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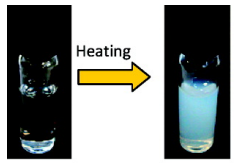
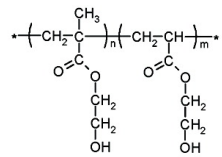
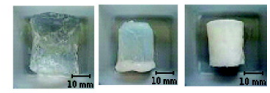


Temperature-responsive soluble copolymers





Hydrogels

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Designing Temperature-Responsive Biocompatible Copolymers and Hydrogels Based on 2-Hydroxyethyl(meth)acrylates

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Free-radical copolymerization of 2-hydroxyethyl methacrylate with 2-hydroxyethyl acrylate can be successively utilized for the synthesis of water-soluble polymers and hydrogels with excellent physicochemical properties, thus showing promise for pharmaceutical and biomedical applications. In the work presented it has been demonstrated that water-soluble copolymers based on 2-hydroxyethyl methacrylate and 2-hydroxyethyl acrylate exhibit lower critical solution temperature in aqueous solutions, whereas the corresponding high molecular weight homopolymers do not have this unique property. The temperature-induced transitions observed upon heating the aqueous solutions of these copolymers proceed via liquid−liquid phase separation. The hydrogels were also synthesized by copolymerizing 2-hydroxyethyl methacrylate and 2-hydroxyethyl acrylate in the absence of a bifunctional cross-linker. The cross-linking of these copolymers during copolymerization is believed to be due to the presence of bifunctional admixtures or transesterification reactions. Transparency, swelling behavior, mechanical properties, and porosity of the hydrogels are dependent upon the monomer ratio in the copolymers. Hydrogel samples containing more 2-hydroxyethyl methacrylate are less transparent, have lower swelling capacity, higher elastic moduli, and pores of smaller size. The assessment of the biocompatibility of the copolymers using the slug mucosal irritation test revealed that they are also less irritant than poly(acrylic acid).

Introduction

Ocular drug delivery is an interesting and challenging opportunity for pharmaceutical development. The eye is protected by a series of complex defense mechanisms, which usually result in poor bioavailability of drugs administered via the ocular route. These include tear production and the blinking reflex in addition to the complex nature of the corneal epithelium which leads to short residence time and reduced drug permeability. The drug bioavailability can be enhanced either by providing prolonged/sustained delivery to the eye or by facilitating transcorneal penetration.[1] The application of soft contact lenses, ocular inserts, viscosity-enhancing/phase transition systems and mucoadhesive polymers have been demonstrated as promising approaches to extend the residence time of various drugs in the eye.[2]

2-hydroxyethyl methacrylate (HEMA) has attracted great attention over several decades as a monomer to develop a number of polymeric materials for ophthalmic applications. The interest to HEMA polymers stemmed from Wichterle and Lim's pioneering paper in *Nature*,[3] which reported the synthesis of HEMA-based hydrogels and their potential for biological applications. Nowadays, hydrogels based on HEMA are commercially produced and widely applied as soft contact lenses. HEMA-polymers have also been used as drug delivery systems, bonding reagents in dentistry, implants, and matrices for immobilization of enzymes and cells.[4,5] The unique features of HEMA-based hydrogels are their biocompatibility and excellent physicochemical and mechanical properties.

HEMA homopolymer, poly(2-hydroxyethyl methacrylate) (PHEMA), has generally been regarded as a hydrophilic but water-insoluble material.[6,7] However, recently Weaver and coworkers[8] demonstrated that linear PHEMA having a degree of polymerization (DP) < 40 is completely water-soluble at 20 °C and pH 6.5, whereas the homopolymers with DP > 40 are only able to swell in water. Moreover, the water-soluble fractions of PHEMA with 30 < DP < 45 exhibited lower critical solution temperature (LCST) in water, that is, their aqueous solutions underwent phase separation upon heating. A statistical copolymerization of HEMA with glycerol monomethacrylate (GMA) also resulted in water-soluble copolymers displaying similar temperature-responsive behavior. For example, an addition of 11 mol % GMA led to a completely water-soluble HEMA-GMA copolymer with a cloud point of 33 °C, which is 5 °C higher than the phase transition temperature observed for PHEMA. These findings look quite promising for the development of novel polymeric forms for ocular drug delivery taking into consideration excellent ocular tolerability of HEMA polymers and the presence of temperature-responsive properties in their aqueous solutions. The LCST behavior of these polymers may be utilized in the design of in situ gelling drug delivery systems, which may exist as a liquid formulation at low temperatures (5−10 °C in a fridge) and undergo phase transition (gelation) upon instillation into the cul-de-sac of the eye due to increased temperature of up to 35−37 °C. This gelation would ensure a good retention of a formulation in the eye, improving drug bioavailability.

2-Hydroxyethyl acrylate (HEA) is the closest analogue of HEMA and its linear homopolymer PHEA can be soluble in water even when its molecular weight is relatively high.[9] However, PHEA does not exhibit temperature-responsive

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behavior in aqueous solutions. In a number of recently published studies we have demonstrated that statistical copolymerization of HEA with relatively hydrophobic monomers such as vinyl butyl ether (VBE)[10] and butyl acrylate (BA)[11] results in copolymers exhibiting temperature-responsive properties in aqueous solutions. The presence of few hydrophobic fragments in the macromolecules of hydrophilic polymers is believed to strengthen the role of hydrophobic effects, which are enhanced upon increase in temperature along with disruption of hydrogen bonds between water and hydrophilic functional groups leading to phase separation in a polymer−water system.

Inspired by a successful attempt of Weaver and co-workers[8] in the synthesis of HEMA-based temperature-responsive polymers, and also by our previous data on LCST behavior of HEA-VBE/BA copolymers,[10,11] we decided to explore the possibility of synthesizing HEMA-HEA with a similar behavior. The analysis of literature revealed only a few papers reporting copolymerization of HEMA and HEA. Jansen and co-workers[12] have studied the copolymerization of HEMA with HEA initiated by ultraviolet irradiation. They followed this copolymerization using real-time infrared spectroscopy, combined with advanced and alternative multivariate statistical data analysis. The reactivity ratios for HEMA-HEA copolymerization were found to be $r_1 = 1.6$ and $r_2 = 0.15$, which indicates higher reactivity of HEMA. However, neither the structure of the resulting copolymers nor their physical properties and solubility were evaluated in this study.

