatum, two rats were sham-operated (craniotomy without a lesion), and 5 rats were used for control NADPH-d staining only. All surgical procedures were performed under Phenobarbital anesthesia (0.2 mg/100 g bodyweight). The animals were placed in a stereotaxic apparatus. A small hole was drilled in the skull and a 1 mm bright cannula was brought into the striatum using Fifková and Maršala [18, 19] coordinates. Small pieces of SM (1.0 × 0.5 × 0.5 mm) inserted in the tip of cannula were push out into the nervous tissue of striatum. Afterwards, the cannula was removed, the skin wound closed and the rats were allowed to survive for 14 days. After this postoperative survival time the rats were sacrificed under deep ether anesthesia and perfused transcardially with 4 % paraformaldehyde in phosphate buffer (pH 7.4). The brains were quickly removed, cut into 1.0 cm slices and stored overnight in the same fixative. The following day the slices were placed into a 30 % sucrose solution in phosphate buffer for cryoprotection. After 3–4 days the slices were cut into 40 μm frontal sections on a freezing microtome. The sections were used for NADPH-d histochemistry and cresylviolet staining.

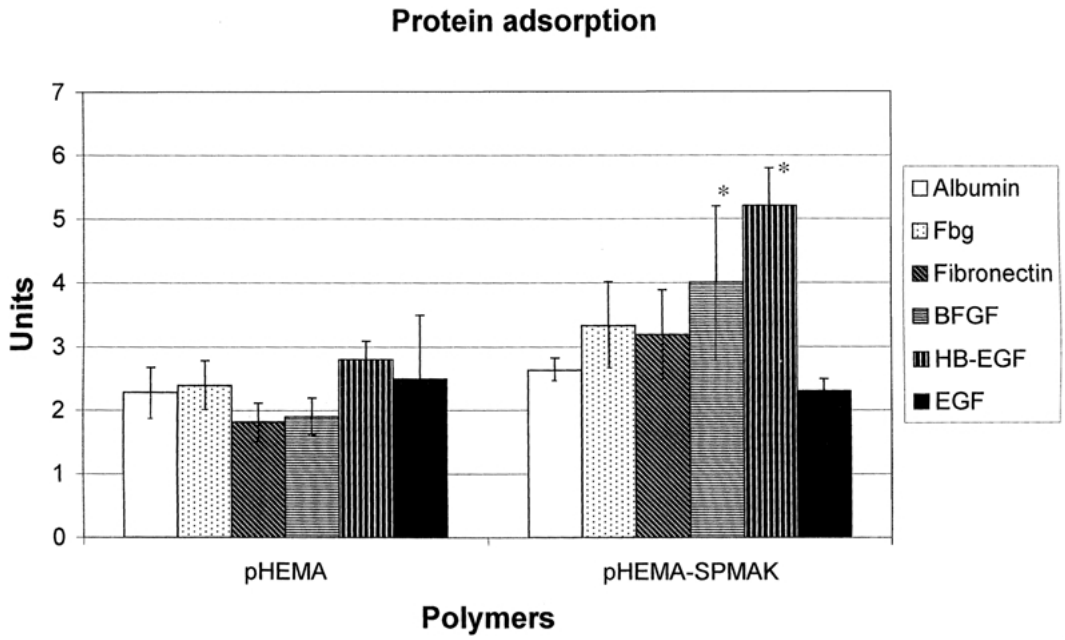

Figure 1 Adsorption of proteins to pHEMA and p(HEMA-*co*-SPMAK). Adsorption of basic fibroblast growth factor (BFGF) and heparin-binding epidermal growth factor-like growth factor (HB-EGF) is statistically significant higher to p(HEMA-*co*-SPMAK) (asterisk, $p \leq 0.05$). Fbg = fibrinogen, EGF = epidermal growth factor.

