Poly(2-oxazoline) Hydrogels Crosslinked with Aliphatic bis(2-oxazoline)s: Properties, Cytotoxicity, and Cell Cultivation

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ABSTRACT: A series of hydrogels from 2-ethyl-2-oxazoline and three bis(2-oxazoline) crosslinkers—1,4-butylene-2,2′-bis(2-oxazoline), 1,6-hexamethylene-2,2′-bis(2-oxazoline), and 1,8-octamethylene-2,2′-bis(2-oxazoline)—are prepared. The hydrogels differ by the length of aliphatic chain of crosslinker and by the percentage of crosslinker (2–10%). The influence of the type and the percentage of the crosslinker on swelling properties, mechanical properties, and state of water is studied. The equilibrium swelling degree in water ranges from 2 to 20. With a proper selection of the crosslinker, Young's modulus can be varied from 10 kPa to almost 100 kPa. To evaluate the potential for medical applications, the cytotoxicity of extracts and the contact toxicity toward murine fibroblasts are measured. The hydrogels with the crosslinker containing a shorter aliphatic exhibit low toxicity toward fibroblast cells. Moreover, the viability and the proliferation of pancreatic β-cells incubated inside hydrogels for 12 days are analyzed. © 2015 Wiley Periodicals, Inc. J. Polym. Sci., Part A: Polym. Chem. **2016**, *54*, 1548–1559

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INTRODUCTION In comparison to conventional flat two-dimensional surfaces, three-dimensional matrices represent more suitable models for cell cultivation[1] and for studying the fragile relationship between cells and their environment. In this context, the development of new convenient materials for cell cultivation *in vitro* is of a huge importance. One of the most common materials used for the cells support are hydrogels.[2] Due to their high water content and soft mechanical properties, they resemble human body tissue quite accurately. Polymers used for the preparation of such hydrogels have to fulfill strict criteria on biocompatibility as well as on economic and reproducible preparation.

One of the most promising synthetic polymers used for hydrogels preparation is poly(2-oxazoline). Although they are structural analogues of poly(amino acids), the tertiary amide group in poly(2-oxazoline) backbone provides an improved stability in human organism.[3] These polymers can be synthesized by living cationic polymerization which enables a preparation of well-defined polymers with a low dispersity.[4] Moreover, it has been shown that poly(2-oxazoline)s exhibit a good biocompatibility toward various cell lines[5–7] as well as fast blood-clearance time and no accumulation in organs when administered intravenously in rodents.[8,9]

Various synthetic routes for the preparation of hydrogels from 2-oxazolines have been described in literature. Christova et al. presented segmented networks from poly(2-oxazoline) macromonomers and acrylates,[10] Dargaville et al. prepared poly(2-oxazoline)s crosslinked with dithiols using UV light.[11] In the work of Farrugia et al.,[12] the latter approach was extended for mild conditions in water, suitable for the encapsulation of fibroblast cells, as demonstrated by the authors. A different approach involving poly(2-oxazoline) copolymers containing amino groups crosslinked with epichlorohydrine was shown in the work of Hartlieb et al.[13] Legros et al. prepared degradable hydrogels and nanogels from partially hydrolyzed poly(2-oxazoline)s crosslinked by 1,6-hexanediol di-glycidyl ether and its analoque containing disulfid.[14] The same authors prepared aldehyde containing copoly(2-oxazoline)s, which were subsequently crosslinked

Additional Supporting Information may be found in the online version of this article.

by bifunctional amines and hydrazines.[15] Preparation of crosslinked poly(2-oxazoline)s were further summarized recently in comprehensive reviews.[16,17]

The ''one-pot'' copolymerization of monofunctional 2-oxazolines with bis(2-oxazoline) as a crosslinker resulting in a formation of a crosslinked network was presented for the first time by Seagusa et al.[18] Thanks to its simplicity and good reproducibility this method has attracted a lot of attention in a wide range of applications. Rueda et al.[19] have prepared hydrogels by copolymerization of 2-methyl-2-oxazoline and 1,4-butylene-2,2′-bis(2-oxazoline) initiated by styrene macroinitiators. A series of poly(2-oxazoline)s hydrogels was prepared by copolymerization of hydrophilic 2-ethyl-2-oxazoline, hydrophobic 2-phenyl-2-oxazoline, and 1,4-phenylene-2,2′-bis(2-oxazoline) as a crosslinker in the work of Kelly et al.[20] Recently, poly(2-oxazoline)s networks crosslinked by 1,4-butylene-2,2′-bis(2-oxazoline) and modified by peptide sequence RGD (arginine-glycine-aspartic acid) were prepared by Schenk et al.[21] When ground, the gels showed an increased adhesion toward cancer cells in comparison to the adhesion toward endothelial cells. Hartlieb et al.[22] introduced poly(2-oxazoline) hydrogels with 1,4-phenylene-2,2′-bis(2-oxazoline) as a crosslinker for long-term stabilization of coagulation factor VIII. Li et al.[23] studied thermoresponsive behavior of hydrogels prepared from 2-isopropyl-2-oxazoline and 1,4-phenylene-2,2′-bis(2-oxazoline).

In order to adjust the swelling properties of such hydrogels various strategies have been implemented: (i) simultaneous copolymerization of monofunctional monomers of various alkyl chain lengths with bis(2-oxazoline) crosslinker[18,21]; (ii) aliphatic and aromatic comonomers crosslinked with bis(2-oxazoline) crosslinker[20]; (iii) usage of macroinitiator[19,24]; and (iv) adjustment of the ratio of a monomer to a crosslinker.[20,21] To the best of our knowledge, the control over the properties of 2-oxazoline-based hydrogels by varying the length of aliphatic chain of the crosslinker, as proposed here, has not yet been presented in literature.

In this article, a preparation of 2-oxazoline-based hydrogels with adjustable properties is presented. The control over hydrogel properties is achieved by (i) changing the aliphatic chain length of bis(2-oxazoline) crosslinker and by (ii) changing the molar ratio of the monomer to the crosslinker. Such hydrogels were subjected to freeze-drying process in order to obtain a porous structure. The swelling properties in various solvents, the mechanical properties, state of water, and cytotoxicity of the samples are analyzed. Finally, selected hydrogels are used as substrates for cultivation of pancreatic β cells.

EXPERIMENTAL

Materials

Adipoyl chloride, sebacoyl chloride, methyl 4-nitrobenzenesulfonate, and 2-chloroethylamine hydrochloride were purchased from Sigma-Aldrich (Steinheim, Germany) and used as received. Suberoyl chloride was purchased from TCI (Zwijndrecht, Belgium). 2-Ethyl-2-oxazoline (Sigma-Aldrich, Steinheim, Germany) was dried over KOH during 48 h and distilled over CaH_2 under reduced pressure. Benzonitrile (Sigma-Aldrich, Steinheim, Germany) was distilled under reduced pressure prior to use. Potassium hydroxide, sodium sulfate, and dichloromethane were purchased from Mikrochem (Bratislava, Slovakia). Methanol (AFT Bratislava, Slovakia), ethanol (Centralchem, Slovakia), and tetrachloromethane (Lachema, Czech Republic) were distilled prior to use. Deionized water (Milipore) was used for swelling experiments.