In the present work we synthesized HEMA-HEA soluble copolymer and hydrogel samples by free-radical copolymerization. The structure of the soluble copolymers was characterized by ^{1}H NMR spectroscopy and gel permeation chromatography. We studied their solubility as a function of temperature and demonstrated the existence of temperature-responsive behavior for water-soluble copolymers of certain composition. To study the structure and properties of hydrogels we used texture analysis, scanning electron microscopy and evaluation of their swelling behavior. The investigation of the mechanical properties of the hydrogels allowed for the calculation of the structure parameters of the networks such as the average molecular weight between cross-links. The porous structure of the hydrogels was probed in solid state after freeze-drying using scanning electron microscopy. The kinetics of swelling of these hydrogels was also studied and the mechanism of water diffusion into the samples was evaluated. The biocompatibility of HEMA-HEA copolymers was estimated using a slug irritation assay.

Materials and Methods

Materials. 2-Hydroxyethyl acrylate, 2-hydroxyethyl methacrylate, phosphate buffered saline (PBS), poly(acrylic acid) (450 kDa), and benzalkonium chloride were purchased from Sigma-Aldrich (U.K.) and used without further purification. According to the manufacturer specification, 2-hydroxyethyl acrylate and 2-hydroxyethyl methacrylate were of ≥97.0% and 99.0% degrees of purity and were stabilized with ~0.001 and 0.025% hydroquinone monomethyl ether, respectively. 2,2-Azoisobutyronitrile (AIBN) was purified by recrystallization from ethanol. Ethanol and cyclohexane were purchased from Fisher Scientific (U.K.) and used without purification. Deionized water was used for preparation of all aqueous solutions. Dimethyl sulfoxide-d_6 used for recording ^{1}H NMR spectra was a product of Cambridge Isotope Laboratories, Inc. (U.S.A.). Dialysis tubes (membranes with molecular weight cutoff 12−14 kDa) were purchased from Medicell International Ltd. (U.K.).

Synthesis of Soluble Copolymers. Three series of soluble HEA-HEMA polymers were synthesized by free-radical polymerization at various monomer ratios in the feed mixture (0−100 mol %). Briefly, 0.0082 g (5×10^{-3} g/mol) of initiator (AIBN) was dissolved in 14 mL of ethanol at room temperature. Then both HEA and HEMA were added into the solution and the mixture was shaken thoroughly until formation of homogeneous solution. The exact amounts of the monomers used for synthesis are shown in Supporting Information. The feed mixture was purged with nitrogen for 15 min to remove dissolved oxygen, then the reaction vials were sealed and placed in water bath at 60 °C for 140 min. To stop the polymerization the vials were cooled down using tap water for 5 min, which resulted in the termination of the initiation reaction, then the reaction mixtures were transferred into dialysis tubes and dialyzed against deionized water for 5−6 days. Then the solutions were freeze-dried in a Heto PowerDry LL3000 Freeze-Dryer (Thermo Scientific), and the polymers were stored in a fridge until further use.

Characterization of Soluble Polymers. The molecular parameters of water-soluble polymers (weight- and number-average molecular weight, intrinsic viscosity) were determined using Viscotek Triple detection GPC/SEC (refractive index, light scattering, and viscosity) system. The samples were analyzed in 0.05 mol/L LiBr dimethylformamide solutions at 60 °C.

The ^{1}H NMR spectra of the polymers were recorded in deuterated dimethylsulfoxide using Bruker AMX 400 MHz spectrometer. The compositions of the polymers were calculated using the following formula:

$$\omega_{\mathrm{HEMA}} = \frac{1}{3} \cdot \frac{I_{0.8}}{I_{4.8}} \cdot 100\% \qquad (1)$$

where ω_{HEMA} is the content of HEMA in copolymer and $I_{0.8}/I_{4.8}$ are the integrals of the chemical shifts at 0.8 and 4.8 ppm, typical for methyl groups in HEMA and OH-groups in both comonomers, respectively.

Dynamic Light Scattering Studies. The temperature-responsive behavior of water-soluble HEMA-HEA polymers in aqueous solutions was studied by dynamic light scattering (DLS) at 10−60 °C using a Malvern Zetasizer Nano-S (Malvern Instruments, UK). Each DLS experiment was repeated in triplicate by preparing and analyzing solutions of each polymer sample separately. The results were statistically treated and presented as an average.

Synthesis of Hydrogels. Hydrogels were synthesized using similar monomer mixtures and concentration of initiator but the polymerization was conducted at 60 °C for 18.5 h. The hydrogels were purified by immersing in deionized water, which was changed daily, for two weeks to remove any unreacted chemicals.

Mechanical Properties of the Hydrogels. The elastic moduli of the fully water-swollen HEMA-HEA hydrogels were determined using TA XT.plus Texture Analyzer (Stable Microsysrems, U.K.) in compression mode at room temperature. The cylindrical shape hydrogel samples with height 9.5−10 mm and contact area 45−55 mm^2 were fixed on the sandpaper and compressed by the upper load cylinder-probe (P/20) at a test speed 0.1 mm/sec. The distance target mode was set up during all experiments. The elastic moduli were determined from the initial parts of the stress/strain curves. Four or five samples were measured for each polymer and the results were statistically treated to calculate the average values and standard deviations.

Determination of Density of the Cross-Linked Polymers. The density of freeze-dried cross-linked polymers used for calculating molecular weight between cross-links was determined in 4.97 mL Gay-Lussac bottle using cyclohexane as a medium as described in Supporting Information.

Determination of the Average Molecular Weight between Cross-Links. The average molecular weight between cross-links M_c was determined as described in reference 13a−c using the following equation:

$$M_c = \rho_p / v_c^* \qquad (2)$$

The density of cross-linking v_c^* was calculated from the results of stress−strain measurements:

$$G = v_c^* \cdot R \cdot T \cdot v_{2,r}^{\frac{2}{3}} \cdot v_{2,s}^{\frac{1}{3}} \quad (3)$$

where G is the elastic modulus of the swollen hydrogel, R is the universal gas constant, T is the absolute temperature (K), and $v_{2,r}$ and $v_{2,s}$ are the volume fractions of polymer in relaxed (after preparation) and swollen states, respectively. These were calculated using the following equations:

$$v_{2,s} = [1 + \mathrm{SD}\rho_p/\rho_s]^{-1} \quad (4)$$

$$v_{2,r} = C_o \cdot M_r/\rho_p \quad (5)$$

where C_o is the total monomer concentration in the feed mixture, M_r is the average molecular weight of the repeating unit in the polymer, and ρ_p and ρ_s are densities of polymer and solvent, respectively. Considering that the swelling experiment was carried out in deionized water, the density of water was taken as a density of solvent.