H. NADPH-diaphorase histochemistry and cresylviolet staining

For the demonstration of NADPH-d activity a modified Scherer-Singler [20, 21] procedure was used. For free-floating sections, half the concentrations of tetrazolium dye and beta-NADPH were enough. In our series we can distinguish two main types of NADPH-d positive neurons and three types of NADPH-d fibres in the neuropil: (i) Golgi-like neurons – blue black with distinct dendrite arborization of the processes, with slightly pale appearance in the nucleus, (ii) Nissl-like neurons – light blue-violet neurons with easily distinguishable nucleus, but without stained processes, and (iii) In neuropil: smooth, spindle or beaded fibers. Studying NADPH-d positivity we traced the changes of this reaction in the NOS positive neurons, characteristic for normal striatum. Every second section was stained with cresylviolet. The location and the extent of the implanted polymers were checked. The structure and presence of neurons and glial reaction in the neighborhood of the implants and in the neighborhood of the cannula tracks were studied in the cresylviolet-stained sections.

Results

The adsorption of BFGF and HB-EGF was significantly more extensive to the p(HEMA-*co*-SPMAK (3 mol %) than to the pHEMA. On the other hand, the adsorption of Alb and Fbg was the same to both of the studied polymers (Fig. 1).

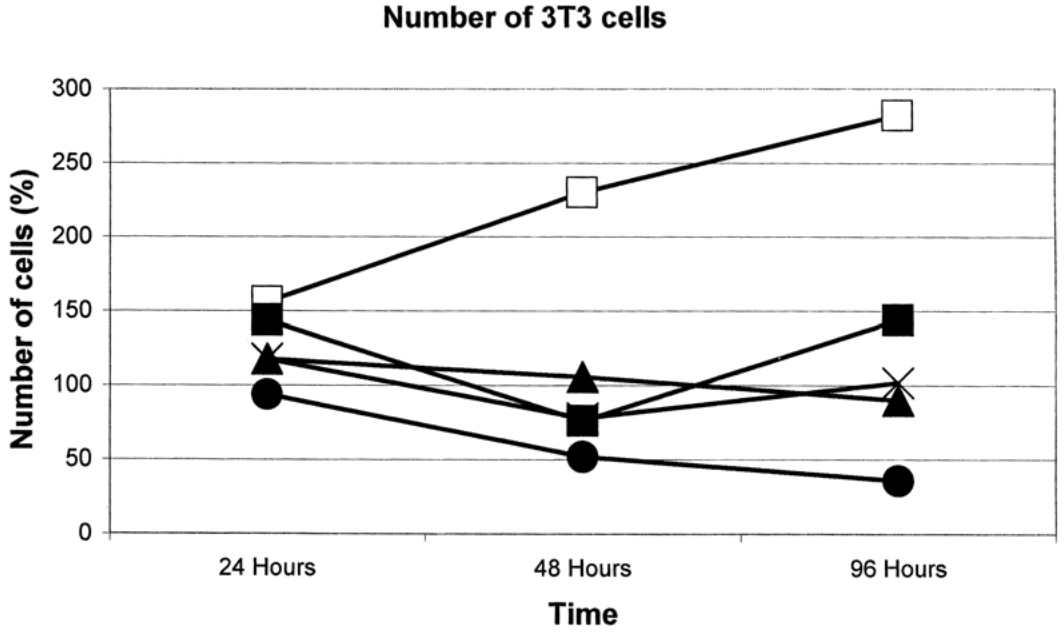

Figure 2 Growth kinetics of 3T3 cells on polystyrene (open quadrangle), pHEMA (black quadrangle), and copolymer p(HEMA-*co*-SPMAK) containing 0.5 mol % SPMAK (triangle), 1.0 mol % SPMAK (X) and 3.0 mol % SPMAK (circle) for 96 h.