Synthesis of Crosslinkers

Bis(2-oxazoline) crosslinkers were prepared according to the procedure described by Néry.[25] Briefly, for the preparation of 1,4-butylene-2,2′-bis(2-oxazoline) (**ButBisOx**), adipoyl chloride (3.97 ml, 27.3 mmol) dissolved in tetrachloromethane (60 ml) was added dropwise into the mixture of KOH (9.2 g, 163.9 mmol) and 2-chloroethylamine hydrochloride (7.9 g, 68.3 mmol) in distilled water (50 ml) at 0 °C. The mixture was stirred overnight at room temperature. The white precipitate was filtered out and dried under reduced pressure at 40 °C (yield 6.34 g, 86.3%). For the cyclization, *N,N*′-bis(2-chloroethyl)adipamide (10 g, 37.16 mmol) was dissolved with KOH (5.21 g, 92.9 mmol) in methanol (250 ml) and refluxed for 6 h. KCl was filtered out and methanol was evaporated. The product was dissolved in chloroform, and then washed four times with brine (50 ml). Chloroform was evaporated and the product was dried for 2 days under reduced pressure at 40 °C. Product was obtained as a yellowish powder (yield 4.27 g, 59%).

1,6-Hexamethylene-2,2′-bis(2-oxazoline) (**HexBisOx**) and 1,8-octamethylene-2,2′-bis(2-oxazoline) (**OctBisOx**) were prepared analogously, starting from suberoyl chloride and sebacoyl chloride, respectively. For NMR and FTIR spectra of monomers, see Supporting Information Figures S1 and S2. The crosslinkers were used without any further purification.

ButBisOx: ^{1}H NMR (400 MHz, DMSO-d_6, δ) 4.10 (t, $J = 9.4$ Hz, 4H; CH_2-O), 3.64 (t, $J = 9.4$ Hz, 4H; CH_2-N), 2.15 (m, 4H; CH_2), 1.60–1.45 (m, 4H; CH_2). Anal. cacld. for $C_{10}H_{16}N_2O_2$: 61.2 C, 14.27 N, 8.22 H; found: 60.2 C, 14.2 N, 8.3 H. M.p. 54 °C.

HexBisOx: ^{1}H NMR (400 MHz, DMSO-d_6, δ) 4.10 (t, 4H; CH_2-O), 3.64 (t, 4H; CH_2-N), 2.14 (t, 4H; CH_2), 1.48 (m, 4H; CH_2), 1.25 (m, 4H; CH_2) Anal. cacld. for $C_{12}H_{20}N_2O_2$: 64.26 C, 12.49 N, 8.99 H; found: 63.43 C, 12.32 N, 8.84 H. M.p. 65 °C.

OctBisOx: ^{1}H NMR (400 MHz, DMSO-d_6, δ) 4.10 (t, 4H; CH_2-O), 3.64 (t, 4H; CH_2-N), 2.14 (t, 4H; CH_2), 1.48 (m, 4H; CH_2), 1.22 (m, 8H; CH_2-CH_2). Anal. calcd. for $C_{14}H_{24}N_2O_2$ in %: 66.63 C, 11.10 N, 9.59 H; found: 64.42 C, 10.71 N, 9.42 H. M.p. 56 °C.

Synthesis of Hydrogels

The hydrogels were prepared by copolymerization of 2-ethyl-2-oxazoline (**EtOx**) with bis(2-oxazoline) (**ButBisOx**, **HexBisOx**, and **OctBisOx**, respectively), using methyl

4-nitrobenzenesulfonate (**MeONs**) (1 mol %) as an initiator in benzonitrile, with five different molar ratios of **BisOx**:**EtOx** (2:98, 3:97, 4:96, 5:95, and 10:90). Typically, for the preparation of 2% **ButBisOx** hydrogel, **ButBisOx** (39.2 mg, 0.2 mmol) and **MeONs** (21.7 mg, 0.1 mmol) were placed in a Schlenk flask and dried in vacuum for 45 min. Subsequently, 2-ethyl-2-oxazoline (0.99 ml, 0.98 mmol) and benzonitrile (1 ml) were added under nitrogen atmosphere and the mixture was stirred for 5 h at 110 °C in oil bath. The yellowish gel was washed in an excess of methanol (2× 24 h), ethanol (1× 24 h), and distilled water (4× 24 h) and freeze-dried. All syntheses were done in triplicates.

Characterization

The ^{1}H and ^{13}C NMR spectra of all compounds were recorded at room temperature on a Varian VXR-400 (Varian) in DMSO-d_6 solutions using tetramethylsilane (**TMS**) as an internal standard. Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR) measurements were performed with FTIR NICOLET 8700 spectrometer (Thermo Scientific, UK) using an ATR accessory equipped with a Ge crystal. For each measurement, the resolution was 4 cm^{-1} and 64 scans.

Scanning Electron Microscopy

The morphology of the freeze-dried samples was examined using Tescan TS 5136 MM microscope (Tescan, Czech Republic) operating at 20 kV in a low vacuum regime ($P = 20$ Pa). Prior to imaging, the dried gels were cut to expose their inner structure. The scanning electron microscopy (SEM) images were obtained using backscattered electrons.

Swelling Properties

For the equilibrium swelling degree (SD) measurements, the samples were immersed in an excess of solvent [CH_2Cl_2, CH_3CH_2OH, dH_2O, phosphate-buffered saline (PBS)] and equilibrated for 24 h. After this time period, no additional swelling was observed. The samples were then gently dried with a paper tissue to remove any remaining solvent from the surface and weighted. SD was calculated using equation

$$\mathrm{SD} = \frac{w_{\mathrm{sw}} - w_{\mathrm{d}}}{w_{\mathrm{d}}}, \quad (1)$$

where w_{sw} is the weight of the swollen sample, w_d is the weight of the dried sample (after freeze-drying). SD is expressed as the mean ± standard deviation from hydrogels from three independent syntheses.

Mechanical Properties

Mechanical properties of hydrogels were determined employing Texture Analyser TA-XT2i (Stable Micro Systems, UK) equipped with a force transducer of a resolution of 1 mN and Texture Expert software version 1.16 used for data acquisition and evaluation. The mobile probe (diameter 3.87 mm) of Texture Analyser in a compression mode was moving vertically at a constant speed 0.1 mm/s. The deformation was set as 98% of the initial position of the probe. The hydrogels were swollen in distilled water and equilibrated for 24 h. Immediately after removing from water, cylinders with a diameter of 4 mm were cut from the samples prior to the measurements of mechanical properties. The initial height H_0 was determined as a deformation in which the force values started to increase. Mechanical properties were determined from stress–strain curves, Young's modulus was calculated for the small deformations (<15%) as a linear fit using least squares method. All data are presented as the mean ± standard deviation from at least five replicates.

Differential Scanning Calorimetry

Thermal analysis was performed using Mettler-Toledo DSC 821^{e} differential scanning calorimeter (DSC) under a nitrogen atmosphere. The thermal behavior of hydrogels samples swollen in distilled water were studied in the temperature interval from −45 to 25 °C at a heating rate 5 °C/min.

The amount of freezing water was calculated using the equation[26]

$$W_{\mathrm{f}}(\mathrm{wt\ \%}) = \frac{\Delta H}{\Delta H_{\mathrm{w}}} \times 100, \quad (2)$$

where ΔH is the melting enthalpy of water in hydrogel calculated from the integral of the endothermic peak at 0 °C and ΔH_w is melting enthalpy of bulk water (333 Jg^{-1})[27].

The amount of non-freezing water was obtained from the equation[27]

$$W_{\mathrm{nf}}(\mathrm{wt\ \%}) = \mathrm{EWC}(\mathrm{wt\ \%}) - W_{\mathrm{f}}(\mathrm{wt\ \%}), \quad (3)$$

where the equilibrium water content (EWC) in water was calculated from the weight of the swollen (w_{sw}) and dry (w_d) hydrogel according to eq 4, i.e.

$$\mathrm{EWC} = \frac{w_{\mathrm{sw}} - w_{\mathrm{d}}}{w_{\mathrm{sw}}} \times 100. \quad (4)$$

DSC was used also for determination of melting points of crosslinkers. Measurements were performed in the temperature interval from 25 to 250 °C at a heating rate 10 °C/min.

