Poly(*N*-isopropylacrylamide-*co*-Poly(ethylene glycol))-Acrylate Simultaneously Physically and Chemically Gelling Polymer Systems

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ABSTRACT: In an effort to create an *in situ* physically and chemically cross-linked hydrogel for *in vivo* applications, *N*-isopropylacrylamide (NIPAAm) was copolymerized with poly(ethylene glycol)-monoacrylate (PEG-monoacrylate) and then the hydroxyl terminus of the PEG was further modified with acryloyl chloride to form poly(NIPAAm-*co*-PEG) with acrylate terminated pendant groups. In addition to physically gelling with temperature changes, when mixed with a multi-thiol compound such as pentaerythritol tetrakis 3-mercaptopropionate (QT) in phosphate buffer saline solution of pH 7.4, this polymer formed a chemical gel via a Michael-type addition reaction. The chemical gelation time of the polymer was affected by mixing time; swelling of the copolymer solutions was temperature dependant. Because of its unique gelation properties, this material may be better suited for long-term functional replacement applications than other thermo-sensitive physical gels. Also, the PEG content of this material may render it more biocompatible than similar HEMA-based precursors in previous simultaneous chemically and physically gelling materials. With its improved mechanical strength and biocompatibility, this material could potentially be applied as a thermally gelling injectable biomaterial for aneurysm or arteriovenous malformation (AVM) occlusion. J Appl Polym Sci 106: 1201–1207, 2007

Key words: hydrogels; injectable biomaterials; Michael-type addition; stimuli-sensitive polymers; cross-linking

INTRODUCTION

Thermo-sensitive polymers have received considerable attention in recent years due to their widespread biomedical applications, which range from drug release systems to implant biomaterials.[1–3] Their characteristic temperature-driven sol–gel transition is advantageous in terms of implantation because it allows the material, in fluid form, to fully conform to its surroundings before gelling *in vivo*.[2–4] Moreover, because these temperature-responsive systems are water-soluble, they can potentially act as alternatives to *in vivo*-gelling materials/embolic liquids[3–6] that are traditionally delivered via water-miscible, but toxic, organic solvents such as dimethyl sulfoxide (DMSO), ethanol, and *N*-methyl pyrrolidone (NMP).[4,7] While these thermo-sensitive polymers exhibit many desirable characteristics, they are constrained by their tendency to creep under low frequency stress, which renders them unsuitable for applications that require long-term functional replacement such as arteriovenous malformation (AVM) or aneurysm occlusion.[4] Thus, there is a need to develop polymer systems that have the ability to address the mechanical limitations of these temperature-responsive materials.

A proposed solution for preventing creep is to create a polymer that does not exclusively form a physical gel, but rather will initially gel physically but then cure with chemical cross-linking to reduce viscoelasticity. Whereas purely physical gels form networks based exclusively on secondary forces, and purely chemical gels are created through chemical bonding[8,9] a hybrid material will be able to utilize both mechanisms for network formation. One such approach uses temperature-triggered gelation to achieve physical cross-linking, and Michael-type addition reaction for chemical gelation. While Cellesi and Tirelli have already used this ''in-tandem'' thermal gelation and chemical cross-linking for cell encapsulation purposes,[10] this approach may also be used to create an *in-situ* gelling polymer that has increased mechanical properties due to its ability to both physically and chemically cross-link, and may be applied as an *in-situ* forming implant.

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Previously, we have copolymerized *N*-isopropylacrylamide (NIPAAm) with hydroxyethyl methacrylate (HEMA) such that it contained functional acrylates that could react with compounds containing thiol groups.[7] The copolymer with acrylates was designed so that physical cross-linking would be triggered by the material's lower critical solution temperature (LCST), and chemical cross-linking would occur over time from the interactions between olefins and nucleophilic thiols via a Michael-type addition reaction.[7,11] The Michael-type addition reaction is a particularly suitable mechanism for chemical cross-linking, not only because it proceeds quickly at room temperature, but also because it uses precursors with lower toxicity.[12] In this study, we copolymerized NIPAAm with poly(ethylene glycol) (PEG) monoacrylate and functionalized the copolymer with pendant acrylate groups on the end of the PEG side chains so as to create a material that has the ability to both physically and chemically cross-link. Because PEG is highly biocompatible and FDA-approved for use in pharmacological applications,[13] this copolymer could ideally be applied as an injectable *in-situ* gelling material whose mechanical properties exceed those of purely physical gels but still allow a temperature-triggered gelation.

