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Synthesis and fabrication of a degradable poly(*N*-isopropyl acrylamide) scaffold for tissue engineering applications

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Abstract

Biodegradable poly(*N*-isopropyl acrylamide) (poly-NIPAM) hydrogels with controlled molecular weight of the parent polymer and its degradation products were synthesized by atom transfer radical polymerization in the presence of a polycaprolactone-based di-chlorinated macroinitiator and polycaprolactone dimethacrylate. The phase transition temperature, swelling, hydrolytic degradability, and mechanical properties at 25 and 37°C were explored. A cytocompatibility study showed good NIH3T3 cell response over 5 days culture on the surface of the hydrogels, demonstrated by a consistent increase in cell proliferation detected by an Alamar Blue assay. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] results suggested that the hydrogels and their degradation products in the concentration range of 1–25 mg/mL were not cytotoxic to NIH3T3 cells. A sphere-templating technique was utilized to fabricate biodegradable polyNIPAM scaffolds with monodisperse, pore size. Scaffolds with pore diameter of $48 \pm 6 \mu\text{m}$ were loaded with A-10 smooth muscle cells and then warmed to 37°C entrapping cells in pores approximately 40 μm in diameter, a size we have found to be optimal for angiogenesis and biointegration. Due to their degradable nature, tunable molecular weight, highly interconnected morphology, thermally controlled monodisperse pore size, and temperature-induced volume expansion–contraction, the polyNIPAM-based scaffolds developed in this work will be valuable in tissue engineering.

Keywords

poly(*N*-isopropyl acrylamide); atom transfer radical polymerization; tissue engineering; scaffold; degradable

INTRODUCTION

Natural and synthetic polymer-based “intelligent” hydrogels have been a subject of special interest in the biomedical field due to their ability to respond to small external stimuli, such as temperature, pH, light, electric fields, and chemical environments with large changes in their properties.^{1–8} The potential of intelligent hydrogels for biological and medicinal applications such as drug delivery, microfluidic actuators, cell entrapment, biosensors, and tissue engineering has been demonstrated.^{1,2,4,9–12} Among this class of hydrogels, thermosensitive poly(*N*-isopropyl acrylamide) (polyNIPAM) is probably the most extensively studied. In aqueous media, polyNIPAM hydrogels exhibit a volume phase transition temperature (VPTT) at around 32–34°C.^{13,14} The crosslinked polyNIPAM network undergoes an abrupt, reversible swelling–deswelling process below and above the VPTT. The VPTT of the hydrogel can be modulated by copolymerization of NIPAM with

hydrophilic or hydrophobic monomers to increase or decrease the transition temperature, respectively.¹⁵ Due to their thermoresponsive nature, biostability, and biocompatibility, polyNI-PAM-based hydrogels are attractive candidates for many biomedical applications.^{10,16–20}

Three dimensional (3D) scaffolds play a central role in tissue engineering.²¹ Most applications of 3D scaffolds in tissue engineering utilize scaffolds with high, interconnected porosity.²¹ Various techniques have been used to fabricate interconnected porous scaffolds such as salt leaching, phase separation, gas foaming, fiber meshes, and electrospinning²¹; however, these methods do not offer precision control of pore size, structure, or interconnectivity. Our research group has developed a sphere-templating technique that results in a scaffold with an interconnected, controllable microstructure where every pore is the same size.²² We determined that a pore size between 30 and 40 μm is optimal for biointegration of the scaffold into host tissue with enhanced angiogenesis and reduced fibrosis.^{23,24} Degradability is an additional important factor in designing scaffolds for tissue engineering.^{25,26} Ideally, the scaffold should provide mechanical and biochemical support during regeneration, and as new tissue forms, it should be degraded and eliminated.

Atom transfer radical polymerization (ATRP) is a controlled radical polymerization (CRP) that allows for the synthesis of polymers with well-defined macromolecular structure, predictable molecular weight (Mw), and a narrow polydispersity index (PDI).^{27,28} By using ATRP, one can control polymer architecture (e.g., copolymer topology, microstructure, and functionality) and thus tune and target physical properties of the polymers toward potential applications.^{29–33}

The aim of this study is to prepare degradable, thermoresponsive scaffolds with controlled porosity. These scaffolds are based on polyNIPAM hydrogels with tightly defined Mw of the parent polymer chains comprising the network and tightly defined Mw degradation products.

In the literature, there are examples of partially degradable polyNIPAM-based hydrogels with degradable crosslinks but nondegradable polymer backbone chains.^{34–48} The concern with these hydrogels is a release of long, nondegradable polyNIPAM chains after cleavage of crosslinkers.

Preparation of polyNIPAM hydrogels with degradable units within both the backbone and the crosslinking sites has only infrequently been mentioned in the scientific literature. Matyjaszewski and coworkers describes the synthesis of polyNIPAM hydrogels by UV polymerization of NIPAM with benzo-2-methylene-1,3-dioxepan in presence of poly(ethylene glycol-*co*-glycolic acid) diacrylate that contributes ester bonds to the backbone and crosslinking sites, respectively.⁴⁹ In our previous work, polyNIPAM-based hydrogels with degradable backbones and crosslinking sites were synthesized by copolymerization of NIPAM with 2-methylene-1,3-dioxepan in the presence polycaprolactone dimethacrylate (PCLDMA).⁵⁰ However, during the synthesis, we did not have control over copolymer topology and Mw of the product.