Cell Lines and Growth Media

For the cytotoxicity and cultivation study mice fibroblasts 3T3 and mouse insulinoma βTC3 cells were used (both DSMZ, Braunschweig). 3T3 cells were cultivated in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), streptomycin (100 μg/ml), penicillin (100 IU/ml). βTC3 cells were cultivated in DMEM supplemented with 15% horse serum (HS), 2.5% FBS, L-glutamine (2 mM), streptomycin (100 μg/ml), penicillin (100 IU/ml), referred further as full DMEM. All chemicals were purchased from Gibco (Life Technologies). The cells were cultivated at 37 °C and 5% CO_2 with saturating humidity in CO_2 incubator. The cells were trypsinized and the medium was changed once every 3 days.

Cytotoxicity Studies

Cell toxicity studies were conducted in accordance with ISO 10993-5 and ISO 10993-12 on mice fibroblast cell line 3T3.

All experiments were done in hexuplicates, with nontreated cells as a negative control.

Toxicity of Extracts

For the toxicity of extracts, the dry gels were sterilized by UV light for 30 min, then immersed in full DMEM medium and sterilized for another 30 min by UV light. The final volume of the extraction medium was 1 ml per 0.1 g of dry gel plus additional volume absorbed by the gel (calculated from the equilibrium SD). The gels were extracted for 24 h at 37 °C. Fibroblast cells were seeded on 96-well plate with the seeding density 5×10^3 cells/well and cultivated for 24 h. The medium was removed from the wells and extracts were added in three different dilutions—1:1, 1:2, 1:5 (extract: total volume). The cells were then incubated for additional 24 h and the viability was measured by MTT cytotoxicity assay as reported elsewhere.[5]

Direct Contact Toxicity

For the direct contact toxicity, cells were seeded on 24-well plate with the seeding density 2×10^4 cells/well and cultivated for 24 h. The dry gels were sterilized by UV light for 30 min, and then immersed in DMEM medium with serum, sterilized by UV light for 30 min, and equilibrated for 24 h. Cylinders of diameter of 6 mm were cut from the gels and gently placed on the top of the cell layer. The gels covered ~14% of the well surface. The cells were incubated with the gels for 24 h, then the gels were removed and the viability was measured by MTT cytotoxicity assay.

Cell Cultivation

For the cell cultivation studies, three types of hydrogels, 2% **ButBisOx**, 2% **HexBisOx**, and 2% **OctBisOx**, were selected. The studies were conducted on murine β-cell line βTC3. Cylinders of diameter of 8 mm were cut from the hydrogels, swollen in full DMEM medium, and sterilized by UV light. The cells were injected into the hydrogel cylinders in seeding number 2×10^4 cells/cylinder and hydrogel cylinders were poured over with the medium. The medium was changed every 3 days during the experiment. In the selected time intervals (day 3, day 7, day 12 after seeding), the viability of the cells was examined by FDA staining and Resazurin assay. For FDA staining, the gels were washed in PBS, then 250 μl of FDA solution (0.024 mg/ml in PBS) and 500 μl of PBS was added to hydrogel cylinder, incubated for 20 min, and then washed with PBS. The cells in hydrogels were visualized using confocal laser scanning microscope (LSM 510 META, Anxiovert 200, Zeiss, Germany, excitation 488 nm, emission 500–550 nm). In case of the Resazurin staining, the gels seeded with cells were relocated to a new well, prior to measurement, to avoid the signal from free unattached cells. Then, 50 μl of stock solution (0.01 mg/ml in PBS) was added to 500 μl of full DMEM media (without phenol red), incubated for 2 h, then the absorbance at 595 and 540 nm was measured on the plate reader (Labsystems Multiskan MS). The experiment was performed in quadruplicates, and the reduction of Resazurin was normalized to the signal on the day of seeding.

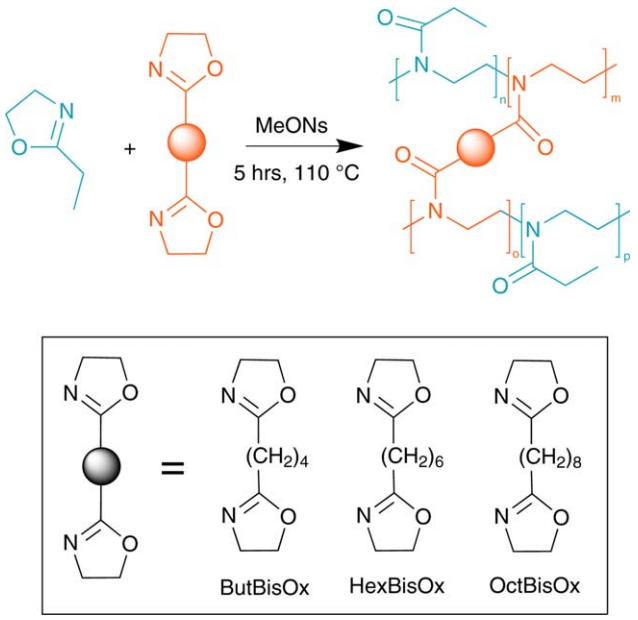

FIGURE 1 Synthesis of hydrogels from 2-ethyl-2-oxazoline and bis(2-oxazoline) crosslinker (2, 3, 4, 5, and 10 mol %). The synthesis was carried out in benzonitrile for 5 h, with 4-methyl nitrobenzenesulfonate as an initiator. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

RESULTS AND DISCUSSION

Synthesis of Hydrogels

Hydrogels were synthesized by "one-pot" random copolymerization of 2-ethyl-2-oxazoline with bis(2-oxazoline) crosslinkers (**ButBisOx**, **HexBisOx,** and **OctBisOx**) in benzonitrile at 110 °C (Fig. 1). Seagusa et al.[18] used acetonitrile for the synthesis of crosslinked poly(2-oxazoline)s. Later, in the work of Rueda et al.,[19] benzonitrile was employed as a solvent. In the more recent works of Schenk[28] and Hartlieb,[22] dichloromethane was used for the preparation of poly(2-

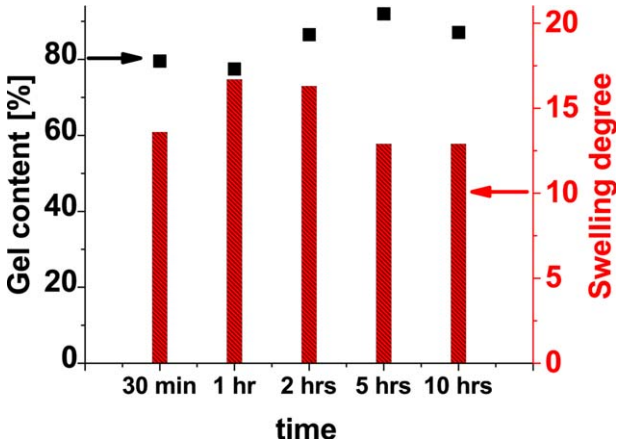

FIGURE 2 Dependence of swelling degree in distilled water (columns) and gel content (squares) on the polymerization time for 2% HexBisOx hydrogel. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

