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Zwitterionic hydrogels crosslinked with novel zwitterionic crosslinkers: Synthesis and characterization

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ABSTRACT

Two novel zwitterionic sulfobetaine dimethacrylate crosslinkers*N*,*N*-bis(methacryloxyethyl)-*N*-methyl-*N*-(3-sulfopropyl)ammonium (CL1) and *N*,*N*-bis(methacryloxyethyl)-*N*-methyl-*N*-(4-sulfobutyl)ammonium (CL2) betaines were synthesized and used for preparation of zwitterionic hydrogels formed from *N*-(methacryloxyethyl)-*N*,*N*-dimethyl-*N*-(3-sulfopropyl)ammonium betaine (SBDMA) via redox-initiated free-radical polymerization. The commercially available crosslinkers *N*,*N*′-methylene bisacrylamide (BIS) and ethylene glycol dimethacrylate (EDMA) were also used. Equilibrium water content, sorption degree, diffusion coefficient of water, state of water, degree of crosslinking and mechanical properties were determined for hydrogels crosslinked using different crosslinking conditions. A minor difference in the spacer length between the charged moieties in CL1 and CL2 crosslinkers, respectively, was shown to influence the hydrogel properties. The CL1 and CL2 crosslinkers with chemical structure similar to SBDMA resulted in hydrogels with higher stiffness, mechanical strength and crosslink density compared to hydrogels crosslinked by BIS and EDMA. This difference was assigned to suppression of the compositional drift during the hydrogel formation when crosslinkers with chemical structure similar to monomer were used. PolySBDMA hydrogels exhibited a low adhesion of RAT-2 fibroblasts-like cells.

1. Introduction

Hydrogels assisting the life sciences in diverse areas of biomedicine and biotechnology represent a significant topic in current polymer science [1–3]. Applications of polymeric hydrogels include such areas as implants, medical devices and diagnostics, drug release, scaffolds and tissue engineering, enzyme and cell engineering and others.

Hydrogels based on synthetic polymers are made of mixtures of monomers and crosslinking agents, with the polymerization process typically carried out to the complete conversion. The chemical similarity of crosslinker and monomer is not always obeyed and, consequently, their different rates of incorporation to the polymeric network lead to compositional drift during hydrogel formation. This may negatively influence hydrogel properties due to the lack of control of the crosslinking process, which increases the heterogeneity of hydrogel network [4,5].

Ethylene glycol dimethacrylate (EDMA) and triethylene glycol dimethacrylate belong to frequently used commercially available crosslinking agents for methacrylate network formation, although their application may be limited due to low solubility in water [6]. *N*,*N*′-methylene bisacrylamide (BIS) is another regularly used crosslinking reagent. However, in application with methacrylic monomers the compositional drift may also impact the hydrogel formation due to different reactivity ratios between acrylate and methacrylate families [5,7]. In some cases, commercially available crosslinkers are not suitable for hydrogel network formation and then the synthesis of new crosslinkers is required [4,5,8]. For example, a hydrophilic crosslinker with a linkage similar to the phosphorylcholine structure was synthesized in order to prepare a hydrogel with methacrylic monomer containing the phosphorylcholine moiety [4]. The resulting hydrogel showed improved mechanical properties compared to the hydrogel prepared in the presence of BIS [6]. In the case of poly(*N*-vinyl pyrrolidone) [8], the pyrrolidone-based crosslinker was synthesized with the aim to eliminate the inconsistencies in the hydrogel properties due to differences in the reactivity ratios between *N*-vinyl pyrrolidone and dimethacrylate crosslinker. These examples demonstrate that the selection of crosslinker is crucial in the strategy used for preparation of hydrogels and in controlling their properties and performance.

Zwitterionic polymers represent a strongly developing class of polymers. They are based on the electrically neutral monomer units

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that formally contain both positive and negative charges on different atoms in a monomer unit. Zwitterionic polymers are of biomimetic character, which makes them suitable for designing non-biofouling materials and surfaces [9–13] with unique physical and chemical properties [14–16]. Zwitterionic monomers are based on various chemical structures and include, most typically, sulfobetaines, carboxybetaines and phosphorylbetaines. Among them, the sulfobetaine monomer (*N*-(methacryloxyethyl)-*N*,*N*-dimethyl-*N*-(3-sulfopropyl) ammonium betaine), SBDMA, is frequently used due to its low price, commercial availability and simple synthetic accessibility [17,18]. This monomer has been used in studies devoted to solution properties [15], kinetics of polymerization [19], reduced protein adsorption [20,21], blood biocompatibility [22,23] and *in vivo* tests [24].

Owing to the significance of zwitterionic hydrogels, we decided to synthesize novel water soluble methacrylate crosslinkers based on the sulfobetaine methacrylate monomer unit with the chemical structure matching the structure of SBDMA monomer. This approach should suppress the eventual compositional drift during the hydrogel formation. The aim was to evaluate the principal physico-chemical properties of the resulting hydrogels and to estimate whether this strategy may lead to enhancement of these properties. It should be mentioned that in parallel to our work, a similar strategy was published on polycarboxybetaine hydrogels by Carr et al. [5]. This paper demonstrated that using a structurally similar crosslinker to the monomer unit was beneficial for both non-biofouling and mechanical properties, which was discussed in terms of the hydrophilicity and homogeneity of the hydrogel network.

Newly designed crosslinkers *N*,*N*-bis(methacryloxyethyl)-*N*-methyl-*N*-(3-sulfopropyl) ammonium betaine (CL1) and *N*,*N*-bis(methacryloxyethyl)-*N*-methyl-*N*-(4-sulfobutyl) ammonium betaine (CL2) are shown in Fig. 1. They are structurally similar to SBDMA monomer also shown in Fig. 1. These crosslinkers differ only in the length of spacer, i.e. propyl vs butyl, separating the sulfate and ammonium moieties. This minor difference is expected to influence the degree of hydrophilicity of the network and, hence, tune the properties of the resulting hydrogel. The feasible synthesis of CL1 and CL2 is described. PolySBDMA hydrogels were characterized in terms of mechanical properties, diffusion coefficient of water, swelling in water as well as in aqueous solution of salts, different water state, degree of crosslinking, mesh size and fibroblast adhesion. Some of these properties were compared to polySBDMA hydrogels prepared by crosslinking with commercially available crosslinkers BIS and EDMA. The majority of work is devoted to hydrogels prepared at the typical crossslinker level ≤ 3 mol% to monomer. Sorption and mechanical properties were determined for hydrogel samples crosslinked by CL1 up to 20 mol% to monomer. This work demonstrates that crosslinkers with a sulfobetaine moiety of the same chemical character as monomer SBDMA can be recommended for formation of polySBDMA hydrogels. In this case, the compositional drift with conversion is expected to be minimized, which cannot be warranted with commercial crosslinkers of a different chemical character.

