In-Situ Injectable Physically and Chemically Gelling NIPAAm-Based Copolymer System for Embolization

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The goal of this work is to make an injectable physically and chemically cross-linking NIPAAm-based copolymer system for endovascular embolization. A copolymer with *N*-isopropylacrylamide (NIPAAm) and hydroxyethyl methacrylate (HEMA) was synthesized and converted to poly(NIPAAm-*co*-HEMA-acrylate) functionalized with olefins. When poly(NIPAAm-*co*-HEMA-acrylate) was mixed with pentaerythritol tetrakis 3-mercaptopropionate (QT) stoichiometrically in a 0.1 N PBS solution of pH 7.4, it formed a temperature-sensitive hydrogel with low swelling through the Michael-type addition reaction and showed improved elastic properties at low frequency compared to physical gelation. This material could be useful for applications requiring water-soluble injection but lower swelling and lower creep properties than available with other soluble in-situ-gelling materials.

Introduction

In-situ-gelling biomaterials have been attractive because of their applications in tissue engineering and drug delivery systems.[1] In-vivo-forming implant systems for water-insoluble hydrophobic polymers provide low swelling but require water-miscible organic solvents such as dimethyl sulfoxide (DMSO), ethanol, and *N*-methyl pyrrolidone (NMP).[2] These hydrophobic polymers provide significant mechanical properties in the implant, low swelling, and constant (zero) order release of hydrophobic drugs. However, ideal, injectable, in-vivo-gelling materials are waterborne rather than delivered in water-miscible organic solvents because of the toxicity of the organic solvents.[2c]

Temperature-responsive systems have been used for vascular embolization and drug delivery applications.[3] When these materials undergo a temperature-driven phase transition after injection they form a gel. These systems provide waterborne delivery but maintain low swelling due to the change in hydrophilicity−hydrophobicity balance. Unfortunately, many temperature-sensitive materials exhibit viscoelastic flow under constant and low-frequency stress.[1d] For biomedical applications including cell culture as well as vascular embolization requiring higher mechanical properties and low-frequency load bearing, the control of mechanical strength is very significant and these materials may require additional chemical curing after the thermal gelling to reduce the viscoelasticity of the materials.[4] One solution is to simultaneously chemically and physically cross-link the gels. Cellesi et al. have described the tandem thermal gelling and chemical cross-linking of telechelic pluronic as a synthetic alternate of physically cross-linking alginate for the encapsulation of cells.[5]

NIPAAm-based copolymers have been designed to undergo simultaneous in-situ physical and chemical cross-linking. Temperature-sensitive NIPAAm-based copolymers independently functionalized with olefin will provide simultaneously physically and chemically cross-linking materials at body temperature by simple mixing with multifunctional thiol compounds or polymers. These materials, when dissolved in water, can be injected and will form a gel immediately due to temperature-driven physical cross-linking. With time, these physically cross-linked gels will be cured by chemical cross-linking through Michael-type addition reactions between the thiols and acrylates. These polymers have potential application as in-situ-gelling materials in applications where viscoelastic creep seen in purely physical gels is not appropriate.

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Materials and Methods

Materials. *N*-Isopropylacrylamide (NIPAAm; Aldrich) was purified by recrystallization from hexanes and dried under vacuum for 4 days. 2,2′-Azobisisobutyronitrile (AIBN; Aldrich 98%) was purified by recrystallization from methanol. Hydroxyethyl methacrylate (HEMA, Aldrich), pentaerythritol tetrakis 3-mercaptopropionate (QT, Aldrich), and acryloyl chloride (Aldrich 96%) were used as received. Anhydrous 1,4-dioxane (Aldrich) and tetrahydrofuran (THF) were used as received. Other solvents used in this experiment were reagent grade and were used as received.

