Hydrogels for Encapsulation of Mammalian Cells

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SUMMARY: In our attempt to prepare new hydrogels for cell encapsulation, we decided to use synthetic copolymers presuming that chemical bonds should bring better mechanical stability of capsules than electrostatic forces between polyanions and polycations in the so far predominantly used materials based on polyelectrolyte complexes. Two series of copolymers of 2-hydroxyethyl methacrylate (HEMA) with alkyl acrylates (methacrylates) or *N*-alkylacrylamides (methacrylamides) were prepared. The copolymers, prepared by solution copolymerization in ethanol, were characterized by swelling in water and PBS solution, viscometric molecular weight, and NMR analysis, and their encapsulation processability was tested. Their characteristics and the first biological tests are the subject of this paper.

Introduction

The immunoprotection cell therapy has a great potential to reduce the ever-increasing lack of human donors for tissue and organ transplantations. Hormone- or protein-secreting cells could be enclosed into polymer microcapsules and subsequently implanted in the human body. In this way diseases caused by failure of secretory cell function could be treated[1-4]. The concept is based on the protection of allo- and xenogenic transplanted cells with semipermeable membrane from the body immune system. The membrane permeability must allow a sufficient intake of low-molecular-weight molecules (oxygen, glucose, ions, amino acids, etc.) necessary for viability and normal function of cells inside the capsule, and, on the other hand, diffusion of desired cellular products in the human body[4-6]. Immunoprotection may allow transplantation of cells without the need for immunosuppression and transplantation of cells from non-human species (xenograft)[7]. So far this method has been applied in small or large animal models of pancreas dysfunction[8], liver failure[9], Parkinson disease[10], Alzheimer disease[11], chronic pain[12] and some other cases.

Various hydrogels for cell encapsulation have been developed; the first material used was a natural polymer based on the ion complex of alginate (polyanion) and polylysine (polycation)[13]. The capsules were applied with good results regarding their biocompatibility, but their stability was limited, which brought a potential risk of the host immunization by the encapsulated cells

 CCC 1022-1360/00/$ 17.50+.50/0

after the capsule breakdown. There were many attempts to improve stability of the alginate-polylysine capsules by their surface modification, e.g., by grafting poly(ethylene oxide)[14]. Many other new types of hydrogels, also based on the principle of polyelectrolyte complexes, have been proposed[15,16]. Usually the complexes are prepared by mixing a water-soluble polymer with negatively charged side chains with multivalent ions of the opposite charge to form a gel that is usually further stabilized by interactions with multivalent polyions of the same charge as those used in formation of the gel. Thus, e.g., hydrolytically stable polyphosphazenes are formed from monomers having carboxylic acid side groups, which are crosslinked by divalent or trivalent cations such as Ca^{2+} or Al^{3+} and then stabilized with a polycation such as poly-L-lysine[16]. Also in this case, an additional stabilization step is required. This is the reason why an intensive research of more stable materials must continue.

Instead of polymeric systems based on polyelectrolyte complexes, which are held together only by physical forces between polyanions and polycations, we decided to use synthetic copolymers, presuming that chemical bonds should result in better mechanical stability of capsules. Synthetic polymers for cell encapsulation must be insoluble under physiological conditions and, on the other hand, soluble in nontoxic solvents. They must comply with many other requirements, including sufficient mechanical stability, high resistance to degradation and hydrolysis, good biocompatibility, non-cytotoxicity, non-carcinogenicity and non-mutagenicity, and good tolerance by cells. It is necessary to define the level of permeability of the polymer to various biologically important molecules including proteins. So far only few synthetic polymers have been used for cell encapsulation[7,17].

PolyHEMA is one of the most frequently used polymers in medicine and its good performance is well known (nontoxicity, good biocompatibility and high resistance to degradation and hydrolysis under physiological conditions). On the other hand, it has also drawbacks in some applications, like calcification resulting from tissue reaction, low mechanical strength and low cell adhesion (cells do not spread and do not proliferate)[18-21].