2. Experimental part

2.1. Materials and reagents

N-(methacryloxyethyl)-*N*,*N*-dimethyl-*N*-(3-sulfopropyl) ammonium betaine (SBDMA, Aldrich, 97%), *N*,*N*′-methylene bisacrylamide (BIS, Sigma–Aldrich, 99%), ethylene glycol dimethacrylate (EDMA, Aldrich, 98%), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMEDA, Fluka, >99%), ammonium peroxodisulfate (APS, Aldrich, >98%), 1,3-propanesultone (Aldrich, 98%) and 1,4-butanesultone

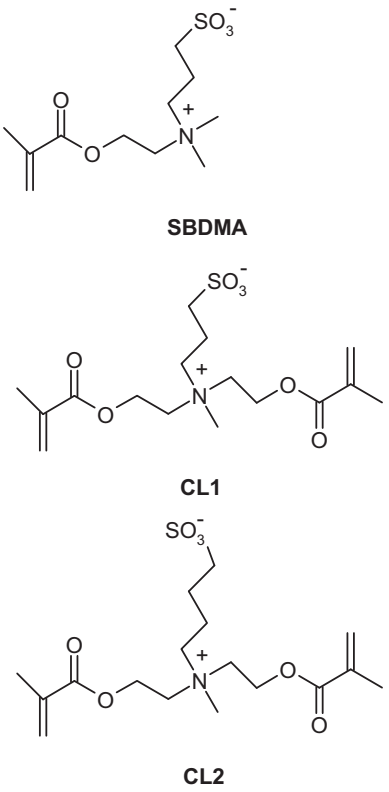


Fig. 1. Chemical structures of monomer *N*-(methacryloxyethyl)-*N*,*N*-dimethyl-*N*-(3-sulfopropyl)ammonium betaine (SBDMA), and crosslinkers *N*,*N*-bis(methacryloxyethyl)-*N*-methyl-*N*-(3-sulfopropyl)ammonium betaine (CL1) and *N*,*N*-bis(methacryloxyethyl)-*N*-methyl-*N*-(4-sulfobutyl)ammonium betaine (CL2).

(Aldrich, >99%) were used as received. *N*,*N*-bis(methacryloxyethyl) methylamine was prepared from methyl methacrylate and *N*,*N*-bis(2-hydroxyethyl)methylamine catalyzed with MeONa [25]. Acetone was dried over $CaCl_2$ and distilled before use. Ultrapure water was obtained from Ultrapure Water System NW Series (Heal Force Bio-Meditech Holdings, Ltd, China). All solvents and salts were of analytical grade.

RAT-2 fibroblasts-like cell line (ECACC, UK, Cat. No. 94050409) was used for testing the cell adhesion properties of prepared hydrogels. Cells were grown on tissue culture polystyrene flasks in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal calf serum, 2 mmol L^{-1} L-glutamine and 4.5 g L^{-1} glucose at 37 °C in humidified atmosphere containing 5% CO_2. All cell culture reagents were purchased from Gibco BRL (Invitrogen Corporation, Germany).

2.2. Synthesis of CL1

The crosslinker CL1 was prepared following Scheme 1. To an ice-cooled solution of 1.020 g (4 mmol) *N*,*N*-bis(methacryloxyethyl) methylamine **1** in 2 mL dry acetone, a solution of 520 mg (4.2 mmol, 0.390 mL) 1,3-propanesultone in 1 mL acetone was added drop-wise. The reaction mixture was stirred at 40 °C for 5 h. The white precipitate formed was filtered and washed with a small amount of dry acetone and diethylether. The filtrate was evaporated and 2 mL of dry acetone were added to the residue. The precipitate was formed at room temperature within one week. The white powder was obtained by centrifugation at a yield of 81%.

^{1}H NMR (300 MHz, CD_3OD): 6.15 d, 2 H, $J = 2.0$ Hz (=C–H); 5.57 d, 2 H, $J = 2.0$ Hz (=C–H); 4.64 t, 4 H, $J = 8.4$ Hz (O–CH_2); 3.88 t, 4 H, $J = 8.4$ Hz (N–CH_2); 3.70 m, 2 H (N–CH_2); 3.25 s, 3 H (N–CH_3);

2.82 t, 2 H, $J = 8.2$ Hz (S–CH_2); 2.24 m, 2 H (CH_2); 1.96 s, 6 H (=C–CH_3).

^{13}C NMR (75 MHz, CD_3OD): 167.7, 137.1, 127.5, 65.9, 63.3, 62.3, 58.9, 19.8, 18.4.

FTIR (cm^{-1}, KBr): 1728 s, 1638 m, 1458 w, 1456 w, 1338 m, 1329 m, 1204 s, 1171 s, 1040 s, 952 w, 938 w, 805 w, 630 w, 539 w, 529 w.

2.3. Synthesis of CL2

Scheme 1 depicts also the synthesis of crosslinker CL2. To an ice-cooled solution of 1.020 g (4 mmol) *N,N*-bis(methacryloxyethyl) methylamine **1** in 2 mL dry acetone, a solution of 571 mg (4.2 mmol, 0.430 mL) 1,4-butanesultone in 1 mL acetone was added drop-wise. The reaction mixture was stirred at room temperature for 5 h. The white precipitate formed was filtered and washed with a small amount of dry acetone and diethylether. The filtrate was evaporated and 1 mL of dry acetone was added to the residue. The precipitate was formed in a freezer at −20 °C for about one week. Centrifugation resulted in a white powder of 64% yield.

^{1}H NMR (300 MHz, CD_3OD): 6.15 d, 2 H, $J = 1.8$ Hz (=C–H); 5.52 d, 2 H, $J = 1.8$ Hz (=C–H); 4.63 t, 4 H, $J = 8.4$ Hz (O–CH_2); 3.87 t , 4 H, $J = 8.4$ Hz (N–CH_2); 3.55 m, 2 H (N–CH_2); 3.24 s, 3 H (N–CH_3); 2.86 t, 2 H, $^3J = 8.2$ Hz (S–CH_2); 1.96 s, 6 H (=C–CH_3), 1.96 m, 2H (CH_2); 1.85 m, 2H (CH_2).

^{13}C NMR (75 MHz, CD_3OD): 167.7, 137.1, 127.5, 64.4, 62.2, 59.0, 51.3, 23.0, 22.1, 18.4.

FTIR (cm^{-1}, KBr): 1717 s, 1635 m, 1474 m, 1458 w, 1319 m, 1293 m, 1237 m, 1202 s, 1162 s, 1044 m, 956 m, 818 w, 793 w, 650 w, 608 w, 571 w, 533 w.

