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An Inverse Thermogelling Bioink Based on an ABA-Type Poly(2-oxazoline) Amphiphile

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ABSTRACT: Hydrogels are key components in several biomedical research areas such as drug delivery, tissue engineering, and biofabrication. Here, a novel ABA-type triblock copolymer comprising poly(2-methyl-2-oxazoline) as the hydrophilic A blocks and poly(2-phenethyl-2-oxazoline) as the aromatic and hydrophobic B block is introduced. Above the critical micelle concentration, the polymer self-assembles into small spherical polymer micelles with a hydrodynamic radius of approx 8–8.5 nm. Interestingly, this specific combination of hydrophilic and hydrophobic aromatic moieties leads to rapid thermoresponsive inverse gelation at polymer concentrations above a critical gelation concentration (20 wt %) into a macroporous hydrogel of densely packed micelles. This hydrogel exhibited pronounced viscoelastic solid-like properties, as well as extensive shear-thinning, rapid structure recovery, and good strain resistance properties. Excellent 3D-printability of the hydrogel at lower temperature opens a wide range of different applications, for example, in the field of biofabrication. In preliminary bioprinting experiments using NIH 3T3 cells, excellent cell viabilities of more than 95% were achieved. The particularly interesting feature of this novel material is that it can be used as a printing support in hybrid bioink systems and sacrificial bioink due to rapid dissolution at physiological conditions.

INTRODUCTION

Thermosensitive water-soluble polymers undergo phase separation due to temperature changes. Phase separation upon heating is characterized by a lower critical solution temperature (LCST).¹ In contrast, in a polymer, which exhibits an upper critical solution temperature (UCST), phase separation takes place upon cooling.^{2–7} Exemplarily, Seuring et al. reported and characterized the UCST-type phase separation properties of a nonionic homopolymer poly(*N*-acryloyl glycinamide) in pure water.⁸ In a more recent study poly(acrylic acid) was copolymerized with acrylonitrile resulting in an UCST-type copolymer combining the driving forces of hydrogen bonding and hydrophobic interactions.⁹ In some cases hydrogel formation instead of phase separation/precipitation is observed. Over the last decades, most described systems in the literature are based on synthetic or natural water-soluble polymers exhibiting thermosensitive gelation upon heating.^{10–15} By introducing thermoresponsive units into the polymer structure, gelation can be induced at room or elevated temperatures as was described many times.^{16,17} Only a few examples can be found in the literature where the gelation occurred upon cooling the sample below a critical temperature (inverse gelation). Particularly prominent examples are natural biopolymers like gelatin, agarose, and pectin/chitosan systems.^{18,19} In 1998 Yoshioka et al. reported a gelatin-*g*-poly(*N*-isopropylacrylamide) graft copolymer which undergoes thermogelation (above 34 °C) and inverse thermogelation (below 10 °C) at 5 wt %.²⁰ In addition to rheological investigations, the hypothetical use of

this system in smart 3D cell culture was discussed. In 2016, an inverse thermogelling ABA-type block copolymer comprising a poly(poly(ethylene glycol) methyl ether methacrylate) middle block and thermosensitive poly(acrylamide-*co*-acrylonitrile) (P(AAm-*co*-AN)) outer blocks was described.²¹ The temperature-dependent sol/gel properties were investigated in the temperature range of 8–80 °C and low polymer concentrations of 3 and 5 wt %. At 8 °C, a clear hydrogel was obtained. The sol/gel transition could be modulated by the polymer composition. By modifying a high molecular weight precursor copolymer comprising *N,N*-dimethylacrylamide and acrylic acid with hydrophobic moieties of *n*-dodecyl amine, an inverse thermogelling hydrogel could be realized.²² At 0 °C, a viscosity of approx. 1 kPas was obtained by varying the sol/gel temperature with different polymer concentrations. However, no defined application as biomaterials has been described here either. Inspired by the water transfer of natural mimosa, Chen and co-workers established a thermoresponsive bilayer system in 2018.²³ One part of the system was an inverse thermogelling hydrogel formed by poly(acrylic acid-*co*-acrylamide) (PAAc-*co*-PAAm) layers. The system was discussed as an actuator for the

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Scheme 1. Synthesis Route^a

^a(A) Synthesis of PhenEtOx (B) adapted from Witte and Seeliger.⁴¹ (B) Synthesis of the ABA-type amphiphile by living cationic ring-opening polymerization of the hydrophilic MeOx (A) and the hydrophobic and aromatic PhenEtOx (B) using methyl triflate (MeOTf) as an initiator. To introduce the terminal Y-functionality, BOC-Pip (P1) and EIP (P2) were used as terminating reagents, respectively.