In our effort to develop new hydrogels suitable for cell encapsulation, we have focused our attention to HEMA copolymers with comonomers eliminating or at least suppressing the mentioned polyHEMA drawbacks. The goal of our work was preparation of new polymeric materials with improved diffusivity of low-molecular-weight substances and improved cell response, which would lead to prolonged viability and secretory activity of the cells. In this paper, we describe preparation and characterization of some new HEMA copolymers with regard to their applicability and processability in encapsulation of cells releasing biologically active molecules.

Materials and Methods

Chemicals: HEMA, methyl methacrylate (MMA), ethyl methacrylate (EMA), butyl acrylate (BA), butyl methacrylate (BMA), *N*-isopropylacrylamide (iPAAm), *N*-isopropylmethacrylamide (iPMAAm), *N-tert*-butylacrylamide (tBAAm), 2,2'-azobisisobutyronitrile (AIBN), poly(ethylene glycol) (MW 200), Ficoll 400 and hexadecane were purchased from Sigma-Aldrich (Prague, Czech Republic), whereas *N-tert*-butylmethacrylamide from Monomer-Polymer and Dajac Laboratories, Inc. (Feasterville, PA, U.S.A.). Petroleum ether and ethanol were obtained from Lachema (Neratovice, Czech Republic). Monomers and ethanol were distilled before polymerization, AIBN was recrystallized from methanol, all the other chemicals were used as received.

Preparation of copolymers: All HEMA copolymers were prepared at three different molar ratios by solution polymerization in ethanol (1:10 w/v) at 70 °C for 4 h. AIBN (0.1 wt-% relative to the monomers), was used as initiator. The polymers were precipitated in petroleum ether and reprecipitated in phosphate-buffered saline (PBS) containing 0.2 g/L KCl, 0.2 g/L KH_2PO_4, 8.0 g/L NaCl, and 2.16 g/L $Na_2HPO_4.12\ H_2O$. The copolymers were characterized by swelling in water and PBS solution, viscometric molecular weight and NMR analysis. Their processability in encapsulation was checked and they were also tested for *in vitro* and *in vivo* biological responses. As a comparative material, copolymer poly(HEMA-*co*-MMA) 75:25 mol/mol[7] was used.

Microencapsulation process: Suitability of the prepared copolymers for encapsulation was tested using a Horvath submerged jet device described elsewhere[22]. Blank microcapsules were prepared by interfacial precipitation from a 10 % copolymer solution in poly(ethylene glycol) (MW 200) using the submerged jet coextrusion technique (Fig. 1). The copolymer solution and Ficoll 400 (instead of cell suspension in Ficoll 400) in a certain volume ratio were pumped through a co-extrusion nozzle, which consisted of two concentric stainless steel needles placed in the middle of a special formed polyMMA unit. Droplets of Ficoll surrounded by the polymer

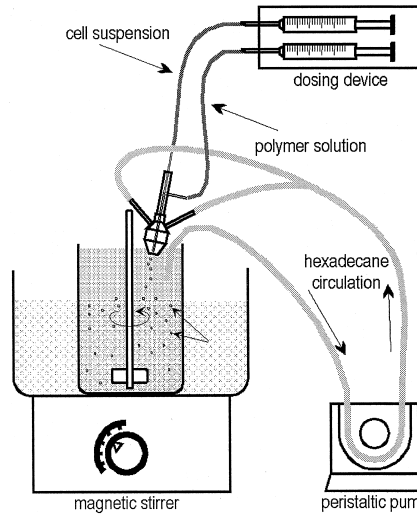

Fig. 1: Schematic diagram of microencapsulation device

solution were sheared off due to the drag force of a hexadecane stream. Passing through the hexadecane layer, the extruded nascent microcapsules stabilized their spherical shape and fell down into a gently stirred precipitation PBS bath. They were then transferred to fresh PBS and washed by suspending using an overhead magnetic stirrer for 30 min. Microcapsules of good spherical shape, about 450 μm in diameter, were collected in Petri dishes (Fig. 2) and washed with fresh PBS for 3 days to remove poly(ethylene glycol). During this conditioning procedure, the microcapsules shrank. In some cases microcapsules became sticky, which complicated further manipulation.

