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Temperature responsive hydrogels enable transient three-dimensional tumor cultures *via* rapid cell recovery

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Abstract: Recovery of live cells from three-dimensional (3D) culture would improve analysis of cell behaviors in tissue engineered microenvironments. In this work, we developed a temperature responsive hydrogel to enable transient 3D culture of human glioblastoma (GBM) cells. *N*-isopropylacrylamide was copolymerized with hydrophilic grafts and functionalized with the cell adhesion peptide RGD to yield the novel copolymer poly(*N*-isopropylacrylamide-*co*-Jeffamine® M-1000 acrylamide-*co*-hydroxyethylmethacrylate-RGD), or PNJ-RGD. This copolymer reversibly gels in aqueous solutions when heated under normal cell culture conditions (37°C). Moreover, these gels redissolve within 70 s when cooled to room temperature without the addition of any agents to degrade the synthetic scaffold, thereby enabling rapid recollection of viable cells after 3D culture. We tested the efficiency of cell recovery following extended 3D culture and were able to recover more than 50% of viable GBM cells after up to 7 days in culture. These data demonstrate the utility of physically crosslinked PNJ-RGD hydrogels as a platform for culture and recollection of cells in 3D. © 2015 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 104A: 17–25, 2016.

Key Words: NIPAAm, thermoreversible scaffold, RGD, cell recollection, glioblastoma

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INTRODUCTION

In vitro cell culture is a key component of biomedical research. Standard cell culture methods utilize plastic surfaces that facilitate cell adhesion and growth across a single, two-dimensional (2D) plane. However, 2D conditions do not fully recapitulate the mechanical and biophysical features of the three-dimensional (3D) microenvironment to which cells are exposed *in vivo*.[1–3] This is of particular concern in cancer research, where interactions between neoplastic cells and the 3D tumor microenvironment drive tumor expansion and malignancy.[4–6] As a result, 3D biomaterial scaffolds and extracellular matrix (ECM) mimics [for example, Matrigel®,[7] collagen,[6] hyaluronic acid,[8] and polyethylene glycol (PEG)[9]] have become popular tools for developing more relevant cancer cell culture methods. However, one key limitation of many biomaterials commonly used in these applications is that recovery of viable cells for postculture analysis can be challenging. These insoluble hydrogel networks may require prohibitive environmental alterations (pH, temperature), the addition of harsh chemical or enzymatic agents, and/or long time periods to dissolve.

The goal of this work was to develop a physically crosslinked scaffold that would enable transient 3D cell culture *via* rapid recollection of cells under mild conditions. Poly(*N*-isopropylacrylamide) (PNIPAAm) exhibits a lower critical solution temperature (LCST) near 30°C in aqueous solution, which enables reversible physical crosslinking where the polymer is soluble at room temperature and gels at 37°C.[10] In the gelled state, PNIPAAm gels undergo notable phase separation and shrinking due to hydrophobic interactions between polymer chains. The equilibrium polymer concentration in a PNIPAAm hydrogel is ~50 wt % for gels initially formed at 5–20 wt %.[10,11] This low equilibrium water content prevents fast dissolution when gels are cooled below the LCST and would also inhibit any recovery of live cells cultured therein. We have reported that the phase separation of PNIPAAm is decreased by the incorporation of Jeffamine® M-1000 acrylamide (JAAm) as a graft comonomer,[11,12] which opens the possibility of developing a fast-dissolving PNIPAAm copolymer scaffold for cell culture.

Other groups have demonstrated the ability to functionalize PNIPAAm with cell adhesion peptides such as RGD for