To investigate the swelling properties of the cross-linked polymers, dry samples were weighed and immersed into excessive amount of deionized water at room temperature. After regular time intervals the samples were taken out, wiped from excessive water and their weight was recorded. The experiment was continued until a constant weight of swollen samples was achieved. The equilibrium swelling degree (SD, g/g) of hydrogels was calculated by the following equation:

$$\mathrm{SD} = (W_s - W_d)/W_d \quad (6)$$

where W_s and W_d are the weights of the swollen and dry gel, respectively.

The average molecular weight of the repeating units in the copolymers was calculated as

$$M_r = \omega_{\mathrm{HEA}} \cdot M_{\mathrm{HEA}} + \omega_{\mathrm{HEMA}} \cdot M_{\mathrm{HEMA}} \quad (7)$$

where ω_{HEA} and ω_{HEMA} are the molar fractions of the monomers in copolymers and M_{HEA} and M_{HEMA} are the molecular weights of HEA and HEMA, respectively.

Porous Structure of the Hydrogels after Freeze-Drying. Scanning electron microscopy (SEM) was used to study the porous structure of the hydrogels. For this purpose small pieces of freeze-dried samples were gold coated on Sputter-coater and then examined using an FEI Quanta FEG 600 environmental Scanning Electron Microscope with an acceleration voltage of 20 kV.

Swelling Kinetics of Hydrogels. Dry cross-linked polymers were weighed, and then immersed in excessive amount of deionized water at room temperature. At regular time intervals, the samples were taken out, wiped of excessive water with a filter paper, weighed, and returned to the swelling medium. The experiment was continued until a constant weight of samples was achieved.

Slug mucosal irritation test. The slug mucosal irritation test was carried out as described by Adriaens et al.[14a,b] with minor changes. *Limax flavus* slugs weighing 3−8 g were sourced locally (Reading, U.K.). Individual slugs were kept in 2.5 L glass beakers lined with a paper towel moisturized with 20 mL PBS solution and left at room temperature for two days before the start of an experiment.

In both positive and negative controls, Whatman filter paper moisturized with 2 mL 1% benzalkonium chloride in PBS and 2 mL PBS solution, respectively, were used to line 90 mm Petri dishes. The samples of HEMA-HEA films (0; 20.0; 33.3; 36.7; 46.7; 53.3 and 100 mol % of HEA content in copolymers) were prepared by casting 0.2 w/w % ethanol solutions of the copolymers into Petri dishes with subsequent vacuum drying. These samples were moistened with 2 mL of PBS solution just before the experiments with slugs were carried out.

Each slug was individually weighed before the experiment and then placed in Petri dishes containing either polymeric sample or positive/negative controls. After a 1 h contact period, slugs were taken out, rinsed with 10 mL of PBS, gently wiped with a tissue paper, and reweighed. The mucus production (MP) was estimated as a slug body weight loss and calculated by the following formula:

$$\mathrm{MP} = (m_b - m_a)/m_b 100\ \% \quad (8)$$

where m_b and m_a are the weights of a slug before and after the experiment, respectively. Each experiment was repeated 3−4 times using different slugs and the results were statistically treated, calculating the average values and standard deviations.

Results and Discussion

Synthesis and Characterization of Linear Polymers. The copolymers were synthesized by free-radical copolymerization initiated by thermal decomposition of 2,2-azoisobutyronitrile. We have chosen this polymerization type because of the intention to synthesize polymers of high molecular weight. Additionally, free-radical polymerization is relatively inexpensive and widely used for industrial production of polymers.

Figure 1 shows the compositions of the copolymers as a function of HEA content in the feed mixture. These compositions were determined by ^{1}H NMR spectroscopy by comparing the chemical shift observed at 0.5−1.0 ppm typical for protons of HEMA methyl group with the signal at 4.8 ppm, characteristic for protons of hydroxyl groups belonging to both monomeric units. The intensity of signals at 0.5−1.0 ppm decreased logically with an increase in HEA content in the feed mixture.

The copolymers are always enriched with HEMA, which indicates its higher reactivity in copolymerization. This result is in good agreement with the reactivity ratios reported for this system by Jansen and co-workers.[12] The higher reactivity of HEMA should result in higher probability for this monomer to form dyads, triads and tetrads in the macromolecules, which may affect their physicochemical behavior.

Water-soluble polymers were analyzed using gel permeation chromatography with triple detection (refractive index, light scattering and viscosity). The molecular characteristics of these polymers are summarized in Table 1. The molecular weights of the polymers broadly range from 291 to 2664 kDa and all samples are highly polydispersed. These data indicate the formation of highly branched macromolecules, which may be caused by the presence of bifunctional admixtures such as ethyleneglycol di(meth)acrylate in both monomers. Additionally, a participation of $-CH_2-CH_2-OH$ groups during radical polymerization can also contribute to branching. Formation of similar branched macromolecules was previously reported for polymerization of HEMA[6] and also for monomers having

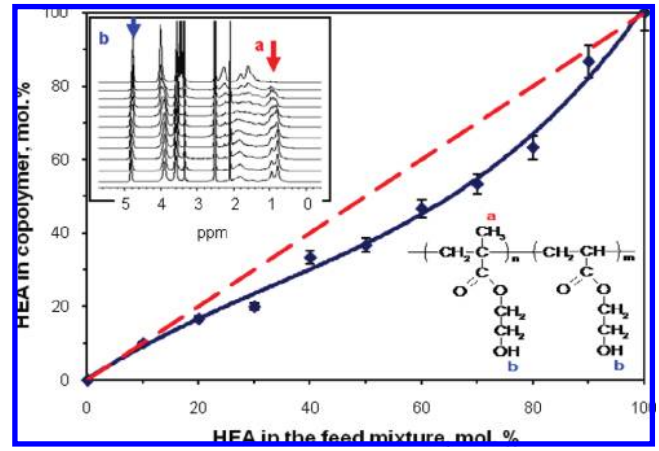

Figure 1. Composition of soluble copolymers as a function of the feed mixture (experimental points were plotted with 95% confidence intervals). Insets: ^{1}H NMR spectra (100 to 0 mol % of HEA) and structural formula of HEMA-HEA copolymers (*n* and *m* show the relative content of monomeric units in the copolymers).

