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Macroporous hydrogels based on 2-hydroxyethyl methacrylate

Part II *Copolymers with positive and negative charges, polyelectrolyte complexes*

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Crosslinked macroporous hydrogels based on 2-hydroxyethyl methacrylate (HEMA)—[2-(methacryloyloxy)ethyl]trimethylammonium chloride (MOETACl) copolymer, HEMA-MOETACl—methacrylic acid (MA) terpolymer, and on a polyelectrolyte complex of HEMA—MA copolymer with poly(MOETACl) were prepared. All the hydrogels were prepared in the presence of fractionated sodium chloride particles. The hydrogels were characterized by the number of pores and the total volume of all pores in unit volume, the average volume and the average diameter of single pore. Morphology of the hydrogels was investigated by confocal and scanning electron microscopy. The hydrogels based on polyelectrolyte complexes were also characterized by chemical composition. Homogeneous (non-porous) hydrogels with the same composition as macroporous hydrogels were prepared and characterized by their biocompatibility.

1. Introduction

Synthetic polyelectrolytes and polyelectrolyte complexes offer very attractive potentials for biomedical applications [1–4] or for study of interactions with biopolymers [5–8], such as electrostatic interactions or selective complexations. Of interest is utilization of these materials for contact lenses [9] or for various implants. In contrast to classic uncharged hydrogels, polyelectrolytes and polyelectrolyte complexes make it possible to modify chemical and physical properties to a wide extent, such as the water content in equilibrium-swollen state and mechanical properties.

The present work continues our previous study dealing with preparation and characterization of macroporous hydrogels based on 2-hydroxyethyl methacrylate–methacrylic acid copolymers [10]. The subject of the present paper is characterization of hydrogels containing both positive and negative charges in polymer chains and a comparison with negatively charged hydrogels. All the materials will be subsequently tested from the viewpoint of biocompatibility and suitability for preparation of implants predominantly into central neural system and urinary tissues. The first studies [11–13] showed that the investigated materials are very promising for the envisaged purpose.

2. Materials and methods

2.1. Preparation of monomers and polymers

Poly{[2-(methacryloyloxy)ethyl]trimethylammonium chloride} (polyMOETACl) was prepared by quaternisation of poly[2-(dimethylamino)ethyl methacrylate] (Fluka) [14] with methyl iodide (Fluka) and its conversion to the Cl form on a column with strongly basic ion exchanger IRA 402 (Fluka).

[2-(Methacryloyloxy)ethyl]trimethylammonium chloride (MOETACl) was prepared analogously. After conversion to the Cl form, water was evaporated in vacuum (20 °C) in the presence of a small amount of hydroqinone and crystallized from a mixture ethanol—diethyl ether.

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Methacrylic acid (MA) (Fluka) was distilled before use.

2.2. Preparation of hydrogels

Three series of macroporous hydrogels were prepared:

Series 1 - HEMA - MOETACl copolymer
Series 2 - HEMA - MOETACl - MA terpolymer
Series 3 - polyelectrolyte complex of HEMA-MA copolymer with linear poly(MOETACl).

The hydrogels were prepared on a pelleting apparatus described earlier [10] using the following mixtures:

2-hydroxyethyl methacrylate	0.67 g
2,2'-azobis(isobutyronitrile)	0.0067 g
sodium chloride	10.02 g
ethylene dimethacrylate	0.019 g
poly(ethylene glycol), MW = 400	3.79 g

Series 1: [2-(methacryloyloxy)ethyl]trimethylammonium chloride 0–14 mol% relative to the total monomer weight
Series 2: [2-(methacryloyloxy)ethyl]trimethylammonium chloride and methacrylic acid 0–14 mol% relative to the total monomer weight. The molar ratio of MOETACl and MA was 1:1
Series 3: methacrylic acid 0–14 mol% relative to the total monomer weight.

The used sodium chloride was screened-fractionated into three fractions with grain sizes below 30 μm, 30–50 μm and 50–90 μm. After thorough mixing of the components, a paste obtained was put into the polymerization chamber of the pelleting apparatus [10], the chamber was closed by a flange with fastening screws and the tightening screws was tightened in a standart way with a force of 10 Nm. The whole pelleting apparatus was thermostatted to 80 °C for 8 h; after cooling, the hard pellet was taken out and weighed. The sodium chloride content (m_{NaCl}) and polymer content (m_H) were calculated from the tablet weight: $m_{NaCl} = 10.02\, m_P/m$, where m_P is the weight of the pellet, m the total weight of monomers (m_M), crosslinker, sodium chloride and poly(ethylene glycol) used for polymerization and $m_H = m_P m_M/m$.