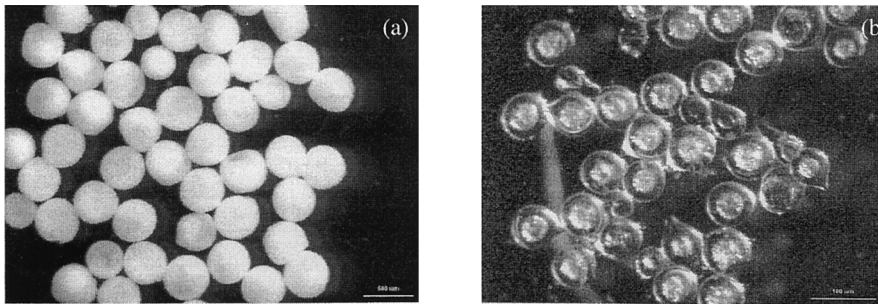

Fig. 2: Microcapsules prepared from: (a) HEMA-EMA copolymer, (b) HEMA-iPMAAm copolymer

In vitro tests: Indirect contact cytotoxicity tests were performed using 3T3 mice fibroblasts. Morphology and growth of cells on the surface of 24-well plates uncoated and coated with the prepared copolymers were compared. The growth of 3T3 fibroblasts was evaluated by counting the total number of cells and the number of dead cells in different time periods (24, 48 and 96 h) by using the Trypan Blue assay. The fibroblast metabolic activity was examined after 96 h by the MTT test and expressed by absorbance values at 570 nm against isopropyl alcohol as blank. Both methods were described in more detail elsewhere[23].

Capsule implantation and histology: Blank microcapsules of HEMA-EMA copolymer were implanted unilaterally deep into the brains of adult Wistar rats. They were sacrificed 2, 4, 12, 24, 36, and 48 weeks after implantation, the brains were removed, fixed in 10 % formalin and cut at the area of implantation site. Vibratome or parrafin-embedded sections were made for a variety of detection methods (see ref.[23]) allowing identification of cell types participating in the host-tissue response.

Results and discussion

The copolymers were characterized by swelling in water and PBS solution, viscometric molecular weight and NMR analysis (Tab. 1). Copolymer HEMA-MMA (75:25 mol-%) taking up ~27 % of water[7] was chosen as a standard. It is evident that nearly all prepared samples, but in particular HEMA copolymers with amide comonomers show significantly higher water uptakes than the standard. Hydrogel swellability of the polymers is the first information about its permeability to low-molecular-weight nutrients and drugs transported through the capsule walls.

Tab. 1. Characteristics of some HEMA copolymers

Copolymer	Monomer ratio in the feed mol/mol	Swelling		Molecular weight $M_\eta.10^{-3}$	Comonomer content (NMR) mol-%
		water wt-%	PBS wt-%		
HEMA-MMA	75 : 25	27.0	23.2	300	21.0
HEMA-EMA	90 : 10	33.9	30.6	320	12.6
HEMA-EMA	80 : 20	28.5	24.8	280	20.7
HEMA EMA	70 : 30	24.3	20.5	330	26.7
HEMA-BA	90 : 10	36.8	31.3	150	9.7
HEMA-BA	85 : 15	35.3	28.1	120	9.8
HEMA-BA	70 : 30	28.8	20.7	150	20.1
HEMA-BMA	90 : 10	31.1	26.1	275	9.8
HEMA-BMA	85 : 15	27.5	23.4	280	13.9
HEMA-BMA	70 : 30	19.2	13.8	290	27.6
HEMA-iPAAm	90 : 10	41.2	39.1	140	3.6
HEMA-iPAAm	80 : 20	40.7	37.1	140	6.2
HEMA-iPAAm	70 : 30	39.8	35.2	150	10.8
HEMA-iPMAAm	90 : 10	39.5	37.2	236	4.3
HEMA-iPMAAm	80 : 20	38.3	37.6	188	10.0
HEMA-iPMAAm	70 : 30	38.2	34.1	183	16.1
HEMA-tBAAm	90 : 10	35.6	32.4	200	5.9
HEMA-tBAAm	80 : 20	31.5	28.4	120	11.2
HEMA-tBAAm	70 : 30	27.6	24.5	115	15.9
HEMA-tBMAAm	90 : 10	35.4	34.4	nd[a)]	4.9
HEMA-tBMAAm	80 : 20	32.3	31.4	nd[a)]	9.3
HEMA-tBMAAm	70 : 30	30.1	26.4	nd[a)]	14.7

[a)]Not determined.