fabrication of intelligent soft materials for bioinspired applications. Nevertheless, the embedding of living cells seems to be challenging as the cells sediment before polymerization and the actual hydrogel formation is performed at 50 °C under ultraviolet light (UV) exposure. The combination of such conditions can be expected to cause significant cytotoxicity. In 2019, Hua et al. reported a multi-thermoreponsive hydrogel system, which offers the possibility of 3D shape transition upon cooling in water and oil systems.²⁴ In the last years, the interest in biofabrication, especially for tissue engineering and regenerative medicine has been rapidly growing into a promising interdisciplinary research field in its own right.²⁵ One major additive manufacturing technique used in the field is extrusion-printing of bioinks.²⁶ Often a hydrogel formulation, which enables printability and cell survival during the printing process, is preferentially used.²⁷ To date, most commonly, hydrogels based on natural biopolymers, such as gelatin, alginate, and hyaluronic acid, are used due to their generally good cytocompatibility.²⁸ However, such systems based on biopolymers can also have limitations such as less-than-ideal printability and often considerable batch-to-batch variations, especially with respect to rheological properties. The synthetic polymer platform formed by cyclic imino ethers, in particular poly(2-substituted-2-oxazoline)s (POx) and poly(2-substituted-5,6-dihydro-4H-1,3-oxazine)s (poly(2-oxazine)s, POzi), was investigated for decades as biomaterials in different applications due to good cytocompatibility and chemical versatility.^{29–32} POx/POzi-based systems were investigated as thermoresponsive materials³² for biomedical applications^{33,34} and drug delivery³⁵ approaches. However, surprisingly few reports can be found that show thermogelation of pure POx/POzi polymers in water. In 2017, Lorson et al. established a thermogelling cytocompatible and printable supramolecular hydrogel based on POx/POzi diblock copolymers.³⁶ The system comprised a hydrophilic poly(2-methyl-2-oxazoline) (PMeOx) block and a thermoresponsive poly(2-*n*-propyl-2-oxazine) (PnPrOzi) block of similar block length. Another thermogelling hydrogel was described by Hoogenboom and Monnery.³⁷ A BAB triblock copolymer bearing poly(2-*n*-propyl-2-oxazoline) (PnPrOx) (B) and hydrophilic poly(2-ethyl-2-oxazoline) blocks (A) showed sol/gel transitions upon heating, but extremely high degrees of polymerization were required. In 2019, Lübtow et al. described a different, much shorter ABA triblock copolymer, which forms stable, but relatively weak and barely printable, thermogelling hydrogels.³⁸ Very recently, the first inverse thermogelling hydrogel formed by POx/POzi-based block copolymers was described.³⁹ Here, the ABA triblock copolymer comprising the hydrophilic PMeOx (A) and aromatic hydrophobic poly(2-phenyl-2-oxazine) (PPheOzi, B) blocks undergoes gelation at lower temperatures. The stable hydrogel was

formed by entangled self-assembled wormlike micelles, which form from spherical micelles upon cooling, leading to gelation. A potential application of such system as a biomaterial ink was mentioned. The limiting factor for using this system as a component in a bioink formulation is its gelation kinetics. Upon cooling, it takes about 1 h to form a hydrogel suitable for printing, which is a rather long time if viable cells are to be embedded. Very recently, a novel gelation mechanism driven by interactions between the hydrophilic PMeOx blocks and the aromatic PPheOzi blocks was suggested for this system.⁴⁰ Due to this interaction, the volume fraction of the hydrophilic to hydrophobic domain changes, leading to the morphology switch. Interestingly, polymers containing very similar 2-phenyl- and 2-benzyl-2-oxazoline and 2-benzyl-2-oxazine did not undergo this gelation. Here, a further structural variation and a new ABA triblock copolymer containing aromatic poly(2-phenethyl-2-oxazoline) (PPhenEtOx, B) is described, which can be seen as a higher homologue or structural isomer of previously studied polymers, and it again undergoes inverse thermogelation in aqueous solution. In contrast to previously studied material, a rapid thermoresponsive sol/gel transition was observed, which facilitates its use as a bioink component, as was demonstrated. The additional two CH₂-groups in the sidechain as well as the different backbones compared to the previously described inverse hydrogel platform, leads to distinct differences in the gelation mechanism as in the present case, the size and morphology of the polymer self-assemblies do not change with temperature.

EXPERIMENTAL SECTION

In general, all substances and reagents for the monomer synthesis and polymerization were purchased from Sigma-Aldrich (Steinheim, Germany) and TCI-chemicals (Eschborn, Germany) and were used as-received without further purification unless otherwise stated. For polymerization, all substances were refluxed over CaH₂ for several hours and distilled prior usage. The solvent benzonitrile (PhCN) was dried over phosphorus pentoxide.

The monomer synthesis of 2-phenethyl-2-oxazoline (Schemes 1A and S1) was carried out as described by Witte and Seeliger.⁴¹ For the reaction, 1 equiv of 3-phenylpropionitrile, 1.2 equiv of amino-ethanol, and catalytic amounts of zinc acetate dihydrate were added to a argon-flushed flask and heated to 130 °C under reflux for several days until the color of the reaction mixture turned brown. Reaction progress was controlled by ¹H NMR spectroscopy. After completion, the mixture was dissolved in dichloromethane and washed three times with H₂O. The organic phase was dried with MgSO₄ and concentrated. The raw product was refluxed with CaH₂ and purified via vacuum distillation under an argon atmosphere yielding a colorless liquid. The resulting compound 2-phenethyl-2-oxazoline was characterized via refractive index, gas chromatography-electrospray ionization-mass spectrometry (GC-ESI-MS) analysis, and ¹H and ¹³C nuclear magnetic resonance (NMR) spectroscopy (see the Supporting Information).

Refractive index measurements of the synthesized monomer 2-phenethyl-2-oxazoline was performed on a RFM 870 refractometer from Bellingham + Stanley at 20 °C (Farnborough, England).

The monomer was further analyzed via mass spectrometry using an Agilent 5977B MDS system coupled with a gas chromatography system Agilent 7820A. The GC system was equipped with an Agilent 19091S-433UI HP-5 ms ultraintert column (30 m × 250 μm × 0.25 μm). The temperature gradient was set from 40 to 300 °C with a constant heat rate of 15 °C/min and a constant flow of 1 mL/min.

The ABA-type copolymers were synthesized following a general procedure based on previous reports (detailed description in the Supporting Information).^{42,43}

NMR was performed on a Bruker Fourier 300 (¹H: 300.12 MHz) spectrometer at 298 K from Bruker BioSpin (Rheinstetten, Germany) and calibrated using the residual protonated solvent signal.