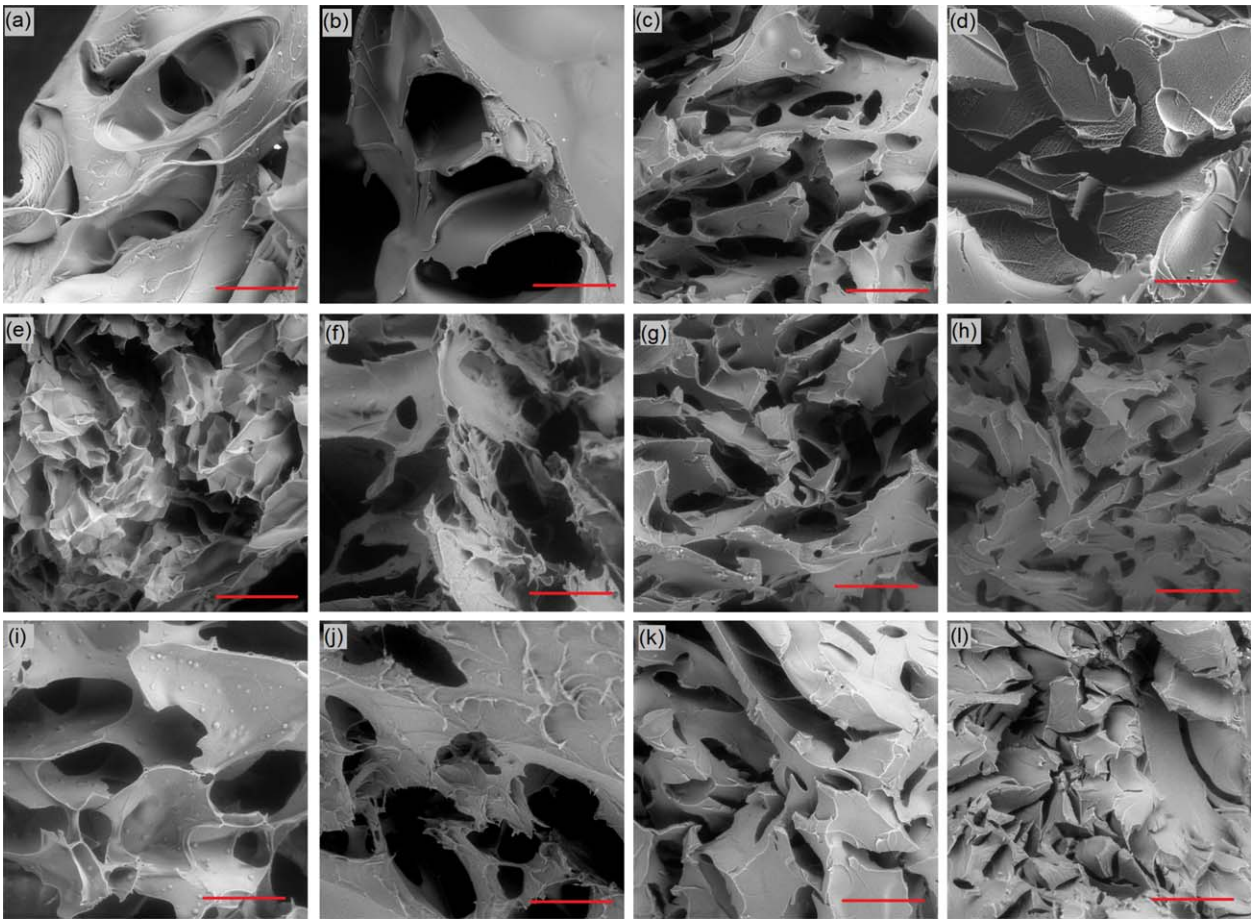

FIGURE 3 SEM micrographs of the morphology of freeze-dried samples. **ButBisOx** (a)–(d), **HexBisOx** (e)–(h), **OctBisOx** (i)–(l), percentage of crosslinker: 2% column 1; 3% column 2; 5% column 3; 10% column 4; scale bar 200 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

oxazoline) hydrogels. However, due to their relatively low boiling point, the polymerizations were conducted under higher pressure in microwave reactor. To overcome this drawback, we decided to use benzonitrile in our reaction conditions, due to its higher boiling point, compared to acetonitrile, which enables us to use higher reaction temperatures at ambient pressure. In addition, bis(2-oxazoline) crosslinkers exhibited good solubility in this solvent. As an initiator, 4-methyl nitrobenzenesulfonate (**MeONs**) was used in our study. **MeONs** was proved to be a suitable initiator for living cationic polymerization of 2-oxazolines, with the propagation rate coefficient slightly lower as that of methyl triflate.[29] In comparison to methyl triflate, **MeONs** exhibits an increased stability during storage and easier synthesis and purification.

First, the equilibrium SD and the gel content was studied as a function of the time of polymerization for the 2% **HexBisOx** hydrogel (Fig. 2). The gelation was observed already after 30 min of polymerization. After 2 h, the gel content was gradually increasing from the value 78 to 92% (maximum at 5 h). The SD decreased from 16.7 for the time 2 h to 12.9. This behavior reflects additional crosslinking in the gel. After 5 h, no changes of SD and minimal changes of gel content were observed. Therefore, we selected 5 h as the polymerization time for all reactions.

For each crosslinker (**ButBisOx**, **HexBisOx**, **OctBisOx**), five different molar percentage were selected: 2, 3, 4, 5, and 10%. For 1% of crosslinker, no gelation under our experimental conditions was observed. All syntheses were carried out in triplicates to ensure the reproducibility of the results. For 2% **ButBisOx**, the averaged gel content from three syntheses was 83%, for 2% HexBisOx and 2% OctBisOx 86%. For all other samples, the gel content was higher than 92%.

Morphology of Dried Hydrogels

The porosity and the pore size of polymeric material has a strong effect on the behavior and the viability of cells.[30,31] In this work, the morphology of freeze-dried samples was examined using scanning electron microscopy. For nonconductive polymeric samples, the coating of sample by conductive (Au) layer before measurement is a common procedure.[32] However, the gold sputtering may not be

efficient method for the thicker 3D structures and charging of the surface may occur during the imaging.[33] To avoid this problem and to simplify the sample preparation, we used low vacuum regime ($P = 20$ Pa) and more energetic backscattered electrons to visualize hydrogel structure without additional coating of the samples. The representative SEM micrographs are shown in Figure 3.

In the electron micrographs, the porous inner structure of hydrogels was revealed. This structure is the consequence of the freeze-drying process, when the ice crystals disrupt the hydrogel structure. The comparison of micrograph of freeze-dried and air-dried samples is shown in Supporting Information Figure S3. With the increasing percentage of the crosslinker, the pores size decreases and the interporous area increases. The exception is 10% **ButBisOx**, where the decreased number of pores but increased size of pores can be seen, compared to 5% **ButBisOx**.

Swelling Properties

The equilibrium SD in various solvents (distilled water, dichloromethane, ethanol, and PBS) was measured to compare the swelling capacity and polarity between hydrogels with three types of crosslinkers and with different crosslinking densities. The results are summarized in Table 1. (For graphical representation, see Fig. 4) The SDs in ethanol, distilled water, and dichloromethane are comparable for all hydrogels (except for 2% **ButBisOx** which swells slightly less in ethanol, see Table 1). This is in accordance with the results published by Schenk et al.[21] The gels can be, thus, referred as amphigels and their ability to swell in ethanol can be further used for the immobilization of therapeutic compounds. It is noteworthy that in previously mentioned work[21] the lowest percentage of **ButBisOx** crosslinker was 4%, referred to as 150:6 EtOx:crosslinker. The SD of such gel in distilled water was presented to be 7.3 (in comparison to 5.5 in our work). Furthermore, the authors claim that the hydrogels with even lower crosslinker percentage (up to 2%) were also prepared; however, they dissolved during purification.

When comparing three different types of crosslinker, it is interesting that despite the observable difference of swelling between **ButBisOx** and **HexBisOx** hydrogels, **HexBisOx** and **OctBisOx** hydrogels swell comparably. The longer aliphatic chain does not influence the SD of hydrogels. Thus, the swelling ability of hydrogels is controlled mainly by the polarity of crosslinker.