Table 1. Molecular Characteristics of Water-Soluble Polymers

HEA in the feed mixture, mol %	HEA in copolymers, mol %[a]	refractive index increment (d*n*/d*c*)	M_w, kDa	M_w/M_n	intrinsic viscosity in DMF, 0.05 M LiBr at 60 °C
100	100	0.0662	308	3.38	0.57
90	87	0.0657	1862	3.79	0.88
80	63	0.0691	291	2.27	0.82
70	53	0.0715	2664	2.90	1.72

[a] Determined by ^{1}H NMR spectroscopy.

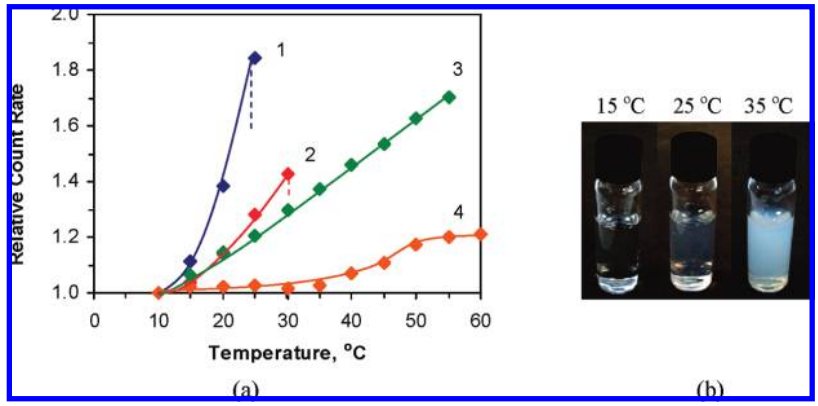

Figure 2. Relative count rate as a function of temperature in 10 mg/mL solutions of HEMA-HEA copolymers containing 53 (1), 63 (2), 87 (3), and 100 mol % (4) of HEA (a); images of 10 mg/mL solution of HEMA-HEA (47:53 mol %) at different temperatures (b).

$-CH_2-CH_2-OH$ and $-CH_2-CH_2-NH_2$ pendant groups.[15a,b] The lack of trend in the M_w data for the sample synthesized from the feed mixture containing 80 mol % of HEA likely results from purification procedure, in which we possibly lost the higher molecular weight fraction due to its poor solubility at room temperature.

Temperature-Responsive Behavior of Linear Copolymers in Solutions. The copolymers containing more than 53 mol % HEA were found to be soluble in water and their solution behavior was studied by dynamic light scattering at different temperatures using relatively dilute solutions (10 mg/mL). All the studied solutions were optically transparent at 10 °C according to the visual inspection; however, their light scattering ability was slightly different. This may have been caused by the difference in their branching, which was not controlled quantitatively in this work. In order to compare the temperature-responsive properties observed for different polymer solutions we present here the normalized (relative) count rate, which was calculated as a count rate at a given temperature against the count rate at 10 °C (Figure 2a). The solutions of the copolymers containing 53 and 63 mol % HEA show a clear phase separation upon heating, which is accompanied by significant increase in a relative count rate. When the temperature reaches 25 and 30 °C for copolymers containing 53 and 63 mol % HEA, respectively, the partial precipitation of the copolymers occurred and made further light scattering measurements meaningless. The typical changes in the solutions appearance upon heating are presented in Figure 2b.

It is interesting to note that the aqueous solution of the copolymer containing 87 mol % HEA did not exhibit any changes in a wide range of temperatures that could be detected by the naked eye. However, the light scattering data still indicated a significant growth in count rate, which is possibly related to the formation of smaller micellar structures compared to larger aggregates formed by copolymers with lower HEA content. Homopolymer PHEA (curve 4) shows only a slight increase in light scattering, which was observed at 40−50 °C,

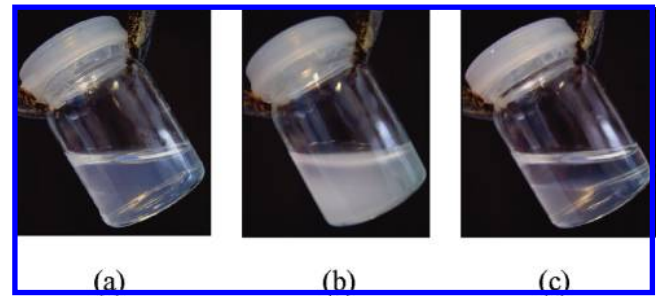

Figure 3. Aqueous solutions of HEMA-HEA (47:53 mol %) at 10 °C (a) and 37 °C (b). Sample (c) was heated to 37 °C and after achieving a phase separation was left cooling at room temperature for 30 min. Concentration of copolymer in solutions is 188.8 mg/mL.

the temperature region where hydrogen bonds between water and functional groups of a polymer are expected to become disrupted.[16]

The dynamic light scattering results clearly indicate the possibility of preparing temperature-responsive systems based on HEMA-HEA copolymers. However, from a practical point of view, it was interesting to achieve temperature-induced sol−gel transitions, which would form the basis for developing in situ gelling systems. For this purpose, we studied the behavior of HEMA-HEA (47:53 mol %) in more concentrated solutions (94.4, 188.8, and 377.5 mg/mL) at two different temperatures 10 and 37 °C, which correspond to the temperatures of a formulation in the fridge and after instillation into the eye. Figure 3 shows temperature-dependent transitions in solutions containing 188.8 mg/mL of the copolymer. The data on 94.4 and 377.5 mg/mL solutions are shown in the Supporting Information.

In the studied concentration range, the solutions became cloudier upon heating but did not lose their ability to flow. Moreover, when the phase separated solution was left cooling at room temperature, a liquid−liquid phase separation was observed with a formation of two clearly distinguishable immiscible liquid layers (Figure 3c). Thus, an increase of the copolymer concentration up to 377.5 mg/mL did not result in temperature-induced gelation. Similar liquid−liquid phase sepa-