Gel permeation chromatography (GPC) was performed on a Polymer Standard Service PSS (Mainz, Germany) SECurity system with the following specifications: pump mod. 1260 infinity, MDS RI-detector mod. 1260 infinity (Agilent Technologies, Santa Clara, California, USA), precolumn: 50 × 8 mm PSS PFG linear M, and 2 columns: 300 × 8 mm PSS PFG linear M (particle size 7 μm; pore size 0.1–1.000 kg/mol) with hexafluoroisopropanol (HFIP, containing 3 g/L potassium trifluoroacetate (KTFA)) as eluent calibrated against PEG standards with molar masses from 0.1 to 1000 kg/mol. The columns were held at 40 °C and the flow rate was set to 0.7 mL/min. Prior to each measurement, samples were dissolved in the eluent and filtered through 0.2 μm PTFE filters (Rotilabo, Karlsruhe, Germany) to remove particles, if any.

Rolling ball viscosity experiments were performed on a LOVIS 2000 M microviscometer from Anton Paar (Graz, Austria) using a LOVIS 1.8 capillary and a steel ball of 1.5 mm diameter. Prior to viscosity measurements the density was determined at 5 and 40 °C using a DMA 4100 M density meter from Anton Paar (Graz, Austria). A temperature scan from 5 °C → 40 °C and 40 °C → 5 °C of a 10, 15, and 20 wt % aqueous sample was performed in an automated angle mode with 3 min of temperature equilibration to establish the temperature-dependent dynamic viscosity.

For pyrene fluorescence assay, 40 μL pyrene solution (25 μM) in acetone was added to glass vials. The solvent was evaporated and 3 mL of polymer solutions of different concentrations in deionized water were added to yield final polymer concentrations of $1 \times 10^{-7} \rightarrow 5 \times 10^{-5}$ M and a fixed pyrene concentration of 5×10^{-7} M. The pyrene fluorescence emission spectra ($\lambda_{\text{excitation}}$: 330 nm at 25 °C) were recorded on a FP-8300 spectrofluorometer system from Jasco (Gross-Umstadt, Germany) between 360 and 400 nm. To determine the critical micelle concentration (CMC), the I_1/I_3 ratios as a function of polymer concentration were fitted using the Boltzmann fit function, which is given by

$$y = \frac{A_1 - A_2}{1 + e^{(x-x_0)/\Delta x}} + A_2 \quad (1)$$

where A_1 and A_2 are the upper and lower limits of the sigmoid, x_0 is the center of the sigmoid, and Δx is directly related to the range where the abrupt change of the sigmoid occurs. The onset of an abrupt decrease is defined as the CMC and can be determined via eq 2, which is given by

$$x(\text{CAC}) = x_0 - 2\Delta x \quad (2)$$

Rheology investigations were recorded on an Anton Paar (Ostfildern, Germany) Physica MCR 301 system utilizing a plate–plate geometry (25 mm diameter) equipped with a solvent trap and Peltier element for temperature control. All aqueous samples were measured after complete dissolution in Millipore water and a concentration of 20 wt % at 5 °C. A temperature sweep from 5 to 40 °C followed by a cooling sequence to 5 °C was carried out in an oscillation mode (angular frequency 10 rad/s and amplitude 0.1%) and a constant heating or cooling rate of 0.1 °C/s. For investigations of viscoelastic behavior, the linear viscoelastic region (LVE) was determined by performing an amplitude sweep (0.02% → 500%) using a fixed angular frequency of 10 rad/s. Subsequently, a frequency

sweep (0.1 rad/s → 100 rad/s) was performed at a fixed strain deformation of 0.1%. For dispense plotting, rheological preconditions of the hydrogel are shear-thinning properties, defined force resistance profile, and fast structure recovery after deformation. For steady shear experiments, the control shear rate mode was used (0.001 1/s → 1000 1/s). The pronounced viscosity η decrease was fitted using the power law expression (eq 3)

$$\eta = K \cdot (\dot{\gamma})^{n-1} \quad (3)$$

where K is the consistency index, n is the flow index, and $\dot{\gamma}$ is the applied shear rate.

During a steady stress sweep (5 Pa → 1500 Pa) the onset value of viscosity decrease is referred as the yield point of the hydrogel system. To investigate the structure recovery properties, two different recovery testing experiments were performed. During the ORO (oscillation-rotational-oscillation) experiment, a low strain deformation of 0.5% is followed by a high shear rate of 100 1/s. In the ROR (rotational-oscillation-rotational) experiment, a low shear rate region of 0.1 1/s is followed by a high strain of 100%.

Dynamic light scattering (DLS) experiments were performed on an ALV SP125 (Langen, Germany) equipped with a He-Ne laser (22 mW, $\lambda = 632.8$ nm) and a single optical avalanche photodiode detector or an ALV CGS-3 multidetection goniometry system (Langen, Germany) equipped with a He-Ne laser (632.8 nm) and eight optical avalanche photodiode detectors with a detector angle distance of 16°. Scattering angles from 30 to 125° were measured with a 5° angle interval for aqueous samples (15, 20, 25, and 40°) and 30 to 70° for a methanol sample (20 °C, correlation time 60 s, average of 3–5 runs). Prior to each measurement, samples were dissolved in methanol (10 g/L) and filtered through 0.02 μm Anotop membrane filters from Whatman GE Healthcare followed by Millex-LG 0.2 μm. After evaporation of methanol, the samples were dissolved in prefiltered (Millex-LG 0.2 μm) Millipore water (containing 2 mM NaNO₃) or methanol. Samples (1 g/L) were filtered (Millex-LG 0.2 μm) into dust-free cuvettes under laminar flow. The decay of the electric field-time autocorrelation function (ACF) was fitted using biexponential fit functions (eq 4) with respect to polydispersities:

$$g_1(t) = a_1 \cdot e^{(-t/\tau_1)} + a_2 \cdot e^{(-t/\tau_2)} \quad (4)$$

with the amplitudes a_i and the decay time $\tau_i = \frac{1}{q^2 D_i}$, q being the absolute value of the scattering vector and D the translational diffusion coefficient which is indirectly proportional to the hydrodynamic radius R_h (Stokes–Einstein equation):

$$R_h = \frac{k_B \cdot T}{6 \cdot \pi \cdot \eta \cdot D} \quad (5)$$

with Boltzmann constant k_B and the viscosity of the solvent η .