EXPERIMENTAL

Materials

NIPAAm (Aldrich) was purified by recrystallization from hexanes and dried under vacuum for 2 days. 2,2′-Azobisisobutyronitrile (AIBN; Aldrich 98%) was purified by recrystallization from methanol. Pentaerythritol tetrakis 3-mercaptopropionate (QT; Aldrich), acryloyl chloride (Aldrich 96%), anhydrous 1,4-dioxane (Aldrich), PEG-acrylate (Aldrich) with molecular weight of 375, and tetrahydrofuran (THF, Aldrich) were used as received. 0.1*M* phosphate buffer saline (PBS) of pH 7.4 was prepared from 2.43 g monobasic phosphate, 11.32 g dibasic phosphate, 3.33 g NaCl dissolved in 1 L of distilled water. Other solvents used in this experiment were of reagent grade and used as received.

Synthesis of poly(NIPAAm-*co*-PEG)-acrylate

NIPAAm (15 g, 133 mmol) and PEG-monoacrylate (2.6 g, 6.98 mmol) were combined with 200-mL THF and AIBN (0.16 g, 0.98 mmol) at 65°C for 20 h under a nitrogen atmosphere to form poly(NIPAAm-*co*-PEG). The copolymer was then precipitated in diethyl ether, filtered and dried under vacuum for 3 days (Yield: 89%). The copolymer was then dialyzed, frozen, and lyophilized. Acrylate addition to the PEG chain on the poly(NIPAAm-*co*-PEG) was accomplished by dissolving the copolymer (6.2 g) in anhydrous THF, adding triethylamine (1.4 mL, 10.12 mmol), followed by drop-wise addition of acryloyl chloride (0.43 g, 5.06 mmol, 1.1 wt % in THF).

Thermo-sensitive properties

The LCST of the polymer was determined with differential scanning calorimetry (DSC). Polymer samples were dissolved at concentrations of 5 wt % in 0.1*M* PBS of pH 7.4 and heated from 0 to 80°C at a rate of 1°C/min with a Multi Cell Differential Scanning Calorimeter (Calorimetry Scientific Corporation, CSC 4100). The control cell contained 0.1*M* PBS of pH 7.4.

Gelation properties

Copolymer samples were prepared as 21 wt % solutions in 0.1*M* PBS of pH 7.4; rheology was performed with a TA Instrument Rheometer (AR 1000) using a 4-cm parallel plate at a 0.5-mm gap for all samples.

To assess temperature-dependant physical gelation, rheometry temperature sweeps from 5 to 45°C were performed on poly(NIPAAm-*co*-PEG) and poly (NIPAAm-*co*-PEG)-acrylate at a frequency of 1 Hz, and oscillatory stress of 10 Pa (linear region). Gel points, defined as the instant at which $\delta = 45^\circ$ or when G' exceeds G'' (i.e., the solid phase becomes dominant), were obtained.[14]

The effects of QT mixing time on the chemical gelation time were determined via the following procedure: poly(NIPAAm-*co*-PEG)-acrylate was combined with QT at a 1 : 1 stoichiometric ratio (1 g copolymer/5.46-μL QT) between the acrylate and thiol groups and mixed between two 1-mL syringes connected by a syringe junction for 10 or 20 s at a rate of 1 stroke per second. About 0.6 mL of the mixed sample was then placed on the rheometer and a 30 min time sweep was performed at a temperature of 20°C to prevent physical gelation, frequency of 1 Hz, and oscillatory stress of 10 Pa. Frequency sweeps from 0.1 to 20 Hz were also performed on the NIPAAm copolymers, with and without QT at various temperatures.