2.4. Formation of polySBDMA hydrogel

SBDMA aqueous solution (2.0 mol L^{-1}, 1.0 mL), various amounts of crosslinkers and 40 μL of 0.22 mol L^{-1} APS aqueous solution (final APS concentration equals to 8.8 mmol L^{-1}) as initiator were placed in a vial. The solution was stirred for 30 min to ensure that all components are dissolved and the solution is homogenous. Argon gas was bubbled for 30 min through the solution to remove oxygen. A 14 μL (0.093 mol L^{-1}) aliquot of TMEDA as the accelerator was added and stirring was continued for an additional 30 s. The solution was then injected between two glass plates of dimensions (in mm) 55 × 15 × 1, with the thickness adjusted with a glass spacer, and purged by an argon gas for about 1 min before injecting the solution. The side walls were secured with adhesive tape. After the gelling process was completed within 24 h at 23 ± 1 °C, the obtained slab-shaped polySBDMA hydrogels were immersed for 4 days in distilled water to remove any low molecular weight compounds. The water was changed several times during this process. PolySBDMA hydrogel slabs were cut with an appropriate cork bore to obtain cylinders used for further characterization. PolySBDMA hydrogels were designated based on the molar concentration of crosslinker to SBDMA in the feed as listed in Table 1.

2.5. NMR spectroscopy

^{1}H NMR and ^{13}C NMR were recorded on a Varian Gemini 300 instrument at 298 K. Chemical shifts are reported in ppm downfield to internal standard TMS (0.00 ppm). The solvent CD_3OD was used as a reference. Working frequency was 300 MHz for ^{1}H and 75.5 MHz for ^{13}C NMR. Coupling patterns were designated as s – singlet, d – doublet, t – triplet, m – multiplet. Coupling constants are given in Hz. Concentration of samples was around 10 mg of crosslinker dissolved in 0.7 mL of solvent.

2.6. FTIR spectroscopy

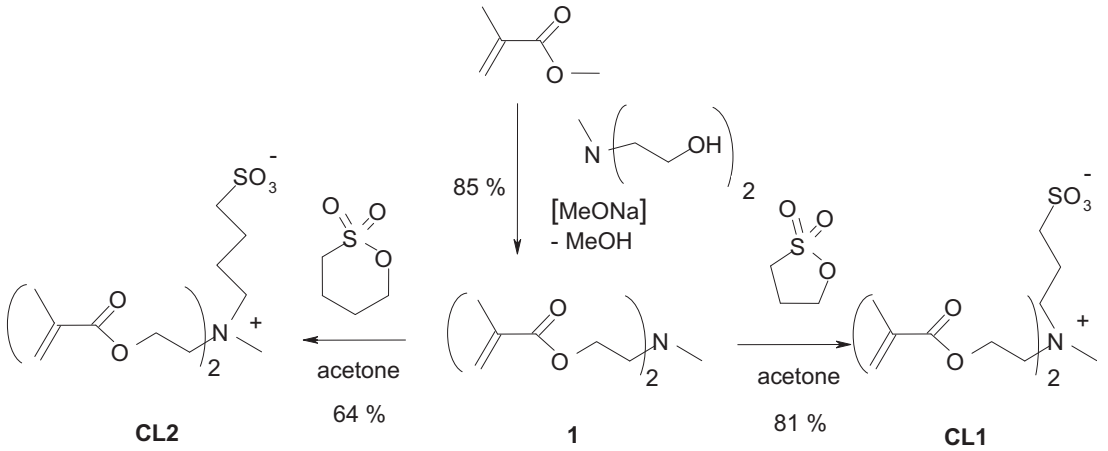
FTIR data were recorded on a Specord M 80 spectrophotometer in KBr pellets with 32 scans. The spectra were characterized in wavenumbers with abbreviations for determination of relative intensities of signals as s – strong, m – medium, w – weak.

2.7. Swelling and sorption measurements

Hydrogel cylinders with diameter of 10 mm were lyophilized for one day and stored in a desiccator over $CaCl_2$ at room temperature before measurement. Lyophilized hydrogels were immersed in water at 24 °C and the water sorption was followed by gravimetry. Before measuring the weight of hydrogel samples during swelling experiments, any excess water on the hydrogel surface was gently wiped off using Kimwipe paper. The equilibrium water content (EWC) in weight percent, which expresses the maximum amount of water swelling the hydrogel at given conditions, was determined from Eq. (1) [6]:

$$\mathrm{EWC} = \frac{W_\infty - W_\mathrm{d}}{W_\infty} \cdot 100 \qquad (1)$$

where W_∞ and W_d are the weights of the swollen hydrogel in equilibrium and of the lyophilized hydrogel, respectively.



Scheme 1. Synthesis of sulfobetaine crosslinkers CL1 and CL2.

Table 1
PolySBDMA hydrogel samples synthesized in the presence of various crosslinkers of various concentrations. Molar concentration of SBDMA was equal to 2.0 mol L^{-1}.

Sample name	Crosslinker	Concentration in feed mol% to monomer
CL1-0.5	CL1	0.5
CL1-1		1
CL1-3		3
CL1-10		10
CL1-20		20
CL2-0.5	CL2	0.5
CL2-1		1
CL2-3		3
BIS-1	BIS	1
EDMA-1	EDMA	1

The rate of water sorption by hydrogel is expressed by the sorption degree (SD), which (in weight percent) is defined by Eq. (2) [26]:

$$\mathrm{SD} = \frac{W_t - W_\mathrm{d}}{W_\mathrm{d}} \cdot 100 \tag{2}$$

where W_t is the weight of wet hydrogel at different time intervals and W_d is the weight of lyophilized hydrogel. The sorption degree at equilibrium was determined after equilibration of hydrogel samples in water, 100 mmol L^{-1} phosphate buffered saline (PBS) and 155 mmol L^{-1} (0.9%) NaCl solutions.

2.8. Differential scanning calorimetry

The differential scanning calorimetry (DSC) measurements were carried out by employing a DSC Mettler-Toledo 821 differential scanning calorimeter. Nitrogen gas was passed through the instrument at a flow rate of 50 mL min^{-1}. Before measurement, the DSC was calibrated from −100 to 250 °C at a heating rate of 5 °C min^{-1} with an indium standard. Hydrogel samples swollen to the EWC were gently wiped with Kimwipe paper to remove surface water, then cut, weighed accurately and immediately sealed in aluminum pans. The sample weight ranged from 4 to 11 mg. DSC curves were obtained by heating from −45 to 20 °C and by cooling from 20 to −45 °C with a heating rate of 10 °C min^{-1}. All experiments were done in duplicate.

2.9. Mechanical properties

Hydrogels were tested on a Texture Analyzer TA-XT2*i* (Stable Micro Systems, Godalming, UK) equipped with a force transducer of 1 mN resolution and Texture Expert software version 1.16 used for data acquisition and evaluation. The mechanical stability was measured on hydrogel cylinders of diameter 3.83 mm in compression mode using a vertically moving mobile probe of diameter 4 mm at a constant speed of 0.6 mm s^{-1}. The cylinders were deformed up to about 90% of their initial height. The initial height values were determined from the compression stress–strain curves based on the probe position at which the force values started to increase. Average values and standard deviations were obtained from the analysis of at least six replicates. Mechanical properties were evaluated from the stress–strain curves using the approach described recently [27].