Additional Supporting Information may be found in the online version of this article.
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the purpose of facilitating 3D cell culture.[13–18] PNIPAAm has also been used to facilitate the formation and subsequent detachment of cellular monolayers and sheets primarily for the purpose of tissue engineering.[19–23] However, to our knowledge, none have developed a fast-dissolving PNIPAAm-RGD hydrogel and demonstrated recovery of live cells grown in a 3D scaffold. To this end, we describe the development of the novel temperature responsive graft copolymer poly(*N*-isopropylacrylamide-*co*-Jeffamine® M-1000 acrylamide-*co*-hydroxyethylmethacrylate-RGD), or PNJ-RGD, that we characterized in transient 3D cell culture. This platform was applied to studying *in vitro* cultures of human glioblastoma (GBM) brain tumor cells. GBM is the most malignant and aggressive primary brain tumor and is associated with poor median survival (12 − 15 months) and substantial morbidities.[24] Previously, we reported that PNIPAAm-hydroxyethylmethacrylate copolymers (PNIPAAm-*co*-HEMA) can be made thiol-reactive following postpolymerization acrylation of hydroxyls.[25] We utilized this approach along with methods reported by Shu et al.[26] to graft a cysteine containing RGD peptide to acrylated poly(NIPAAm-*co*-JAAm-*co*-HEMA) *via* Michael addition. The resulting PNJ-RGD copolymer formed a physically crosslinked hydrogel that was rapidly reversible when cooled below the LCST. These hydrogels supported physiological behaviors and recollection of viable GBM cells maintained in an adherent 3D culture.

MATERIALS AND METHODS

Materials

All chemicals were reagent grade and purchased from Sigma-Aldrich unless otherwise stated. *N*-isopropylacrylamide (NIPAAm; Tokyo Chemical Industry, Portland, OR) was recrystallized from hexane, and 2,2′-Azobisisobutyronitrile (AIBN) was recrystallized from methanol. Tetrahydrofuran (THF) used in polymer synthesis and gel permeation chromatography was HPLC grade. Jeffamine® M-1000 was generously donated by Huntsman Corporation (Salt Lake City, UT). The CGRGDS peptide (63.5% purity, 954 Da MW) was purchased from American Peptide Company (Sunnyvale, CA). All cell culture reagents were purchased from Invitrogen (Carlsbad, CA) or Fisher Scientific (Anthem, AZ) unless otherwise stated.

Synthesis of temperature responsive PNIPAAm copolymers

Jeffamine® M-1000 acrylamide (JAAm) was synthesized from Jeffamine® M-1000 and acryloyl chloride as described previously.[12] PNIPAAm copolymers were synthesized by free radical polymerization of NIPAAm, JAAm, and hydroxyethylmethacrylate (HEMA) monomers to yield poly(NIPAAm-*co*-JAAm-*co*-HEMA), or PNJH. NIPAAm (9 g), JAAm (1 g), and HEMA (200 mg) were dissolved in 100 mL of anhydrous benzene in a dried flask and heated to 65°C. After bubbling with nitrogen for 20 min, AIBN (95 mg) was added to initiate polymerization, and the reaction proceeded under positive nitrogen pressure for 18 h. PNJH polymer precipitated during the reaction and was redissolved in an equal volume of acetone, precipitated in cold diethyl ether, collected by filtration, and vacuum dried overnight. For the purpose of evaluating the effect of JAAm content on polymer properties, poly(NIPAAm-*co*-JAAm) copolymers were synthesized through the same method with a 100 : 0, 95 : 5, or 90 : 10 ratio of NIPAAm : JAAm by weight in the feed.[11] These copolymers are abbreviated as PNIPAAm, PNJ5, and PNJ10, respectively.

Poly(NIPAAm-*co*-JAAm-*co*-HEMA-acrylate), or PNJHAc, was synthesized by reacting PNJH with acryloyl chloride to convert hydroxyl groups to pendent acrylates.[25] The polymer was dried by heating to 60°C overnight under vacuum and dissolved at 15 wt % in THF with 5 molar equivalents of triethylamine relative to HEMA repeat units. Acryloyl chloride predissolved in THF was added dropwise to the stirring solution on ice and under nitrogen to achieve a final polymer concentration of 10 wt %. After 6 h, TEA-HCl salts were removed by filtration and the copolymer was precipitated in ether, filtered, and vacuum dried. The product was purified by dialysis against deionized water at 4°C with a 3,500 MWCO membrane for 3 days. Polymer was lyophilized for storage at −20°C.

Conjugation of integrin adhesion peptide

Poly(NIPAAm-*co*-JAAm-*co*-HEMA-RGD), or PNJ-RGD, was formed by Michael addition of an RGD peptide containing a terminal cysteine residue onto the acrylates of the polymer. PNJHAc was dissolved at 3 wt % in chilled PBS and Cys-Gly-Arg-Gly-Asp-Ser (CGRGDS) TFA salt was added in a threefold molar excess of the available acrylate groups. The solution was titrated to pH 8 and stirred for 24 − 48 h at 4°C. The polymer was then purified by dialysis (3,500 Da MWCO) against deionized water at 4°C for 1 week and lyophilized to obtain PNJ-RGD. Dry polymers were sterilized by ethylene oxide gas.