Swelling test

Poly(NIPAAm-*co*-PEG)-acrylate was mixed with QT at 1 : 1 stoichiometric ratios between the thiol and acrylate groups, immersed in a 37°C water bath, and allowed to gel for 24 h. The gel was extracted and cut into cylindrical cross sections. A cross section was then incubated in 0.1*M* PBS of pH 7.4 at a tem-

perature of 4°C. After 1 h, the sample was removed from solution, and was weighed after removal of excess surface water. The same procedure was performed for samples in 0.1*M* PBS of pH 7.4 at temperatures of 20 and 37°C. Each polymer sample was then lyophilized and weighed again. The swelling ratio Q_s was then calculated as $(W_s - W_0)/W_0 \times 100$, W_s being the weight of the swollen polymer, and W_0 being the weight of the dried polymer.

Molecular identification

The composition of the copolymers was identified through ^{1}H NMR (Gemini 300 MHz). Polymer samples were dissolved in D_2O.

Molecular weight

Molecular weight was determined using static light scattering (Wyatt Minidawn, Santa Barbara, CA) in conjunction with gel permeation chromatography (GPC). GPC (Shimadzu, micro-styragel HR-5 column) was performed on the copolymers with THF mobile phase and a flow rate of 0.8 mL/min.

Cytotoxicity

An indirect contact study was conducted to test cytotoxicity of the hydrogels. A sample of the poly(NIPAAm-*co*-PEG)-acrylate (16 wt %) was prepared stoichiometrically in cell culture media and mixed with QT between syringes for 30 s. Millicell culture plate inserts (12 mm, Costar Corporation, 0.4 μm pore size polycarbonate membrane filter (PIHP01250)) were filled with 100 μL of the sterile pregelled solution. The hydrogels were cross-linked inside of the Transwell inserts by exposing them to 37° for 1 h. The 3T3 cells were seeded into each well of a 24-well plate, and then 0.5 mL of cell culture medium was added to each well. The cells were cultured in this environment for 7 days and then Promega (Celltiter 96 Aqueous One Solution Cell Proliferation Assay) was added after removal of the inserts. Three hours later the media was aliquotted with a micropipette into 96-well plates, 120 μL at a time and then their absorbance was measured at 490 nm. For the control, polyNIPAAm was used, along with wells that contained no material.

RESULTS AND DISCUSSION

Poly(NIPAAm-*co*-PEG) was prepared by free radical polymerization, using the scheme shown in Figure 1(A). When attempting to synthesize NIPAAm and polyethylene-oxide (PEO)-methacrylate through free radical polymerization, Virtanon et al. reported that the reaction resulted in gel formation.[15] During synthesis of the NIPAAm-*co*-PEG polymer for this study, a gel was formed during the reaction when dioxane was used. However, when the materials were reacted in THF, there was no such unwanted cross-linking. The composition of the copolymer was confirmed by ^{1}H NMR. The mole ratio of NIPAAm and PEG was calculated from the integration ratio between the methyl protons (6H)($(CH_3)_2$CHNHCO—) of NIPAAm and the methylene protons (2H)(H $OCH_2CH_2(OCH_2CH_2)_n$ OCH_2CH_2OCO—) of PEG appearing at 1.1 and 4.3 ppm, respectively, in Figure 2. Poly(NIPAAm-*co*-PEG)-acrylate was synthesized by allowing terminal OH groups of PEG to react with acryloyl chloride as shown in Figure 1(A). The total conversion was calculated using the integration ratio of the 1-ethylene protons (3H) (CH_2=CH—$COOCH_2CH_2O$—)) of PEG-acrylate at around 6.2 ppm, and (6H)((CH_3)$_2$ CHNHCO—) of NIPAAm at around 1.1 ppm and the integration ratio of the methylene protons (CH_2=CH—$COOCH_2CH_2O$—) of PEG-acrylate at around 4.4 ppm and methylene protons (2H) ($HOCH_2CH_2(OCH_2CH_2)_nOCH_2CH_2OCO$—, CH_2=CH—$COOCH_2CH_2(OCH_2CH_2)_nOCH_2CH_2OCO$—) of unreacted PEG and reacted PEG at around 4.3 ppm. The copolymers synthesized are found in Table I. For poly(NIPAAm-*co*-PEG) at 95 : 5 feed ratio, the real ratio was 94.9 : 5.1 and NMR data exhibited that the ratio of PEG to PEG-acrylate was 1.6 : 3.5, giving approximately a 69% conversion.