The crosslink density and distance between crosslinks were determined for hydrogel samples using their swelling characteristics and mechanical properties. The elastic modulus is related to the effective network chain concentration of the swollen hydrogel. The crosslink density ν_e/V can be calculated from Eq. (3) [28,29]:

$$\frac{\nu_\mathrm{e}}{V} = \frac{\sigma\left(\frac{\varphi_2}{\varphi_0}\right)^{2/3}}{RT\left(\alpha - \alpha^{-2}\right)} \tag{3}$$

where σ is the strain stress or compression stress, φ_2 is the volume fraction of polymer at the equilibrium (in the fully swollen hydrogel; it was calculated from the change of the volumes of dried and fully swollen cylinders), and φ_0 is the volume fraction of polymer in the relaxed state (non-swollen, but not dehydrated hydrogel), R is the gas constant, T is the absolute temperature and α is the deformation ratio related to the stress determined as the ratio of elastically deformed length L to initial length L_0 of the hydrogel. Crosslink densities were arbitrarily calculated from 40% strain and averaged crosslink distance (mesh size), ξ, was estimated from the crosslink density using Eq. (4) [30]:

$$\xi = \left(\frac{\nu_\mathrm{e} N_\mathrm{A}}{V}\right)^{-1/3} \tag{4}$$

where N_A is Avogadro's number. The deformation of 40% was chosen arbitrarily to estimate the effectiveness of crosslinkers.

2.10. Cell adhesion

Cell adhesion tests were carried out using polySBDMA thin hydrogel films (CL1-1 in Table 1) deposited on cover glass slips by a spin-coating technique. Cover glass slips were cleaned with Piranha solution for 10 min and then washed extensively with deionized water and dried in oven at 120 °C prior to use. The spin-coated films were prepared as follows: A stock solution was prepared consisting of 2 mol L^{-1} of SBDMA, 1 mol% to monomer content of CL1 and 0.013 mol L^{-1} of APS in distilled water. 300 μL of stock solution was mixed with 7 μL TMEDA for 20 s and the solution was dropped onto the cover glass slip mounted to a spin coater. The spin-coating was performed for 30 s at 5000 rpm. Then the cover glass slip was transferred to a Petri dish, where the hydrogel was formed for 70 min under the argon atmosphere. Consequently, the slip was immersed in distilled water for 2 days and stored at 4 °C. During this period distilled water was exchanged periodically. The film on a cover glass slip was sterilized by autoclaving prior to cell adhesion tests. The thickness of films of around 3 μm was determined by a confocal laser scanning microscopy.

Growing RAT-2 fibroblasts-like cells at concentration of 1×10^5 per plate in DMEM were seeded on a 60 mm tissue culture polystyrene Petri dish to which a glass cover slip with the spin-coated polySBDMA hydrogel was placed. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 5 days. Proliferation of RAT-2 fibroblasts-like cells on the sample surface and their morphology were analyzed using optical microscopy (XDS-IM1, Optika microscopes) by a digital camera (Canon, DS 126071) using 10× and 2.5× objectives. Cell density on the hydrogel surface and on the non-coated cover glass slip, used as a positive control, was determined using a hemocytometer (Glaswarenfabrik Karl Hecht KG, Germany).

3. Results and discussion

3.1. Synthesis of CL1 and CL2

The synthesis of new crosslinkers was carried out from dimethacrylate **1** according to Scheme 1. **1** was prepared from commercially available *N*,*N*-bis(2-hydroxyethyl)methylamine and methyl methacrylate with azeotropic distillation of methanol catalyzed with MeONa following the previously reported method [25]. A chromatographic separation provided product of 92% yield,

which is slightly higher than reported (85%). Synthesis of CL1 and CL2 was done in acetone with a sufficient yield of 81 and 64%, respectively. Crosslinkers were characterized by means of ^{1}H NMR, ^{13}C NMR and FTIR (both contained in the Supplementary Data file).

^{1}H NMR spectra for CL1 and CL2 demonstrate the purity of synthesized crosslinkers. In the FTIR spectra of CL1 and CL2, strong peaks are observed at about 1320 and 1640 cm^{-1}, which indicate the presence of methacrylic C=C double bonds. Esteric carbonyl is detected at about 1720 cm^{-1}. Peaks referring to quaternary ammonium and organic sulfate are seen at about 1030 and 1170 cm^{-1}, respectively.

3.2. Formation of polySBDMA hydrogels

PolySBDMA hydrogels were prepared using SBDMA concentration of 2 mol L^{-1} (56 wt%) and various crosslinkers of concentrations (in mol% to monomer), given in Table 1, by redox-initiated free-radical polymerization using as a redox couple initiator APS and accelerator TMEDA. Polymerizations were carried out to reach complete conversion that was confirmed by Soxhlet extraction of prepared discs showing no solid residues in the extracted fraction. Moreover, FTIR analysis of the freeze-dried hydrogels did not show the presence of peaks from C=C double bonds at 1640, 1320 and 1300 cm^{-1} (data not shown), which also demonstrates that the xerogels did not contain any residual monomer. Thus the solid content of prepared hydrogels is approximately 56–62% given by the amounts of monomer and crosslinkers. After polymerization, hydrogels were colorless with transmittance over 95% for around 1 mm thick slabs within the wavelength range between 400 and 800 nm determined by UV–VIS spectrometry (data not shown). The hydrogels exhibited uniform and smooth surfaces without the occurrence of bubbles or other imperfections. Their shape was stable in aqueous solutions demonstrating that an effective network was formed and that the hydrogels exhibit a high capacity for water swelling.

3.3. Determination of the state of water by DSC

The state of water in hydrogels is a material property that can be correlated with applications especially in biorelated fields, where the aqueous environment determines the performance of proteins and cells [31]. In swollen hydrogels, states of water are classified to freezing free water, freezable-bound water, and non-freezing water [32]. Freezing free water does not participate in hydrogen bonding and/or complexation with the polymer chains. Freezable-bound water is the intermediate water that interacts only weakly with polymer chains and affects transport properties [6,33]. It exhibits the phase transition temperature lower than 273 K. The non-freezing water is the fraction of water bonded to the hydrophilic sites of the polymer chain through hydrogen bonds. This water does not exhibit a detectable phase transition over the temperature range from 200 to 273 K [32] and may influence the biocompatibility of a hydrogel [34].

In our study, DSC was employed as the most common technique [6] to determine the content of total freezing (W_{tf}) water, in weight percentage, in hydrogels from Eq. (5):

$$W_{tf} = W_{ff} + W_{fb} = \frac{\Delta H}{\Delta H_W} \cdot 100 \quad (5)$$

where W_{ff} and W_{fb} are the weight percentages of free freezing and freezable-bound water, respectively, ΔH is the melting enthalpy of water in the hydrogel and ΔH_W is the melting enthalpy of bulk water equal to 333 J g^{-1} [32].