For transmission electron microscopy (TEM) investigations, the polymer was dissolved in ultrapure water to a final concentration of 20 g/L. For the negative stain method, 400 mesh copper–rhodium grids (maxtaform) with a homemade carbon layer were glow-discharged in air for 1.5 min at medium power in a Harrick PDC-002 plasma cleaner. The 20 g/L sample was diluted (1/125) and 8 μL was incubated on the grids for 1 min before blotting (Whatman filter paper No 50). Subsequently, the grids were washed with water (3×) and with 2% (w/v) uranyl acetate (3×). For imaging, a single-tilt room temperature holder in a FEI Tecnai T12 Spirit transmission electron microscope equipped with a LaB₆ emitter at 120 kV was used. Images were recorded with an Eagle CCD camera under low-dose conditions. The micrographs were binned two times resulting in a pixel size of 4.4 Å/pix at the specimen level.

The temperature-dependent ¹H NMR analysis was performed on a Bruker Avance III HD 600 spectrometer (Karlsruhe, Germany) operating at 600.4 MHz with a BBFO 5 mm probe. ¹H NMR experiments at different temperatures (5–40 °C) of a 20 wt % hydrogel sample in D₂O/H₂O (1:1) were performed without sample spinning and 16 scans. The sample was kept for 10 min at the desired temperature prior to each measurement. Temperature calibration was

done using 4% MeOH in MeOD and 80% ethylene glycol in DMSO- d_6 . All recorded spectra were referenced using the temperature-dependent HDO signal. For a more quantitative characterization of the temperature-induced phase transition, the fraction p was calculated with the integrals $I(T)$ and $I(T_0)$ at the respective temperatures T and T_0 using the following equation:⁴⁴

$$p = 1 - \frac{I(T)}{I(T_0) * \frac{T_0}{T}} \quad (6)$$

The highest signal intensity was measured at 40 °C, which was chosen to be T_0 . Reductions of signal intensity caused by inhibited molecular mobility due to gelation and viscosity increase were quantified by values of $p < 0$.

For small-angle X-ray scattering (SAXS) experiments, an in-house setup at the Chair of X-ray Microscopy at the University of Würzburg was used, which was built by Fraunhofer EZRT (Fürth, Germany). It consists of a MicroMax-007 HF X-ray source (Rigaku, Japan) and an EIGER R 1M detector unit (Dectris, Switzerland). The sample-detector distance can be varied between 5 cm and 3.5 m, which corresponds to possible Q -values between 0.005 and 5 Å⁻¹. The complete setup is operated in a vacuum below 0.1 mbar to reduce air scattering. The sample solution (20 wt %) was placed in quartz capillaries (inner diameter: 1 mm, wall thickness: 10 μm) (Hampton Research, Aliso Viejo, California), which were positioned perpendicularly to the X-ray beam. The presented experiments were done at sample-detector distances of 57, 565, and 1560 mm with an integration time of 15 min for the shortest distance and 240 min for the two longer configurations. All distances were calibrated using a silver behenate standard sample. Data were acquired for different temperatures between 5 and 40 °C. To achieve thermal equilibrium, the sample was kept at the desired temperature for 15 min prior to each measurement. The SAXS data, which were obtained at the two largest distances, were calibrated in terms of absolute intensities using glassy carbon as a secondary calibration standard.^{45,46} The scattering curves of the hydrogel were obtained by azimuthal integration and corrections taking the sample thickness, X-ray transmission, detector accuracy, setup geometry, and solvent scattering into account, by following the standard procedures described in the literature.⁴⁷ A detailed description of the fitting procedure using a hard sphere model can be found in the Supporting Information.

For scanning electron microscopy (SEM) of the native hydrogel structure, we investigated a cryogenic sample preparation procedure. For this, hydrogel samples were placed between two aluminum holders ($d = 3$ mm), both containing a notch with a diameter of 2 mm, enclosing the sample and rapidly frozen in slush nitrogen (SN) at −210 °C. The samples were then transferred into a sputter coater with a Leica EM VCT100 cryo-shuttle at −140 °C (Leica Microsystems ACE 400, Wetzlar, Germany). Here, the upper half of the sample was knocked off to create a fresh fractured surface and freeze-etched at −85 °C for 15 min under vacuum ($<1 \times 10^{-3}$ mbar). The samples were finally sputtered with 3 nm platinum and transferred with the cryo-shuttle into the SEM chamber. The morphology of the fractured surfaces was imaged at −140 °C, by detecting SE using acceleration voltages of 2 or 8 kV.

To investigate the printability of 20 wt % hydrogel samples, a compact bench-top 3D bioprinter (BioX, Cellink, Sweden) working on the principle of an extrusion-based technology was used. The printhead and the print bed were cooled to 8 °C prior to each fabrication process. The printing speed was set to 10 mm/s and a pressure of 120–160 kPa was applied (nozzle: 25 G, stainless steel, length of 6.35 mm). First, the printing resolution was investigated by increasing the strand-center to strand-center distance stepwise from 0.5 mm → 0.75 mm → 1 mm → 1.5 mm → 2 mm (layer height 0.25 mm). To investigate the steadiness of the hydrogel, a strand collapse test was investigated as described by Ribeiro et al.⁴⁸ The distance between the two edition points increased from 0.1 → 0.2 → 0.4 → 0.9 → 1.7 cm. Finally, real 3D-printing was performed by printing a 20-layered tubular construct of 5 mm total height and 5 mm diameter using the parameters already described.