When poly(NIPAAm-*co*-PEG)-acrylate was mixed with a multifunctional thiol compound, chemical cross-linking occurred between the acrylate and thiol groups through a Michael-type addition reaction. The mechanism, in Figure 1(B), shows a nucleophilic thiol attacking the double bond adjacent to the carbonyl forming a covalent bond between the two entities. Figure 1(C) shows the chemical cross-linking network that is formed when poly(NIPAAm-*co*-PEG)-acrylate is allowed to react with QT, which contains four thiol functional groups. Poly(NIPAAm-*co*-PEG)-acrylate itself is soluble in water at temperatures lower than its LCST and in organic solvents such as THF, acetone, and ethanol. However, when an aqueous solution of poly(NIPAAm-*co*-PEG)-acrylate is mixed with QT and allowed to cure chemically, it forms an opaque solid.

The LCST and the gelation temperature found with rheology of copolymers 1 and 2 can be seen in Table I. Although the addition of comonomers to NIPAAm can result in large deviations from the typical LCST of PolyNIPAAm (32°C),[1,15] results from this study indicate that the conditions and concentrations under which PEG and PEG-acrylate were added to NIPAAm minimally affect the copolymers' LCSTs and gelation temperatures; both copolymers demonstrated LCST properties at ~30°C (Fig. 3). Vir-