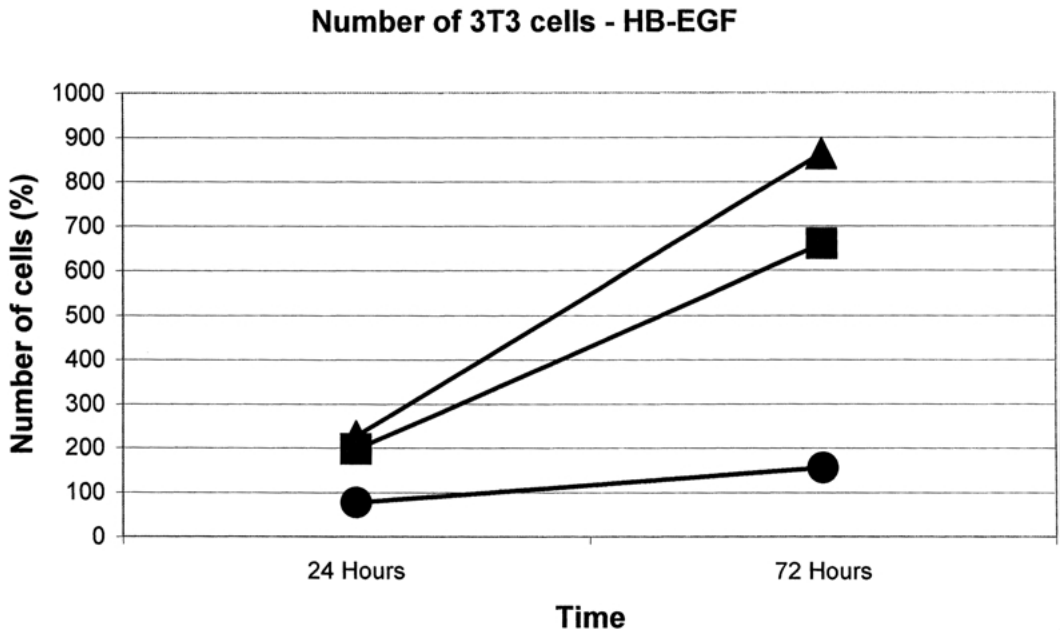

Figure 3 Growth of 3T3 cells on polystyrene (triangle), pHEMA (quadrangle) and copolymer p(HEMA-*co*-SPMAK) containing 3.0 mol % SPMAK (circle) for 72 h.

The growth of 3T3 cells was very similar on the surface of all the studied hydrogels pHEMA and p(HEMA-*co*-SPMAK) (0.5, 1.0 and 3 wt %) and much lower than on the polystyrene of tissue culture grade (Fig. 2). The preadsorption of HB-EGF on polystyrene and pHEMA accelerated the growth of 3T3 cells, but it had only minimal influence on the growth of these cells on p(HEMA-*co*-SPMAK) (3 mol %) (Fig. 3). The growth of human keratinocytes on the surface of all studied materials even preadsorbed with HB-EGF was very poor (Fig. 4). The adherence of leukocytes to pHEMA and p(HEMA-*co*-SPMAK) (3 mol %) was very similar and significantly lower than to the glass. Pretreatment of glass and pHEMA with plasma strongly stimulated adhesion of leukocytes to these surfaces. Plasma pretreatment of p(HEMA-*co*-SPMAK) (3 mol %) had no stimulatory effect on leukocytes adhesion (Fig. 5). The classical foreign-body reaction was observed around the pHEMA implants.

The implanted strips were colonized predominantly with macrophages with high tendency to fusion into foreign-body giant multinucleate cells. On the other hand, the extent of the foreign-body reaction induced by p(HEMA-*co*-SPMAK) (3 mol %) was very low, almost without presence of foreign-body giant multinucleate cells (Fig. 6).

In close surroundings of both synthetic polymers used in our experiments, typical neurons stainable with cresylviolet were *always* present. The NADPH-d positive neurons were absent in the pHEMA implant