Synthesis of Poly(NIPAAm-*co*-HEMA) and Poly(NIPAAm-HEMA-acrylate). NIPAAm-based copolymers with NIPAAm/HEMA 95:5 were synthesized by radical polymerization in 1,4-dioxane as in the literature.[6] Briefly, the total monomer concentration was 0.1 g mL^{-1} in 1,4-dioxane (10 wt %). AIBN was used as an initiator (initiator/total amount of monomers $= 7 \times 10^{-3}$ mol/mol). Batches of approximately 15 g were prepared. Nitrogen was bubbled through the solution at room temperature for 15 min prior to the addition of the initiator to reduce oxygen content in the polymerization reaction. The copolymerization was conducted at 65 °C for 20 h under a nitrogen atmosphere. Subsequently, the copolymer was precipitated in an excess of diethyl ether, filtered, and then dried under reduced pressure. The copolymers were dissolved in water, dialyzed for 3 days, and lyophilized. Yield = 85%. ^{1}H NMR (D_2O): δ = 4.2−4.0 (H^8), 4.0−3.6 (H^3, H^9), 2.3−1.2 (H^1, H^2, H,^{6} H^7), 1.2−0.9 (H,^{4} H^5). An amount of 5 g of poly(NIPAAm-*co*-HEMA)(43.9 mmol) was dried for 24 h under reduced pressure at 60 °C to minimize moisture and then dissolved in anhydrous THF for 1h. A volume of 1.2 mL of triethylamine (4 equiv per OH group) was added to the solution. The solution was cooled to around 4 °C in an ice bath, and then 0.4 mL of acryloyl chloride (2 equiv per OH group) diluted in 30 mL of THF was added dropwise to the solution. The mixture was stirred at this temperature for 3 h and

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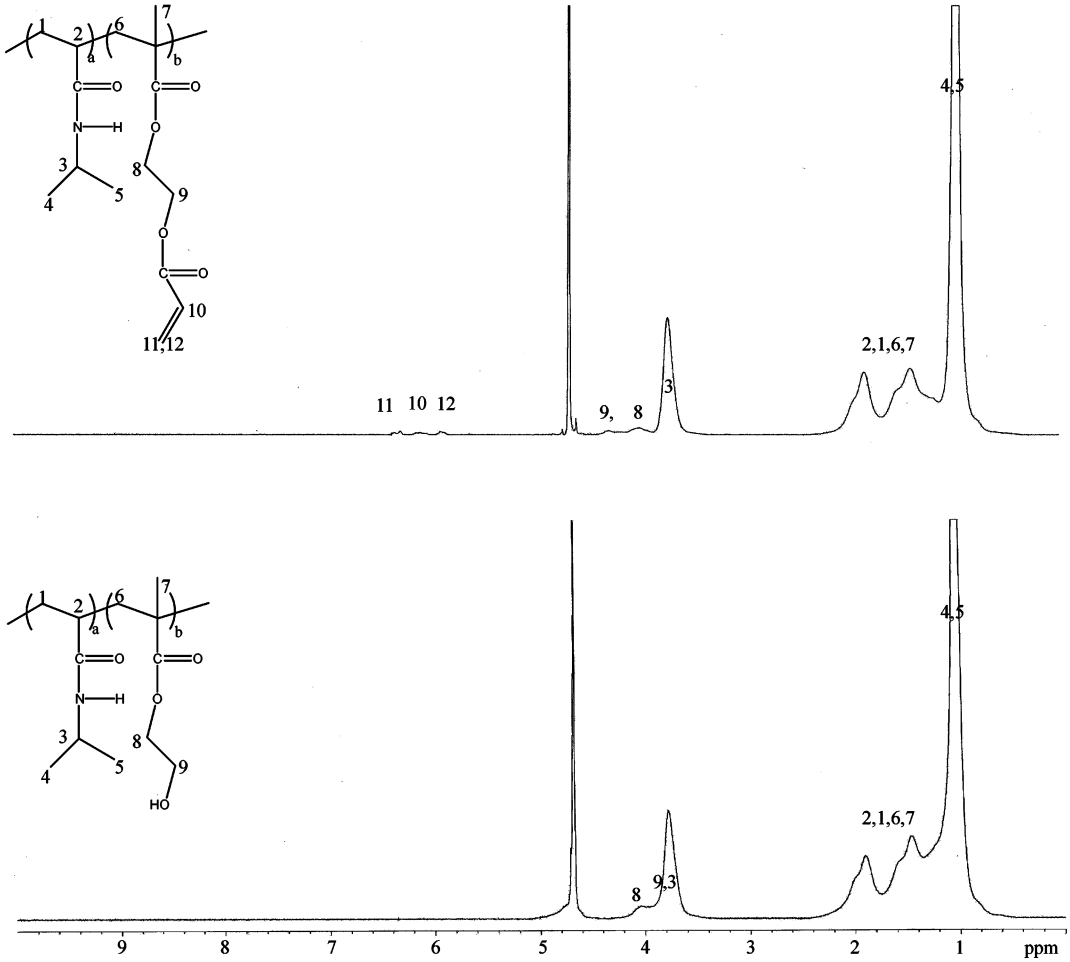


Figure 1. ^{1}H NMR spectra of copolymers **1** and **2**.

then at room temperature for an additional 12 h. The triethylammonium chloride salt was removed by filtration. The remaining mixture was precipitated in 10-fold excess ether, filtered, and then dried under vacuum for 1 day. After drying, the polymer was dissolved in water, dialyzed over 3 days against 3000 MWCO at 5 °C, and then lyophilized to obtain the final product. Conversion = 56% (from 1 H NMR data). Yield = 70% ^{1}H NMR (D_2O): δ = 6.4−5.8 (H,^{11} H,^{12} H^{10}), 4.4−4.2-(H^9), 4.2−4.0 (H^8), 4.0−3.6 (H^3), 2.3−1.2 (H^1, H^2, H,^{6} H^7), 1.2−0.9 (H,^{4} H^5).