^{1}H NMR characterization

^{1}H NMR spectroscopy (Varian Inova, 400 MHz) was used to determine the chemical composition of all polymers following each synthesis. All polymer samples were prepared at a concentration of 10 mg/mL in D_2O. NIPAAm repeat units were calculated by integration of the peak associated with the lone isopropyl proton [$\delta = 3.84$, 1H, $(CH_3)_2$-C*H*-NHCO] in reference to peaks associated with oxyethylene protons in JAAm [$\delta = 3.63$, 76H, CH_2CH_2O], methylene protons in HEMA [$\delta = 4.09$, 4H, HO—C*H2CH2*—OCO], and methylene protons in HEMA-acrylate [$\delta = 4.09$, $\delta = 4.38$, 4H, CH_2=CH—COO—CH_2CH_2—OCO].[11,25] Peaks corresponding to the terminal olefin protons in HEMA-acrylate, [$\delta = 5.97$, $\delta = 6.18$, $\delta = 6.41$, 3H, OCO—C*H*=CH_2], were integrated in respect to the other monomer peaks to determine the degree of acrylation to be ∼88% of the HEMA groups (^{1}H NMR spectrum provided in Supporting Information Fig. 1). Addition of the CGRGDS peptide to the PNJHAc polymer by Michael addition was confirmed by characteristic shifts from the methylene protons in aspartic acid [$\delta = 2.58$, 2H, OHCO—CH_2—CH], methylene protons in serine [$\delta = 3.86$, 2H, OH—CH_2—CH], and methylene protons in arginine [$\delta = 3.09$,

2H, $NH_2CNHNH—CH_2—CH_2$].[27] Peptide concentration was calculated to be ~140 μmoles of RGD per gram of PNJ-RGD copolymer (^{1}H NMR spectra provided in Supporting Information Fig. 2).

Molecular weight and polydispersity determination

The molecular weight and polydispersity of the PNIPAAm copolymers were determined by gel permeation chromatography (GPC) using refractive index detection (Shimadzu) in conjunction with static light scattering (MiniDawn, Wyatt Tech. Corp.). Samples were run through two columns in series (Waters Styragel HR 4 and 6) to separate polymer chains by size exclusion. Measurements were made using HPLC grade THF as the mobile phase running at a flow rate of 1 mL/min.

LCST determination

The LCST was evaluated by cloud point measurement and rheometry. For cloud point assays, PNIPAAm copolymers ($n = 3$) were dissolved at 0.1 wt % in PBS at pH 7.4 in cuvettes and heated in a water bath from 20 to 40°C in 1°C increments and 40 to 55°C in 5°C increments. Samples remained at each temperature for at least 120 s prior to each measurement. Absorbance readings in the visible range (450 nm) were recorded with a UV/Vis spectrometer (Pharmacia Biotech Ultrospec 3000). The maximum absorbance was defined as the highest absorbance prior to polymer precipitation (determined by first measurement at which absorbance decreased). The LCST was defined as the temperature at which 50% of the relative maximum absorbance was recorded during the assay. For rheometry, PNIPAAm copolymer solutions were prepared at 16.7 wt % in 150 m*M* PBS (pH 7.4) and placed on a parallel plate rheometer (MCR-101, Anton-Paar) that provided real-time temperature control over the measurement stage. In all tests, an oscillatory 0.5% shear strain deformation was applied at 1 Hz frequency without normal force control to measure the sample storage (G') and loss modulus (G''). Polymer solutions were allowed to equilibrate to 4°C on the rheometer and were then measured during controlled heating (0.5°C/min) to 37°C to induce gelation.