The pellet was washed with water and physiological solution (five times for Series 1). Hydrogels of Series 2 were first washed with 1% NaOH solution and then with water and physiological solution, hydrogels of Series 3 successively with 1% NaOH solution, water, 1% poly(MOETACl) solution (2 weeks), water and physiological solution. After washing with water, elemental analysis of dry hydrogel was performed. Its volume (V_H) was calculated from the dimensions of the swollen macroporous hydrogel in physiological solution.

Also, strips of homogeneous (non-porous) hydrogels without sodium chloride of the same concentrations of MOETACl and MA as with macroporous hydrogels were prepared. After their washing (analogously to macroporous gels), the volume fraction of dry polymer in equilibrium-swollen hydrogel (Z_V) was determined. These strips were prepared by the crosslinking polymerization of monomers, initiator, crosslinker and poly(ethyleneglycol) in a thermostated block [15] of hard-aluminium flow forms with an area of 10 × 10 cm, fitted with reinforced polypropylene foils and firmly closed using screw clamps. The thickness of the unswollen original samples corresponded to the thickness of the silicone seals used. The polymerization proceeded for a period of 16 h at 80 °C.

2.3. Implantation into the rat

Strips of homogenous (non-porous) polymers were implanted subcutaneously to interscapular region of five male rats (200 g, Wistar strain, Anlab, Prague, Czech Republic) for each experimental series under sterile condition at inhalation aether anaestehsia such described [15]. Five weeks later, the animals were sacrificed, the implants with surrounding tissue were removed and fixed with paraformaldehyde. The soft tissue was routinely processed for embedding to paraplast (Sigma, Prague, Czech Republic), cut for 5 μm thin sections and stained with hematoxylin-eosin. The connective tissue capsules surrounding the implants were histologically characterized and the thickness of capsule was measured using Optiphot-2 microscope (Nikon, Prague, Czech Republic) equipped with CCD camera and computer assisted image analyzer LUCIA (Laboratory Imaging, Prague, Czech Republic). The gels were stained with hematoxilin and inspected as whole-mount preparation.

2.4. Characterisation of hydrogels

Macroporous hydrogels were characterized by the following quantities, the calculations of which were given in the previous paper [10]:

(1) The number of pores in macroporous hydrogel in 1 cm^3 of sample, i.e. the number of sodium chloride particles (n):

$$n = m_{NaCl}/[\rho_{NaCl} \cdot 4/3\pi \cdot (d/2)^3 \cdot V_H],$$

where ρ_{NaCl} is the density of sodium chloride (2.16 g·cm^3) and d is the average diameter of NaCl particles (15, 40 and 70 μm).

(2) Total volume of all the pores in hydrogel relative to 1 cm^3 of sample, i.e. the volume of physiological solution in all pores (V_V):

$$V_V = [V_H - m_H/(\rho_P.Z_V)]/V_H,$$

where ρ_P is the density of dry polymer (1.2 g·cm^3).

(3) Diameter of single pore in macroporous hydrogel (d_H):

$$d_H = 2[3V_V/n(4\pi)]^{1/3}.$$

All these calculations were made under the simplifying assumption of the spherical shape of sodium chloride particles, i.e. the irregular shape of NaCl crystals was approximated by spheres of average diameter given by the size of screen mesh of the screen used for fractionation. These diameters were 15, 40 and 70 μm. A

further assumption is 100% conversion of the polymerization, i.e. the weight of the dry matter of the hydrogel phase is equal to the weight m_H.

(4) In Series 3, the composition of hydrogel based on elemental analysis (Cl, N).

2.5. Morphology of hydrogels

The morphology of the prepared hydrogels was studied by laser scanning confocal microscope (Leica) and low-vacuum electron microscope AquaSEM (Tescan, Czech Republic). Laser scanning confocal microscope (LSM) enables to observe samples containing water (e.g., samples in solution), while low-vacuum electron microscope (LVSEM) makes it possible to observe samples containing ice (for example flash-frozen samples) [10].