To evaluate the cytocompatibility of POx/alginate (20:1 wt %) bioinks, the viability of embedded NIH 3T3 cells (ATCC, Germany, 1 Mio/mL) was investigated. As a reference, sodium alginate (VIVA Pharm, PH176, 1 wt %) was used. After homogenous cell distribution at 37 °C a simple one-layered square structure (Figure S6) was printed at 10 °C with a printing pressure of 50 kPa and a printing speed of 5 mm/s. Stabilization after printing was performed using a 0.1 M CaCl₂ aqueous solution (10 min). The cross-linked scaffolds were incubated for 24 h at 37 °C in cell culture medium (DMEM high glucose (Gibco from Sigma-Aldrich)) supplemented with 1% pen/strep, 2% glutamine (Thermo Fisher), and 10% bovine calf serum from Corning under controlled conditions (5% CO₂, 95% relative humidity). To assess the cell viability, printed NIH 3T3 cells were stained inside the scaffolds using Calcein AM (Invitrogen, Thermo Fisher) after 24 h of cultivation, whereas blue nuclei acid stain (DAPI) (Thermo Fisher) was used to visualize nuclei of all embedded cells. ImageJ software was used to determine the cell viability using automatic cell counting in three fluorescence images ($n = 3$) of three different samples ($n = 3$) obtained with an epifluorescence microscope (Zeiss Observer, Germany) using the following equation:

$$\text{cell viability} = \frac{[\text{number of living cells}]}{[\text{number of cell nuclei}]} \quad (7)$$

RESULTS AND DISCUSSION

Two batches of an ABA-triblock copolymer PMeOx₃₅-*b*-PPhenEtOx₁₅-*b*-PMeOx₃₅ (=A-PPhenEtOx-A) (Scheme 1B) were synthesized by living cationic ring-opening polymerization (LCROP) and characterized by ¹H NMR spectroscopy (Figures S2 and S5) and GPC (Figures S1 and S4). The successful termination using different terminating agents (P1: 1-Boc-piperazine (BOC-Pip), P2: ethyl isonipecotatate (EIP)), the comparable degree of polymerization of approx 90 repeating units, and a ratio of PMeOx/PPhenEtOx of 4 were verified by the corresponding signal intensities in ¹H NMR spectra.

Via GPC, the number average molar mass M_n and the dispersity $\mathcal{D}$ (M_w/M_n) of both batches were compared after completion of every single block and purification of the final polymer product. Both batches show reasonably narrow molar mass size distributions ($\mathcal{D}$ (P1) = 1.12, $\mathcal{D}$ (P2) = 1.17) and an increase in the number average molar mass during the polymerization reaction, indicative of the living polymerization. The primary structural difference between these novel polymers in comparison to the previously described aromatic amphiphiles is one additional methylene group in the sidechains of every repeat unit in the central B block, which leads to significantly altered physicochemical properties. Very similar to previously described ABA-type amphiphiles, the novel polymers form stable nanoscale micelles in aqueous solutions above the CMC.^{42,49,50} The investigated A-PPhenEtOx-A polymer exhibited a rather low CMC of 0.3 μM as determined by pyrene fluorescence measurement in dependency of the polymer concentration (Figure 1A,B). The hydrodynamic radius of these nanoscale aggregates was determined via DLS at different scattering angles (Figure 1C,D). In the nonselective solvent, methanol, the hydrodynamic radius of an individual polymer chain was determined as ca. 2.4 nm. In contrast, in the selective solvent water, the polymers self-assemble into micelles with a hydrodynamic radius of ca. 8 nm. The low dependence on the scattering angle (30 → 125°) suggests spherical micelles of low polydispersity (Figure 1D). The spherical morphology of the micelles could be further corroborated by means of TEM (Figure 1E).

Inspired by our previous finding for a similar ABA triblock,^{39,40} we investigated the thermoresponsive properties.

Figure 1. Self-assembly of A-PPhenEtOx-A at low polymer concentration in aqueous solution. (A) Characteristic pyrene emission spectra at 25 °C and polymer concentrations of 10^{-7} M (red) and 10^{-5} M (blue). (B) I_1/I_3 intensity ratio of the pyrene signal at different polymer concentrations fitted with the Boltzmann fit function (R^2 : 0.99707). (C) Autocorrelation functions of DLS experiments of 1 g/L aqueous polymer solution containing 2 mM NaNO_3 at 20 °C and different scattering angles (30 → 125° in 5° steps). (D) Inverse hydrodynamic radius in dependency of the scattering vector q^2 of 1 g/L aqueous polymer solution containing 2 mM NaNO_3 (circle) compared to 1 g/L polymer solutions in methanol as nonselective solvent (square). (E) TEM image from aqueous polymer solution stained with uranyl acetate showing spherical micelles.

Indeed it was observed that when the concentration exceeded 20 wt %, a hydrogel was formed at low temperature (Figure 2A,B (P2), Figure S3 (P1)). However, investigating this phenomenon in more detail, crucial differences in gel strength, critical concentration, and temperature compared to the previously described material (PMeOx_{35} - b - PPhEOzi_{15} - b - PMeOx_{35}) were found. The thermoresponsive behavior was evaluated by rolling ball viscosimetry at different concentrations (10, 15, and 20 wt %) combined with a heating (5 °C → 40 °C) and cooling (40 °C → 5 °C) ramp. At 10 and 15 wt %, the viscosity decreases steadily with increasing temperature ($\eta_{5\text{ °C}}(10\text{ wt \%}) = 9\text{ mPas} \rightarrow \eta_{40\text{ °C}}(10\text{ wt \%}) = 3\text{ mPas}$, $\eta_{5\text{ °C}}(15\text{ wt \%}) = 50\text{ mPas} \rightarrow \eta_{40\text{ °C}}(15\text{ wt \%}) = 11\text{ mPas}$) without any notable features. The cooling and heating ramps yield very similar values. In contrast, at 20 wt % the viscosity increases very fast below a critical temperature of approx. 22 °C eventually trapping the ball, whereupon no further values are obtained (Figure 2C). Increasing the temperature, the polymer solution again flows freely forming a moderately viscous solution with dynamic viscosity values of approx. 70 mPas at 40 °C. A notable hysteresis