Viscoelastic characterization

Rheometry was performed to measure the viscoelastic properties of the PNJ-RGD copolymer over a range of concentrations during heating and cooling. Solutions were prepared by dissolving PNJ-RGD at 4.2, 8.3, or 16.7 wt % in PBS. Concentrations were selected starting at the maximum concentration that permitted solution flow and decreasing by a factor of 2 until the solution did not gel. The storage and loss modulus in the solution and gel states were assessed by a multistep temperature controlled protocol. In all tests, an oscillatory 0.5% shear strain deformation was applied at 1 Hz frequency, and normal force control was used at temperatures above the LCST to maintain consistent contact between the gel and rotating head. Polymer solutions were allowed to equilibrate to 4°C on the rheometer and were measured during controlled (0.5°C/min) and sustained heating (37°C for 1 h) followed by rapid and sustained cooling (4°C for 1 h).

Cell and tumor spheroid culture

The human GBM cell line U118 and U118 cells expressing green fluorescent protein (U118-GFP) were generously gifted by colleagues at the Translational Genomics Institute (TGen, Phoenix, AZ). Cells were cultured in antibiotic free DMEM + 10% FBS. Multicellular GBM spheroids (5,000 cells/spheroid) were formed in 4 days by a hanging drop culture.[28]

Cytocompatibility of PNJ-RGD hydrogels

PNJ-RGD was dissolved in culture media at 8.3 wt % with U118 cells dispersed uniformly throughout the mixture (400,000 cells per mL polymer solution). Samples were plated in ~70 μL aliquots ($n = 6$ per time point) in a 96-well plate. After incubating overnight at 37°C, an equal volume of cell culture media was added to each sample and exchanged for fresh media every 2–3 days. Cells were stained with fluorescent calcein AM (2 μ*M*; 488 nm excitation/535 nm emission) and ethidium homodimer-1 (4 μ*M*; 528 nm excitation/617 nm emission) using a Live/Dead assay kit (Invitrogen) at 1, 3, and 14 days. Live and dead cells were quantified through fluorescence detection *via* fluorescence confocal microscopy. 3D stacks of images were collected through 300 μm with a step size of 10 μm. Cells within the images were quantified using an automated cell counting algorithm described in our previous work.[29]

Three dimensional (3D) cell culture

PNJ10 and PNJ-RGD were dissolved at 16.7 wt % in cell culture media overnight at 4°C. The elastic strength of gels at this concentration is similar to hydrogels that we have previously observed to promote proliferation and invasion of this cell line.[29] The polymer solution was transferred in approximately 70 μL aliquots ($n = 6$) to a 96-well tissue culture plate and incubated at 37°C to form hydrogels. A single multicellular U118-GFP spheroid was seeded on top of the gel in each well and incubated overnight to allow for attachment. An equal volume of cell culture media was added to each well and exchanged with fresh media every 2–3 days. Cellular behavior was analyzed using 3D fluorescence confocal microscopy (Leica SPE II) of live cells. Prior to imaging, samples were cooled to room temperature (making the gels transparent), and images were captured through a depth of 500 μm with a step size of 10 μm. Each individual spheroid was tracked separately to allow for analysis of behaviors observed over the culture period.

Recollection of 3D cultured cells

PNJ-RGD samples were prepared as for the Live/Dead assay and seeded uniformly with U118-GFP cells at low (86,000 cells/mL), medium (186,000 cells/mL), or high (357,000 cells/mL) densities. The experiment was replicated in three separate assays. Samples were transferred in 70 μL aliquots ($n = 6$ per time point) into a 96-well tissue culture plate and incubated at 37°C. Cells were recollected from the