Differential Scanning Calorimetry

In general, three different states of water were described for hydrated polymers.[34] The free water, with the freezing temperature around 0 °C, is not bound to the polymeric chains. The nonfreezing, or bound water, is bound to the polymeric chains, and therefore exhibits no transition on the DSC heating curve. The third type is so-called intermediate or freezing-bound water, for which the cold crystallization peak below 0 °C on the DSC heating curve is typical. It was suggested that non-freezing water may play a key role in protein adsorption resistance, and thus lead to increasing biocompatibility of polymeric materials.[26] In our study, the state of water in hydrogels was evaluated using DSC. The representative DSC heating curves are shown in Figure 5. It is noteworthy that a small exothermic peak appears on DSC curves around −12 °C for the highest percentage of crosslinker (Supporting Information Fig. S4). From the DSC and X-ray diffraction study of hydrated poly(2-methoxyethylacrylate), the exothermic peak at lower temperatures was associated with the formation of hexagonal ice crystals,[35] thus, with the presence of freezing-bound water. However, the reported crystallization temperature was around −45 °C. The shift to the higher temperatures may be due to different chemistry of poly(2-methoxyethylacrylate) compared to poly(2-ethyl-2-oxazolines) where hydrogen bound between water molecules and amidic group can be found. Since this suggested explanation needs a further study, we limit our discussion to the freezing and nonfreezing water content.

The results from DSC measurements are summarized in Table 2. For all three types of crosslinkers, we observe a decreasing amount of free water with the increasing percentage of crosslinker. 2% **ButBisOx** hydrogel contains $73.2 \pm 2.4\%$ of free water, in comparison to $29.8 \pm 3.8\%$ for 10% **ButBisOx** hydrogel. Interestingly, the percentage of crosslinker has a stronger influence on the state of water than the polarity of crosslinker. The opposite observation was made by Kasak et al.[36] for zwitterionic hydrogels, where the chemistry of crosslinker was the major factor influencing the state of water.

When comparing the amount of free water in hydrogels with their morphology (see Section Morphology of dried hydrogels), the decreasing free water content with increasing crosslinking percentage may be explained by the decrease of the pore size of hydrogels.

Mechanical Properties

One of the most important properties of a material used in contact with cells is its mechanical stiffness. As it was shown in the pioneering work of Disher et al., the mechanical stiffness of the substrate influences the behavior of cells.[37] Moreover, the differentiation of the stem cells can be influenced by the mechanical properties of their environment.[38] On soft substrates ($E \approx 1$ kPa), the pluripotent stem cells differentiate toward neural cells, while on rigid surfaces ($E \approx 100$ kPa), the stem cells differentiate toward osteoblasts.

In our work, we have examined the mechanical properties of hydrogels swollen in distilled water by using Texture Analyser in compression mode. For the sake of clarity, the presented data are from one synthesis of every type of hydrogels. The comparison between different syntheses of the same hydrogel composition is shown in Supporting Information Figure S5. The mechanical properties of hydrogels are summarized in Table 3.

With increasing crosslinking density, the Young's modulus of hydrogels increases for all three crosslinkers. This is a

TABLE 1 Equilibrium Swelling Degree of Hydrogels in Various Solvents, Mean ± SD from Three Independent Syntheses

	%	CH_2Cl_2	CH_3CH_2OH	dH_2O	PBS
ButBisOx	2	18.3 ± 1.0	13.2 ± 1.5	21.3 ± 0.9	13.4 ± 1.3
	3	10.0 ± 1.0	7.0 ± 0.7	8.0 ± 0.2	7.7 ± 0.7
	4	7.3 ± 0.2	4.8 ± 0.2	5.5 ± 0.3	5.0 ± 0.1
	5	5.7 ± 0.4	4.0 ± 0.2	4.7 ± 0.3	4.4 ± 0.2
	10	3.4 ± 0.2	2.1 ± 0.2	2.3 ± 0.1	2.4 ± 0.1
HexBisOx	2	14.4 ± 0.5	10.5 ± 0.9	13.2 ± 0.1	10.3 ± 0.3
	3	7.8 ± 0.1	5.7 ± 0.4	6.3 ± 0.7	5.7 ± 0.5
	4	6.2 ± 0.2	4.1 ± 0.3	4.5 ± 0.3	4.2 ± 0.3
	5	4.5 ± 0.3	3.0 ± 0.2	3.2 ± 0.1	3.1 ± 0.3
	10	3.1 ± 0.05	1.9 ± 0.1	2.0 ± 0.1	2.1 ± 0.0
OctBisOx	2	14.0 ± 3.3	10.5 ± 0.5	12.5 ± 0.9	10.7 ± 0.1
	3	10.8 ± 3.6	6.9 ± 2.1	7.6 ± 2.5	6.8 ± 1.8
	4	6.2 ± 0.3	3.9 ± 0.3	4.0 ± 0.4	4.0 ± 0.2
	5	5.2 ± 0.1	3.1 ± 0.1	3.2 ± 0.1	2.9 ± 0.1
	10	3.1 ± 0.1	1.8 ± 0.0	1.7 ± 0.2	1.8 ± 0.2

general trend for hydrogels described also in the work of Anseth et al.[39] The exception were 10% **HexBisOx** and 10% **ButBisOx**, where the Young's modulus slightly decreased (from 66.7 ± 16.8 to 51.4 ± 16.2 kPa and from 70.3 ± 20.3 to 62.3 ± 21.3 kPa, respectively). On the other hand, the decrease of compression at break with increasing crosslinking density was observed for **ButBisOx** (from 48 ± 6 to 34 ± 11%) and **HexBisOx** hydrogel (from 44 ± 6 to 28 ± 4%). The trend was opposite for **OctBisOx** crosslinker. The compression at break increased from 32 ± 9 to 43 ± 3%. For the comparison of all hydrogels, the stress at 20% (σ_{20}) deformation was arbitrary selected. The increase of σ_{20} with the increase of crosslinked density may be observed. For the 10% of crosslinker, σ_{20} of **OctBisOx** hydrogel is 44.2 ± 7.4 kPa, which is 1.7-fold higher than σ_{20} of **HexBisOx** hydrogel (25.9 ± 6.4 kPa). However, the SD in water of 10% **HexBisOx** (2 ± 0.1) does not differ dramatically from the SD of 10% **OctBisOx** (1.7 ± 0.2). The differences in Young's modulus as well as in compression at break thus could not be explained by the crosslinking densities of hydrogels. Since the high reaction yields imply successful incorporation of bis(2-oxazoline) crosslinkers into the polymeric chains, we hypothesize that this effect is caused by sterical reason. Crosslinker with the longest aliphatic (**OctBisOx**) enables crosslinking between distant polymeric chains whereas crosslinkers with shorter aliphatic chains (**ButBisOx, HexBisOx**) reacts preferentially with closer molecules, leading to the generation of loops and blind ends. These structures do not contribute to the mechanical stiffness of a material. A similar behaviour was observed for polyacrylamide gels, where the maximum of Young's modulus was observed at 5% of the crosslinker. A further increase of the crosslinker led to a decrease of the modulus.[40]