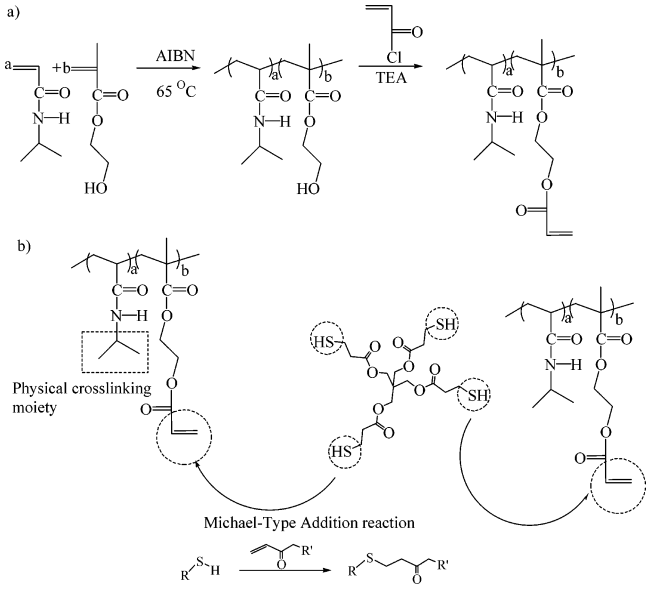
Molecular Weight. The molecular weights of the synthesized polymers were determined by gel permeation chromatography (Shimadzu HPLC) in conjunction with static light scattering (Wyatt miniDawn−Santa Barbara, CA). The copolymers were dissolved in THF at 3 mg mL^{-1}, and gel permeation chromatography (GPC) was performed with Waters Ultrastyragel columns HR4 and HR6 in series. Spectrometric grade THF was used as the mobile phase flowing at 1 mL min^{-1}.

Gelation Properties. Each copolymer was dissolved at a concentration of 20 wt % in 0.1 M phosphate-buffered saline (PBS) of pH 7.4 to measure temperature-dependent physical gelling properties. To measure both physically and chemically gelling properties, poly-(NIPAAm-*co*-HEMA-acrylate) was mixed with QT such that there was stoichiometry between the thiol and acrylate groups. Quantification of the elastic or storage modulus for these materials was investigated using a TA Inst rheometer. The components, 1.0 g of polymer and 4.9 μL of QT, were combined in a 3-cm^3 syringe and mixed by hand (60 strokes/min) in two 3-cm^3 plastic syringes with a syringe junction for 10 and 20 s and then transferred to the rheometer. The polymer solution, 0.6 mL, was placed between parallel plate geometry with a 40-mm diameter and a gap of 0.5 mm at various temperatures. Also, a temperature sweep was performed from 5 to 45 °C with a heating rate of 2 °C min^{-1}. The data were collected at a frequency of 1 Hz and a controlled stress of 10 Pa (linear viscoelastic region). The sol-to-gel transition temperature was defined as the temperature at which storage modulus (G') is equal to loss modulus. A gel (elastic solid) can be defined when G' is higher than G'' as storage modulus (G') is dominant at the gel phase while loss modulus is dominant at the sol phase.[5−7]

Swelling Test and ESEM Imaging. A sample of the poly(NIPAAm-*co*-HEMA-acrylate) (16 wt %)/QT was prepared stoichiometrically at pH 7.4 and was mixed between syringes for 1 min, and incubated at 37 °C for 24 h until it completely gelled. A cylinder-shaped gel was then extracted from the syringe and then lyophilized. The obtained gel was incubated in 0.1 M PBS at pH 7.4 at a temperature of 37, 20, and 5 °C for 3 h. The degree of swelling is defined as $[100(W - W_0)/W_0]$, where W is the weight of the swollen gel, and W_0 is the weight of the dried polymer. For imaging, the sample was cut with a razor blade, mounted, and placed in the environmental scanning electron microscope (FEI XL 30 EFSEM). The sample was uncoated and was imaged at a working distance of 10.1 at 105×, 418×, and at 1672× as well as other magnifications to capture informative structure.