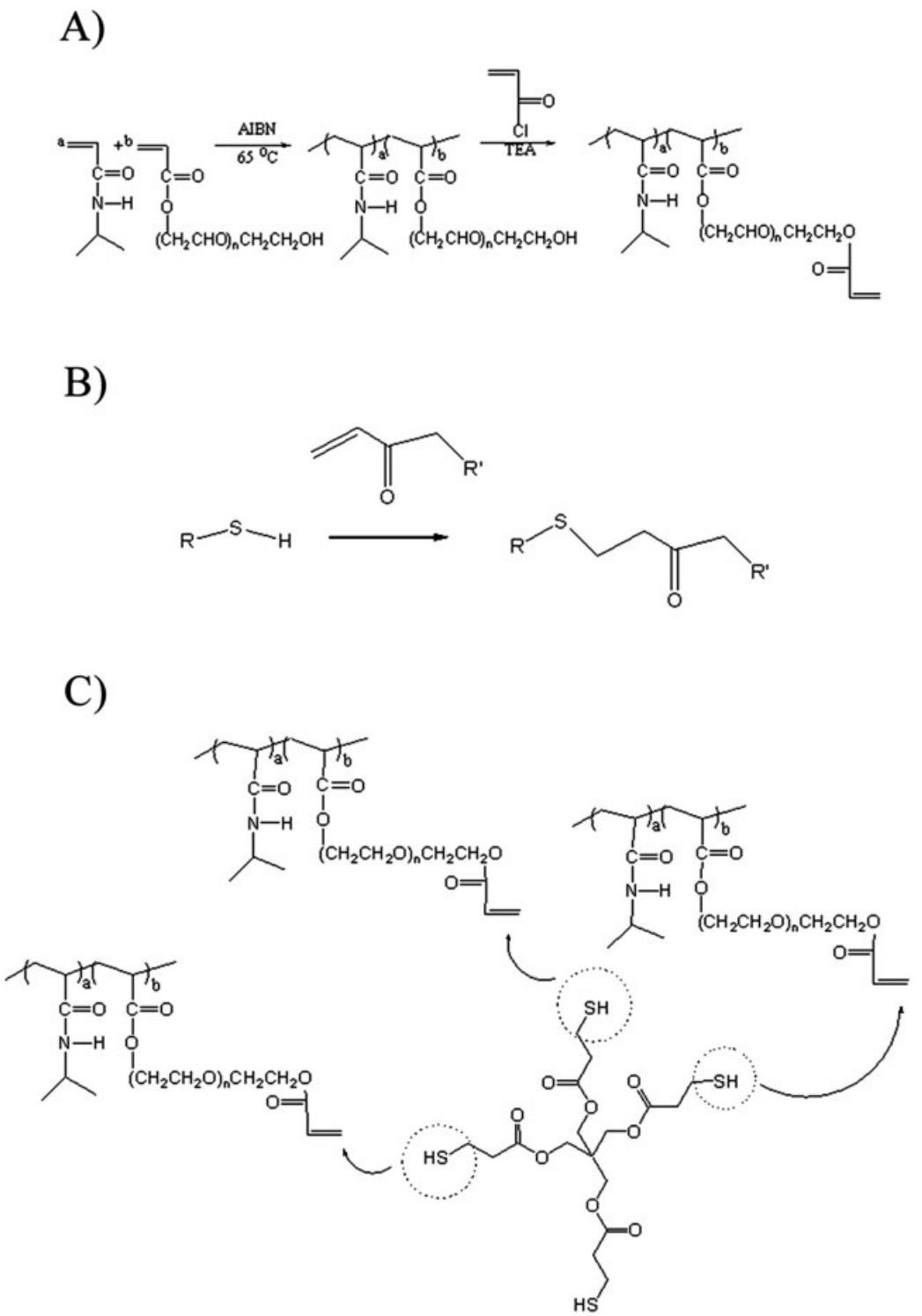

Figure 1 (A) Polymer synthesis scheme; (B) Michael-type addition reaction; (C) Chemical cross-linking network between functionalized copolymer and QT.

tanen, et al synthesized NIPAAm graft PEO polymers with similar weight percents and reported LCST values that closely match those of our polymers.[15] GPC determined that the copolymers had a molecular weight of 3.4×10^5 g/mol and polydispersity index (PDI) of 2.1.

The poly(NIPAAm-*co*-PEG) and poly(NIPAAm-*co*-PEG)-acrylate copolymers themselves formed purely physical gels at temperatures above their LCSTs. From the rheology temperature sweeps in Figure 4, it is evident that the gelation of copolymers 1 and 2 was temperature-dependent. At lower temperatures,