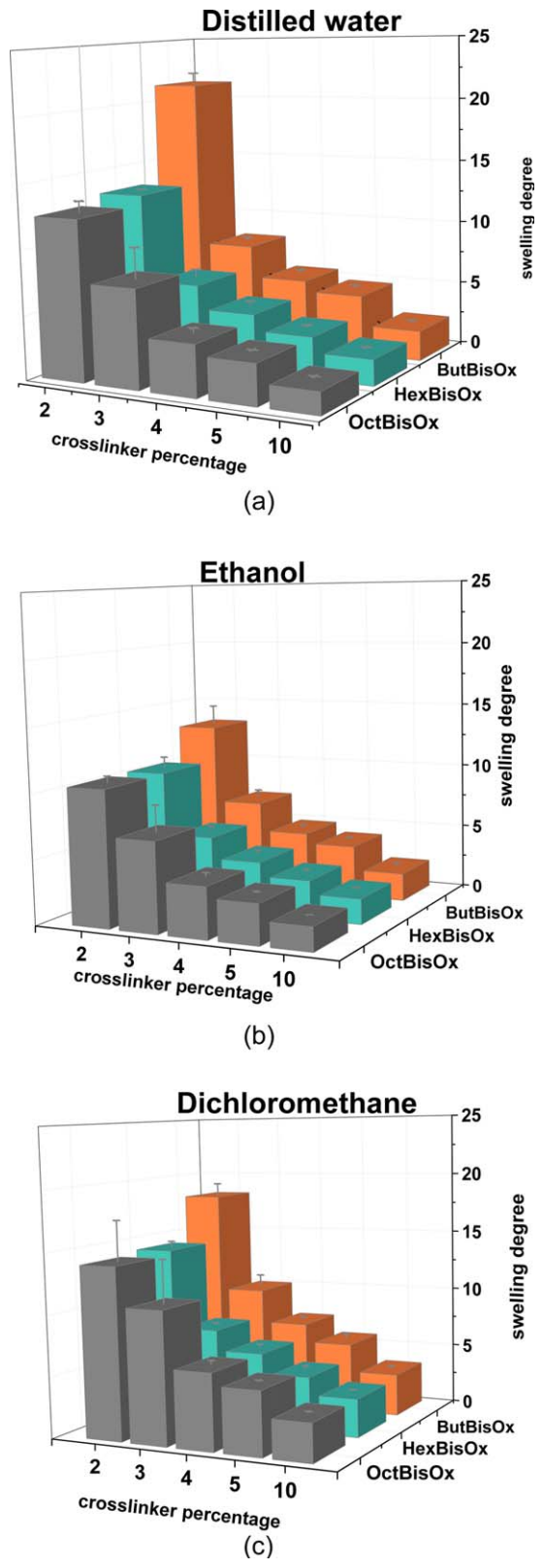

FIGURE 4 Equilibrium swelling degrees of hydrogels in distilled water, ethanol, and dichloromethane, mean ± SD, $n = 3$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

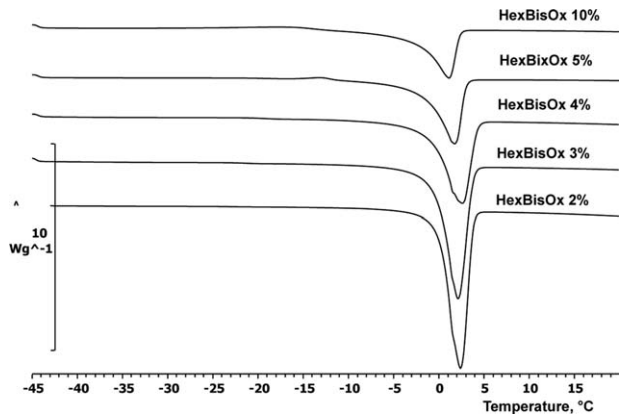

FIGURE 5 The representative DSC heating curves for **HexBisOx** hydrogel with different crosslinker percentage.

The representative stress-deformation curves are depicted in Figure 6.

Cytotoxicity Studies

The direct contact toxicity was evaluated on 3T3 mice fibroblast cell line after 24 h cultivation in contact with hydrogels covering approximately 14% of the well surface. Figure 7 represents the percentage of viable cells compared to non-treated controls (cells cultivated without hydrogels).

The highest viability (100.4 ± 8.4%) was exhibited by cells grown in contact with 2% **ButBisOx**, which is the softest and the most hydrophilic hydrogel. Hydrogels prepared by more hydrophobic crosslinkers (**HexBisOx** or **OctBisOx**) exhibited decrease in cell viability (Fig. 6). In addition, the increase in the crosslinker concentration (> 2%) also results in a decreased cell viability (e.g., 55.8 ± 8.6% for 10% **HexBisOx**). These effects may be due to the higher stiffness of hydrogels causing the defects in the cell membranes and/or by the leakage of low molecular chemical compounds.

To further analyze the toxic effect of low molecular compounds, the toxicity of hydrogel extracts toward murine 3T3 fibroblasts was studied. The gels were extracted for 24 h in full DMEM medium at 37 °C. The toxicity studies were performed on extracts from all hydrogels (Supporting

TABLE 2 The States of Water of Poly(2-oxazoline) Hydrogels

	%	EWC (%)	W_f (%)	W_{nf} (%)
ButBisOx	2	95.4	73.2 ± 2.4	22.2 ± 2.4
	3	89.5	52.3 ± 12.0	37.2 ± 12.0
	4	84.5	50.7 ± 6.1	33.8 ± 6.1
	5	80.2	35.0 ± 0.7	45.2 ± 0.7
	10	72.2	29.8 ± 3.8	42.3 ± 3.8
HexBisOx	2	92.9	71.8 ± 1.7	21.1 ± 1.7
	3	87.1	62.7 ± 6.3	24.4 ± 6.3
	4	81.8	49.9 ± 4.1	32.0 ± 4.1
	5	76.8	40.2 ± 2.1	36.6 ± 2.1
	10	67.4	25.4 ± 6.9	42.0 ± 6.9
OctBisOx	2	92	75.0 ± 2.4	17.0 ± 2.4
	3	91.3	51.2 ± 3.1	40.1 ± 3.1
	4	80.7	43.0 ± 6.8	37.7 ± 6.8
	5	76.6	40.9 ± 3.4	35.7 ± 3.4
	10	60.7	25.6 ± 2.4	35.1 ± 2.4

EWC, equilibrium water content, W_f, free water content; W_{nf}, nonfreezing water; the data are presented as a mean ± SD from three measurements

TABLE 3 Mechanical Properties of Hydrogels

	%	E (kPa)	σ_{20} (kPa)	σ_{br} (kPa)	ε_{br} (%)
ButBisOx	2	11.5 ± 4.0	2.4 ± 1.2	8.3 ± 2.8	48 ± 6
	3	26.0 ± 3.2	4.2 ± 1.6	10.2 ± 4.0	36 ± 7
	4	34.2 ± 8.5	5.4 ± 2.1	13.9 ± 6.0	37 ± 10
	5	66.7 ± 16.8	11.5 ± 3.4	23.0 ± 5.4	34 ± 5
	10	51.4 ± 16.2	19.4 ± 7.2	40.1 ± 14.4	34 ± 11
HexBisOx	2	21.9 ± 3.9	5.0 ± 0.9	18.3 ± 6.4	44 ± 6
	3	23.2 ± 4.7	5.5 ± 0.9	16.2 ± 5.3	39 ± 4
	4	26.4 ± 5.5	6.1 ± 1.3	18.1 ± 9.0	37 ± 9
	5	70.3 ± 20.3	18.1 ± 6.7	48.4 ± 22.8	31 ± 3
	10	62.4 ± 21.3	25.9 ± 6.4	48.3 ± 10.6	28 ± 4
OctBisOx	2	23.7 ± 4.2	5.0 ± 1.0	19.7 ± 4.1	32 ± 9
	3	33.9 ± 8.7	6.7 ± 1.7	19.7 ± 3.1	34 ± 4
	4	39.4 ± 11.7	10.1 ± 3.8	23.5 ± 4.8	36 ± 5
	5	65.8 ± 14.1	17.3 ± 5.0	40.0 ± 9.5	41 ± 3
	10	85.6 ± 17.1	44.2 ± 7.4	96.7 ± 34.5	43 ± 3

E, Young's modulus; σ_{20}, stress at 20% deformation; σ_{br}, stress at break; ε_{br}, compression at break; mean ± SD ($n \geq 5$).