Cytotoxicity. A sample of the poly(NIPAAm-*co*-HEMA-acrylate) (16 wt %)/QT was prepared stoichiometrically at pH 7.4 and was mixed between syringes for 30 s. The material was placed in a sterile centrifuge tube and was aliquotted with a micropipet into 96-well plates, 50 μL at a time. Five replicates were created in each row. An indirect contact methodology was also used for cytotoxicity of the hydrogels. Transwell inserts (6.5 mm, Costar Corporation, 3-μm pore size polycarbonate membrane filter) were filled with 50 μL of sterile pre-gelled solution. The hydrogels was cross-linked inside of Transwell inserts by exposure to 37 °C for 1 day. The 3T3 cells were seeded into each well, and then 0.1 mL of cell culture medium was added to each well. The cells were cultured in this environment for 2 days. The samples were allowed to

Scheme 1



sit for 2 h after the Promega (Celltiter 96 Aqueous One Solution Cell Proliferation Assay) was added and then had their absorbance measured.

Results and Discussion

Poly(*N*-isopropylacrylamide) is a polymer that exhibits a LCST (lower critical solution temperature) of about 32 °C.[8] At temperatures below the LCST, the polymer is soluble in aqueous solutions, preferring an unfolded conformation. Above the LCST, the polymer becomes insoluble and acquires a folded conformation. At a sufficient concentration, poly(*N*-isopropylacrylamide) undergoes a sol-to-gel transition with temperature because the chain collapse leads to physical cross-linking due to chain entanglement. One advantage of the poly(*N*-isopropylacrylamide)-based copolymers compared to the pluronics is that the free radical polymerization mechanism used to synthesize the materials allows more flexibility in gel properties due to the abundance of comonomers that can be polymerized with *N*-isopropylacrylamide. To obtain a copolymer with physical gelling properties and OH functional groups as a starting material for a further modification, NIPAAm (thermosensitive moiety) and HEMA were used.

Poly(NIPAAm-*co*-HEMA) was prepared by free radical polymerization in Scheme 1a. The compositions of the copolymers were calculated from the ^{1}H NMR spectra. The mole ratio of NIPAAm and HEMA was calculated from the integration ratio between the methyl protons (6H)($(CH_3)_2CHNHCO-$) of NIPAAm and the methylene protons (2H)($HOCH_2CH_2OCO-$) of HEMA appearing at 1.1 and 4.1 ppm, respectively, in Figure 1. Poly(NIPAAm-*co*-HEMA-acrylate) was synthesized by allowing terminal OH groups of HEMA to react with acryloyl chloride as shown in Scheme 1a. The total conversion was calculated using the mole ratio of the methylene protons (2H) ($CH_2=CH-COOCH_2CH_2OCO-$)) of HEMA-acrylate, and the methylene protons (2H) ($HOCH_2CH_2OCO-$ and $CH_2=CH-COOCH_2CH_2OCO$) of HEMA and HEMA-acrylate, appearing at around 4.3 and 4.1 ppm, respectively. The copolymers synthesized are found in Table 1. For poly(NIPAAm-*co*-HEMA), the ratio was 94.6:5.4, and NMR data showed that the ratio of HEMA to HEMA-acrylate was 2.4:3.0, giving approximately a 56% conversion.

The gel temperature of the poly(NIPAAm-*co*-HEMA) determined by rheometry ($\delta = 45°$) was 27 °C at 20 wt % PBS solution of pH 7.4, while that of poly(NIPAAm-*co*-HEMA-acrylate) was 23 °C at the same condition. HEMA-acrylate is more hydrophobic than HEMA due to the conversion of the hydrophilic $-OH$ group to $-OCOCH=CH_2$. The gelling of the copolymer was driven by association of hydrophobic groups (isopropyl groups of NIPAAm) through a change in temperature. The frequency dependence of the dynamic moduli for copolymer **2** (20 wt %) at 20 and 37 °C is also presented in Figure 2b. At 20 °C, the loss modulus, G'', was larger than the storage modulus, G', in the frequency range 0.1 to 20 Hz. At 37 °C, G' was larger than G'', indicating that the polymer formed a gel through temperature-driven physical cross-linking.