Prior to observation in the confocal microscope, the hydrogel samples were immersed for 1 min in a solution of Lucipher Yellow (Sigma-Aldrich) and washed with physiological solution for 10 min. The sample was then scanned using an objective with water immersion.

LVSEM samples were prepared in two ways. Preparation 1: a small piece of the hydrogel (approx. 3 × 3 × 0.5 mm) was cut out from the middle of the hydrogel in the physiological solution with a sharp blade, removed from the solution and flash-frozen in liquid nitrogen; the frozen sample was fastened to the heating stage at −15 °C and observed in the LVSEM microscope [10]. Preparation 2: the second preparation is a modification of the preparation number 1 described above; it was used for some samples that showed artifacts on the surface. The artifacts were either tiny crystals of NaCl, which covered the surface, or collapsed structures not showing the pores clearly. If the artifacts on the surface were observed, the microscope was opened and the top of the sample, which was still fastened to the heating stage at −15 °C and frozen, was cut off using liquid-nitrogen cooled-blade. The top surface, displaying inner structure of the sample without artifacts, was observed in the microscope.

3. Results and discusion

Fig. 1 shows the dependence of volume fraction of dry polymer in equilibrium-swollen, homogeneous, i.e. nonporous hydrogel on the ionic component content (x, mol%) in copolymers of Series 1–3 (mol.%). For Series 1, x is the molar content of MOETACl in copolymer, for Series 2 that of MOETACI and MA, and for Series 3, x is the MA content in the copolymer before the reaction with linear poly(MOETACl). As we consider the application of macroporous hydrogels for implants in neural tissue, all their properties were investigated in physiological solution.

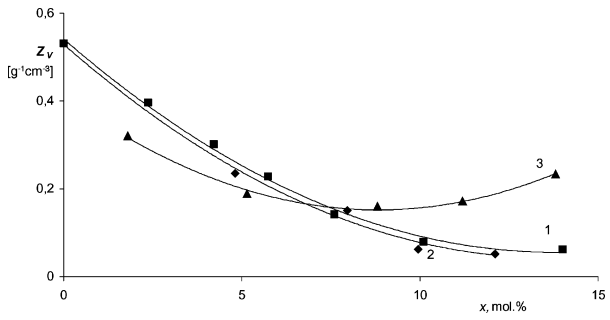

Figure 1 Dependence of volume fraction of dry polymer in equilibrium-swollen hydrogel on the hydrophilic comonomer content for Series 1–3.

It follows from Fig. 1 that the mole fraction of the polymer decreases with the charged group content monotonically and without perceptible differences for Series 1 and 2 and less steeply than in an analogous dependence for methacrylic acid [10]. For a polyelectrolyte complex (Series 3), the dependence shows a flat minimum in the region around 9 mol% of MA, i.e. the hydrophilicity of the complex is highest just in this region. A higher concentration of the ionogenic component could not be used, because the hydrogels spontaneously disintegrated. The Z_V values must have been measured for subsequent calculations of characteristics of macroporous hydrogels.

Fig. 2 shows the dependences of specific pore number of hydrogels, i.e. the number of pores (both communicating and noncommunicating) in 1 cm^3 of hydrogel, on the ionogenic component content in polymer chain for three different fractions of the used sodium chloride. The measured dependences correspond to the relationship Z_V vs. x in Fig. 1; at a constant sodium chloride content in the polymerization mixture, the pore density is determined by the volume of walls, i.e. by the