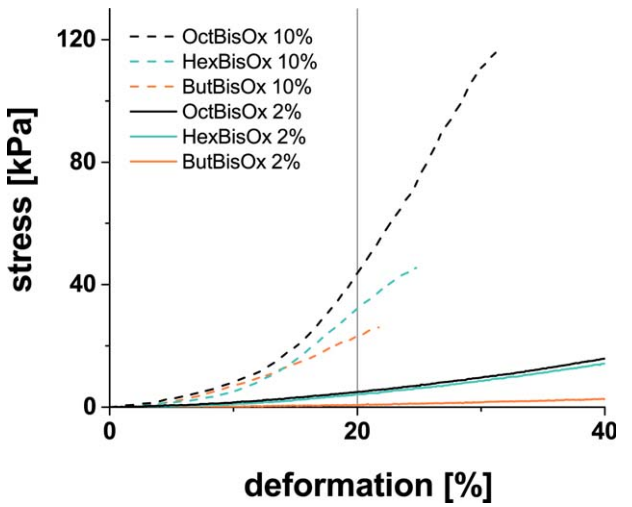

FIGURE 6 Representative stress-deformation curves of **ButBisOx** (orange), **HexBisOx** (blue), and **OctBisOx** (black) hydrogels containing 2% (solid) and 10% (dashed) of crosslinker. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

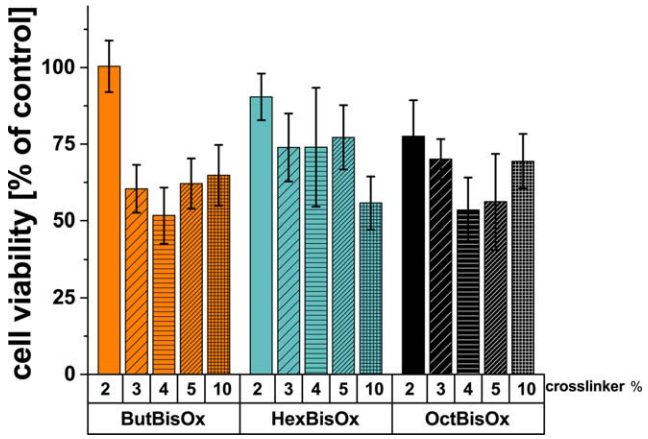

FIGURE 7 Contact toxicity of hydrogels toward 3T3 fibroblasts evaluated by MTT test, mean ± SD from hexuplicates. The hydrogels differ in composition (But-Hex-Oct) and in concentration (2–10%) of the crosslinker. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Information Table S6). In Figure 8, the median from three hydrogels from different syntheses for every concentration and type of crosslinker is presented. The extracts leading to the decrease of cell viability to 75% and lower was considered as toxic in our study. The viability of the cells incubated in extracts prepared from the **ButBisOx** and **HexBisOx** crosslinker is not compromised, and reached 87% and higher. The extracts from **OctBisOx** hydrogels were not toxic up to 3% of the crosslinker percentage, however, higher crosslinking density led to the decreased viability of cells (30.5 ± 1.77% for 4% **OctBisOx**). In general, and in agreement with the contact cytotoxicity results, the lowest cell viability was observed for the cells incubated with extracts of hydrogel made of 4% of crosslinker, this effect is most pronounced for **HexBisOx** hydrogels. This effect may be caused by the presence of low molecular compounds, monomers and of benzonitrile which are present in the extracts as demonstrated by HPLC (Supporting Information Fig. S7) and which is, in general, difficult to wash out from denser networks. In addition, benzonitrile can interact via intermolecular Van der Waals forces with more hydrophobic crosslinkers. It was shown that 2-oxazoline monomers,[41] as well as concentrations of benzonitrile above 1 mg/ml (Supporting Information Fig. S8) are toxic. The toxicity of extracts depends on a combination of several factors. The factors influencing the toxicity include: (i) the efficiency of the purification procedure (ii) the SD of hydrogel in water (the more swollen hydrogel is, the higher amount of benzonitrile can be entrapped in 1 g of its weight), and (iii) the release rate of compounds into the medium.

Cell Cultivation

For the cell cultivation study, the hydrogels with 2% of crosslinker were selected, due to their low toxicity in both contact and extract studies. The viability of cells cultivated inside the hydrogels was evaluated by Resazurin assay and FDA staining for 12 days. Nowadays, Resazurin dye is trademarked as alamarBlue® and it is used for the investigation of viability and proliferation of mammalian cells.[42] The

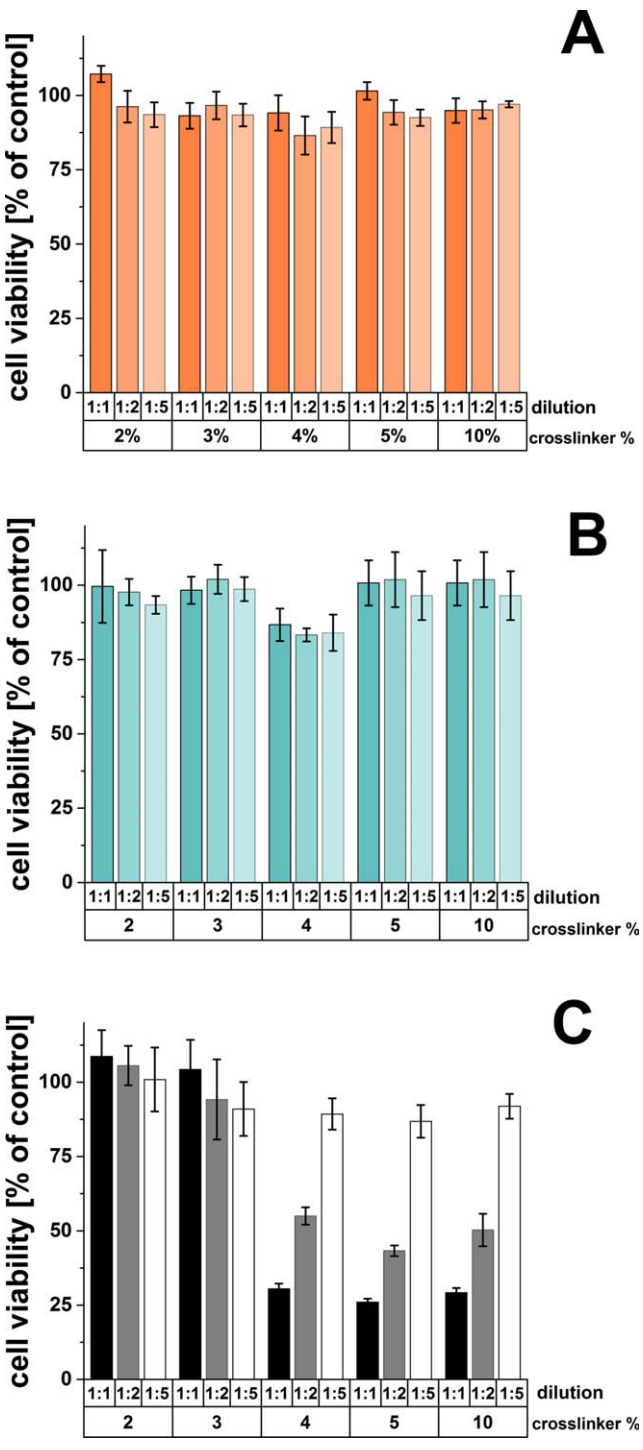

FIGURE 8 Toxicity of extracts from hydrogels evaluated by MTT test on 3T3 fibroblast cell line (seeding density 5×10^3 cells per well) after 24 h of incubation, mean ± SD from hexuplicates. Hydrogels were crosslinked by **ButBisOx** (A), **HexBisOx** (B), and **OctBisOx** (C) crosslinker. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