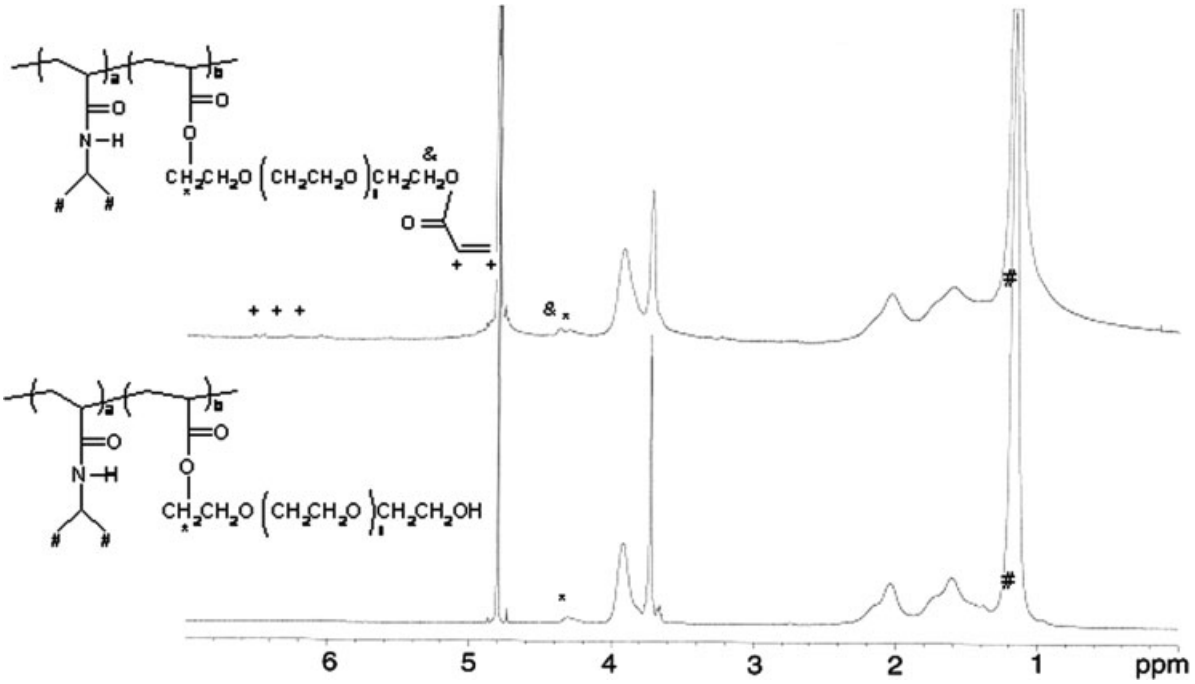

Figure 2 ^{1}H NMR spectra (solvent, D_2O) of NIPAAm copolymers in various stages of synthesis. Top: poly(NIPAAm-*co*-PEG)-acrylate. Bottom: poly(NIPAAm-*co*-PEG).

G'' was larger than G'; however, a sol–gel transition occurred as temperature increased to 27 and 28°C, respectively, for the two copolymers, and the storage modulus G' became dominant, suggesting that a gel formed due to temperature-triggered physical cross-linking.

When copolymer 2 was mixed with QT at 1 : 1 acrylate-thiol ratios and mixing time was varied at 10 and 20 s, the effect on gelation kinetics was evident, seen in Figure 5. As expected, longer mixing times resulted in faster gelation time, which can be attributed to the higher degree of homogenization resulting from longer mixing[7]; gelation occurred at 245 s at 20-s mixing time, while for 10-s mixing time gelation occurred at 411 s. When compared to the previously characterized poly(NIPAAm-*co*-HEMA)-acrylate system, this polymer system had a slightly faster gelation time for both the 10 and 20 s mixing times. This behavior may be due to the better solubilizing effects of PEG on the QT.

Swelling of the chemically cross-linked polymer was found to be temperature dependent, as seen in Figure 6. At 4°C, the polymer gel demonstrated a high degree of swelling, at 456%. However, as temperature increased to 20 and 37°C, the polymer gel swelling decreased to 387 and 319%, respectively. From previous studies, it was noted that other thermo-sensitive polymer systems, such as poly(NIPAAm-*co*-HEMA)-acrylate demonstrated a high degree of volume change with temperatures.[7] This copolymer, in contrast, swelled less at each temperature change relative poly(NIPAAm-*co*-HEMA)-acrylate. Such swelling behavior can be attributed to PEG's affinity and ability to bind to water; because the PEG arm is longer than HEMA, the polymer forms a gel network with more space when mixed with QT and is thus able to hold more water at various temperatures. This results in a smaller extent of shrinking, which then makes the polymer system behave more like a hydrogel.

TABLE I
Synthesized Copolymers

	Actual Compositions (mol %)			Rheology Gelation	LCST	$M_W \times 10^5$	
N	NIPAAm	PEG	Acrylate	Temperature (°C)[a]	DSC[b] (°C)	(g/mol)	PDI
1	94.9	5.1	–	27.0 ± 0.2	31.6 ± 0.1	3.4	2.1
2	94.9	1.6	3.5	28.0 ± 1.0	30.1 ± 0.1		

[a] Rheology was performed at 21 wt % concentrations in PBS.
[b] DSC was performed at 5 wt % concentrations in PBS.