As shown in Scheme 1b, temperature-sensitive NIPAAm-based copolymers independently functionalized with acrylates can react with an equimolar amount of thiol groups such as QT through a Michael-type addition reaction, which provides in situ simultaneously physically and chemically cross-linking materials. When these materials dissolved in aqueous solutions are

Table 1. Copolymers Synthesized

	compositions (mole ratio)			gel point		
N	NIPAAm	HEMA	HEMA-acr ylate	(°C)	MW ($\times 10^{-5}$)	PDI
1	94.6	5.4		27	1.6	1.6
2	94.6	2.4	3.0	23		

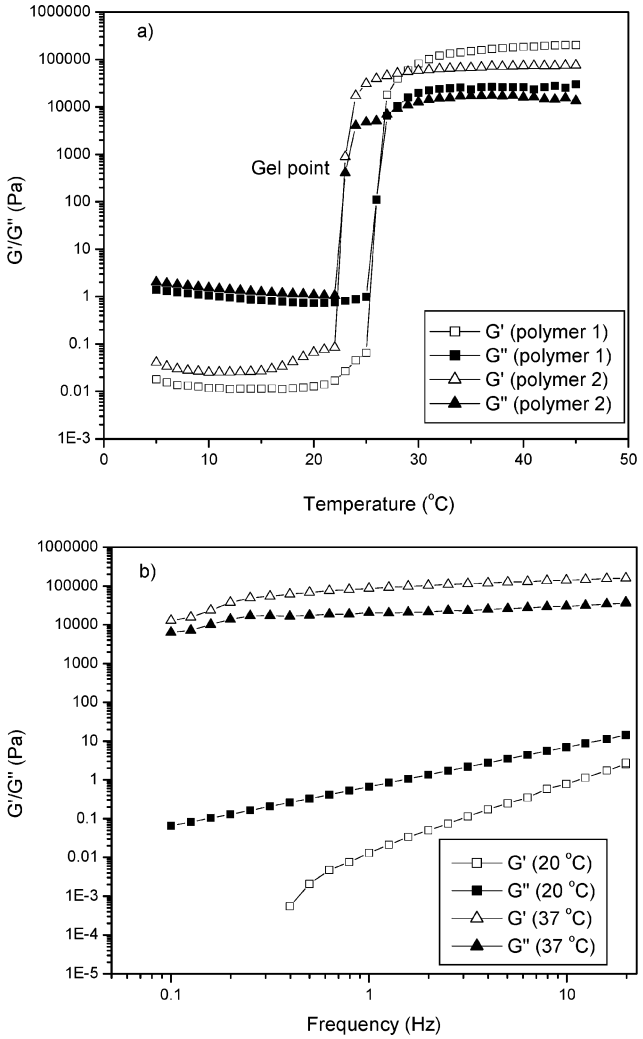


Figure 2. (a) Temperature dependence of the dynamic moduli of 20 wt % solution of copolymers **1** and **2** in 0.1 M PBS of pH 7.4 at frequency (1 Hz); (b) frequency dependence of the dynamic moduli of 20 wt % solution of copolymer **2**.

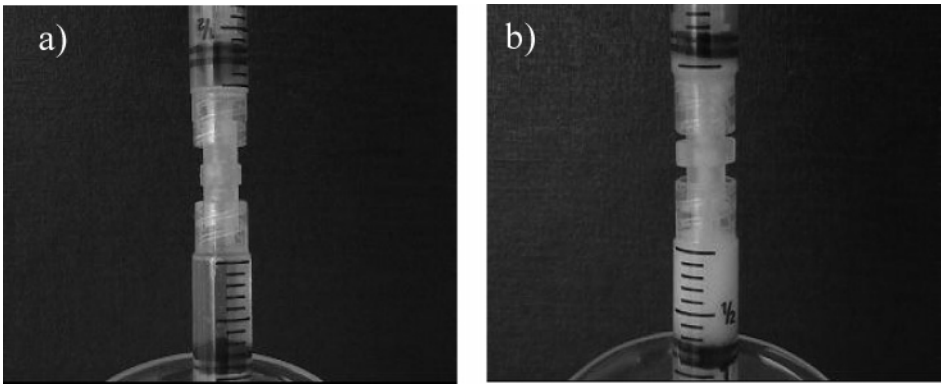


Figure 3. Preparation of polymer samples (a) before mixing, and (b) during mixing.