Here, we report the preparation of a thermoresponsive scaffold based on a degradable polyNIPAM hydrogel with defined Mw of the polyNIPAM backbone and its degradation products. This polymer has potential applications in tissue engineering. First, polyNIPAM hydrogels with degradable units within the polymer backbone and at the crosslinking sites were synthesized by ATRP of NIPAM in the presence of di-functional chlorinated polycaprolactone-based macroinitiator (Cl-PCL-Cl) and PCLDMA. The VPTT of the polyNI-PAM hydrogels over a range of molecular weights was measured and the mechanical properties of the hydrogels as functions of Mw and temperature were explored at

25 and 37°C. Swelling properties, hydrolytic degradation, cytotoxicity, and cytocompatibility of the hydrogels were also examined. Second, a sphere-templating technique was applied to fabricate a scaffold with monodisperse, interconnected morphology based on the aforementioned hydrogels. A preliminary study was performed to demonstrate the potential of the material for tissue engineering where the scaffold was successively seeded with smooth muscle cells (SMCs) and cellular infiltration and morphology were investigated.

MATERIALS AND METHODS

Materials

The following chemicals were purchased from Sigma and were used without further purification: NIPAM (97%), poly-caprolactone diol ($M_n \sim 530$), methacryloyl chloride, triethylamine (TEA) (99.5%), dimethylsulfoxide (DMSO) anhydrous (99.9%), CuCl ($\sim 99\%$), 2-chloropropionyl chloride, tris(2-aminoethyl) amine (96%), sodium borohydride. Uncrosslinked poly(methyl methacrylate) (PMMA) microspheres (Kupa; sphere diameter 100 μm); HPLC-grade dichloromethane, acetonitrile, and acetone were purchased from EMD Chemicals; Vybrant MTT cell proliferation assay kit (Invitrogen); Dulbecco's modified Eagle medium (DMEM) (Invitrogen); cell line A-10 and ATCC-formulated DMEM (ATCC®); Alamar Blue (AlamarBlue®, AbD Serotec, UK).

Analytical methods

^1H -NMR spectra were obtained with a Bruker AV-300 spectrometer. For chloroform-*d*, chemical shifts are expressed in parts per million downfield from tetramethylsilane used as an internal standard.

Average M_w and M_w distribution of linear degradable polyNIPAM and its degradation products were determined by a Tosoh Corp gel permeation chromatograph with TSGel α -3000 and α -4000 columns (Tosoh Bioscience, Montgomeryville, PA). DMF containing 0.1% wt/vol LiBr at 60°C was used as the mobile phase at a flow rate of 1 mL/min and the system was calibrated with PMMA standards. Mechanical properties of the hydrogels were evaluated from dog bone-shaped samples ($4.5 \times 0.65 \times 20 \text{ mm}^3$) that were punched from swollen and collapsed hydrogel films. Mechanical properties were obtained by tensile tests using a Universal Testing Machine (Instron 5543) possessing a load cell of 10 N and occupied with a water bath at 25 or 37°C. The crosshead speed was set to stretch at 10% strain/min until failure. At least, five samples were used for each measurement. The Young's modulus was calculated as the ratio of stress to strain of the initial linear region of the stress-strain diagram. The VPTT of the polyNIPAM hydrogels was determined by differential scanning calorimetry (DSC) (Netzsch DSC 200). The hydrogel sheets, swollen to equilibrium, were punched into 3 mm diameter disks and excess water on the disk surface was removed with wet filter paper. Then, the samples were placed in an aluminum pan and sealed with an aluminum lid. The samples were scanned from 25 to 50°C at a heating rate of 3°C/min under dry nitrogen. The VPTT was defined as the onset temperature of the endotherm. Scaffold and cell morphologies were examined by scanning electron microscopy (SEM; FEI SEM XL Siron, Hillsboro, OR). Dry samples were Au/Pd sputter coated for 60 sec (SPI Supplies, West Chester, PA). Details regarding additional equipment used during this research are described later.

Synthesis of di-functional macroinitiator (Cl-PCL-Cl)

Polycaprolactone diol ($M_n \sim 530$, 5 g, 9.4×10^{-3} mol) and TEA (6.5 mL, 0.047 mol) were dissolved in anhydrous tetrahydrofuran (150 mL), and the solution was cooled to 4°C. Chloropropionyl chloride (2.3 mL, 0.024 mol) was added dropwise under N_2 atmosphere

and the reaction mixture was stirred at room temperature (RT) for 18 h. The white precipitate was removed by filtration and the solvent was evaporated. The material was then dissolved in dichloromethane and washed with saturated NaHCO_3 , 1N HCl, and H_2O . The organic phase was dried over MgSO_4 , filtered, and evaporated to produce yellow oil (yield 92%). ^1H NMR (CDCl_3): δ 1.30 (m, $-\text{COOCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{COO}-$), 1.52 (m, $-\text{COOCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{COO}-$ and $\text{ClCH}(\text{CH}_3)\text{COO}-$), 2.35 (m, $-\text{COOCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{COOCH}_2\text{CH}_2\text{O}-$), 3.75 (m, $-\text{COO}-\text{CH}_2\text{CH}_2-\text{O}-\text{CH}_2\text{CH}_2-\text{OOC}-$), 4.0–4.6 (m, $-\text{COOCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{COOCH}_2\text{CH}_2\text{O}-$ and $\text{ClCH}(\text{CH}_3)\text{COO}-$).