ration or coacervation was previously reported by Miyazaki and Kataoka[17] for statistical copolymers of *N*,*N*-dimethylacrylamide-*co*-*N*-phenylacrylamide, by Yin and Stöver[18] for *N*,*N*-dimethylacrylamide-*co*-glycidyl methacrylate and more recently by Maeda and co-workers[19a,b] for *N*-isopropylacrylamide-*co*-2-hydroxyisopropylacrylamide. Unlike the systems mentioned above, the classical temperature-responsive homopolymer of *N*-isopropylacrylamide (NIPAAM) in aqueous solutions undergoes a sharp coil-globule transition at 32 °C and precipitates as a solid. The gelation behavior was also reported for block copolymers. For example, Pluronics (triblock copolymers of ethylene glycol-propylene glycol-ethylene glycol)[20] have been widely used for formulating in situ gelling systems. Sugihara and co-workers[21] observed temperature-induced gelation in aqueous solutions of diblock copolymers based on 2-(2-ethoxy)ethoxyethyl vinyl ether and 2-methoxyethyl vinyl ether. A rapid gelation occurred upon heating of 20 wt % aqueous solution of the diblock copolymer up to 42 °C resulting in transparent physical gel. Zhao and co-workers[22] reported a successful attempt to prepare in situ gelling copolymers based on *N*-isopropylacrylamide (NIPAAM)-HEMA triblock copolymers. A free-standing opaque gels were formed upon heating of 1 − 10% solutions of $HEMA_{40}$-$NIPAAM_{640}$-$HEMA_{40}$ and $NIPAAM_{320}$-$HEMA_{80}$-$NIPAAM_{320}$ (here, subscripts show the degree of polymerization). Based on the analysis of the above-mentioned studies and our own experimental data, it can be hypothesized that random copolymers exhibiting temperature-responsive properties undergo liquid−liquid phase separation (coacervation) and do not form gels even in concentrated solutions. Gelation can be achieved for copolymers having more regular structure such as block copolymers (for example, Pluronics). However, this hypothesis has to be confirmed experimentally as at the moment there are many studies published on novel temperature-responsive polymers but only a few reports about the mechanism of their phase separation (liquid−liquid or liquid−solid). Additionally, the architecture of polymers such as linear vs branched macromolecules may play an important role in the mechanism of phase separation. Recently, Rimmer and co-workers[23a,b] reported that the highly branched temperature-responsive NIPAAM-based polymers exhibited lower cloud points compared to the equivalent linear macromolecules. In the future, we will attempt to synthesize block copolymers based on HEMA and HEA and study their behavior in aqueous solutions. We expect that the solutions of these block copolymers will be able to form gels upon heating.

Synthesis and Characterization of HEMA-HEA Hydrogels. The cross-linked copolymers formed when the polymerization was left to proceed for 18.5 h. It can be hypothesized that the cross-linking occurred because of the presence of bifunctional admixtures in both monomers.[8] Additional contribution to the cross-linking process may be due to transesterification reactions with participation of hydroxyl groups of HEMA or HEA.[24] The yield of the copolymers increased with higher HEMA content in the feed mixture (Figure 4). After washing the unreacted monomers the cross-linked samples were left in water to reach equilibrium swelling. The equilibrium swelling degrees of the hydrogels were found to be dependent on the monomer ratios in the feed mixture. Because it was not possible to determine the real monomer ratio in the hydrogels, here we will discuss the properties of the samples as a function of the composition of the feed mixtures. When the content of HEA in the monomer mixture was less than 40 mol % the hydrogels exhibited relatively low swelling capacity (~3 g of water per 1 g of dry polymer). A further increase of HEA

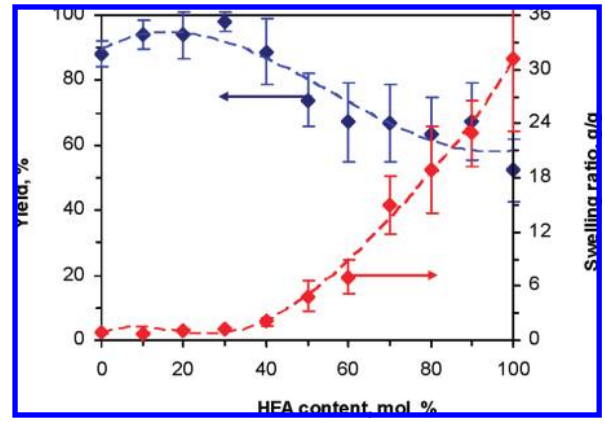

Figure 4. Yield and equilibrium swelling degree of hydrogels in distilled water at room temperature as a function of HEA content in the monomer mixture.

content in the feed mixture resulted in a significant increase in the equilibrium swelling degree up to 32 g/g for PHEA homopolymer hydrogel.

The transparency of the fully swollen hydrogels was also greatly dependent on the monomer ratio in the feed mixture (Figure 5). The hydrogels formed from the monomer mixtures containing more than 70 mol % of HEA were totally transparent. When HEA content in the monomer mixture was 60 and 50 mol % the samples appeared translucent, and when it was lower the hydrogels were white and not transparent. The opacity of the hydrogels enriched with HEMA results from poor hydrophilicity of branched polymers, formed at earlier stages of polymerization causing a microphase separation.[25]

The evaluation of hydrogels' mechanical properties is extremely important to assess their suitability for a particular application. For example, when these hydrogels are to be used as implants or organ substitutes they should be mechanically compatible with the surrounding tissues. Moreover, the knowledge of the mechanical characteristics of hydrogels can be used to estimate the parameters of networks such as the average molecular weights between cross-links.[26] In the present study the mechanical properties of HEMA-HEA swollen hydrogels were estimated using a compression test. An example of the dependences of hydrogel compression as a function of applied force (stress−strain curves) can be found in the Supporting Information. These dependences were used for calculating the elastic moduli of the networks with subsequent determination of the average molecular weights between cross-links. The data on both parameters are presented in Figure 6 in logarithmic form, showing nearly linear trends. The elastic modulus determined for PHEMA is comparable with the literature data for similar hydrogels. For example, Wilder and co-workers[27] synthesized a series of PHEMA hydrogels with varied concentration of diethylene glycol dimethacrylate as a cross-linker (0.5−6.0%). The elastic moduli determined for these samples increased with cross-linker content in the feed mixture and ranged between 500 − 2500 kPa. Clearly, these gels exhibited better mechanical properties than our PHEMA samples (283 ± 24 kPa), which is likely to be due to more efficient cross-linking in the presence of a bifunctional cross-linker and lower water absorption.