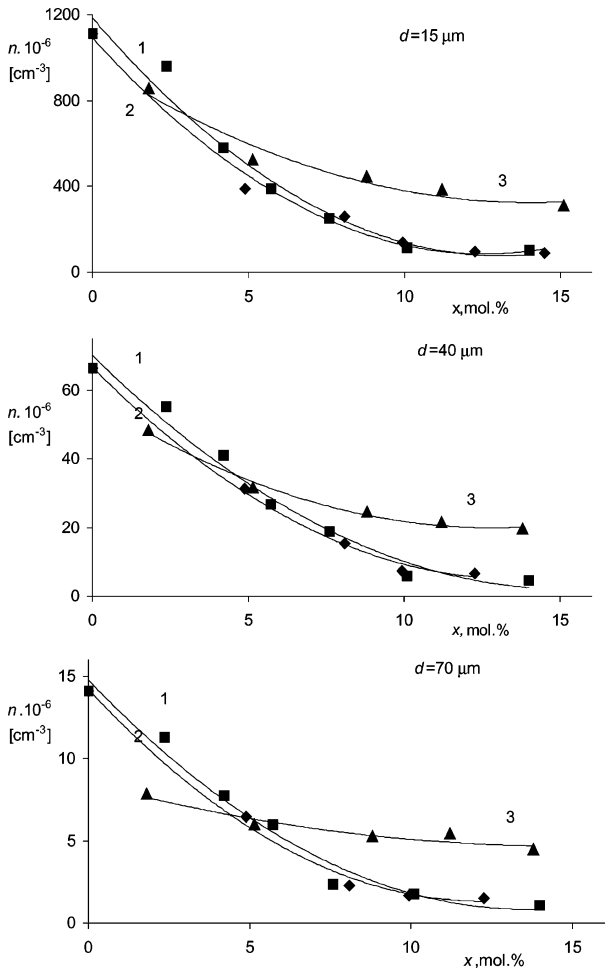

Figure 2 Dependence of the specific number of pores in macroporous hydrogels on the hydrophilic comonomer content for Series 1–3 and for three sizes of sodium chloride particles.

degree of its swelling. Since Z_V for Series 1 and 2 are almost identical, the specific pore number of both hydrogels is also the same. The considered dependence for the polyelectrolyte complex is less steep, which is in accord with the relationship Z_V vs. x. At the same time, the specific pore number in hydrogels increases with decreasing diameter of the used sodium chloride particles (d) because the number of its particles in volume unit grows at a constant NaCl mass in the reaction mixture.

The dependence of the specific pore volume (i.e. the pore volume in 1 cm^3 of macroporous hydrogel) on the ionogenic component content in the copolymer is given in Fig. 3. In contrast to the preceding characteristics, the V_V is only little affected by the size of the used sodium chloride particles; the measured curves for individual fractions differ only little and the investigated quantity slightly decreases with the sodium chloride particle size, especially for the polyelectrolyte complex (Series 3). At the same time, the difference between the series of hydrogels is perceptible. The least specific pore volume exhibits the polyelectrolyte complex, on the curves of which a minimum at an ionogenic component content of 5–7 mol% is perceptible similarly to the dependence Z_V vs. x (Fig. 1). The same minima, but considerably deeper, were observed previously [10] for macroporous hydrogels based on HEMA—MA copolymer. A different behaviour of Series 1 and 2 hydrogels is apparent: for the MOETACl comonomer (Series 1), the specific pore volume increases with increasing hydrophilicity, but the trend for the hydrogels bearing both charges is opposite.

The dependences of average values of diameter of single pore in swollen macroporous hydrogels are shown in Fig. 4 for all the three investigated sodium chloride fractions. It follows from Fig. 4 that d_H increases monotonically with increasing ionogenic component content in the measured range, the growth being the steepest in Series 1 and the least steep in Series 3. At low contents of the ionogenic component in the copolymer, the calculated pore diameter is smaller than the particle size (d) of the used NaCl; at higher contents, $d_H > d$ for Series 1 and 2. d and d_H coincide in the range 7–10 mol% for Series 1 and 2; for the polyelectrolyte complex, $d_H < d$ in the whole measured range.

Macroporous hydrogels based on the polyelectrolyte complex (Series 3) were further characterized by their composition. The synthesized gel consists of three parts:

(1) Basic skeleton of crosslinked HEMA—MA copolymer (MA^- in ionized form).
(2) A part of linear poly ($MOETA^+$), whose functional groups are electrostatically bonded to MA^-.
(3) An unreacted part of linear poly ($MOETA^+$).