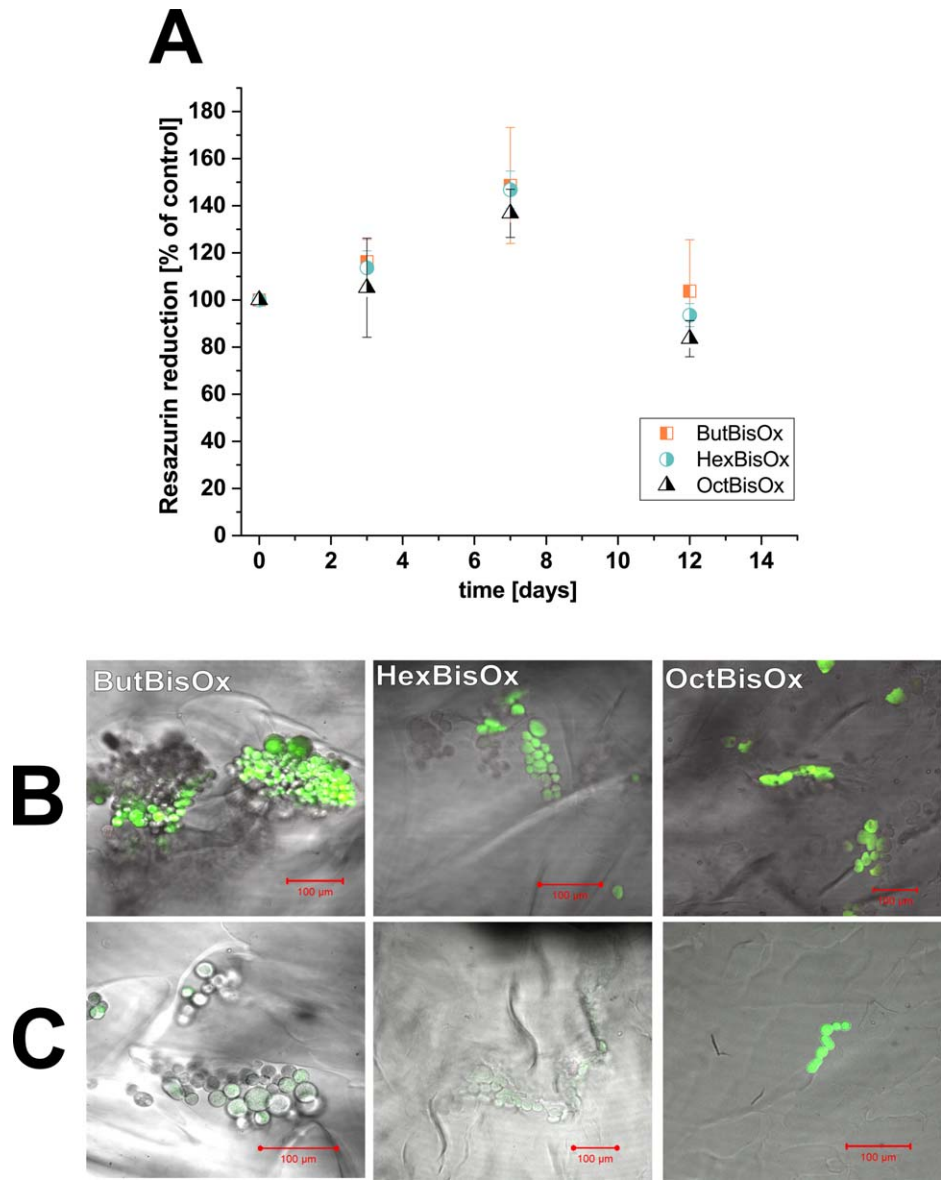

FIGURE 9 Cultivation of pancreatic β-cells inside hydrogels, seeding density 2×10^4 cell/hydrogel cylinder, resazurin staining of cells (A), FDA staining after 7 days (B), and 12 days (C) of incubation. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

oxidized blue Resazurin is reduced in mitochondria to pink fluorescent Resofurin, which enables to measure metabolic activity of the cells. Moreover, as Resazurin does not disrupt the mitochondrial respiratory chain, it is non-toxic for the cells, thus, it can be used for a long-time monitoring of cell viability study. During the first 7 days of cultivation, an increase in the signal of reduced Resazurin is observed in all three types of hydrogels indicating the cell proliferation [Fig. 9(A)]. The Resazurin reduction is comparable in all three types of hydrogels; $148.6 \pm 24.6\%$ for 2% **ButBisOx** hydrogels, $146.8 \pm 8\%$ for 2% **HexBisOx** hydrogels and $136.8 \pm 10.2\%$ for 2% **OctBisOx** hydrogels, normalized to the value at the beginning of the experiment. However, after 12 days of cultivation, the reduction of Resazurin decreased to $103.8 \pm 21.9\%$ for 2% **ButBisOx** hydrogels, $93.6 \pm 4.9\%$ for **HexBisOx** hydrogels and $83.6 \pm 7.7\%$ for **OctBisOx** hydrogels. These results are in agreement with the FDA fluorescence staining, where, in comparison to the hydrogels after 12 days of cultivation [Fig. 9(C)], the hydrogels after 7 days of cultivation contained higher number of viable cells stained green [Fig. 9(B)]. The highest loss in cell viability is observed for **OctBisOx** hydrogels with the longest aliphatic chain. The decrease in cell viability can be due to the confined space and insufficient nutrients supply.[43] The morphology of the cells cultivated in hydrogels was spherical and cells formed aggregates, what is particularly visible for 2% **ButBisOx**. Due to the anti-fouling properties of poly(2-oxazoline)s,[44] the cell–cell interactions were promoted over the

cell-surface interactions, what led to the formation of spherical aggregates of cells.

CONCLUSIONS

Poly(2-oxazoline)-based hydrogels have recently been recognized as promising scaffolds for the cultivation of fibroblast,[12] as well as cancer cell lines.[45] Up to date, the cultivation studies were limited to the poly(2-oxazoline)s crosslinked by thiol-ene click reaction. In our work, we focused on the hydrogels prepared by the copolymerization of (2-ethyl-2-oxazoline) with bis(2-oxazoline) crosslinkers.

We synthesized a series of hydrogels by copolymerization of 2-ethyl-2-oxazoline with three different bis(2-oxazoline)s – 1,4-butylene-2,2′-bis(2-oxazoline), 1,6-hexamethylene-2,2′-bis(2-oxazoline), and 1,8-octamethylene-2,2′-bis(2-oxazoline). The bis(2-oxazoline) crosslinkers with the longer aliphatic chain were used for the preparation of hydrogels for the first time. Hydrogels showed a comparable SD in distilled water, ethanol, and dichloromethane. The SD in water varied with the type and molar percentage of crosslinker from 21.32 ± 0.88 for 2% **ButBisOx**, to 1.72 ± 0.16 for 10% **OctBisOx**. Young's modulus varied from 11.5 ± 4 kPa for 2% **ButBisOx** to 85.6 ± 17.1 kPa for 10% **OctBisOx**. The hydrogels with lower crosslinking density and a shorter aliphatic chain of crosslinker were well tolerated by fibroblast 3T3 cells in both contact and extract toxicity studies. The pancreatic βTC3 cells were cultivated within the hydrogels for 12 days and formed spherical aggregates. The modification of hydrogel samples to promote the cell adhesion will be the subject of the ongoing studies.

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