The higher swellability the better transport conditions through the hydrogel membrane in both directions; however, there must exist a limiting pore diameter threshold, enabling the capsule to keep the basic function, to protect encapsulated cells against attaching by the host immune system. The limiting molecular weight cut-off value for HEMA-MMA copolymer of about 100 000 has been found[24]. To define the threshold of molecular weight cut-off as well as the copolymer permeability to various biologically important molecules is the task we are working on. Molecular weights of the copolymers are very important quantities with respect to the processability of copolymers by encapsulation. Our experiments showed that an optimum molecular weight was about 300 000 for HEMA copolymers with alkyl acrylates or methacrylates. Obviously, the low molecular weight of HEMA-BA copolymers was a reason why capsules prepared from this hydrogel have collapsed within two days. The usability of HEMA-BA copolymers in encapsulation process should be further directed to obtaining higher-molecular-weight products by controlling the polymerization towards higher conversion. On the other hand, in the case of HEMA copolymers with acryl- and methacrylamides, much lower molecular weights were sufficient for obtaining mechanically stable capsules. Besides the higher swellability, another advantage of amide hydrogels has been found; the capsules prepared from these copolymers became transparent when the solvent (PEG 200) was washed out. The hydrogel transparency is also one of requirements, as it would enable direct microscopic investigation of encapsulated cells.

The results of NMR measurements indicate real proportions of monomeric units in the copolymers. These values show concentration of the hydrogel comonomers, which should improve the mentioned inappropriate polyHEMA properties. From Tab. 1 it is evident that the copolymerization reactivities of all comonomers, but in particular those of all amides, were lower compared with the HEMA reactivity.

The cell responses to the surfaces of all copolymer samples were intensively studied. Daily microscopic controls showed that in all cases the morphology of cells did not change, but the samples differed in the degree of cell spreading. The *in vitro* test results of 3T3 fibroblast adhesion, proliferation and metabolic activity on surfaces of the studied hydrogels are summarized in Tab. 2 as the mean values of six measurements. The best results were obtained on poly(HEMA-*co*-EMA) and poly(HEMA-*co*-BA) samples, as the cells adhered well to the copolymer surfaces, they spread, their growth was satisfactory and showed the highest metabolic activity. Unfortunately, with respect to the already mentioned capsule breakdown, mechanical properties of HEMA-BA copolymer should be improved by controlling the polymerization reaction to obtain a higher-molecular-weight copolymer.

Tab. 2. Adhesion, growth and metabolic activity of fibroblasts on the (co)polymer surfaces

(Co)polymer	24 h		48 h		96 h		MTT test
	No of cells $\times 10^{-3}$	Dead cells %	No of cells $\times 10^{-3}$	Dead cells %	No of cells $\times 10^{-3}$	Dead cells %	absorbance at 570 nm
HEMA	51	55.6	38	23.7	72	12.5	0.391
HEMA-MMA[a)]	57	21.1	74	25.6	117	5.1	0.273
HEMA-EMA[b)]	69	17.4	153	19.6	246	6.1	0.768
HEMA-BA[b)]	120	37.5	131	13.0	252	19.0	0.711
HEMA-BMA[b)]	66	31.8	141	24.8	102	25.0	0.284
HEMA-iPAAm[b)]	63	41.2	92	23.0	117	7.7	0.165
HEMA-iPMAAm[b)]	88	29.3	84	26.3	81	14.8	0.267
HEMA-tBAAm[b)]	110	34.3	119	13.5	204	17.6	0.378
HEMA-tBMAAm[b)]	96	23.6	99	12.2	193	11.5	0.339

[a)] HEMA:MMA molar ratio in the copolymerization mixture 75 : 25.
[b)] HEMA:comonomer molar ratio in the copolymerization mixture 70 : 30.

The first results of biological *in vivo* investigation with blank capsules prepared from poly(HEMA-*co*-EMA) showed that the response of recipient tissue was mainly caused by injury during implantation while in the surroundings of microcapsules the reaction was much smaller (Fig. 3). The acceptable tissue reaction around implanted capsules included glial and connective tissue components in which reticular fibres prevailed (Fig. 4). The long-term *in vivo* tests proved the HEMA-EMA copolymer to be very stable, as the implanted capsules persisted intact in parenchyma for one year without any copolymer degradation changes.