Figure 2. Increasing concentration of A-PPhenEtOx-A leads to hydrogel formation. (A) Pictures of aqueous solutions at 5 °C and different polymer concentrations (10 → 15 → 20 wt %). (B) Photographic images of aqueous solution at 40 °C and a polymer concentration of 20 wt %. (C) Temperature-dependent viscosity measurements (rolling ball system) at different polymer concentrations (blue and filled symbols: cooling, red and open symbols: heating).

of approximately 3 °C was observed in this experiment. In addition, we characterized the inverse gelation by rheology (Figure S7). Again, a hysteresis of the gel temperature T_{gel} ($G' = G''$) between heating ($T_{\text{gel}} = 25\text{ °C}$) and cooling ($T_{\text{gel}} = 22\text{ °C}$) can be seen. The gelation is fully reversible and a stable hydrogel ($G' > G''$) below T_{gel} was observed.

Compared to the recently described ABA triblocks featuring a PPhEOzi hydrophobic B block, there are notable differences in the present system. First, a significantly higher concentration is needed ($c_{\text{gel}}[\text{A-PPhEOzi-A}] = 5\text{ wt \%}$ ³⁹ vs $c_{\text{gel}}[\text{A-PPhenEtOx-A}] = 20\text{ wt \%}$). Second, the critical temperature is lower ($T_{\text{gel}}[\text{A-PPhEOzi-A}] = 32\text{ °C}$ ³⁹ vs $T_{\text{gel}}[\text{A-PPhenEtOx-A}] = 22\text{--}25\text{ °C}$). Third, the gelation kinetics is much faster compared to the previous system (slow gelation³⁹ ↔ rapid gelation).

To investigate the thermoresponsive aggregation on a molecular level, temperature-dependent ^1H NMR experiments were conducted (Figure 3A,B). At 5 °C, the signals attributed to the polymer backbone (3.45 ppm) and CH_3 -sidechain (2.00 ppm) of the hydrophilic building blocks are visible, albeit broad and featureless. In the aromatic region (6.92 ppm) of the spectrum, no signals are visible at 5 °C. This can be readily explained by very short transversal relaxation times T_2^* of the moieties in the hydrophobic micellar core, which are densely packed and apparently less hydrated so that efficient spin–spin relaxation can occur. As previously discussed, a gradual increase of the temperature from 5 to 40 °C causes liquefaction, and the signals of the hydrophilic as well as the hydrophobic part become narrower and more defined (Figure 3A,B) as molecular mobility increases. Most interestingly, this is particularly visible in the aromatic region of the spectrum. An increasing, albeit still featureless signal is visible with increasing temperature, which is caused by more flexible units of the hydrophobic core. Likewise, the reversibility of aggregation could be demonstrated by a subsequent cooling sequence, whereby the initial aggregation is achieved (Figure 3B). A more quantitative insight was obtained by comparing the integrated NMR intensities of different polymer signals as a function of temperature (Figure 3C). A p -value (eq 6) of zero indicates the highest mobility in the liquid

Figure 3. Temperature-dependent aggregation by means of ^1H -NMR spectroscopy and SAXS experiments. ^1H NMR spectra of a 20 wt % hydrogel sample ($\text{H}_2\text{O}/\text{D}_2\text{O}$, 1:1) at different temperatures (A) 5 °C $\rightarrow$ 40 °C, (B) 40 °C $\rightarrow$ 5 °C. (C) p -Values for different polymer signals as a function of temperature. (D) SAXS of a 20 wt % aqueous hydrogel sample at different temperatures (5, 15, 20, 25, 30, and 40 °C). After the initial measurement, the sample was cooled again to 5 °C (E). (F) Correlation length ξ as a function of temperature obtained from the scattering curves (error bars: numerical error).

state (40 °C). The aromatic CH-protons are most strongly influenced yielding high p -values (close to 1 = little signal intensity left) in the gel state. Liquefaction due to increased temperature caused an increasing signal resulting in a steady p -value decrease up to 40 °C for the hydrophobic signals. The previously described slow gelling system based on A-PPheOzi-

A³⁹ showed a constant p -value in the gel state and a rapid p -value decrease upon liquefaction. The hydrophilic shell and the backbone are affected to a much lesser extent, similar to previous reports on the aggregation behavior of thermoresponsive phase separation of different POx-based polymers.⁴⁴ In contrast to DLS, the polymer sol/gel could be investigated at concentrations needed for hydrogel formation (20 wt %) using SAXS. The scattering curves suggest a feature size of 16–18 nm with only small changes with changing the temperature (Figure 3D). However, the small changes observed proved to be fully reversible as evidenced by scattering curves at 5 °C being superimposable after a heating/cooling cycle to 40 °C (Figure 3E). To characterize the scattering curves in more detail, hard-sphere model fit functions were employed (detailed information in the Supporting Information and Table S1). In particular, we focused to obtain the correlation length ξ , the distance in which neighboring micelles correlate with each other. Interestingly, ξ increased with decreasing temperature with a strong increase in particular between 15 and 5 °C. An increased correlation length means that the micelles interact over a greater distance. Interestingly, on comparing the temperature-dependent rheology and correlation length it, becomes apparent that the correlation is not completely straightforward.