DSC measurements were accompanied by swelling experiments of lyophilized samples to determine EWC values from Eq. (1) that combine total freezing and non-freezing water in the polymeric network. The EWC values and DSC results were then used to determine the weight percentage of non-freezing water (W_{nf}):

$$W_{nf} = EWC - W_{tf} \quad (6)$$

Fig. 2 shows DSC endothermic curves of the first heating run for hydrogel samples between −45 and 20 °C. The peaks corresponding to freezable-bound water (lower temperature shoulder) and freezing free water (higher temperature shoulder), respectively, are overlapped and shifted to temperatures above 0 °C due to the fast heating rate used in this study. These data are, nevertheless, sufficient to determine the melting enthalpy calculated from the area under the integrated endothermic curves from −15 to 15 °C. The content of total freezing water was determined from Eq. (5), which, together with the equilibrium water content, provides information on the content of non-freezing water from Eq. (6). Table 2 summarizes obtained EWC, W_{tf} and W_{nf} data in addition to W_{tf}/W_{nf} ratios in order to compare the characteristics of hydrogels prepared in the presence of various crosslinkers. It should be mentioned that integration of exothermic curves from cooling runs between 20 and −45 °C resulted in lower values of enthalpy (data not shown). These values were not used for further analysis as they were assigned to artifacts (water evaporation and condensation) similar to those discussed in the work of Ahmad and Huglin [32].

EWC values for all hydrogels with the lower crosslinker content up to 3 mol% to monomer are about 60 wt% with some slight systematic differences depending on both type and concentration of crosslinker. For hydrogels formed in the presence of CL1 and CL2, EWC decreases by about 4 wt% for both crosslinkers upon increasing their concentration. This follows the expected increase in the degree of crosslinking and, therefore, the slightly reduced capability of the hydrogels to be swollen by water. An increase in CL1 level to 10 and 20 mol% to monomer further reduces the EWC values to around 47 and 42 wt%, respectively.

A higher water content for hydrogels prepared using CL1 compared to CL2 (at the same crosslinker concentration) reflects the more hydrophobic character of CL2 (4-sulfobutyl pendant) than CL1 (3-sulfopropyl pendant). This trend of somewhat different hydrophilicity of hydrogels prepared in the presence of CL1 and CL2, respectively, is further seen with respect to W_{tf} and W_{nf} representing the state of water related to the EWC values via Eq. (6).

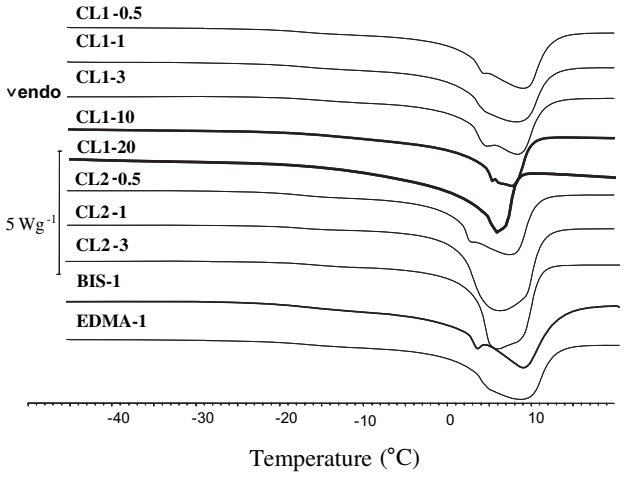


Fig. 2. DSC endothermic curves of first heating run for polySBDMA hydrogels prepared from 2 mol L^{-1} SBDMA concentration and various concentrations of crosslinkers in mol % to monomer.

Table 2
Equilibrium water content, EWC, total freezable water, W_{tf}, and non-freezing water, W_{nf}, for zwitterionic polySBDMA hydrogels prepared from 2 mol L^{-1} SBDMA and various concentrations of CL1 and CL2 (mol% to monomer) and 1 mol% to monomer of BIS and EDMA crosslinkers.

Sample	EWC (wt%)	W_{tf} (wt%)	W_{nf} (wt%)	W_{tf}/W_{nf}
CL1-0.5	63.0	47.3	15.7	3.0
CL1-1	61.5	43.4	18.1	2.4
CL1-3	59.8	41.6	18.2	2.3
CL1-10	47.1	28.8	18.3	1.6
CL1-20	42.2	18.6	23.6	0.8
CL2-0.5	60.8	49.1	10.8	4.5
CL2-1	58.9	48.2	10.7	4.5
CL2-3	57.3	47.9	9.4	5.1
BIS-1	60.8	41.4	19.4	2.1
EDMA-1	60.5	48.1	12.4	3.8

For CL1 the W_{tf} values decrease from 47.3 to 18.6 wt% with increasing CL1 concentration from 0.5 to 20 mol%. The W_{tf} values at the crosslinker concentration up to 3 mol% to monomer are significantly lower than W_{tf} values for hydrogels prepared in the presence of CL2 crosslinker. This indicates that hydrogel crosslinked by CL2 is less hydrophilic than that crosslinked by CL1. It is interesting to note that only a minor difference in the crosslinker chemistry between CL1 and CL2 represents a significant factor influencing the state of water, and interactions of water with the hydrogel network. In this sense, Table 2 shows that W_{nf} values are higher and ratios W_{tf}/W_{nf} are lower for hydrogels prepared using CL1 compared to CL2. The duplicate experiments exhibited less than 2% variation, hence, the discussed differences among the data in Table 2 are outside the error range. In summary, these data demonstrate that a higher degree of hydrogen bonding interactions with water molecules occurs for hydrogels crosslinked with CL1 than with CL2.

EWC, W_{tf} and W_{nf} values in Table 2 are also shown for hydrogels prepared in the presence of 1 mol% to monomer of BIS and EDMA crosslinkers. The hydrogel prepared in the presence of BIS is more hydrophilic than that prepared in the presence of EDMA. Then the state of water in the former hydrogel is similar to the hydrogels in the presence of CL1 and the state of water for the latter hydrogel is closer to the situation observed for hydrogels prepared in the presence of CL2. This observation can be related to the chemical structures where the methylene bisacrylamide moiety of BIS is more hydrophilic than is the ethylene glycol dimethacrylate moiety of EDMA.

3.4. Diffusion coefficient of water in hydrogels

Fig. 3 reveals the experimental data for hydrogels crosslinked by all crosslinkers obtained from the water sorption experiments. The weight of hydrogel after placing the lyophilized hydrogel samples in water was determined at different time intervals until the saturation level was reached. Overall, the crosslinker concentration seems to be a more significant factor for sorption of water than the selection of crosslinker. Results shown in Fig. 3 can be used for calculation of the diffusion coefficient of water in hydrogels for the case when diffusion controls the transport process [35]. Generally, the sorption behavior in polymers is a complex process. It can range from pure Fickian to pure relaxation behavior [35–37] and is controlled either by Fickian diffusion or by segmental relaxation depending on which of these processes is slower. This phenomenon can be analyzed using various models [38–41].