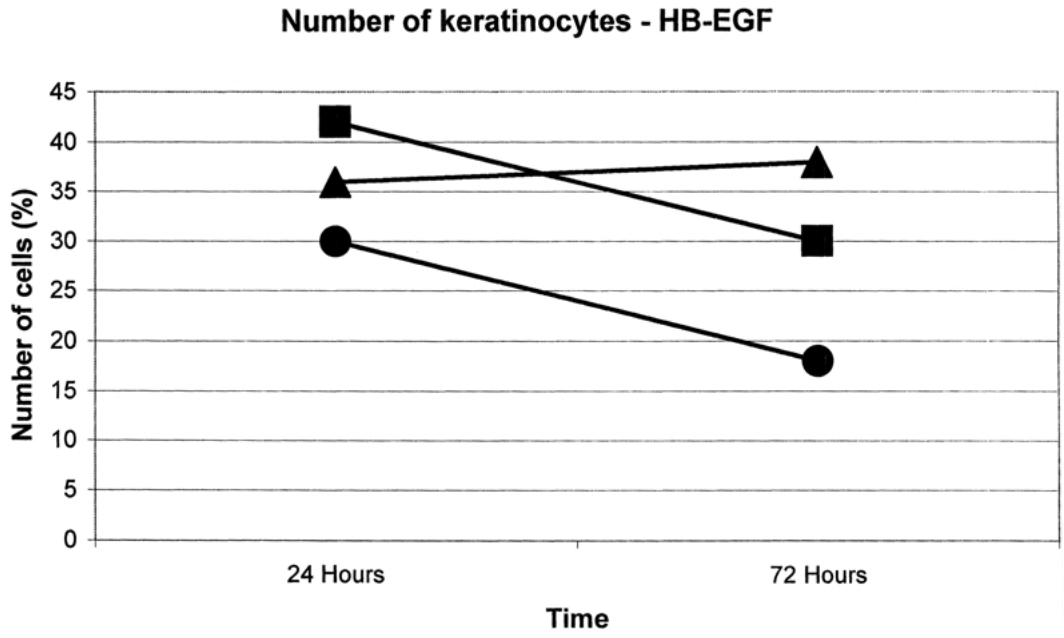

Figure 4 Growth of human keratinocytes on polystyrene (triangle), pHEMA (quadrangle) and copolymer p(HEMA-*co*-SPMAK) containing 3.0 mol % SPMAK (circle) for 72 h.

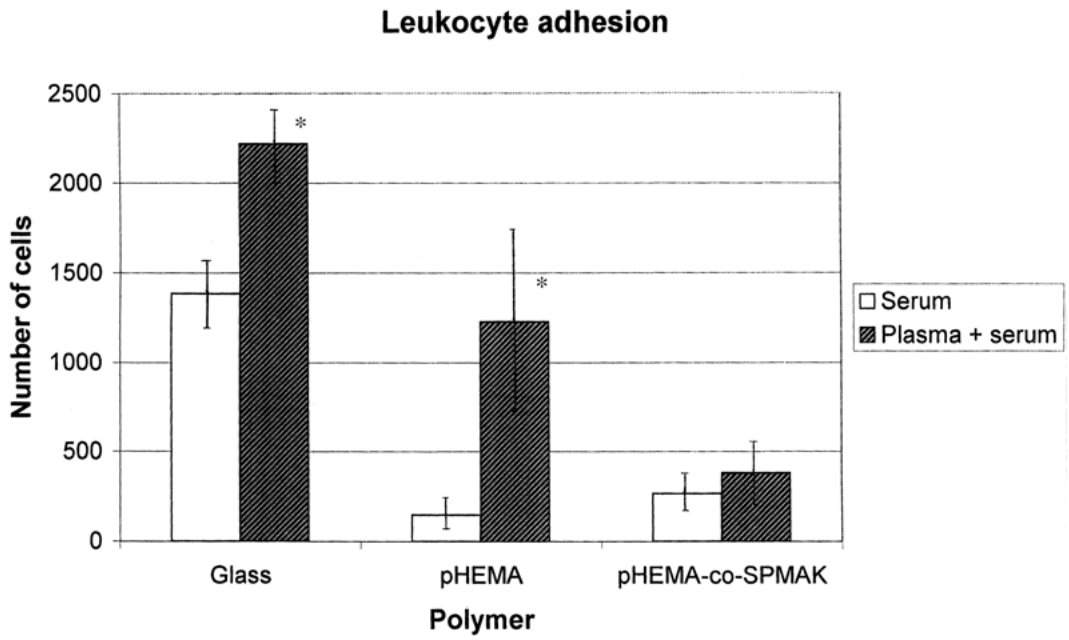

Figure 5 Adhesion of leukocytes to glass coverslips, pHEMA and copolymer p(HEMA-*co*-SPMAK) containing 30 mol % SPMAK. Preincubation of glass and pHEMA with plasma significantly ($p \leq 0.01$) significantly stimulated the cell adhesion (asterisk).

vicinity although single positive fibers were present. The smaller than typical ones NADPH-d positive neuronal bodies without signal in processes were detected in brain tissue surrounding the p(HEMA-*co*-SPMAK) (3 mol %). At the contact point of polymers and nervous tissue the glial response was not detectable. A weak glial reaction was found in the surrounding of the cannula track. In some few cases the glial response slightly passed from cannula track also toward the peak of implant area (Fig. 7).