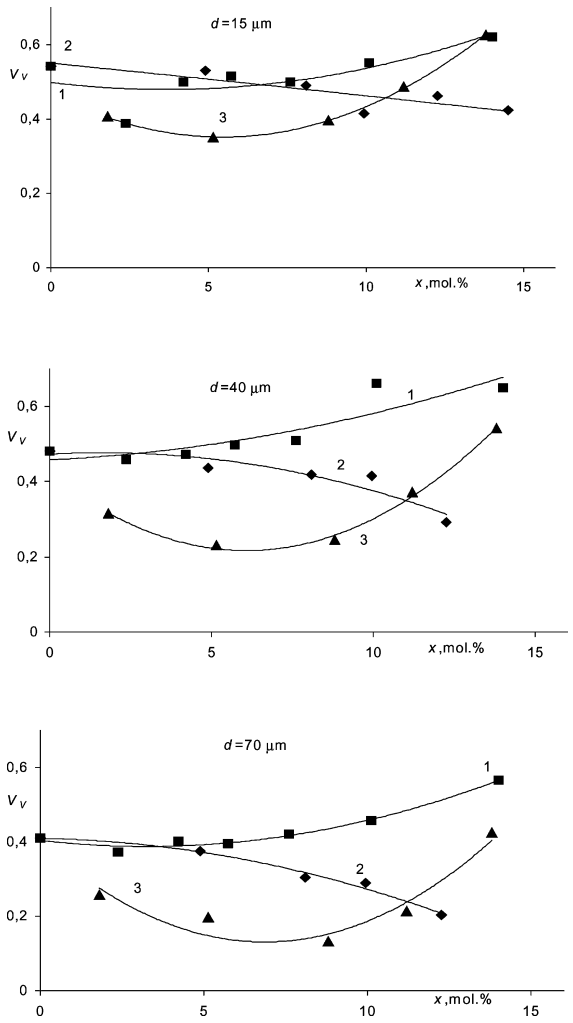

Figure 3 Dependence of the specific pores volume in macroporous hydrogels on the hydrophilic comonomer content for Series 1–3 and for three sizes of sodium chloride particles.

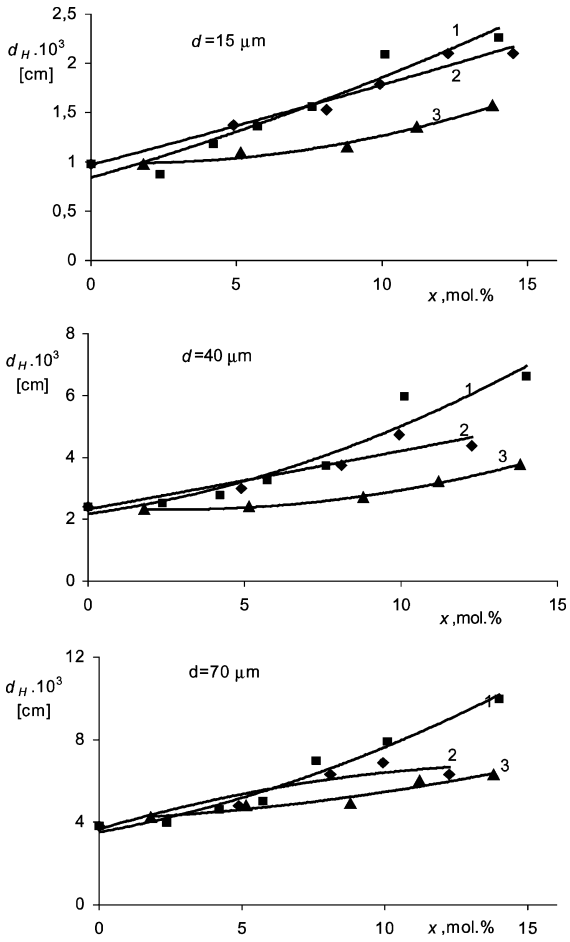

Figure 4 Dependence of the single-pore diameter in macroporous hydrogels on the hydrophilic comonomer content for Series 1–3 and for three sizes of sodium chloride particles.

TABLE I Elemental analysis and composition of Series 3 hydrogels for various sizes of NaCl particles and methacrylic acid contents (x)