Synthesis of PCLDMA

Polycaprolactone diol ($M_n \sim 530$, 25 g, 0.047 mol) and TEA (19.7 mL, 0.14 mol) were dissolved in anhydrous dichloromethane (150 mL), and the solution was cooled to 4°C .⁵¹ Methacryloyl chloride (13.8 mL, 0.14 mol) was added drop-wise under N_2 , and the reaction mixture was stirred at RT for 18 h. The formed solid was filtered off, and the dichloromethane solution was washed with 0.1N HCl, saturated NaHCO_3 and H_2O . The organic phase was dried over MgSO_4 , filtered, and evaporated to produce orange oil. Purified PCLDMA was obtained by precipitation into cold hexanes as a slightly yellow wax (yield 82%). The degree of methacrylation was determined with ^1H NMR spectroscopy by the ratio between the integral area under the peak at δ 6.12 (s, 2H, olefinic, cis) and 3.70 (m, 4H, $-\text{OCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{O}-$).

Synthesis of tris[2-(dimethylamino)ethyl]amine (Me_6TREN)

Tris(2-aminoethyl) amine (TREN) (3 mL, 19.9×10^{-3} mol) and acetic acid were dissolved in 600 mL of acetonitrile.⁵² Aqueous formaldehyde (37 wt %, 49 mL, 660×10^{-3} mol) was added to the solution, and the mixture was stirred at room temperature for 1 h. The reaction mixture was placed in an ice bath and sodium borohydride (10 g, 13.4×10^{-3} mol) was slowly and carefully added. After being stirred for 48 h at RT, the solvent was evaporated to obtain a yellow solid; 3M NaOH solution was added to dissolve the solid (final pH = 11), and the product was extracted three times with dichloromethane. The organic phase was dried over MgSO_4 , filtered, and evaporated to obtain a yellow oil (yield 88%). ^1H NMR (CDCl_3): δ 2.2 (s, 18H, $-\text{CH}_3$), 2.32 (m, 6H, $-\text{NCH}_2\text{CH}_2\text{N}(\text{CH}_3)_2$), and 2.60 (m, 6H, $-\text{NCH}_2\text{CH}_2\text{N}(\text{CH}_3)_2$).

Synthesis of degradable polyNIPAM hydrogels by ATRP

Degradable polyNIPAM-10, polyNIPAM-20, and polyNIPAM-40 hydrogels with backbone target M_w of 10,000, 20,000, and 40,000, respectively, were synthesized. Table I summarizes molar ratios between reagents to obtain hydrogels with the desired M_w by ATRP. In a typical procedure to synthesize degradable polyNIPAM-20 hydrogel based on a backbone with target M_w of 20K, NIPAM (1 g, 0.00884 mol), Cl-PCL-Cl (36 mg, 5×10^{-5} mol), and PCLDMA (0.186 g, 0.00026 mol, 3% mol/mol_{NIPAM}) were dissolved in DMSO (1 mL), and the solution was purged with Ar for 1 h. Then, under inert atmosphere, CuCl (50 mg, 0.0005 mol) was dissolved in the purged solution followed by the addition of Me_6TREN (115 μL , 0.0005 mol). The reaction mixture was cast into a mold (consisting of two microscope slides separated by a 1 mm thick Teflon spacer) and polymerized at RT for 18 h. The polymer sheet was then removed from the mold and washed with acetone and water to extract impurities and residues of unreacted reagents to obtain a transparent degradable polyNIPAM-20 hydrogel.

Linear degradable polyNIPAM with theoretical M_w 's of 10,000, 20,000, and 40,000 was synthesized as described above but in absence of the crosslinker. After polymerization, the material was purified by dialysis against water, lyophilized, dissolved in chloroform,

precipitated from cold diethyl ether, and dried under vacuum to obtain linear poly-NIPAM as a white powder.

Swelling study

PolyNIPAM hydrogel sheets were punched into 8 mm disks. The disks were lyophilized to determine the dry weight (W_{dry}). The samples were then swollen in distilled water at 4, 25, and 37°C for 24 h to reach an equilibrium state. For each temperature, the excess water on the swollen hydrogel surface was removed with wet filter paper and the weight of the swollen sample (W_{swollen}) was determined (in triplicate). The swelling percent was calculated using

$$\text{Swelling (\%)} = [(W_{\text{swollen}} - W_{\text{dry}}) / W_{\text{dry}}] \times 100 \quad (1)$$