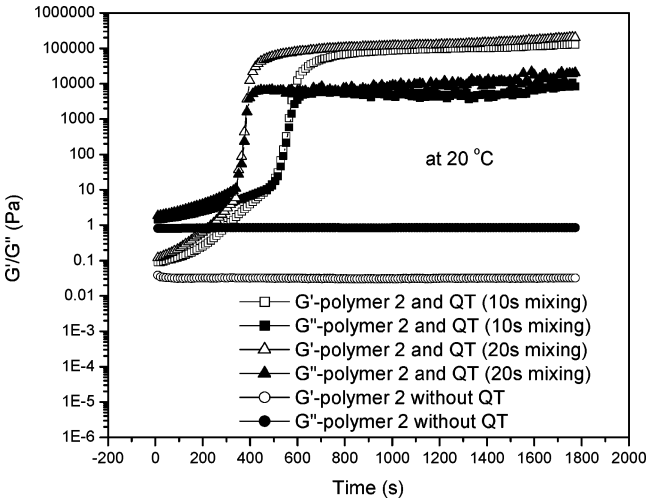


Figure 4. Gelation behavior of copolymer **2** with and without QT when they mixed at pH 7.4 at room temperature. The system with QT gelled at about 350 and 500 s, depending on mixing time.

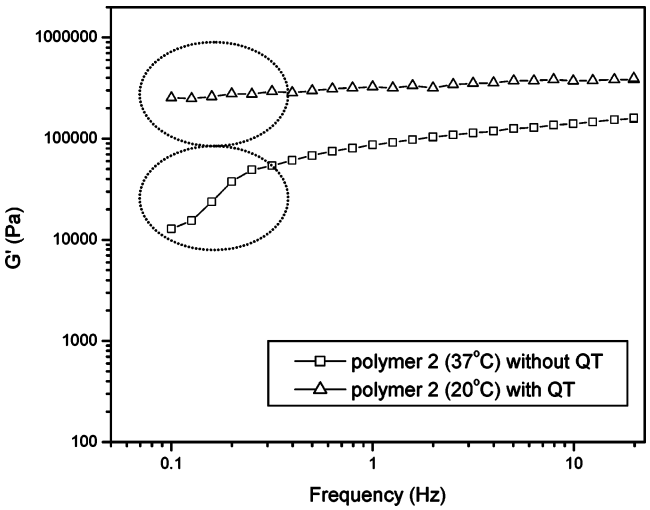


Figure 5. Frequency sweep of chemically cross-linked polymer gel with QT and only physically cross-linked polymer gel (at 37 °C).

injected into the body, they will form a gel immediately due to temperature-driven physical cross-linking. With time, these physically cross-linked gels will be cured by chemical cross-linking through Michael-type addition reactions between the thiols and acrylates.[5,9] As shown in Figure 3, 1.0 g of polymer and 4.9 μL of QT were combined in a 3-cm^3 syringe and mixed by hand in two 3-cm^3 plastic syringes with a syringe junction for 10 and 20 s and then transferred to the rheometer.

The time dependence of the dynamic moduli for copolymer **2** (20 wt % solution) with and without QT at 20 °C is presented in Figure 4. That copolymer **2** without QT remained a sol was evident because G'' was higher than G'. The mixture consisted of poly(NIPAAm-*co*-HEMA-acrylate) and QT with an acrylate and thiol functional molar ratio of 1:1. The polymer mixture formed a gel within 600 s owing to chemical cross-linking through Michael-type addition reactions without physical cross-linking, after 10- or 20-s mixing of acrylate and thiol precursors at 20 °C. Obviously, increase in mixing time caused an acceleration of the gelation of the polymer mixture; for the 10-s mixing time, the polymer mixture formed a gel at around 500 s; for the 20-s mixing time, the polymer mixture gelled at about 350 s. The increase in mixing time seems to contribute to a more homogeneous polymer mixture in order to increase collision between thiol groups and acrylate groups. In addition to the mixing time, the pH and buffer strength of the PBS buffer can both also be used to increase or decrease the chemical gelation kinetics. Our system formed a gel quickly in a neutral buffered solution (pH 7.4). More basic solutions are required to get comparable gelation kinetics in PEGDA (poly(ethylene glycol) diacrylate) or PPODA (poly(propylene glycol) diacrylate) polymers cross-linked with QT due to the lower water solubility of the precursors in these systems.[9]

Figure 5 shows the frequency dependence of the dynamic moduli for copolymer **2** with and without QT. Copolymer **2** alone formed a gel at 37 °C without chemical curing due to physical cross-linking and exhibited low-frequency strength loss due to viscoelasticity. On the other hand, copolymer **2** chemically gelled with QT, mixing for 30 s, and incubated for 1 h at room temperature, showed improvement of its low-frequency properties. Physical gels formed by secondary forces such as chain entanglement, hydrophobic interaction, ionic interaction, and van der Waals forces are easily injected but form weak viscoelastic solids. In comparison, chemical gels formed by covalent bonds result in stronger, less viscoelastic solids.[10] However, chemically cross-linked gels can be difficult to inject with hydrophobic polymers and can show high swelling and low mechanical properties in hydrophilic polymers. NIPAAm-based copolymers functionalized with olefins and thiols will provide a new class of in-situ-gelling materials having advantages over exclusively physical or chemical gels. These materials in aqueous solutions can be injected and will form a gel in situ due to temperature-driven physical cross-linking. With time, these physically cross-linked gels will be further cured by chemical cross-linking through Michael-type addition reactions between thiols and acrylates. These materials may be useful in endovascular embolization as in-situ-gelling materials allowing water-soluble injection but lower swelling/improved viscoelastic properties compared to traditional in-situ-gelling materials.