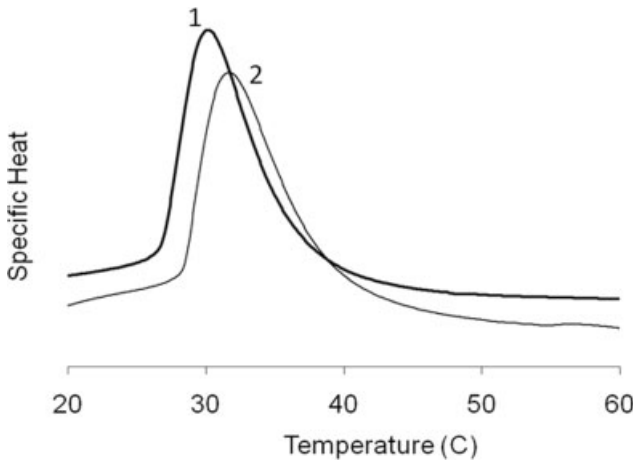

Figure 3 LCST determined by DSC at 5 wt % concentration in pH 7.4 PBS, (n = 4) for poly(NIPAAm-*co*-PEG) (2) and poly(NIPAAm-*co*-PEG)-acrylate (1).

Comparatively, there are some differences in gelation behavior between the poly(NIPAAm-*co*-PEG)-acrylate system and the poly(NIPAAm-*co*-HEMA)-acrylate system. The gel point of this poly(NIPAAm-*co*-PEG)-acrylate system remained fairly consistent before and after the addition of the terminal acrylate pendant groups, whereas there was a larger drop in gel temperature for poly(NIPAAm-*co*-HEMA)-acrylate after the addition of acrylate. The decrease in LCST of the poly(NIPAAm-*co*-HEMA)-acrylate system is due to the substitution of the hydrophilic end group (OH) of HEMA with the hydrophobic acrylate; in contrast, the end group substitution of

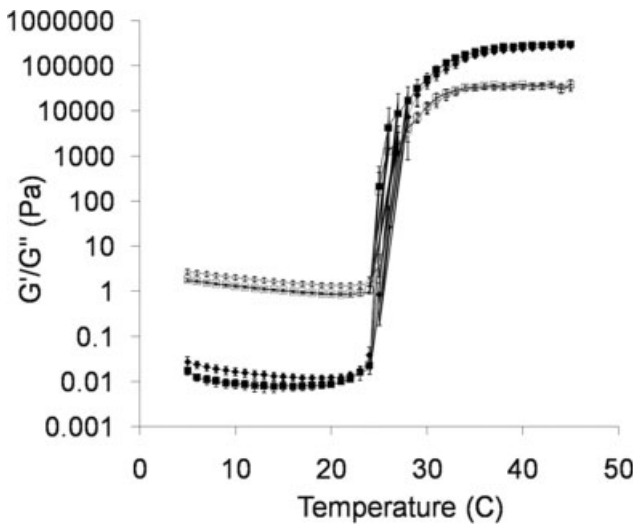

Figure 4 Rheology temperature sweep showing temperature-dependant physical gelation of both poly(NIPAAm-*co*-PEG) [■G'; □G''] and poly(NIPAAm-*co*-PEG)-acrylate [◆G'; ◇G''] at 21 wt % in 7.4 pH PBS (n = 3).

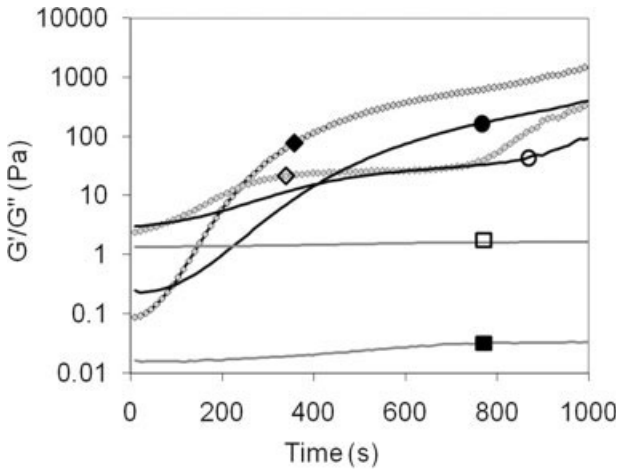

Figure 5 Rheology time sweep showing time-dependant chemical-gelation behavior based on different mixing times (21 wt % in 7.4 pH PBS, 20C) (gel point, 20-s mixing = 245 s; 10-s mixing = 411 s) for poly(NIPAAm-*co*-PEG)-acrylate mixed with QT for 10 s [●G'; ○G''], or for 20 s [◆G'; ◇G''], or without QT [■G', □G''].