Hydrolytic degradation

PolyNIPAM hydrogel sheets were punched into 8 mm disks. The disks were lyophilized to determine the original dry weight (W_1). Then, the dry disks were placed in 0.007N NaOH solution, and the samples were shaken at 25 and 37°C. For both temperatures, hydrogels samples (triplicates) were taken at different points in time, washed with H₂O, and lyophilized to determine dry weight after degradation (W_2). The degradation was evaluated by calculating the percent of weight loss using

$$\text{Weight loss (\%)} = [(W_1 - W_2) / W_1] \times 100 \quad (2)$$

Cytocompatibility and cell adhesion

PolyNIPAM hydrogels were punched into 8 mm disks, sterilized with 70% ethanol, washed with sterile PBS, and soaked in DMEM at 4°C overnight prior to seeding. Afterward, disks immersed in DMEM media were placed at 37°C for 24 h to allow shrinkage of the material. The shrunken disks then were removed from the media and placed into a 96-well plate to fit exactly the diameter of the well and to serve as a surface for cell culture; 180 μ L of cell suspension (1×10^4 cells/well) was added, and cells were cultured on the surface of the hydrogel for 5 days. Cytocompatibility of the material was evaluated by measuring proliferation of NIH3T3 cells cultured on the surface of the hydrogel by an Alamar Blue assay. At each time point, DMEM media was replaced with 180 μ L DMEM media containing 10% vol/vol of Alamar Blue reagent, followed by 4 h incubation at 37°C. A 100- μ L aliquot from each well was transferred into a 96-well plate and absorbance was measured at 570 and 600 nm using a microplate reader (tunable VERSAmax microplate reader; Molecular Devices, CA). Cell proliferation was evaluated following manufacturer's guidelines using:

$$\text{Reduction of Alamar Blue (\%)} = [(O2 \times A1) - (O1 \times A2)] / [(R1 \times N2) - (R2 \times N1)] \times 100, \quad (3)$$

where O1 [molar extinction coefficient (E) of oxidized Alamar Blue at 570 nm] = 80,586; O2 (E of oxidized Alamar Blue at 600 nm) = 117,216; R1 (E of reduced Alamar Blue at 570 nm) = 155,677; R2 (E of reduced Alamar Blue at 600 nm) = 14,652; A1 = absorbance of test wells at 570 nm; A2 = absorbance of test wells at 600 nm; N1 = absorbance of negative control well (media plus Alamar Blue but no cells) at 570 nm; N2 = absorbance of negative control well (media plus Alamar Blue but no cells) at 600 nm.

To visualize cell adhesion, at each time point, the media was removed, the hydrogel's surface was washed with warm sterile PBS and then fixed with 2% glutaraldehyde solution at 37°C. At the end of fixation, the sample was washed with DI water and immediately frozen in liquid nitrogen and lyophilized. The samples were observed under SEM to explore cell adhesion and morphology.

Cytotoxicity

Cytotoxicity of the hydrogels and their degradation products were evaluated by using an MTT assay.

Hydrogels cytotoxicity—Hydrogels were sterilized with 70% ethanol, washed with sterile PBS, and placed in DMEM for 24 h. At the same time, 0.5 mL of NIH3T3 mouse fibroblasts (1×10^4 cells/well) were seeded in 24-well culture plates for 24 h. The media then was removed from the wells and replaced with 0.5 mL of scaffold eluent. The cells were incubated with media eluted from the scaffold for an additional 24 h, then washed with phenol red-free DMEM media, and afterward exposed for 4 h to 0.5 mL of phenol red-free DMEM media containing 10% of MTT solution (5 mg/mL MTT reagent in PBS); 1 mL of DMSO was then added with vigorous pipetting to extract a blue product. Tissue culture polystyrene (TCPS) and latex were used as the negative and positive controls, respectively, and were treated similarly as above; a 200 μ L aliquot from each well was transferred into a 96-well plate and absorbance was measured at 550 nm with a microplate reader (tunable VERSAmax microplate reader; Molecular Devices).

Degradation product cytotoxicity—Hydrogels were hydrolyzed in 0.1N NaOH. The solution was neutralized and degradation products were extracted with chloroform. The organic phase was dried over MgSO_4 , filtered, and chloroform was evaporated to obtain a white powder. The degradation products were dissolved into media at concentrations ranging from 150–1 mg/mL and the cytotoxicity at different concentrations was evaluated according the procedure for the scaffold eluent, described in the paragraph above.

Sphere-templated scaffold fabrication

Step 1. Template preparation—PMMA microspheres were fractionated to the size of $46 \pm 8 \mu\text{m}$ with an ATM model L3P Sonic Sifter. The beads were transferred to a mold (composed of two microscope slides separated by a 1 mm thick Teflon spacer) and sonicated for 10 min for optimal packing. The beads were then sintered for 20 h at 140°C to obtain PMMA templates with neck sizes (interconnects between the beads) of 30% of the bead diameter.

Step 2. Scaffold fabrication—Under an inert atmosphere, PMMA templates were infiltrated with the reaction mixture composed of NIPAM (1 g, 0.00884 mol), Cl-PCL-Cl (36 mg, 5×10^{-5} mol) and PCLDMA (0.186 g, 0.00026 mol, 3% mol/mol_{NIPAM}), CuCl (50 mg, 0.0005 mol), and Me₆TREN (115 μ L, 0.0005 mol) dissolved in DMSO (1 mL). The mixture was polymerized at RT for 18 h, and the PMMA template infused with polymerized polyNIPAM-20 hydrogel was removed from the mold and placed in dichloromethane to dissolve the PMMA beads. The scaffold obtained, composed of a degradable polyNIPAM gel, was washed in acetone and then hydrated in distilled water.