In fact, the correlation length only increases strongly below the T_{gel} , at 15 °C and below, while gelation ($G' \geq G''$) occurs already at 22 °C. Interestingly, below 15 °C, the p -value for the pMeOx shows a marked increase, indicating reduced mobility. Looking more closely at the temperature-dependent rheology, the longer correlation lengths coincide with the formation of a plateau for both G' and G'' . In contrast, the values for G' and G'' at T_{gel} are about three orders of magnitudes lower than at this plateau. It appears at this point that the hydrogel formation of A-PPhenEtOx-A is caused by densely packed spherical micelles with reduced mobility at low temperature due to increased intermicelle correlation, somewhat resembling the aggregation behavior of Pluronic F127. However, Pluronic F127 forms a hydrogel upon heating because of a thermoresponsive poly-(propylene glycol) block whereas A-PPhenEtOx-A does so upon cooling and does not contain a polymer block which is thermoresponsive in itself. Accordingly, it appears obvious that on the molecular level, the thermogelation of A-PPhenEtOx-A and Pluronic F127 is not related. Also, comparing to the wormlike hydrogel system comprising A-PPheOzi-A⁴⁰

Scheme 2. Self-Assembly of the ABA-Type Triblock Copolymer and Inverse Thermogelation^a

^a(A) At very low polymer concentration single polymer chains are present. The aqueous polymer solution has a low viscosity. Above the CMC, the amphiphile self-assembles into spherical micelles. (B) Increase in concentration above T_{gel} leads to a micellar solution with moderate viscosity and macromolecular mobility. Decrease in temperature below T_{gel} leads to an increase in the intermicelle correlation, physical network formation, and a macroscopic gelation. (C) Schematic illustration of the different correlation lengths at different temperatures. Upon cooling, the correlation of individual micelles increases.

Figure 4. Rheological characterization for dispense plotting applications and *cryoSEM* analysis of the A-PPhenEtOx-A hydrogel at 5 °C. (A) Amplitude sweep of the 20 wt % hydrogel at 5 °C and an angular frequency of 10 rad/s with storage moduli (G' = square), loss moduli (G'' = circle), and damping factor (filled square) is shown. (B) Frequency sweep with a fixed amplitude of 0.1%. (C) Viscosity (triangle) and shear stress (filled square) in dependency of the applied shear rate for a 20 wt % hydrogel. (D) Viscosity (triangle) and shear rate (filled square) as a function of the applied shear stress in the steady shear stress experiment. (E) ORO recovery test of the hydrogel. (F) ROR three-step recovery. (G–I) *CryoSEM* images of the 20 wt % hydrogel at 1 k $\times$, 2 k $\times$ and 10 k $\times$ magnification.

with different slope regions in the SAXS pattern, significant differences were obtained. To summarize, the amphiphile self-assembles into spherical micelles (R_h (15 °C) = 8.5 ± 0.1 nm, R_h (25 °C) = 8.4 ± 0.1 nm, and R_h (40 °C) = 8.5 ± 0.2 nm) at all investigated temperatures above the CMC (0.3 μ M). In comparison, the previously described inverse gelling system with PheOzi instead of PhenEtOx as hydrophobic repeat units showed a pronounced temperature dependency of the apparent R_h (R_h (15 °C) > 100 nm) connected to a change in micellar morphology from spherical to worm.⁴⁰ Here, the increased concentration leads to the formation of highly concentrated dispersion of spherical micelles, similar to the situation well known for Pluronic F127. However, in contrast to the present system, Pluronic F127 only forms micelles above the critical temperature of the poly(propylene glycol) blocks. Decreasing the temperature resulted in reduced micellar mobility which was observed due to an increasing correlation length between

neighboring micelles, consequently leading to the inverse gelation (Scheme 2).

As previously mentioned, stimuli-responsive hydrogels have been utilized in different biomaterial applications.⁵¹ Recently, different approaches were established to overcome limitations in the so-called biofabrication window, especially in bioprinting.⁵² One approach is to improve printability by support materials and sacrificial materials, while retaining cell viability by utilizing biological components, cell-friendly cross-linking, and mild printing conditions. The search for suitable hydrogel platforms is an ongoing challenge due to multiple specific and sometimes contradictory requirements. Appropriate gelation kinetics, good printability, and cytocompatibility are key requirements for hydrogels during bioink design. Therefore, the viscoelastic properties of the present inverse thermogelling platform were thoroughly investigated via rheology. In the pronounced viscoelastic region (LVE, end of LVE and flow point ($G' =$

Figure 5. Extrusion printing of a A-PPhenEtOx-A 20 wt % hydrogel (A–D) and a hydrogel blend of A-PPhenEtOx-A and alginate (E–H) at 8 °C (nozzle 25 G, speed: 10 mm/s). (A, E) Filament fusion test to visualize printing resolution. (B, F) Filament collapse test by printing bridges of increasing distances. (C, D, G, H) Printed 20 layer (5 × 5 mm cylindrical tube structures) constructs (top and side view).

Figure 6. Cytocompatible bioprinting of POx/alginate-based bioinks using NIH 3T3 cells (A). (B, C) Single and merged fluorescence images of embedded NIH 3T3 cells in POx/alginate (upper row) and alginate (bottom row) stained with DAPI (blue) and Calcein AM (green) after printing, stabilization with CaCl_2 , and 24 h of incubation. (D) Cell viability of NIH 3T3 cells in three different printed scaffolds of alginate and POx/alginate hydrogel blend.