Discussion

Copolymer p(HEMA-*co*-SPMAK) significantly supported the adsorption of BFGF and HB-EGF in comparison with pHEMA. The adsorption of other tested proteins such as Alb, Fbg, Fibro and EGF were influenced by the hydrogel chemical structure only non-significantly. The adhesion of 3T3 cells and human keratinocytes was very low in comparison with the polystyrene (tissue culture grade) and pHEMA. Even the preadsorption of HB-EGF, which is strongly adsorbed to p(HEMA-*co*-SPMAK), has no stimulatory effect on the cell adhesion and proliferation of 3T3 cells and human keratinocytes. The macromolecules of extracellular matrix, namely glycosaminoglycans, represent the very good substrate for the interactions with growth factor and even stimulate their functional activity [22–24]. Although, the capacity of p(HEMA-*co*-SPMAK) to bind BFGF and HB-EGF is quite high, the structural

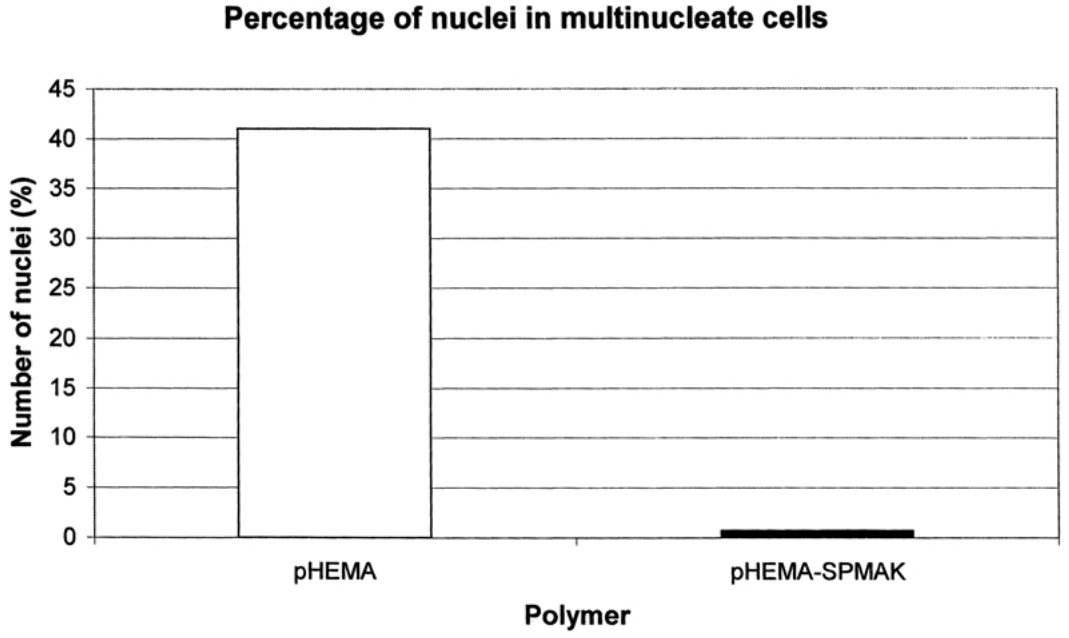

Figure 6 Formation of foreign-body multinucleate giant cells is significantly reduced ($p \leq 0.001$) on surface of copolymer p(HEMA-*co*-SPMAK) containing 3.0 mol % SPMAK.