Cell seeding study

The polyNIPAM-20-based scaffold with $48 \pm 6 \mu\text{m}$ pore diameter was punched into 6 mm disks, sterilized with 70% ethanol, washed with sterile PBS, and soaked in culture media [standard high glucose (4.5 g/L) DMEM with 1.5 g/L sodium bicarbonate buffering system

(for use in 5% CO₂ environment) supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic] at 4°C overnight. Prior to loading with cells, excess media was removed from the scaffold's surface with wet filter paper. Afterward, the disks were placed on dry filter paper and four 50 µL aliquots of the A-10 cell suspension (3×10^6 cells/mL) was added to the top of each disk (triplicates). The scaffolds were then placed into a 24-well plate and 1 mL of prewarmed media at 37°C was added. The cells were cultured for 7 days, and the media was changed every other day. The scaffolds were then fixed with a methanol-glacial acetic acid solution (9:1 vol/vol) for 30 min at 37°C. For histological analysis, samples were dehydrated through graded ethanol/xylene at 37°C and then embedded in paraffin. Paraffin blocks were sectioned (5 µm thick) onto charged Superfrost® plus slides (VWR International, West Chester, PA), deparaffinized, rehydrated, and stained with hematoxylin and eosin. Images were obtained with a Nikon E800 upright microscope equipped with MetaMorph® software (version 6.0; Molecular Devices, PA). For observation under SEM the samples' cross-sections (approximately 1 mm thick) were deparaffinized, rehydrated, frozen in liquid nitrogen, and lyophilized.

RESULTS AND DISCUSSION

Degradable polyNIPAM hydrogels

Figure 1(A) illustrates the synthesis of degradable polyNI-PAM hydrogel by ATRP in the presence of a di-functional PCL-based initiator and a PCL-based crosslinker. In general, ATRP of acrylamides, such as NIPAM monomer, was reported to be challenging, plausibly due to (a) deactivation of the copper catalyst through binding to monomeric or polymeric amide groups, (b) displacement of the terminal halide atom from the propagating chain by amide, and (c) low values of the ATRP equilibrium constant.⁵³ Polymerization in the presence of more stable alkyl chlorides rather than bromides as initiators, in combination with Me₆TREN, which is a stronger coordinating ligand that forms a powerful CuCl/Me₆TREN catalyst, improved the ATRP of acrylamides.^{54–56} In the present work, we synthesized a PCL-based di-alkyl chloride to serve as an initiator and used an excess of CuCl/Me₆TREN complex to catalyze the reaction. Figure 1(B) shows that the liquid reaction mixture before polymerization converts to a gel in later stages of polymerization. During the study, degradable polyNIPAM-10, polyNI-PAM-20, and polyNIPAM-40 hydrogels based on a polyNI-PAM backbone with theoretical Mw of 10,000, 20,000, and 40,000, respectively, were synthesized. Figure 2 presents example images of polyNIPAM-20 hydrogel before (A) and after purification at 25°C (B) and 37°C (C). Visual inspection of the polyNIPAM-20 hydrogel reveals a clear and transparent material at 25°C, a temperature below the VPTT, after purification and removal of copper ions [Fig. 2(B)]. Figure 2(C) shows the opaque appearance and shrinkage of the purified hydrogel at 37°C, a temperature above the VPTT.

The synthetic strategy described in Figure 1 has number of advantages. First, ATRP of NIPAM in the presence of di-functional PCL-based initiator and PCL-based crosslinker leads to the formation of a polyNIPAM hydrogel with degradable sites in the backbone and in the polymer network crosslinks. The obtained hydrogel is designed to degrade via hydrolysis of ester bonds contributed by PCL fragments, which are integrated throughout the entire hydrogel's structure. Second, ATRP permits precise control of polyNIPAM Mw tuning the physical properties of the hydrogel for specific applications. Moreover, since ATRP is a controlled polymerization, polyNIPAM chains should grow at the same rate from both sides of di-functional initiator, meaning that Mw of the degradation products should be half of the Mw of the parent polymer.

In order to prove controlled polymerization, linear degradable polyNIPAM with theoretical Mw of 10,000, 20,000, and 40,000 were synthesized. Table II summarizes the Mw and PDI

of the linear polymers and their degradation products. The M_w of the linear polymers before degradation demonstrates controlled polymerization with low polydispersity. The M_w of the degradation products are about half of the M_w of the parent polyNIPAM with a slight increase in PDI. These data support the suggestion that polyNIPAM chains grow at the same rate from both sides of the di-functional macroinitiator.