A typical dependence for the water sorption experiment is shown in Fig. 4. M_t and M_∞ are the characteristic water contents in a hydrogel in time t and at the equilibrium, respectively. According to the Ritger–Peppas theory [37,38], these experimental data can be divided into two regions. The region of "short times" corresponds to the early stage of diffusion, for which the short-time approximation is valid for the first 60% of the equilibrium water content. The region of "long times" corresponds to the late stage of diffusion. In order to study the water transport mechanism in the hydrogel, the Ritger–Peppas model described by Eq. (7) was considered to fit the experimental data:

$$\frac{M_t}{M_\infty} = kt^n \tag{7}$$

here M_t/M_∞ is the fractional water content, k is the kinetic constant, t is the diffusion time and n is the time exponent that can be related to the solute transport. For a thin hydrogel film, and when $n = 0.5$, the water diffusion mechanism is described by Fickian diffusion. The comparison of the model with the experimental data for sample CL1-0.5 is shown as an inset in Fig. 4.

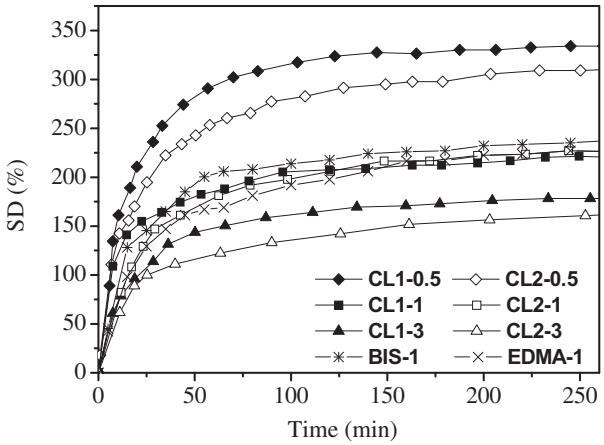


Fig. 3. The sorption degree (SD) dependence on time for polySBDMA hydrogels crosslinked with all crosslinkers used in this study (in mol% to monomer). Lines connect the experimental data points to guide the eye.

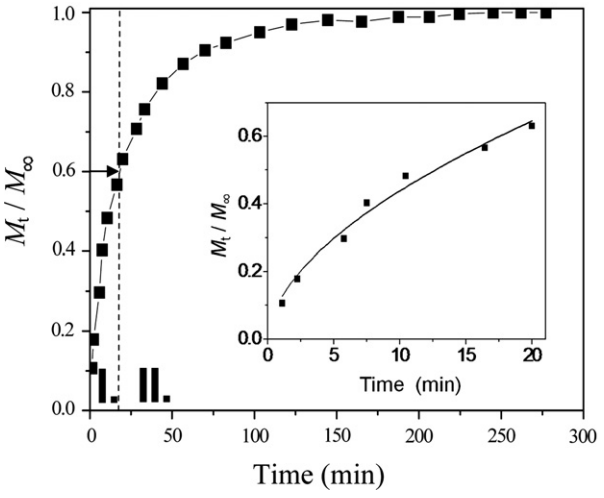


Fig. 4. Water sorption experiment for sample CL1-0.5 showing the fractional water content M_t/M_∞ in hydrogel as a function of time: (I) short-time approximation, and (II) long-time approximation ranges. Line connects the experimental data points to guide the eye. Inset: Comparison of experimental data with model given by equation (6) for the fractional water content M_t/M_∞ in hydrogel as a function of time for sample CL1-0.5 in the short-time range.

The n values for the investigated hydrogels are summarized in Table 3. They are all close to the value of 0.5, which suggests that the water diffusion into hydrogels can be considered as Fickian. Then, the water diffusion into the hydrogel in the region of short times can be described by Eq. (8) [37]:

$$\frac{M_t}{M_\infty} = 2\left(\frac{Dt}{L^2}\right)^{0.5} \quad (8)$$

where L is the sample thickness and D is the diffusion coefficient. Eq. (8) is restricted to thin layers. In our studies, the ratio of sample thickness to diameter is ~1/15, which can be considered as the diffusion of water into a planar sheet. This validates using of Eq. (8) to estimate diffusion coefficients of water in investigated hydrogels, which are contained in Table 3.

The data listed in Table 3 can be summarized as follows: the increase in crosslinker concentration is associated with lowering the sorption degree and slightly decreased diffusion coefficients of water in hydrogels. In correlation with the DSC and EWC data, the diffusion of water is slower (i) into less hydrophilic hydrogels crosslinked by CL2 compared to CL1, and (ii) into the hydrogels with a higher degree of crosslinking. The results for hydrogels crosslinked by commercial crosslinkers also follow this line of explanation, *i.e.*, a higher value of diffusion coefficient for BIS compared to EDMA reflects the differences between hydrophilic character of these crosslinkers.

Typical diffusion coefficients for diffusion of water in polymers are in the order of 10^{-11} to 10^{-10} $m^2 s^{-1}$ [42]. Hence, the diffusion coefficients of about 2×10^{-10} $m^2 s^{-1}$ determined in this work demonstrate that the water diffusion in prepared sulfobetaine hydrogels is relatively fast. For comparison, the same approach as used in this work for characterization of the buffer sorption process into the hydrophobically modified *N,N*-dimethylacrylamide hydrogels [43] and resulted in the early- and late-time diffusion coefficients ranging from 7.3×10^{-11} to 1.35×10^{-10} $m^2 s^{-1}$ and 6.5×10^{-11} to 1.21×10^{-10} $m^2 s^{-1}$, respectively. The values of diffusion coefficients of water determined in our work also agree with those determined for (*N*-isopropylacrylamide-co-acrylamide-co-2-hydroxyethyl methacrylate) hydrogels ranging from about 2.5×10^{-10} to 5.0×10^{-10} $m^2 s^{-1}$ [44], glycol chitosan superporous hydrogels ranging from 8.3×10^{-10} to 1.7×10^{-9} $m^2 s^{-1}$ [45], and the network of poly(vinyl alcohol) and poly(*N*-vinyl pyrrolidone) crosslinked by radiation ranging from 1.8×10^{-11} to 4.4×10^{-11} $m^2 s^{-1}$ [46].

Studies on sorption degree of water in hydrogels are extended by the determination of the sorption degree at equilibrium in the presence of simple electrolytes in order to demonstrate the anti-polyelectrolyte behavior of zwitterionic polymers [14,15]. Fig. 5 shows the swelling ratio for hydrogels immersed and equilibrated in water, 100 mmol L^{-1} PBS and 155 mmol L^{-1} saline solution. Non-surprisingly, the sorption degree is higher in the presence of simple electrolytes in aqueous solutions, which disrupt intra- and intermolecular associated structures typical for zwitterionic polymers [14–16]. Thus the sorption degree at equilibrium depends on the level of crosslinker as well as is proportional to the concentration of simple electrolytes. This also points out that identification of subtle differences among the hydrogels may not be possible in case when the zwitterionic hydrogels are studied in the presence of simple electrolytes.

Table 3
Sorption degrees at equilibrium, time exponents, n, and diffusion coefficients, D, of water for polySBDMA hydrogels prepared using different crosslinking conditions.