d (cm)	x (mol%)	%Cl	%N	m_3	m_2	m_1	x_3	x_2	x_{HEMA}	x_{MA^-}	x_c
0.0015	5.14	0.74	0.68	0.036	0.048	0.916	0.028	0.047	0.862	0.047	0.016
0.0015	8.8	0.72	0.99	0.035	0.087	0.878	0.027	0.070	0.810	0.079	0.015
0.0015	11.2	0.68	1.12	0.033	0.105	0.862	0.028	0.107	0.732	0.119	0.014
0.0015	13.8	0.76	1.44	0.037	0.140	0.823	0.022	0.131	0.702	0.129	0.016
0.004	5.14	0.22	0.83	0.011	0.091	0.898	0.009	0.050	0.879	0.047	0.016
0.004	8.8	0.38	1.2	0.018	0.129	0.853	0.014	0.099	0.796	0.077	0.015
0.004	11.2	0.25	1.19	0.012	0.134	0.854	0.009	0.102	0.776	0.098	0.014
0.004	13.8	0.49	1.6	0.024	0.173	0.803	0.018	0.132	0.713	0.124	0.013
0.007	5.14	0.30	1.01	0.015	0.110	0.875	0.012	0.055	0.872	0.046	0.016
0.007	8.8	0.46	1.18	0.022	0.123	0.855	0.017	0.094	0.797	0.077	0.015
0.007	11.2	0.71	1.36	0.034	0.133	0.833	0.026	0.101	0.762	0.096	0.014
0.007	13.8	0.49	1.62	0.024	0.176	0.800	0.019	0.124	0.720	0.125	0.013

Due to the fact that sodium was not found in the complexes by elemental analysis, all carboxylic acid groups have ammonium groups as counterions and the reaction time of two weeks is sufficient for quantitative reaction of carboxylic and ammonium ions inside the hydrogel. The amounts of individual parts of the polyelectrolyte complex can be calculated from elemental analysis, expressed as mass fractions m_1, m_2, m_3 and subsequently as mole fractions $x_3, x_2, x_1 = x_{HEMA} + x_{MA^-} + x_c$, ($x_{HEMA}, x_{MA^-}, x_c$ are the mole fractions of HEMA, MA and crosslinker), corresponding to the composition of the complex:

$$m_3 = \%\mathrm{Cl}.\mathrm{M}_{\mathrm{MOETA}} + /\mathrm{M}_{\mathrm{Cl}}/100$$

$$m_2 = (\%\mathrm{N}/\mathrm{M}_{\mathrm{N}} - \%\mathrm{Cl}/\mathrm{M}_{\mathrm{Cl}}) \cdot \mathrm{M}_{\mathrm{MOETA}^+}/100$$

$$m_1 = 1 - m_2 - m_3,$$

where %Cl and %N are the contents of chlorine and nitrogen in dry samples, measured by elemental analysis, M_{MOETA^+}, M_{Cl} and M_N are molecular weights of [2-(methacryloyloxy)ethyl] trimethylammonium, chlorine and nitrogen, respectively. The results of elemental analysis and the calculated mass fractions of individual parts are given in Table I. After calculation of mole fractions $x_3, x_2, x_{HEMA}, x_{MA^-}$ and x_c, corresponding to parts 3, 2, HEMA, MA^- and crosslinker, respectively, we obtain the resulting composition of the hydrogel based on the polyelectrolyte complex.

Theoretically, $x_{MA^-} = x_2$ and the given values should not depend on size of NaCl particles. From Table I follows that the values somewhat differ, the real correlation being $x_{MA^-} = 0.98\ x_2 - 0.21$, $R^2 = 0.91$. The deviation can be interpreted as satisfactory and can be ascribed in particular to errors in elemental analysis (Cl and N contents are very low).

Morphology of hydrogels was investigated by confocal and electron microscopy. The confocal microscope makes it possible to observe prepared hydrogels

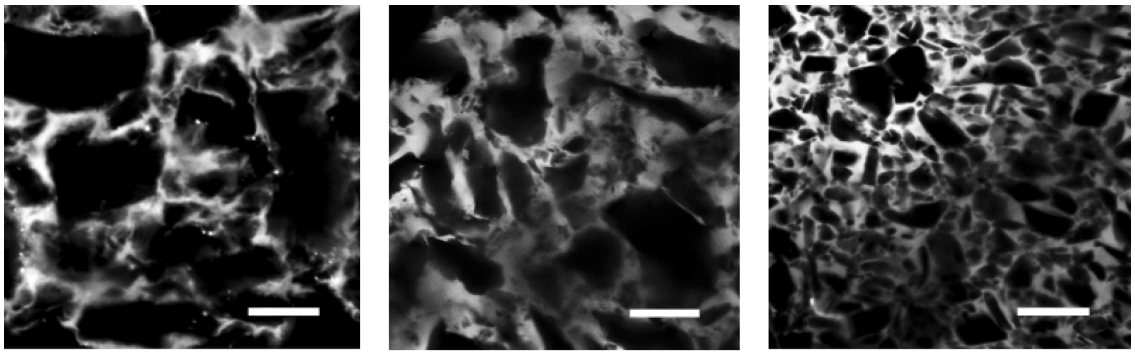

Figure 5 Optical sections of hydrogels of Series 1, 2, 3, hydrogels, NaCl fraction 50–90 μm, 9.94 mol% of ionic comonomer, scale 50 μm.