The concept of using a di-functional initiator for ATRP of NIPAM was reported previously. For example, Huang and coworkers⁵⁷ used a di-brominated polyfluorene-based macroinitiator for ATRP of NIPAM to obtain a thermally responsive linear fluorescent copolymer. However, in this study, the M_w of the synthesized copolymer was not investigated. Li and coworkers⁵⁸ utilized a di-brominated poly[(R)-3-hydroxybutyrate]-based initiator to synthesize a degradable linear thermoresponsive copolymer that forms micelles in water. Guan and coworkers⁵⁹ employed a di-brominated PCL-based initiator to synthesize a linear degradable thermosensitive copolymer with a transition temperature (TT) below 37°C before degradation and a TT above 37°C after degradation, thus producing soluble degradation fragments in body fluid. Both Li and coworkers and Guan and coworkers studied the M_w of the copolymers before degradation. However, the M_w of the degradation products was not discussed. In all the aforementioned studies only linear but not crosslinked polyNIPAM copolymers were synthesized using brominated initiators, which are not optimal for ATRP of acrylamides.⁵³

The VPTT of degradable polyNIPAM-10, polyNIPAM-20, and polyNIPAM-40 hydrogels based on polyNIPAM backbones with theoretical M_w of 10,000, 20,000, and 40,000, respectively, were determined by DSC and defined as the onset temperature of the endotherms, as shown in Figure 3. The VPTT of the polyNIPAM-10, polyNIPAM-20, and polyNIPAM-40 hydrogels are 31.7, 32.4, and 34.6°C, respectively. The results show an increase in VPTT of the hydrogels as the M_w of the backbone increases. To the best of our knowledge, the effect of M_w on the VPTT of crosslinked polyNIPAM hydrogels has not been reported. In general, VPTT of polyNIPAM is governed by the relative hydrophobicity of the bulk, which could be affected, for instance, by copolymer composition of the backbone as well as by the hydrophobic/hydrophilic nature of the crosslinker and end-groups. Here, we can assume that the increase in M_w decreases the effect of the hydrophobic PCL unit incorporated within polyNIPAM backbone and the hydrogel's bulk is more hydrophilic compared to the hydrogels with lower M_w , which leads to the increase in VPTT.

Figure 4 shows the swelling of polyNIPAM-10, polyNIPAM-20, and polyNIPAM-40 hydrogels at temperatures below and above VPTT. Figure 4 demonstrates the same tendency for all hydrogels, which is a decrease in swelling as temperature increases. A significant decrease in swelling occurs at 37°C, due to intensive dehydration of the polymer network after undergoing the phase transition. The thermoresponsive behavior is consistent with previously reported data regarding polyNIPAM-based materials.^{17,36,40,49}

Figure 5 demonstrates the accelerated degradation of polyNIPAM-10, polyNIPAM-20, and polyNIPAM-40 at 25°C that was monitored by measuring weight loss of the hydrogel in 0.007N NaOH as a function of time. Figure 5 shows that polyNIPAM-10, polyNIPAM-20, and polyNIPAM-40 exhibit weight loss over the time and after 4 days of accelerated degradation the initial weight decreases by 34, 27, and 15%, respectively. Figure 5 also demonstrates that after approximately 2.5 days polyNIPAM-40 exhibits lower weight loss as compared to polyNIPAM-10 and polyNIPAM-20. We assume that, as degradation progresses and crosslink sites are cleaved, the hydrogel's polymer chains can move more freely. Thus, polyNIPAM chains with higher M_w might fold and sterically shield degradable sites within the backbone thus slowing the degradation rate. The degradation of the

polyNIPAM-10, polyNIPAM-20, and polyNIPAM-40 hydrogels at 37°C was also explored and the materials exhibited approximately 6–8% weight loss after 3 weeks of degradation. A slow degradation of these thermally collapsed hydrogels could be explained by the significant increase in their hydrophobicity at 37°C. Significant loss of water, in addition to the initial hydrophobic character of the hydrogels from the PCL fragments, create the collapsed, hydrophobic network with limited access to its degradation sites. In general, when these polymer systems are applied toward specific applications, we can tune the polymer accordingly to appropriate degradation times.

We have also studied changes in mechanical properties of polyNIPAM hydrogels as the temperature is elevated from 25°C to body temperature. Figure 6 presents the mechanical properties of polyNIPAM-10, polyNIPAM-20, and polyNIPAM-40 at 25 and 37°C. Figure 6(A) shows the elastic modulus of the polyNIPAM hydrogels with different Mw in the swollen and collapsed states. It can be seen that in going from 25 to 37°C, the Young's moduli of polyNIPAM-10 and polyNIPAM-20 increase from 0.35 ± 0.01 and 0.64 ± 0.04 MPa, respectively, to 0.49 ± 0.04 and 0.86 ± 0.02 MPa. This behavior, which is related to the increase in polymer density as temperature is elevated above the VPTT, correlates with previously reported findings.^{56,60} Interestingly, polyNIPAM-40 does not follow the trend described above and instead exhibited a slight decrease in the Young's modulus as temperature increased: 1.02 ± 0.04 and 0.91 ± 0.01 MPa at 25 and 37°C, respectively. Figure 6(B,C) shows ultimate tensile strength (UTS) and maximum strain of the hydrogels in their swollen and collapsed states. There is an increase in UTS of polyNIPAM-10, polyNIPAM-20, and polyNIPAM-40 from 0.10 ± 0.01 , 0.37 ± 0.07 and 0.45 ± 0.02 MPa, respectively, at 25°C to 0.38 ± 0.07 , 0.59 ± 0.08 and 0.56 ± 0.02 MPa at 37°C. The same tendency was noted for maximum strain values of polyNIPAM-10, polyNIPAM-20, and polyNIPAM-40: an increase from $30 \pm 5\%$, $73 \pm 2\%$, and $62 \pm 2\%$, respectively, at 25°C to $105 \pm 16\%$, $109 \pm 13\%$, and $96 \pm 6\%$ at 37°C. This behavior is reasonable and shows that decreasing water content increases the mechanical strength of the polymer.⁶¹ In general, increasing the temperature above the VPTT affects the mechanical properties of polyNIPAM-based hydrogel with lower Mw (10,000) more significantly in comparison to the hydrogels with higher Mw (20,000 and 40,000). Additionally, increasing Mw of polyNIPAM hydrogel from 20,000 to 40,000 does not result in improved mechanical performance of the material. We assume we have reached the plateau in the relationship of Mw to mechanical properties.