The elastic moduli of HEMA-HEA hydrogels decreased with higher HEA content in the feed mixture, which means that the samples become softer. The average molecular weights between cross-links increased with the growth of HEA in the samples, that is, longer cross-linking chains are formed in more hydro-

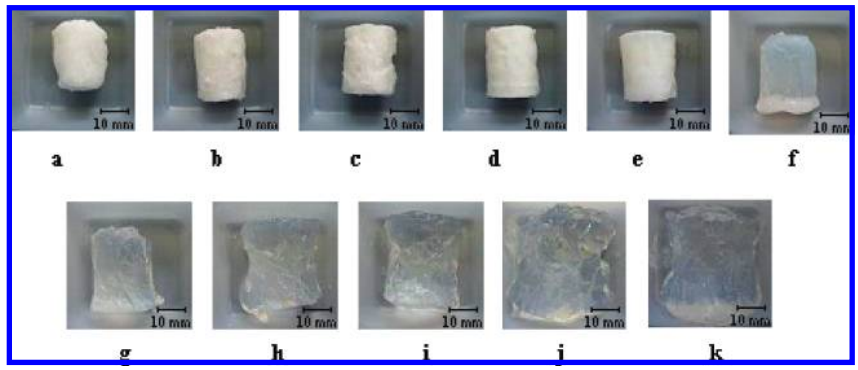

Figure 5. Images of hydrogels containing 0 (a), 10 (b), 20 (c), 30 (d), 40 (e), 50 (f), 60 (g), 70 (h), 80 (i), 90 (j), and 100 mol % HEA (k) in the feed mixture.

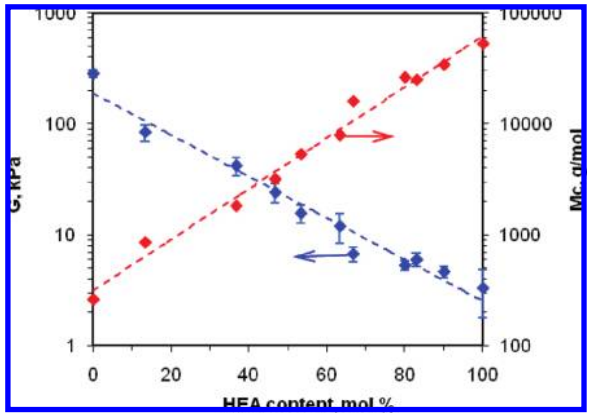

Figure 6. Elastic moduli of hydrogels and molecular weights between cross-links as a function of HEA content in the monomer mixture.

philic networks. However, the absolute values of M_c determined in our study appear to be relatively small, compared to the literature data, for example, for copolymers of HEMA with acrylamide.[13] It can be related to the method we used for drying our samples. The density of freeze-dried samples can be significantly lower than the values determined for samples, which were vaccuum-dried. In this case, the M_c values, calculated using eq 2, may be understated. It would be interesting to perform a comparative study and estimate the effect of drying method on the network structural parameters, which we may explore in the future.

The swelling behavior of the hydrogel, whose composition roughly corresponds to the composition of one of the temperature-responsive soluble copolymers, was studied at different temperatures (Figure 7). Practically no difference could be observed between the samples at 10 and 20 °C; however, the sample heated up to 37 °C shows reduced transparency. At 50 °C, a contraction of the network is observed, but its degree is not as high as reported for well-known temperature-responsive hydrogels, for example, based on poly(*N*-isopropyl acrylamide).[28] HEMA units in the copolymers are believed to contribute to the hydrophobic effects causing the phase separation in aqueous solutions of soluble copolymers and contraction of hydrogels. While, the hydrophobic interactions mediated by HEMA are sufficient to cause a collapse of a single macromolecule in solution, they are relatively weak to affect the conformation of macromolecules forming a network. A better degree of sample contraction might be achieved when HEMA and HEA are copolymerized and cross-linked in the presence of porogens leading to so-called superporous hydrogels.[29] In superporous hydrogels the chains of copolymers should experience less conformation restrictions and are expected to contract more efficiently.

The porous structure of the hydrogels synthesized in the present work was estimated by analyzing freeze-dried samples by scanning electron microscopy (SEM). The SEM images of selected hydrogel samples are shown in Figure 8. The PHEA homopolymer was highly hygroscopic and absorbed water from air immediately after freeze-drying resulting in very sticky sample. By this reason it was not possible to obtain an image showing its porous structure. For the rest of the samples, a clear porous morphology was observed. As is expected pore size changes with the composition of the feed mixture. Larger pores are observed for hydrogels containing more HEA in copolymers. For example, the pore size found for the sample synthesized from the feed mixture containing 80 mol % HEA was around 304 ± 66 μm, whereas the size of pores in PHEMA-based sample is approximately 65 ± 21 μm. The data on porosity of the hydrogels are consistent with the results on their transparency and swelling capacity, that is, the samples having larger pores are more transparent and exhibit higher degrees of swelling.

Swelling Kinetics of Hydrogels. The kinetics of hydrogels swelling in water was studied using gravimetric approach and a selection of results is presented in Figure 9a. The equilibrium swelling degrees are achieved approximately after 50−100 h of swelling. To have an insight into the mechanism of water sorption by hydrogels the kinetic data were analyzed by fitting into the semiempirical equation proposed by Ritger and Peppas:[30]

$$\frac{M_t}{M_0} = k \cdot t^n \tag{9}$$

where M_t is a water intake (g) at time t (min) and M_0 is the weight of the dry polymer, k is a characteristic constant of a hydrogel, and n is a characteristic exponent of the transport mode of the penetrant (water). It should be noted that this equation is valid only at $M_t/M_0 \leq 0.6$. The value of n provides information about the water sorption mechanism. When $n = 0.5$ the sorption of water by the samples obeys Fick's law. In this case diffusion is a rate limiting factor, that is, it is significantly slower than the rate of relaxation of the polymer chains. When $0.5 < n < 1.0$, it indicates a non-Fickian process, in which the water uptake is controlled by both diffusion and relaxation of polymeric chains. If $n = 1$ then the water uptake is predominantly controlled by chain relaxation, that is, the rate of penetrant diffusion is greater than the rate of relaxation of the polymer chains.

The experimental results obtained in the present work were analyzed by plotting the data in $\log(M_t/M_o) - \log(t)$ coordinates to give a linear correlation at $M_t/M_o < 0.6$ and n values were determined as a slop of this plot (see the

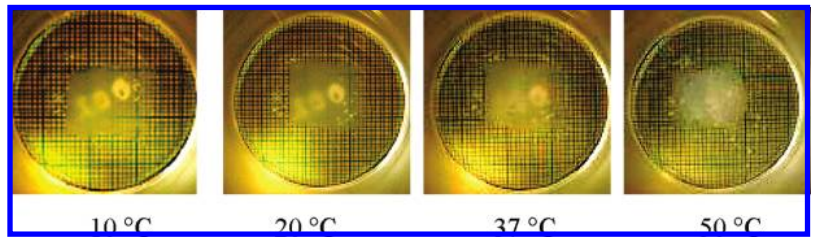

Figure 7. Temperature-responsive behavior of HEMA-HEA hydrogel (feed mixture 30:70 mol %).