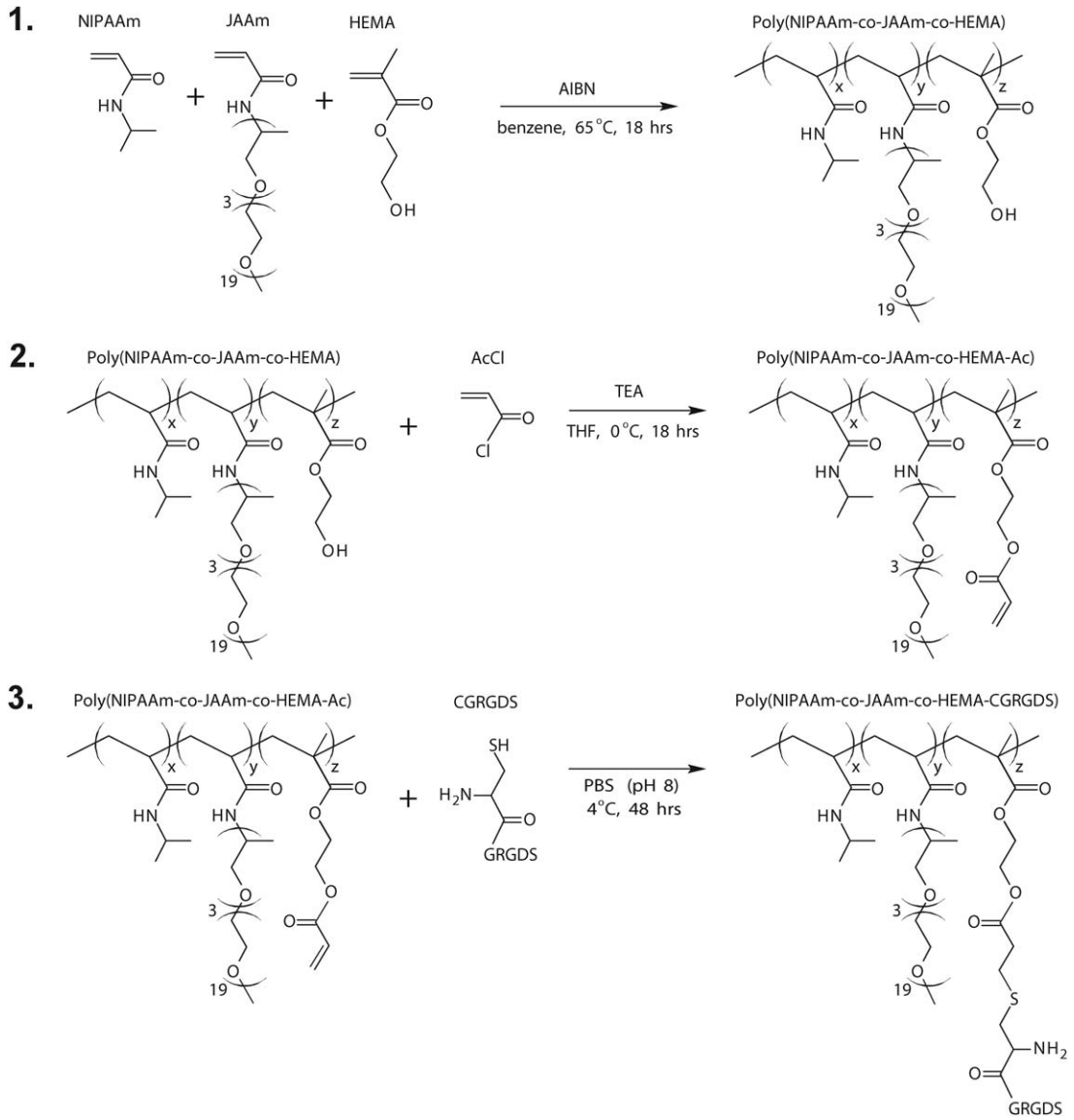

FIGURE 1. Three-step synthesis of PNJ-RGD. (1) PNJH is synthesized through free radical polymerization. (2) Acrylation of the HEMA side chain to form PNJHAc. (3) Conjugation of the CGRGDS peptide through Michael addition of the thiol-containing cysteine residue to form PNJ-RGD.

hydrogels after 1, 2, 3, and 7 days of 3D culture. Only cells seeded at the medium density were collected at 7 days. To recollect cells, the plate was first cooled to room temperature to liquefy the hydrogels and allow removal from the well plate. The samples were diluted in 2 mL of media, mixed with a micropipette, and centrifuged at 1,100 RPM for 5 min at 4°C. The pellet was resuspended in 2 mL of 0.25% trypsin-EDTA. This solution was cycled between the gel (37°C) and soluble (4°C) states for 5 min to improve activity and penetration of the enzyme into the copolymer network. The solution was diluted in media and passed through a 100-μm cell strainer (Fisher Scientific). Cell viability was quantified in the liquid media fraction for the recollected cells and in the gel fraction for the gel encapsulated cells using CellTiter-Glo (Promega) with luminescence measured in a microplate reader (Tecan).