hydroxyl group of PEG with the hydrophobic acrylate does not change the gelation temperature of the copolymer because inside ethylene oxide groups of PEG are still available to hydrogen bond with water. On the whole, while these two polymer systems share similar cross-linking mechanisms, there are subtle differences in behavior regarding the gel point as well as swelling behavior.

The poly(NIPAAm-*co*-PEG)-acrylate crosslinked with QT was tested for cytotoxicity, using an indirect contact assay. The result of this study is shown in Figure 7, and indicates that the cell numbers in the media after 1 week of incubation with the physically and chemically cross-linked polymer were statistically similar to those of both polyNIPAAm and the control without polymers. Preliminarily, this

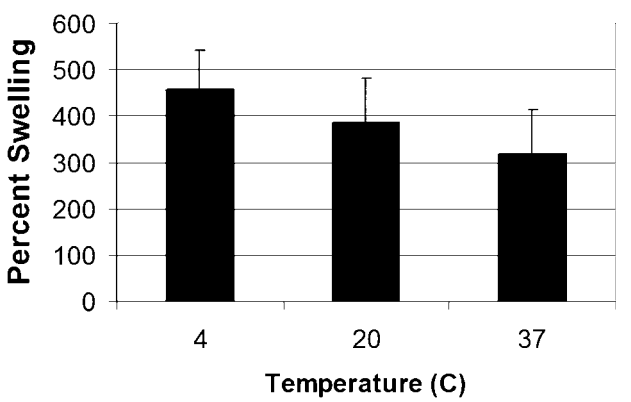

Figure 6 Temperature-dependent swelling of poly(NIPAAm-*co*-PEG)-acrylate cross-linked with QT (pH 7.4); swelling ratios after 1 h, n = 3 at 4, 20, and 37°C.

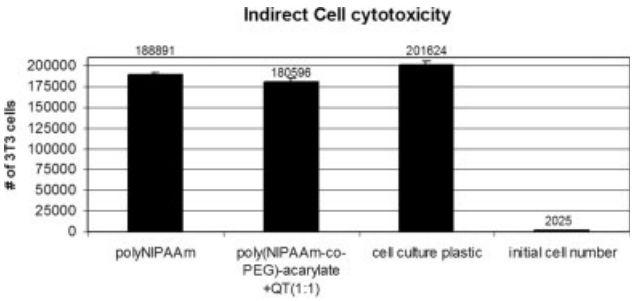

Figure 7 Indirect contact assays on 3T3 cells using poly (NIPAAm-*co*-PEG)-acrylate crosslinked with QT; cell count after 7 days, $n = 3$.

study shows that the material has favorable biocompatibility properties.

CONCLUSION

A simultaneously physical and chemical cross-linking polymer was developed by modifying poly(NIPAAm-*co*-PEG) with acryloyl chloride and mixing it with a tetrafunctional thiol compound. This system demonstrates lower critical solution properties at $\sim$ 27–28°C, which were confirmed by DSC and rheology. Moreover, this system exhibits unique gelation behavior; physical cross-linking is triggered by temperature changes and chemical cross-linking can be simultaneously achieved through a Michael-type addition reaction between the acrylate groups and the thiol groups in the tetrafunctional QT. For this particular system, swelling is temperature-dependent, and although it has a high degree of swelling, volume changes are small in between temperature changes. This combination of properties may render this polymer useful as a biomaterial for *in-situ* gelling applications, such as AVM or aneurysm occlusion.

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