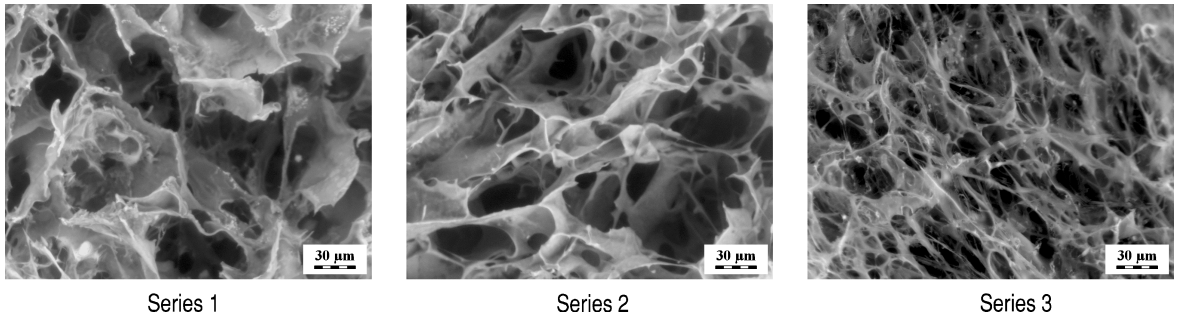

Figure 6 LVSM micrographs of hydrogels of Series 1, 2 and 3, NaCl fraction 50–90 μm, 9.94 mol% of ionic comonomer.

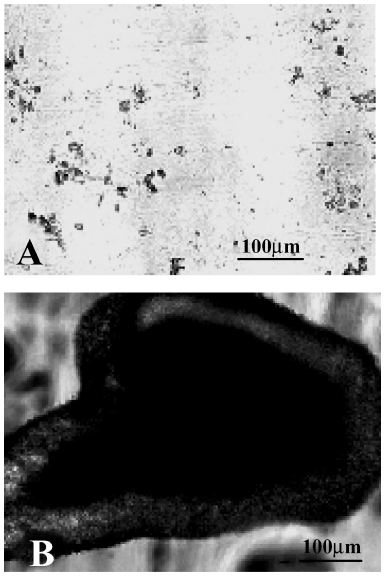

Figure 7 Surface of hydrogel poorly colonized with cells (Series 1A) and calcified defects (Series 3B). Hematoxilin, bar is 100 μm.

in physiological solution, hence in a medium, in which no distortion of their structure occurs. On optical sections obtained using a confocal microscope, there can be observed the pore size in hydrogel samples and an approximate ratio of the water volume in pores to the volume of hydrogel walls. In Fig. 5, optical cuts slices of Series 1–3 hydrogels with a hydrophilic component content of 9.94 mol% are juxtaposed, used sodium chloride fraction was 50–90 μm.

Fig. 6 shows LVSEM micrographs of hydrogels of Series 1, 2 and 3. The content of the hydrophilic component in the hydrogels was 9.94 mol% and the NaCl particle sizes range from 50–90 μm.

LVSEM has two advantages and one disadvantage in comparison with LSM. The first advantage is higher resolution of the electron microscope, which makes it possible to observe smaller details. The second advantage is a higher depth of focus, which allows observing structure in three dimensions, whereas LSM sees just a relatively thin cross-section of the sample. Nevertheless, the important disadvantage of LVSEM studies of the hydrogels is that they are observed in the frozen state, rather than in their natural state in the physiological solution. That is why the LSM is essential for confirmation of the LVSEM results. Regardless of some artifacts observed on LVSEM micrographs, which were mentioned in the Experimental, the authors believe that the final electron micrographs show the real structure of the hydrogels. This is confirmed by several facts: (a) qualitative agreement between the LVSEM and LSM micrographs was achieved, (b) the sizes of pores are in accord with theoretically calculated values (cf. Fig. 5) and (c) the LVSEM micrographs agree qualitatively with those obtained in our previous work on similar systems [10]. It follows from the Fig. 5 and 6 that hydrogels Series 1 and 2 have comparable sizes of pores. In contrast to the hydrogel Series 3 has smaller pores and thiner walls among the pores. It qualitatively corresponds to the calculated values of d_H (Fig. 4) which are similar for Series 1 and 2 and are higher that at hydrogel Series 3. Also, the thiner walls among the pores at Series 3 corresponds to the smaler Z_V for this Series (Fig. 1).