G'') marked with red lines), a constant storage modulus G' of 14.8 ± 0.3 kPa was obtained with a low damping factor of 0.3 ± 0.02 , which suggest a stable viscoelastic hydrogel (Figure 4A). Interestingly, the hydrogel exhibits a profound dependency on the frequency (Figure 4B). Increasing the frequency leads to increased G' and decreased G'' values, i.e., the solid-like character is amplified with the dampening factor reaching values <0.03 at 500 s^{-1} . To utilize a hydrogel as 3D-printable ink in different printing applications, three major rheological considerations must be addressed, namely, pronounced shear-thinning, defined force resistance, and fast structure recovery properties.⁵³ With the increasing shear rate, the viscosity of the present hydrogel decreases following a power law expression with a flow index of 0.05 ± 0.004 , indicating very pronounced shear-thinning (Figure 4C). Even at very low shear rates of 0.001 s^{-1} , no viscosity plateau is reached and shear stress values of ca. 200 Pa are measured at this point. In contrast, at very high shear rates of 1000 s^{-1} , the viscosity values are very low (ca. 1 Pa s) with shear stress values increasing up to 750 Pa. To obtain the yield point and the force resistance of the hydrogel, the viscosity was plotted as a function of the applied shear stress and split up in three regions (Figure 4D). Below the critical stress, almost constant viscosity values between 10^4 and 10^5 Pa s with very low shear rates (between 10^{-4} and 10^{-5} 1/s) were determined (1). At a certain stress, the viscosity decreases very fast with increasing shear rate values to low viscosity values (2). The onset of 440 Pa is determined via two tangents. At high shear stress

values of $800 \rightarrow 1500$ Pa, plateau values for the viscosity and shear rate of 0.1 Pa s and $10^3 \rightarrow 10^4 \text{ s}^{-1}$ are reached (3). Structure recovery was tested using a three-step test, designed as ORO and ROR experiments, altering low and high strain regimes (Figure 4E,F). In both cases, the structure recovered very fast and the initial values were obtained during the experiments, which we interpret to show rapid and complete structure recovery. To obtain insights into the hydrogel morphology, we conducted cryogenic scanning electron microscopy (cryoSEM) (Figure 4G–I). A rather homogenous porous structure with pores in the range of several hundred nanometers was obtained. In comparison to a recently described hydrogel based on a A-PPhOzi-A triblock copolymer, the presently described A-PPhenEtOx-A hydrogel has significantly smaller pores.³⁹ The strong frequency dependence in combination with smaller pore sizes compared to the previously published hydrogels consisting of wormlike micelles further underlines the differences between the individual gelation mechanism. In summary, the novel hydrogel shows promising viscoelastic characteristics for bioprinting.

Based on favorable rheological properties, the printability of the hydrogel was investigated using extrusion-based printing (Figure 5A–D). Additionally, a hydrogel blend comprising the POx-based hydrogel and alginate was prepared, printed, and stabilized with CaCl_2 (Figure 5E–H). Alginate hydrogels are widely used in biofabrication as alginate is easily cross-linked by the addition of CaCl_2 after printing to stabilize the constructs.

However, the printability of pure sodium alginate without the addition of viscosity enhancers or modifications is very low.⁵⁴ First, we screened for the highest achievable resolution by printing hydrogel lines with a decreasing line distance, from 2 → 1.5 → 1 → 0.75 → 0.5 mm (Figure 5A,E). Even at a layer distance of 0.75 mm every individual strand could be distinguished and no strand fusion was observed. Additionally, the collapse of suspended filaments was investigated.⁴⁸ A free hanging hydrogel strut over a total distance of 1.7 cm could be realized without significant strand sag (Figure 5B,F). Based on these promising results, a first real 3D construct was printed (Figure 5C,D,G,H, Supporting Movie 1). A 20-layered cylindrical construct with a diameter of 5 mm and a total height of 5 mm was printed. The structure stayed intact, and no significant collapse was observed. The structure of the stabilized POx/alginate tubular construct was preserved even after 24 h of incubation in PBS solution at 37 °C (Figure 5G,H).

Finally, a first bioprinting process was performed and the cytocompatibility was investigated using the POx/alginate (20:1 wt %) hydrogel blend in comparison to pure alginate (1 wt %) (Figure 6A–D) after 24 h. After dissolving the polymers in cell culture medium, the cells were dispersed in the homogeneous free flowing viscous liquid at 37 °C. The relatively high viscosity of the bioink precursor at 37 °C (see Figure 2) prevented significant cell sedimentation. The bioink blend was transferred to the precooled bioprinter equipped with a cooling system. Using the pronounced inverse gelation of A-PPhenEtOx-A as described above, cell-laden constructs were obtained and stabilized via cross-linking of alginate using CaCl₂ aqueous solution. After 24 h of incubation, cell staining was performed and quantified. Overall, the bioprinting process of POx-based bioinks was found to be highly cytocompatible (cell viability >95%) and no significant difference with respect to cytocompatibility was observed for the hydrogel blend in comparison to plain alginate solution. Plain A-PPhenEtOx-A was not tested for cytocompatibility during bioprinting. On the one hand, it is known from the literature that very similar polymers do not show cytotoxic properties up to a concentration of 100 g/L.^{39,42} On the other hand, addition of 1 wt % alginate should not have any significant effect in terms of cytocompatibility.

CONCLUSIONS

Here, a new ABA-type amphiphile based on poly(2-oxazoline)s with an aromatic hydrophobic central block of poly(2-phenethyl-2-oxazoline) B was described. Above the CMC, small spherical nanoscale micelles were confirmed. At or above 20 wt %, an inverse and fast sol/gel transition was observed upon cooling, due to densely packed micelles and investigated via temperature-dependent ¹H NMR and SAXS experiments. Favorable rheological properties, such as pronounced viscoelastic solid-like properties, shear-thinning, and structure recovery properties led to very good 3D-printability of the investigated hydrogel. In a hydrogel blend comprising the readily printable POx-based polymer and the biopolymer alginate, stable constructs were obtained. First cytocompatible bioprinting at approximately 10 °C for a A-PPhenEtOx-A/alginate hybrid system concluded the study and opens a wide range of different applications in the field of biofabrication.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.biomac.1c00427>.

Detailed description of monomer and polymer synthesis and characterization and an overview and detailed description of SAXS analysis (PDF)

Additional movie of the extrusion printing process (MP4)

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Notes

The authors declare the following competing financial interest(s): L.H. and R.L. are listed as inventors on a patent pertinent to some materials in the present work.

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