In order to explore the cytocompatibility of the developed materials, polyNIPAM-10, polyNIPAM-20, and polyNIPAM-40 hydrogels were used as surfaces for culturing NIH3T3 cells. An Alamar Blue test was performed to follow the metabolic activity of the cells, as demonstrated in Figure 7. An increase in the percentage of reduced Alamar Blue can be noticed for all types of polyNIPAM-based hydrogels, indicating an increase in cells proliferation during 5 days in culture. Figure 8 shows SEM images of the polyNIPAM-20 hydrogel disk before the cell seeding and after 5 days in culture. In Figure 8(B), the uniform texture on the surface of the disk [compared to the smooth surface of the material before cell addition, Fig. 8(A)] is attributed to almost complete coverage of the surface with cells after 5 days in culture. Cell morphology and distribution at different points on the disk can be seen in the magnified images [Fig. 8(C–E)]. The images are consistent with the results of the Alamar Blue test and also demonstrate cell adhesion to the biodegradable polyNIPAM material.

The cytotoxicity of the degradable hydrogels and their degradation products was evaluated by an *in vitro* colorimetric MTT test. The main degradation products of the polyNIPAM-based hydrogel are polyNIPAM chains terminated with chlorine with a Mw that is half of the Mw of its parent polymer backbone, as supported by the GPC results in Table II. Figure

9 shows results of the MTT test for the polyNI-PAM-20 hydrogel and its degradation products. From the data presented in Figure 9, it can be seen that the parent material is not cytotoxic toward NIH3T3 cells. The degradation products at high concentrations show slight cytotoxicity, while in the concentration range between 1 and 25 mg/ mL, the degradation products are not cytotoxic. The same results were obtained for polyNIPAM-10 and polyNIPAM-40 hydrogels (data not shown).

Degradable PolyNIPAM scaffolds

Degradable thermoresponsive scaffolds based on polyNI-PAM-20 hydrogel were fabricated by the sphere-templating technique. The technique involves forming a crosslinked polymer matrix around a template of sinter-fused, monodisperse-sized PMMA microspheres and removing the template to generate a porous biomaterial.⁵⁰ This specific approach to sphere-templating, developed by Ratner and Marshall,²² was adjusted to the specifics of the monomer mixture used here⁵⁰ and allowed for the fabrication of a monodisperse, highly interconnected scaffold with thermally controllable pore size. The typical morphology of the scaffold based on polyNIPAM-20 hydrogel is demonstrated in Figure 10. In our previous study, we emphasized the change in pore size of a polyNIPAM-based scaffold going from RT to body temperature.⁵⁰ In particular, we were interested in a scaffold with a pore size large enough to facilitate efficient seeding with cells at RT that, at 37°C, shrinks to reduce the pore diameter to 35–40 μm , which has been found in our laboratory to be optimal for vascularized, relatively nonfibrotic healing into host tissue.^{23,24} In our current study, we fabricated polyNIPAM-20-based scaffolds with various pore sizes to investigate the effect of temperature on their pore size at this particular bulk composition. Scaffold with a pore diameter of $48 \pm 6 \mu\text{m}$ at room temperature showed linear shrinkage of approximately 15% at body temperature leading to decrease in pore diameter to $40 \pm 5 \mu\text{m}$, which is in the range for optimal healing and integration.

Figure 11 schematically describes a preliminary *in vitro* study to demonstrate the potential of our scaffold for tissue engineering with cell-seeded scaffolds. A polyNIPAM-20-based scaffold with a $48 \pm 6 \mu\text{m}$ pore diameter was loaded by a filter paper-capillary wicking method with A-10 SMC at RT and then the cells were cultured within the contracted scaffold at 37°C. After 5 days in culture the distribution, infiltration, adhesion, and morphology of the A-10 cells were examined.