RESULTS

Polymer synthesis and characterization

PNIPAAm copolymers with varying amounts of JAAm, HEMA, and RGD were synthesized as outlined in Figure 1. Characterization data is summarized in Table I and described in detail below. The incorporation of the constituent monomers and the RGD peptide was confirmed by ^{1}H NMR analysis (spectra provided in Supporting Information Figs. 1 and 2). Quantification of peaks corresponding to the amino acid sequence indicated that PNJ-RGD was functionalized with 140 μmoles RGD per gram of polymer. Molecular weight determination indicated that all copolymers exhibited M_W >500,000 Da. The molecular weight of PNJ-RGD was not measured by GPC due to poor solubility in THF.

From cloud point data [Fig. 2(A)], PNIPAAm homopolymer displayed the lowest LCST (26.5°C), and the addition of

TABLE I. Characterization of PNIPAAm Copolymers by NMR, GPC, Cloudpoint, and Rheology

Polymer	Monomer Ratios NIPAAm: JAAm: HEMA (wt %)		Peptide Content (μmol/g polymer) CGRGDS	Molecular Weight Determination		LCST Determination (°C)	
	Feed	Composition		M_W (10^6 Da)	Dispersity (M_W/M_n)	Cloudpoint	Rheology
PNIPAAm	100 : 0 : 0	100 : 0 : 0	–	1.127	1.382	26.5	22.6
PNJ5	95 : 5 : 0	97.3 : 2.7 : 0	–	1.257	1.223	27.0	23.6
PNJ10	90 : 10 : 0	92.1 : 7.9 : 0	–	1.303	1.351	28.5	28.1
PNJHAc	88.2 : 9.8 : 2.0	90.7 : 8.2 : 1.1	–	0.5488	1.411	27.5	26.6
PNJ-RGD	88.2 : 9.8 : 2.0	90.7 : 8.2 : 1.1	140	0.5944		31.3	29.1

JAAm increased the LCST in PNJ5 (27°C) and PNJ10 (28.5°C) proportional to the incorporation of the hydrophilic monomer. Conversely, the addition of hydrophobic pendant acrylate groups in PNJHAc resulted in a slight decrease in LCST (27.5°C). PNJ-RGD exhibited the highest LCST (31.3°C), which is attributed to increased hydrophilicity of the copolymer imparted by the RGD peptide. The phase transition was also studied by measuring the shear storage (G') and loss moduli (G'') of PNIPAAm copolymer gels at high concentration (16.7 wt %) during temperature ramp [Fig. 2(B)]. The LCST was determined to be the temperature at which the storage and loss moduli exponentially increase and invert, that is, $G' > G''$. This inversion was observed as expected in the PNJ5, PNJ10, and PNJ-RGD formulations. However, G' remained greater than G'' for PNIPAAm homopolymer and PNJHAc even below the LCST, indicating that these solutions behaved like viscous gels at all temperatures. Therefore, the LCST was defined for these samples as the temperature of at least two-fold increase in the shear storage modulus. The copolymers showed the same trend in LCST transition as measured by cloud point, with PNIPAAm having the lowest LCST (22.6°C) and LCST increasing with JAAm content for PNJ5 (23.6°C) and PNJ10 (28.1°C). PNJHAc again had a lower LCST (26.6°C) than PNJ10, and PNJ-RGD displayed the highest transition point (29.1°C).

Dissolution kinetics were evaluated by rheology while rapidly cooling hydrogels below the LCST (samples were maintained at 37°C for 1 h and then rapidly brought to 4°C) [Fig. 3(A)]. In general, copolymers with greater hydrophilic content redissolved more quickly upon cooling. However, PNIPAAm, PNJ5, PNJHAc, and PNJ10 showed poor dissolution, maintaining viscoelastic solid behavior ($G' > G''$) and exhibiting shear storage moduli that exceeded 900 Pa for 30 min after cooling. This indicates that water was unable to effectively penetrate the gel structure and solvate the polymer. PNIPAAm gels did not return to solution when cooled and, interestingly, exhibited signs of stiffening, as evidenced by a brief increase in storage modulus [Fig. 3(A)]. Conversely, PNJ-RGD showed much improved dissolution kinetics, returning to a predominantly liquid state ($G'' > G'$) within 70 s of cooling below the LCST. Collectively, these data demonstrate that increasing the hydrophilic content of NIPAAm copolymers enables complete phase reversal and rapid dissolution upon cooling below the LCST.