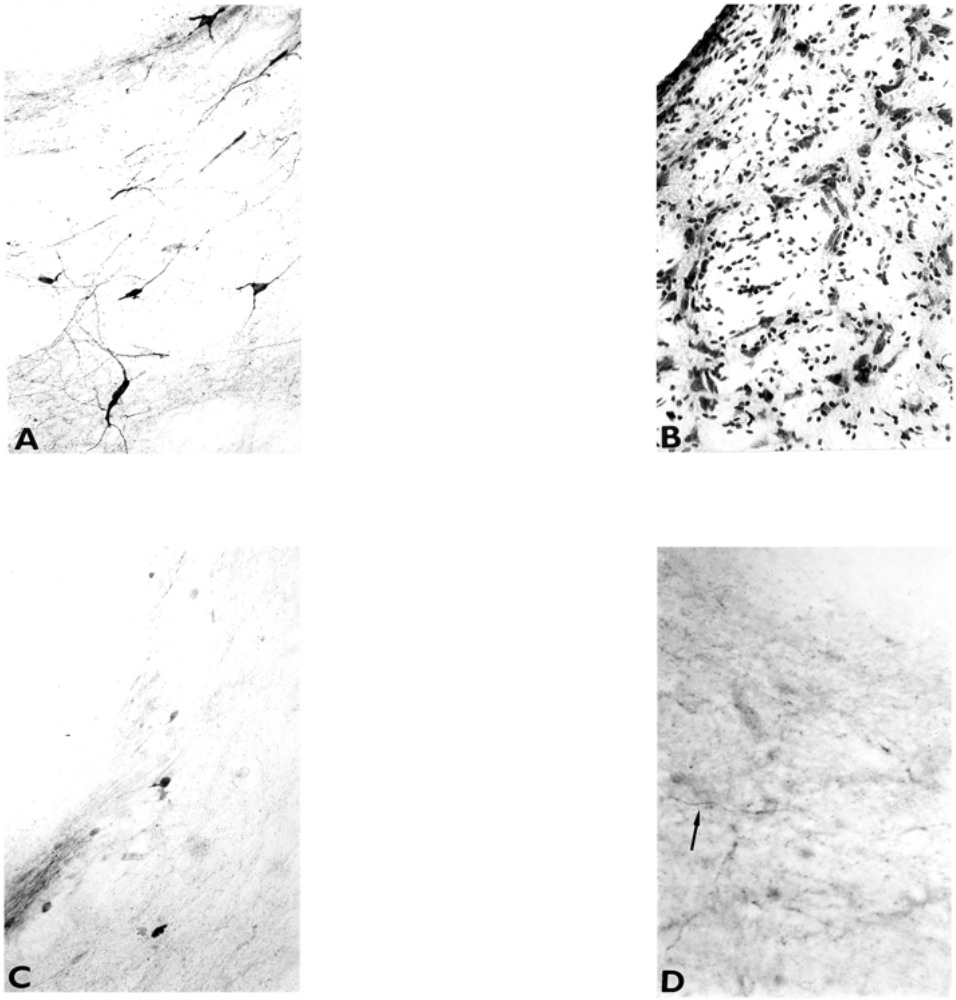

Figure 7 The borders between cannula track (A) or polymer implant (B–D) in the striatum of the rat. The typical NADPH-d positivity in striatal neurons in control lesioned animal without implant (A), normal cresylviolet staining around the pHEMA implant (B), the border between p(HEMA-*co*-SPMAK) and striatum with neurons without NADPH-d positivity in their processes (C), NADPH-d reaction in neighborhood of pHEMA implant; NADPH-d positive neurons absent and only single NADPH-d positive fibres are present (arrow) (D). Magnification × 180.

difference between the glycosaminoglycans and synthetic hydrogels can explain the no efficiency of adsorbed growth factor to influence cell proliferation. The very low adhesion and extent of proliferation of both cell types on surface of p(HEMA-*co*-SPMAK) can be explained with higher swelling and wettability of this copolymer in comparison with pHEMA. The p(HEMA-*co*-SPMAK) is much more better tolerated after the subcutaneous implantation into the rat than pHEMA. The very similar biocompatibility was observed in copolymer of HEMA with sodium salt of methacrylic acid [10, 11, 13–15].

The extent of leukocyte adhesion to both polymers *in vitro* is almost the same in presence of human serum. If the pHEMA and p(HEMA-*co*-SPMAK) surface is preadsorbed with human plasma the adhesivity of pHEMA for these cells was significantly elevated. This phenomenon can be induced by the adsorption of some bioactive compound of plasma to pHEMA surface and it explains the difference of subcutaneous biocompatibility of both polymers. Both the implanted polymer materials induced only minimal advertise reaction of brain tissue (glial response). However, the functional phenotype of surrounding neurons (NADPH-d) was ''more normal'' in brain tissue surrounding the p(HEMA-*co*-SPMAK) implants. Hypothetically, the NADPH-d positive neurons disappeared (died) after implantation or they only leave their NADPH-d positivity.

Acknowledgments

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