Sample	Sorption degree (wt%)	n	$D.10^{10}$ ($m^2 s^{-1}$)
CL1-0.5	334	0.56 ± 0.04	2.5
CL1-1	220	0.58 ± 0.06	2.3
CL1-3	178	0.56 ± 0.07	2.0
CL2-0.5	310	0.55 ± 0.07	2.1
CL2-1	226	0.62 ± 0.04	1.6
CL2-3	164	0.58 ± 0.08	1.5
BIS-1	230	0.60 ± 0.05	2.3
EDMA-1	218	0.59 ± 0.06	1.8

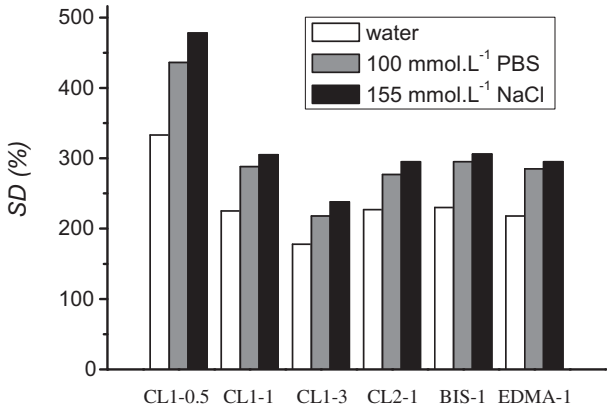


Fig. 5. Sorption degree at equilibrium for polySBDMA hydrogels crosslinked with various crosslinkers (for CL1 at various crosslinker concentrations between 0.5 and 3 mol%, and for CL2, BIS and EDMA at crosslinker concentration of 1 mol%) in water, 100 mmol L^{-1} PBS and 155 mmol L^{-1} NaCl.

3.5. Mechanical properties and degree of crosslinking

Mechanical properties of the investigated hydrogels prepared in the presence of different concentrations of crosslinkers synthesized in this work (CL1 and CL2) as well as commercially available crosslinkers (BIS and EDMA) are contained in Table 4.

Increased crosslinker concentration leads both to higher stiffness and hardness (represented by Young's modulus), pronounced strength (expressed by stress parameters σ_b and σ_{40}), whereas deformability of hydrogels (ε_b) decreases. This is a common behavior not only for hydrogels [47] but also for crosslinked rubbers as well as thermoplastics [48,49]. The comparison between hydrogels crosslinked by CL1 and CL2 at the same crosslinker concentration does not lead to an unambiguous conclusion about the effect of each of these crosslinkers. The stress at arbitrary 40% deformation, σ_{40}, was selected as another quantity since it enables the comparison among all the hydrogels in this study (Table 4).

Fig. 6 and the data in Table 4 represent the comparison of mechanical properties for hydrogels crosslinked by various crosslinkers at the equal crosslinker concentration of 1 mol% based on

Table 4
Mechanical properties of hydrogels prepared by using different crosslinking conditions: Young's modulus, E, elongation at break, ε_b, stress at break, σ_b, stress at deformation of 40%, σ_{40}, crosslink density, ν_e/V, and crosslink distance, ξ.

Sample	E (kPa)	ε_b (%)	σ_b (kPa)	σ_{40} (kPa)	ν_e/V (mol m^{-3})	ξ (nm)
CL1-0.5	63 ± 12	57 ± 1	251 ± 22	110 ± 17	46 ± 7	3.3 ± 0.5
CL1-1	127 ± 15	46 ± 1	241 ± 58	162 ± 26	59 ± 10	3.0 ± 0.5
CL1-3	147 ± 64	46 ± 3	550 ± 72	366 ± 68	122 ± 23	2.4 ± 0.5
CL1-10	437 ± 224	72 ± 2	2185 ± 170	695 ± 95	280 ± 44	1.8 ± 0.2
CL1-20	1450 ± 375	85 ± 4	4400 ± 260	1235 ± 115	348 ± 53	1.7 ± 0.6
CL2-0.5	64 ± 9	57 ± 1	297 ± 72	95 ± 2	41 ± 1	3.5 ± 0.1
CL2-1	90 ± 35	55 ± 3	574 ± 61	281 ± 34	105 ± 13	2.5 ± 0.3
CL2-3	226 ± 72	41 ± 3	404 ± 31	383 ± 51	130 ± 17	2.3 ± 0.3
BIS-1	13 ± 1	81 ± 2	532 ± 66	43 ± 6	16 ± 2	4.7 ± 0.6
EDMA-1	52 ± 12	70 ± 2	436 ± 99	79 ± 8	29 ± 3	3.8 ± 0.4

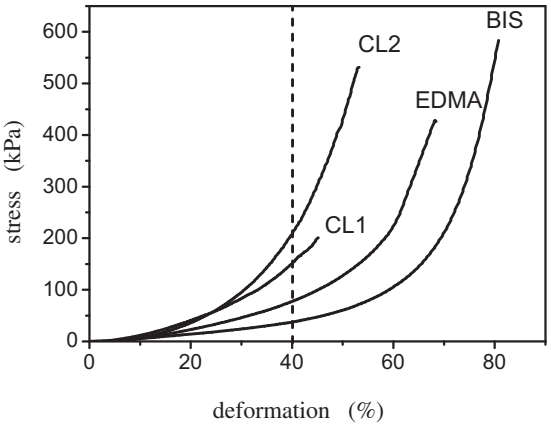


Fig. 6. The compression stress–deformation curves for hydrogels crosslinked with various crosslinkers at concentration of 1 mol% on monomer content. Dashed line indicates stress values σ_{40} at the arbitrary deformation of 40%.

monomer content. Commercial crosslinkers form a more elastic network and softer hydrogels compared to hydrogels crosslinked by CL1 and CL2. This is implied from the about 1.3-fold higher elongation at break as well as from the Young's moduli, which are lower by about one order of magnitude and about two-fold in case of the hydrogels crosslinked by BIS and EDMA, respectively. Since the stress steeply increases with increased deformation, σ_b values do not show any systematic variation with the type of crosslinkers, i.e., the most deformable hydrogels show the highest values of the stress at break. On the other hand, the comparison of stress values at the lower deformation, arbitrarily set to 40%, clearly identifies that the stress σ_{40} required to achieve this deformation is several times higher for hydrogels crosslinked by CL1 and CL2 than for those crosslinked by EDMA and BIS. Although mechanical properties for hydrogel samples crosslinked by CL1 and CL2 exhibit some differences, overall it may be stated that, at the same crosslinker concentration between 0.5 and 3 mol%, the hydrogels do not differ significantly. Data contained in Fig. 6 and Table 4 also indicate that hydrogels crosslinked by EDMA are stronger than hydrogels crosslinked by BIS. These data are confirmed by quantities characterizing the level of crosslinking: the crosslink density and the distance between crosslinks. At the same concentration of crosslinker of 1 mol%, both indicate a more effective crosslinking reaction, i.e. higher crosslink densities and lower crosslink distance, in the presence of CL1 and CL2 than in the presence of commercial crosslinkers with different chemical structure than SBDMA monomer.