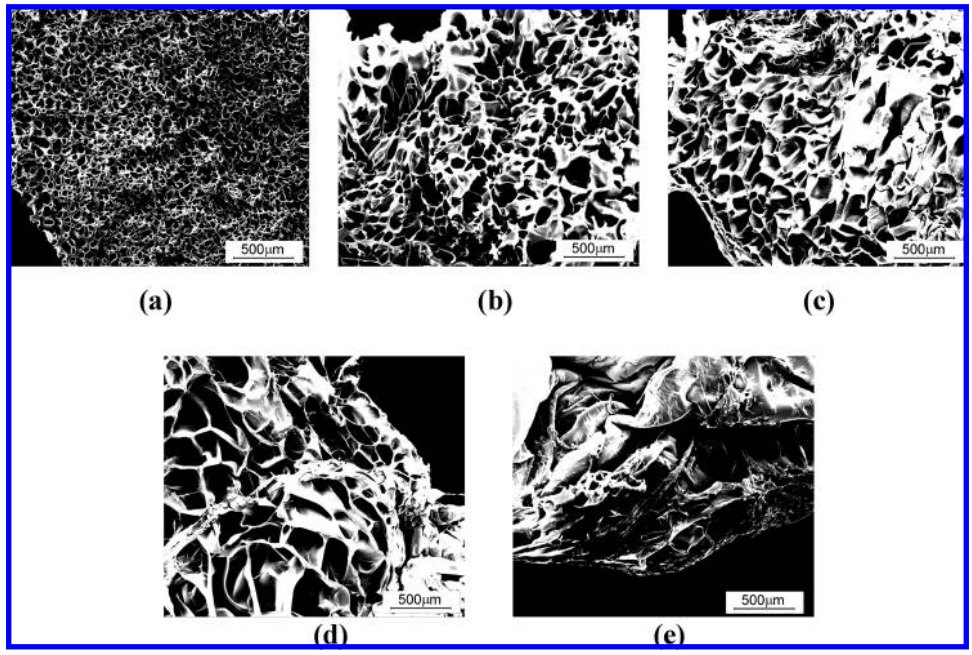

Figure 8. Porous structure of freeze-dried samples based on HEMA-HEA copolymers containing 0 (a), 50 (b), 70 (c), 80 (d), and 100 mol % of HEA (e) in the feed mixture.

exemplary plots in the Supporting Information). The n values determined for HEMA-HEA hydrogels are plotted in Figure 9b as a function of HEA content in the monomer mixture. There is a nearly linear dependence of n on HEA content in the feed mixture and the samples synthesized from the feed mixtures with up to 70 mol % HEA show Fickian diffusion (n ranges within 0.33−0.45). The Fickian diffusion of water into PHEMA hydrogels has also been previously reported by George et al.,[31] who synthesized PHEMA hydrogels by polymerizing HEMA in the presence of 0.5 wt % ethylene glycol dimethacrylate as a cross-linker and determined n to be equal to 0.48. Tomic et al.[32] have also reported n for PHEMA hydrogels equal to 0.44 and n values of 0.5−0.53 for HEMA copolymers with more hydrophilic itaconic acid. The Fickian diffusion of water into samples containing up to 70 mol % of HEA is possibly related to a limited mobility of polymer chains. When the content of HEA in the feed mixture grows it results in an increase of n. In the samples obtained from the monomer mixtures with more than 80 mol % of HEA the n values are larger than 0.5 and the contribution of the relaxation processes becomes higher. Similar observations were reported by Hariharan and Peppas[33] and George et al.[31] for copolymers of HEMA with more hydrophilic diethylamino ethyl methacrylate and ethylene glycol methacrylate phosphate, respectively. The hydrogels containing higher levels of hydrophilic comonomers show non-Fickian water uptake.

Biocompatibility of HEMA-HEA Copolymers. All new excipients intended for application in ocular drug delivery should pass a series of vigorous tests to assess their biocompatibility. One of the tests is the assessment of the ability of a material to cause an eye irritation. Traditionally, this is performed using so-called Draize test, where a material (chemical) is placed into conjuctival sac of one eye of an albino rabbit and the other eye serves as a control.[34a,b] The ocular response is evaluated at different time intervals looking at opacification of the cornea, swelling or hemorrhage of the iris and redness or discharge from the conjunctiva. However, this test has been heavily criticized because of its inhuman nature and a number of limitations.

Many modifications and alternatives to the Draize eye test have been developed during the past decade to reduce the number of animals by refining the test procedures and to replace their use with cell culture models or lower organisms (e.g., inveterbrates). Adriaens and Remon have developed a new test for screening irritation potential of chemicals using terrestrial slugs.[14a,b,35] Slugs release mucus to aid their locomotion and in response to irritation. Adriaens and Remon estimated the effect of 28 reference substances on the mucosal tissue of the slugs by measuring the amount of mucus produced and the concentration of proteins released as a result of exposure to the studied chemicals. This test was found to be a reliable and promising method for estimating the ocular irritancy of chemicals. The mucosal irritation potency of some polymeric formulations based on Carbopol, poly(acrylic acid) (PAA) and starch blends was also estimated using slug mucosal irritation test (SMIT) by the same group.[36,37] In the above-mentioned studies, Adriaens and co-workers used *Arion lusitanicus* slugs for SMIT experiments; however, recently they also published a paper

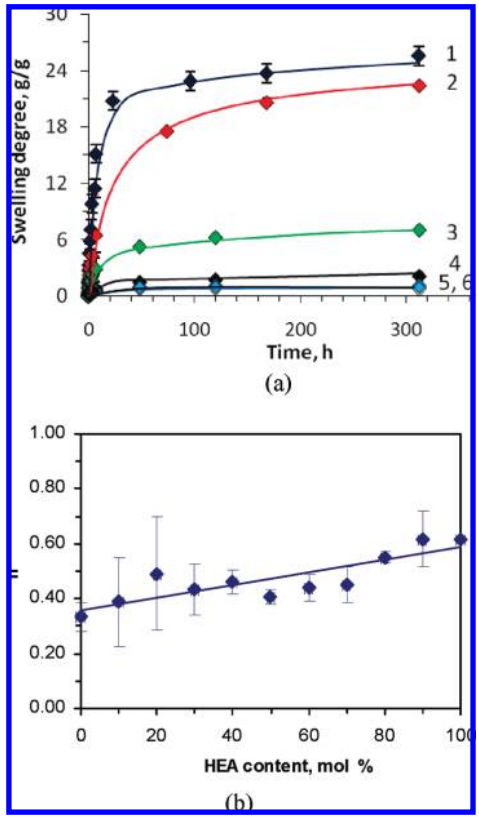

Figure 9. (a) Kinetics of HEMA-HEA hydrogels swelling. HEA content in the feed mixtures: 100 (1), 80 (2), 60 (3), 40 (4), 20 (5), and 0 mol % (6); (b) effect of HEA content in the feed mixture on the exponent *n*.