To further evaluate the mechanical changes induced during the phase transition, PNJ-RGD gels were subjected to multistage rheological measurements at various concentrations with controlled heating and cooling cycles [Fig. 3(B)]. Data were collected for 4.1, 8.3, and 16.7 wt % samples heated from 4°C to 37°C (0.5°C/min) to estimate LCST and gel strength. Samples were then maintained at 37°C to allow

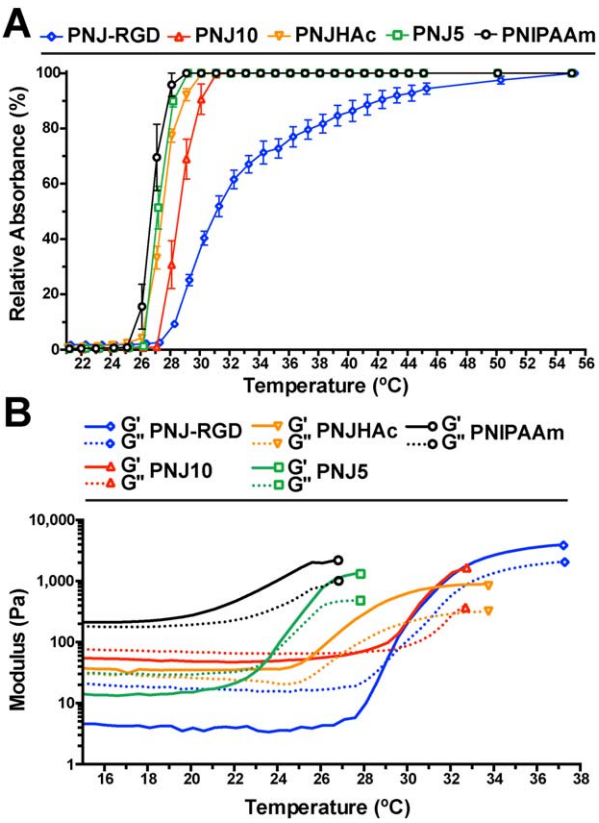

FIGURE 2. LCST determination for PNIPAAm copolymers by (A) cloudpoint and (B) rheology. A: Cloudpoint measurements are reported as the absorbance at 450 nm relative to the maximal absorbance for 0.1 wt % polymer solutions at increasing temperatures. The LCST is defined as 50% relative absorbance. Error bars represent standard deviation. B: Rheology measurements of the storage (G') and loss modulus (G'') were collected for 16.7 wt % polymer solutions under controlled heating (0.5°C/min). The LCST is defined at the point of G' and G'' inversion or exponential increase.