From electron micrographs it is evident that pores in all investigated hydrogels are predominantly

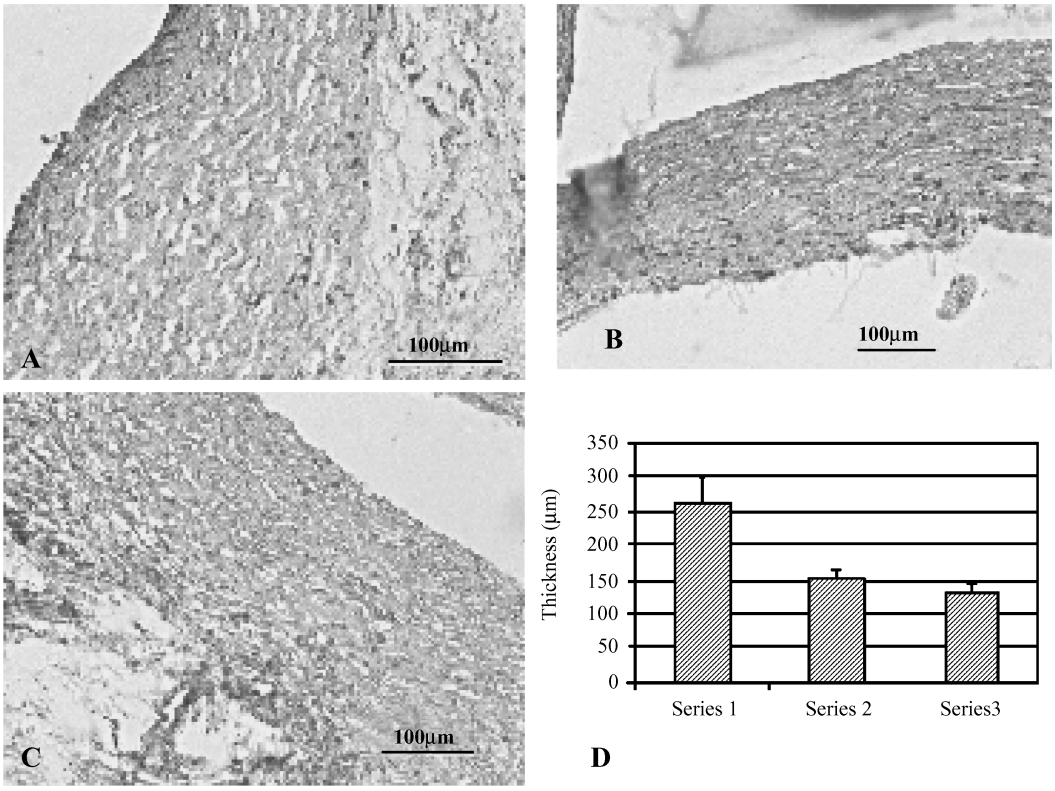

Figure 8 Connective tissue covering the implant of Series 1(A), 2 (B) and 3(C) and mean thickness of capsules (D). Hematoxilin and eosin, bar is 100 μm.

comunicating and hence the described materials are suitable from the morphological viewpoint for the envisaged purpose, i.e. as implants in neural tissues, possessing a potential of neural and glioma cells growing through implants. In subsequent studies, we are going to investigate in detail the influence of chemical structure of the prepared hydrogels on their suitability for the intended application.

Concerning the *in vivo* biological behavior of tested polymers, the surface was only poorly colonized with cells such as macrophages and fibroblasts (Fig. 7(A)). The numerous calcified defects were observed on all series of studied polymers (Fig. 7(B)). The connective tissue surrounding the implant surface was formed by collagen fibers with very low extent of inflammatory cell infiltration. The implants of Series 1 were surrounded with capsule which thickness was higher than in other implants (Fig. 8). The observed results are in good agreement with our previous results [16], which demonstrated a favorable biocompatibility of anionic polymers. In conclusion the studied materials were well tolerated by host organism.

Acknowledgments

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