reporting the comparison of two slug species (*Arion lusitanicus* and *Limax flavus*).[38] *Limax flavus* slugs were found to express more mucus in response to an irritation compared to *Arion lusitanicus*

In the present work, we adapted the methodology reported by Adriaens et al.[14,35] and used *Limax flavus* slugs to estimate the ability of HEMA-HEA soluble copolymers to cause mucosal irritation. For this purpose, polymeric films based on HEMA-HEA copolymers of different compositions were prepared by casting from ethanol solutions on Petri dishes. After ethanol was evaporated in a vacuum oven at room temperature, the films were formed and used for testing. A 2% solution of benzalkonium chloride (BAC) in phosphate buffer saline (PBS) was used as a positive control and PBS solution alone was used as a negative control. The polymeric films were moisturized with 2 mL of PBS. In all experiments with HEMA-HEA polymers slugs released colorless mucus, which is a first indication of their relatively good biocompatibility. In experiments with a positive control, slugs experienced a severe irritation, releasing approximately 33 ± 14% of yellow mucus (Figure 10). A considerable variability of the data obtained from experiments with the positive control is explained by slugs' increased activity and tendency to avoid a contact with an irritant chemical. It should be noted that the amount of mucus released by slugs in our experiments with positive and negative control (33 ± 14 and 3.6 ± 1.0%, respectively) are slightly higher than the data reported by Dhondt et al.[38] They reported that *Limax flavus* slugs express 20.6 ± 4.5 and 1.4 ± 0.8% of mucus in positive and negative control experiments, respectively. The observed variability can be explained by slug population-specific effects demonstrated by Dhondt et al.[38] in the same study by comparing

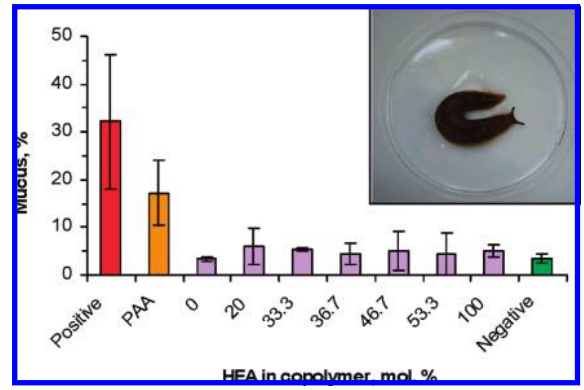

Figure 10. Mucus production by slug *Limax flavus* in response to the contact with PAA and HEMA-HEA copolymers. Inset: slug *Limax flavus* in experiment.

Arion lusitanicus slugs collected in Belgium and Switzerland. Taking into account this variability we also estimated the mucosal irritancy of the films based on poly(acrylic acid) as a third reference. This polymer is widely used in ophthalmic formulations but is known to cause a minor irritation.[39,40] The slugs exposed to poly(acrylic acid) released 17.2 ± 1.0% of mucus, confirming the ability of this polymer to irritate mucosal epithelia.

The ability of PHEMA to irritate slugs' mucosa was found to be even lower than the results obtained in a negative control experiment (3.3 ± 0.5 and 3.6 ± 1.0% of released mucus, respectively). This result was expected as PHEMA is known to be a highly biocompatible material with respect to ocular epithelia even for chronical exposure as it is widely used as a material for contact lenses.[4] The surface of a filter paper moisturized with PBS solution used as a substrate in negative control experiment is rougher compared to PHEMA gel, so it caused slugs to release more mucus to aid their locomotion. A slight increase in the amount of mucus secreted by slugs was observed for HEMA-HEA copolymers compared to PHEMA samples. However, the amount of mucus released by slugs upon their exposure to HEMA-HEA copolymers is nearly 2−3 times lower compared to the effect of the films based on PAA. Hence, the copolymers can be potentially nonirritant materials, promising for applications in ophthalmology. However, further biological testing is required to prove their complete biocompatibility.

Conclusions

Free-radical copolymerization of 2-hydroxyethyl methacrylate and 2-hydroxyethyl acrylate results in the formation of branched copolymers with high molecular weight at lower degrees of conversion and cross-linked networks at later stages. The branching of copolymers is believed to be due to the presence of bifunctional admixtures in the monomers or alternatively because of chain transfer reactions to $-CH_2-CH_2-OH$ groups during radical polymerization. The branched copolymers are soluble in water when the content of 2-hydroxyethyl acrylate is higher than 53 mol %. The water-soluble copolymers exhibit lower critical solution temperature in aqueous solutions. Above this temperature their solutions undergo a liquid−liquid phase separation.

The structure and properties of the cross-linked copolymers (hydrogels) are dependent on the monomer ratios in the feed mixtures. Higher content of 2-hydroxyethyl methacrylate in the monomer mixtures results in formation of less transparent

hydrogels, having smaller pore size, higher elastic moduli and lower swelling capacity. The uptake of water by the hydrogels rich in 2-hydroxyethyl methacrylate is mainly determined by Fickian diffusion, whereas the role of chain relaxation processes becomes more important for more hydrophilic networks. The hydrogels of certain composition exhibit temperature-responsive properties, that is, they undergo contraction upon heating.

The evaluation of the copolymers using the slug mucosal irritation test demonstrated their nonirritant nature; however, further biological testing is required to prove complete biocompatibility and to explore the possibility of their application in ophthalmology.

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Supporting Information Available. Detailed description of the methodology used for determination of cross-linked copolymers density, composition of the feed mixtures used in synthesis of copolymers, images of aqueous solutions of the copolymers undergoing phase transition, example of stress−strain curves obtained for the hydrogels, and example of $\log(M_t/M_0) - \log(t)$ plots used for analysis of hydrogels' swelling kinetics. This material is available free of charge via the Internet at http://pubs.acs.org.

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