Apart from obtaining quantities characterizing the mechanical properties and crosslink density of hydrogels, these data are significant also from the point of view of characterization of the mechanism of crosslinking. The swelling and sorption as well as state of water characteristics of polySBDMA hydrogels have already indicated some differences among hydrogels prepared using synthesized and commercial crosslinkers, respectively. Importantly, mechanical properties seem to demonstrate more clearly the benefit of using crosslinkers CL1 and CL2. The stronger CL1- and CL2-crosslinked hydrogels show that crosslinkers chemically similar to the monomer unit are advantageous because they minimize the compositional drift during crosslinking reaction to complete conversion. Consequently, the hydrogel network should be more homogeneous than in the case when chemically different crosslinkers, such as BIS and EDMA, are used for preparation of a hydrogel. The compositional drift may be either more significantly (assumed for BIS; an acrylamide type of monomer with a chemically very different character to SBDMA) or less significantly (assumed for EDMA; a methacrylate type of monomer as is SBDMA) taking part during the hydrogel formation. The polySBDMA hydrogel samples crosslinked with either BIS or EDMA should be viewed as hydrogel networks with a more significant fluctuation in the network density that is reflected in the mechanical properties (Table 4). The reactivity ratios for acrylamide with methacrylates determined in the past [50] as well as for acrylate and methacrylate monomers determined recently more precisely by pulsed-laser-initiated copolymerizations [7] suggest that the compositional drift occurs during formation of the hydrogels and, hence, influences the hydrogel properties. This should be considered in the preparation strategies for polySBDMA hydrogels and, naturally, other hydrogels.

This phenomenon anticipated from our work has recently been demonstrated for polycarboxybetaine hydrogels [5], where the concentration of crosslinker was in the range from 2 to 100 mol%. We increased the level of CL1 crosslinker up to 20 mol% to monomer for which, similarly as in Ref. [5], crosslink density increases and crosslink distance decreases upon increasing the CL1 concentration. This is accompanied by significantly increased stiffness and strength of formed hydrogels, which emphasizes the possibility to tune the physical properties of sulfobetaine hydrogels by using newly prepared CL1 and CL2 crosslinkers. This is not possible by using commercial crosslinkers such as BIS and EDMA due to their limited solubility. The future work will be carried out to recognize the behavior of polySBDMA hydrogels prepared and tested in the solutions of simple electrolytes to further correlate the information on polysulfobetaines to polycaboxybetaine hydrogels [5].

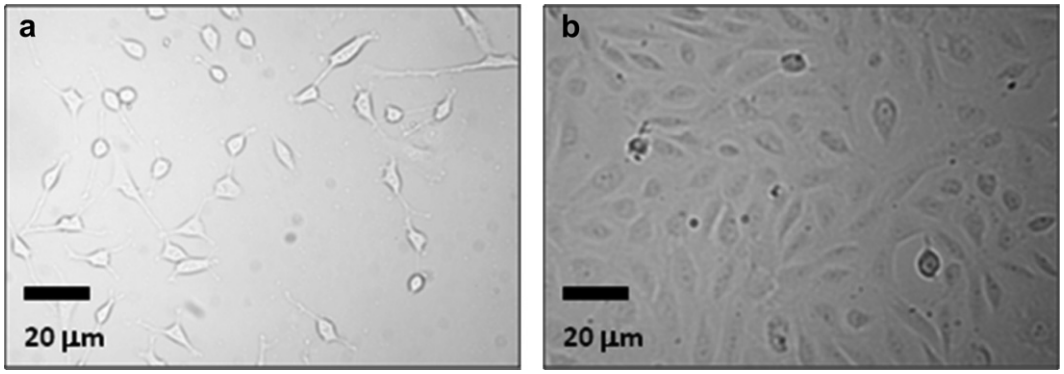


Fig. 7. Microscopic picture of a cover glass slip spin-coated by polySBDMA hydrogel (a) and non-coated cover glass slip used as a control (b) after 5 days of cultivation with the RAT-2 fibroblasts-like cells.

3.6. Cell adhesion to the hydrogel surface

Numerous factors influence the affinity of cells to polymeric surfaces including general chemistry of monomers and crosslinkers [51], hydrophobic and hydrophilic properties [52], presence of functional groups [53], and others [54]. Zwitterionic hydrogels are known as polymers with non-biofouling properties [5,9,16,24,55], which was also tested in this work.

Fig. 7 demonstrates the adherence of RAT-2 fibroblast-like cells to the hydrogel layer prepared by spin-coating on a cover glass slip using the CL1-1 recipe (Table 1). The cell adhesion to the non-coated slip served as a positive control. After cultivation for 5 days, the density of cells on a hydrogel surface was around 2.2×10^4 cells/cm^2 with 5% confluence of cells exhibiting a round-like shape (Fig. 7a), while the control sample reveals the cell density of around 4×10^5 cells/cm^2 and 90% confluence of firmly attached cells spread on the surface (Fig. 7b). This behavior was consistently seen for hydrogels formed by spin-coating on a glass cover slip as well as for hydrogel slabs, which demonstrates low biofouling of polysulfobetaines crosslinked by newly synthesized crosslinker. It should be noted that at this low level of crosslinker, the crosslinker chemistry should not play a significant role since the hydrogels consist almost exclusively of the zwitterionic polymers. The beneficial effect of the zwitterionic crosslinker compared to the non-zwitterionic crosslinker can be expected at the higher levels, where the non-zwitterionic crosslinker is expected to interrupt the restructuring of water within the zwitterionic polymer network thus reducing the non-biofouling character as demonstrated recently [5].

4. Conclusion

The goal of this work was to contribute to the topic of synthesis and characterization of zwitterionic hydrogels and to identify new features leading to more controlled properties of this class of hydrogels. Two novel sulfobetaine dimethacrylate crosslinkers were synthesized and applied for preparation of polysulfobetaine hydrogels based on the sulfobetaine monomer of a similar chemical structure as crosslinkers. The crosslinkers differ slightly in terms of the length of spacer between the charged groups, i.e., 3-sulfopropyl vs. 4-sulfobutyl spacers. Such a small difference in the crosslinker structure introduces a detectable effect on the equilibrium water content, state of water, diffusion coefficient of water, mechanical properties and degree of crosslinking even at a crosslinker concentration not exceeding 3 mol% to monomer. These differences can be ascribed to the affinity of water to the hydrogel network depending on the chemical structure of sulfobetaine crosslinker. The diffusion of water in selected hydrogels was found to obey Fickian diffusion. Diffusion coefficients of about 2×10^{-10} m^2 s^{-1} reveal some correlation with degree of crosslinking and type of crosslinker. In addition, no limitations in the solubility of CL1 and CL2 crosslinkers can be used to control the mechanical properties, water content and degree of crosslinking of polySBDMA hydrogels, which cannot be done using commercial crosslinkers, such as BIS and EDMA, due to their limited solubility in water.

This work on polysulfobetaine hydrogels demonstrates, in addition to those on polycarboxybetaine [5] and polyphosphobetaine [6] ones, that similar chemical structure of crosslinker and monomer is a prerequisite for enhancement of mechanical properties and maintaining the non-biofouling character of zwitterionic hydrogels.

Acknowledgements

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.polymer.2011.04.056.

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