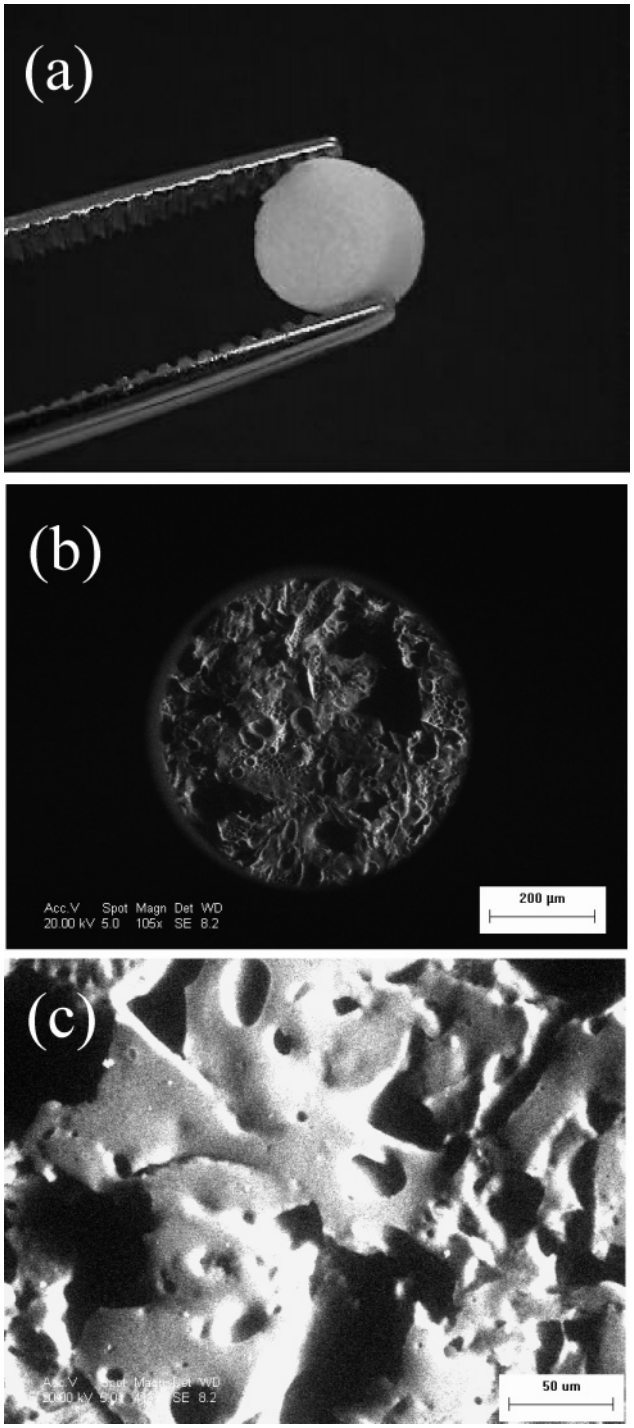


Figure 6. ESEM image of chemically cross-linked gel with QT (a) macroscopic observation, (b) 105×, and (c) 418×.

This chemically cross-linked polymer gel showed a porous structure with various pore sizes as seen in Figure 6. In our previous papers, PEGDA/QT showed a dispersion-type structure due to phase segregation of large organic-rich droplets during curing, while PPGDA/QT exhibited reverse emulsion-type structure showing the continuous, homogeneous organic phase with small aqueous phase domains interspersed. Our system is composed of aqueous solution (84 wt %), 16 wt % polymer, and a small amount of cross-linker (QT), so the morphogenesis of the normal oil-in-water emulsion-type gels can be described as follows: a small amount of QT was dispersed in water giving a low-viscosity solution, and then QT reacted with acrylates of the polymer through the Michael-type addition reaction while