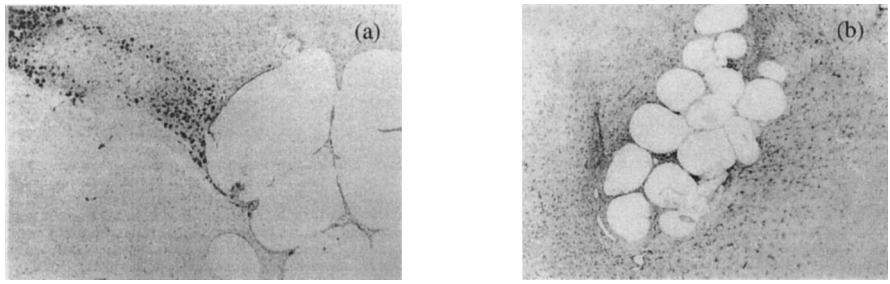

Fig. 3: Paraffin-embedded section of brain tissue 2 weeks following implantation of poly(HEMA-*co*-EMA) microcapsules into the brain: (a) The presence of siderophages in the injection tract, visualised by Perl's reaction, shows the extent of injury caused by implantation procedure; (b) Immunohistochemical staining showed that the highest density of GFAP-immunopositive reactive astrocytes was observed along the injection tract.

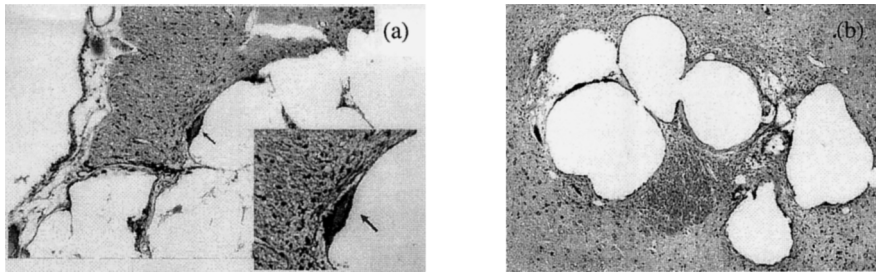

Fig. 4: Paraffin-embedded section of brain tissue 6 months after implantation of poly(HEMA-*co*-EMA) microcapsules into the brain: (a) Hematoxylin-eosin-stained section shows that the capsules are only sporadically surrounded by multinucleated foreign body giant cells (arrows); (b) Masson Green trichrome stained section proved only minimum collagen components resulting from a tissue reaction around implanted capsules.

Conclusion

In an attempt to prepare new hydrogels for cell encapsulation with improved properties regarding diffusivity and with better cell response to the material, new types of HEMA copolymers were prepared, characterized, tested for encapsulation processability and their *in vitro* or *in vivo* biological properties were evaluated.

The obtained results show that nearly all copolymers, but in particular HEMA copolymers with amide comonomers, showed better swellability in water and PBS, indicating better diffusivity for nutrients and drugs compared with the poly(HEMA-*co*-MMA) 75:25 mol-% standard.

From almost all new copolymers, microcapsules with a good spherical shape were prepared; unfortunately, some of them were sticky, which made manipulation with them difficult and limited their usage in implantation. Mechanical properties of microcapsules from poly(HEMA-*co*-BA) were not satisfactory as their shape collapsed within two days. This was probably caused by a low molecular weight of the copolymer.

Experiments with 3T3 fibroblasts showed that their adhesion and proliferation were higher on HEMA copolymers compared to HEMA homopolymer. The best cell responses and metabolic activity were found with poly(HEMA-*co*-EMA) and poly(HEMA-*co*-BA); microcapsules from the former copolymer were implanted into the rat brain and tests showed very positive results from the point of view of copolymer biocompatibility and mechanical stability.

Acknowledgement

Support of this work by Grant Agency of the Czech Republic (grant No. 304/98/0267) is gratefully acknowledged. The authors are grateful to J. Spěváček, M. Netopilík and F. Lednický

(all from the Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic) for NMR spectra, viscometric measurements evaluation and micrographs.

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