Figure 12 shows that after 5 days in culture A-10 cells infiltrated approximately one third of the scaffold. The magnified images show appropriate cell morphology and infiltration through the interconnecting throats. Figure 13 presents SEM images of the scaffold's cross-section, demonstrating cell attachment and infiltration. These images are consistent with the histology images. To note, in our previous, study we demonstrated complete infiltration of the polyNIPAM scaffold with NIH3T3 cells compared to approximately one third of the polyNIPAM-based scaffold infiltrated with A-10 cells in the present study.⁵⁰ The difference in cell seeding efficiency is likely because A-10 cells are considerably larger than NIH3T3 cells, which makes their infiltration throughout the porous scaffold structure more challenging. Penetration of cells uniformly throughout the scaffold would be desirable, and we will continue to refine seeding protocols to achieve this goal.

CONCLUSIONS

In this study, degradable polyNIPAM hydrogels with controlled Mw of the chains that comprise the crosslinked starting polymer and the degradation products were synthesized by ATRP and characterized. Then a porous, interconnected polyNIPAM-20-based scaffold with a defined, mono-disperse pore diameter of $48 \pm 6 \mu\text{m}$ at RT was fabricated using the sphere-templating technique. To demonstrate the potential of the material for tissue engineering, the

scaffold with thermally controlled pore size was seeded with SMCs at 25°C and then cultured at 37°C in the scaffold with a pore size of $40 \pm 5 \mu\text{m}$. Expansion-contraction of the scaffold as a function of temperature could mimic a radial force within a native artery. Therefore, the thermally-responsive scaffold might serve as a bioreactor for culturing SMCs by mechanically conditioning the cells through alternating the temperature between 37 and 25°C. A future study will focus on a bioreactor based on thermally cycling the scaffold. We conclude that, due to degradable nature, tunable Mw, highly interconnected morphology, thermally controlled monodisperse pore size, and temperature-induced volume expansion-contraction, this polyNIPAM-based scaffold developed here is a promising candidate for applications in tissue engineering.

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FIGURE 1.

Synthesis of a degradable polyNIPAM hydrogel by ATRP (A) and images of the reaction mixture before and after polymerization (B). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 2.

Degradable polyNIPAM-20 hydrogel before (A) and after purification at 25°C (B) and 37°C (C). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 3.
DSC thermograms of the polyNIPAM-10, polyNIPAM-20, and polyNIPAM-40 hydrogels.

FIGURE 4. Swelling (%) of polyNIPAM-10, polyNIPAM-20, and polyNI-PAM-40 as a function of temperature.

FIGURE 5. Degradation of polyNIPAM-10, polyNIPAM-20, and polyNI-PAM-40 in 0.007N NaOH at 25°C.

FIGURE 6. Mechanical properties of polyNIPAM-10, polyNIPAM-20, and polyNIPAM-40 at 25 and 37°C.

FIGURE 7.
Percent reduction of Alamar Blue for NIH3T3 cells cultured on the surface of polyNIPAM-10, polyNIPAM-20, and polyNIPAM-40 hydrogels during 5 days.

FIGURE 8. SEM images of polyNIPAM-20 hydrogel's disk before (A) and after (B) seeding and culturing for 5 days with NIH3T3 cells. C–E: Magnified SEM images of different areas of the seeded hydrogel.

FIGURE 9. Cytotoxicity of the polyNIPAM-20 hydrogel and its degradation products at various concentrations. TCPS and latex were used as negative and positive controls, respectively. Absorbance of the samples has been normalized to the TCPS's value.

FIGURE 10. SEM images of polyNIPAM-20-based scaffold with $72 \pm 8 \mu\text{m}$ pore diameter at various magnifications.

FIGURE 11.
Schematic presentation of the *in vitro* experiment.

FIGURE 12.

Light microscope images of a histological section of A-10 cells loaded into a polyNIPAM-20 scaffold with $48 \pm 6 \mu\text{m}$ pore diameter. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 13. Representative SEM images of A-10 cell loaded polyNIPAM-20-based scaffold cross-sections (different areas of the scaffold) with a pore diameter of $48 \pm 6 \mu\text{m}$ (at 25°C).

TABLE I
Molar Ratios of the Reagents to Obtain Degradable PolyNIPAM Hydrogels With Target Mw of PolyNIPAM Backbone

Abbreviation	Degradable PolyNIPAM Hydrogel		Molar Ratios of Reagents			
	Target Mw of the PolyNIPAM Backbone, (g/mol)	NIPAM	Cl-PCL-Cl	CuCl	Me ₆ TREN	PCLDMA
PolyNIPAM-10	1.0×10^4	1	88	10	10	0.03
PolyNIPAM-20	2.0×10^4	1	177	10	10	0.03
PolyNIPAM-40	4.0×10^4	1	353	10	10	0.03

TABLE II

Mw of the Degradable Linear PolyNIPAM and Its Degradation Products

	[NIPAM]/[Initiator] (mol/mol)	Before Degradation			After Degradation		
		Mn (g/mol) (Theory)	Mn (g/mol) (GPC)	PDI Mw/Mn (GPC)	Mn (g/mol) (GPC)	PDI Mw/Mn (GPC)	PDI Mw/Mn (GPC)
polyNIPAM-10	88	1.0×10^4	1.17×10^4	1.38	5.43×10^3		1.65
polyNIPAM-20	177	2.0×10^4	2.29×10^4	1.14	1.38×10^3		1.16
polyNIPAM-40	353	4.0×10^4	3.53×10^4	1.09	1.89×10^3		1.17