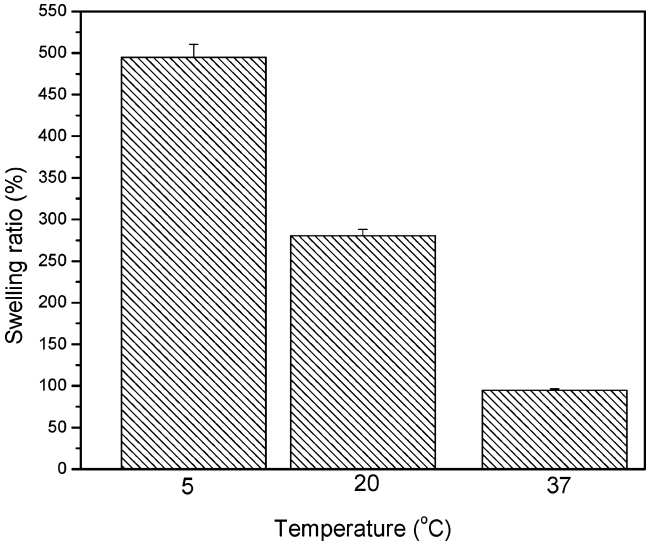


Figure 7. Swelling of chemically cross-linked polymer with QT gel at different temperatures.

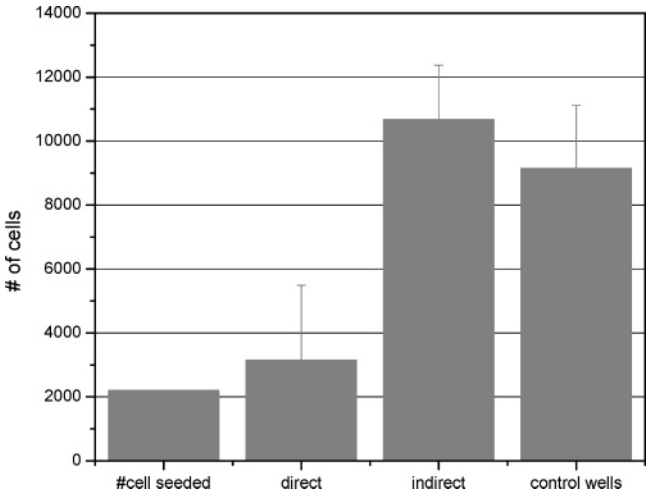


Figure 8. Modified MTT results for direct and indirect contact assays using copolymer **2** cross-linked with QT.

the polymer formed a continuous gel.[9] Upon reaction of the QT with the NIPAAm/HEMA acrylate, the resultant cross-linked polymer is completely swellable below the LCST. Above the LCST, the cross-linked polymer shows a more hydrophobic nature, reducing swelling and protecting the material from hydrolysis.

Figure 7 shows the swelling test of the chemically cross-linked gel. When copolymer **2** was cured chemically with QT, this gel swelled depending on temperature in 0.1 N PBS of pH 7.4. As the temperature of the solution increased, the swelling extent of the polymer gel decreased due to the LCST. At 5 °C the gel swelled up to 500%, while at 20 and 37 °C the polymer gel showed around 275 and 100% swelling, respectively. This result is similar to that of other temperature-sensitive gels.[5]

The copolymer **2** with QT was tested for cytotoxicity in direct contact assays and indirect contact assays. Figure 8 shows MTT results for the direct and indirect contact assays. The numbers of cells in the media, treated by incubation with the material, were statistically similar to the control numbers. In separate experiments, cells in direct contact showed that the cells were unable to attach to the material, but that the material was not cytotoxic. The cells were not able to attach and proliferate, but all the cells originally introduced in the samples were observed viable after the 36-h period. In the indirect cytotoxicity studies,

cells indirectly exposed to copolymer **2** cross-linked with QT showed statistically similar viability to control cells. Further evidence of the biocompatibility of the in-situ Michael-type addition reaction has been demonstrated in various studies.[5c,9b,c]

Conclusion

N-Isopropylacrylamide (NIPAAm) was synthesized with HEMA to form poly(NIPAAm-*co*-HEMA) with −OH functional groups. This copolymer was allowed to react with acryloyl chloride to obtain poly(NIPAAm-HEMA-acrylate). Poly(NIPAAm-*co*-HEMA-acrylate) was dissolved in 0.1 N PBS of pH 7.4 and reacted with QT to form simultaneously physically and chemically cross-linkable injectable gels. The gel time depended on mixing time. The formed gel reduced the creep in the material compared to physically formed gels without QT. This gel showed good cell viability during direct and indirect contact cytotoxicity tests. In-vivo animal study for endovascular embolization will be conducted in the future.

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