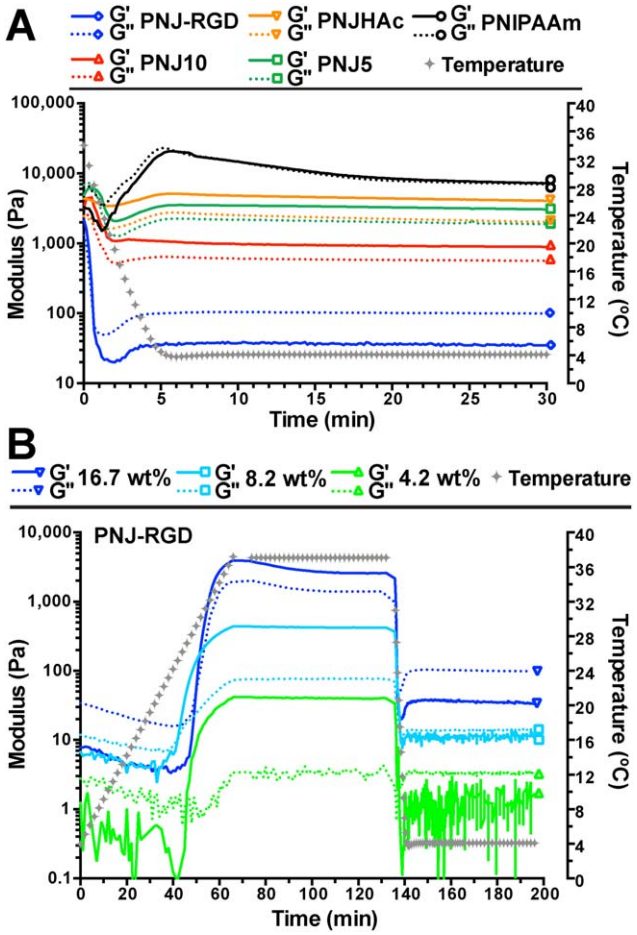

FIGURE 3. Viscoelastic characterization of PNIPAAm copolymers by temperature controlled rheology. A: Storage (G') and loss modulus (G'') measurements were made on 16.7 wt % polymer samples heated to 37°C for 1 h and rapidly cooled to 4°C. The degree of hydrogel dissolution is measured by the decay in G' and G''. B: Mechanical properties of PNJ-RGD hydrogels at three concentrations during controlled heating and rapid cooling. Hydrogel viscoelasticity is dependent on polymer concentration, but all samples displayed reversible gelation kinetics at the LCST. Temperature is displayed on the right axis.

the gel to equilibrate, and then cooled rapidly to 4°C to estimate dissolution time. The LCST was consistent between the samples and viscoelastic strength, measured by the storage and loss modulus, increased with concentration. At 37°C, the hydrogels formed viscoelastic solids with storage moduli (G') ranging from 40 Pa (4.1 wt %) to 3 kPa (16.7 wt %). Most importantly however, all PNJ-RGD gels were capable of rapid ($\sim$70 s) phase reversal to a soluble state when cooled below the LCST following prolonged (1 h) gelation. Thus, unlike PNIPAAm homopolymer, PNJ-RGD exhibits tunable mechanical properties through a rapidly reversible phase transition. These data motivated us to further evaluate PNJ-RGD as a platform for recollection of live cells cultured in 3D.

3D scaffolds for transient cell culture

The primary goal of these experiments was to evaluate if PNJ-RGD hydrogels could promote cell viability and physiological behavior while also enabling the recovery of viable cells from 3D culture. To examine material cytocompatibility, U118 GBM cells were dispersed uniformly in PNJ-RGD hydrogels. A fluorescent live/dead assay was performed within the gel to analyze cell viability through 3D space with fluorescence confocal microscopy. 3D stacks of live/dead confocal images were collected after 1, 3, and 14 days of culture [Fig. 4(A)]. These images were analyzed by a previously developed automated image processing method to identify and count the number of live and dead cells. [29] Over 14 days in culture, the hydrogels were found to consistently support a viable fraction of cells that was greater than 85% of the total number of cells counted [Fig. 5(A)]. Elongated cell morphologies were observed in PNJ-RGD, confirming that pendent RGD sites on the polymer allowed for cell attachment [Fig. 4(A)].

Proliferative and invasive behaviors of GFP-expressing GBM cells within PNJ10 and PNJ-RGD hydrogels were assayed by 3D fluorescence confocal microscopy. Multicellular spheroids tracked during culture showed markedly different behaviors on gels with RGD versus gels without RGD [Fig. 4(B)]. Cultures on PNJ-RGD showed increased cellular invasion, altered morphologies, and increasing spheroid size at successive time points. In contrast, spheroids in the PNJ10 hydrogels exhibited minimal changes in size and morphology and also did not produce invasive cells over the times measured here.

Recollection of live cells from PNJ-RGD cultures was enabled by the rapid gel dissolution after cooling. In preliminary experiments (results not shown), we attempted to remove live cells from hydrogels by simply cooling gels below the LCST with no further treatment. However, we observed that cells cultured for any extended period of time (>18 h) secreted extracellular matrix proteins that prevented their complete release from the gel. To improve cellular detachment from the matrix, the hydrogels were treated with trypsin. Recovered cells and those that remained attached to the gel were quantified with a luminescence viability assay to determine the recollection efficiency [Fig. 5(B)]. Using this method, live cells were successfully recovered from the hydrogels after $1-7$ days in culture. Recollected cells maintained normal proliferation and morphology when reintroduced to standard 2D culture (results not shown). Cells cultured for 1 day in PNJ-RGD were recollected with the highest efficiency, with $\sim$79% of the live cells composing the recollected fraction. As culture times increased, the combined collection efficiency declined but remained above 50% after up to 7 days in culture. High initial cell seeding densities were also observed to have a slight negative effect on recollection efficiency compared with the lower initial cell numbers (Supporting Information Fig. 3).

DISCUSSION

Standard 2D tissue culture plastic provides reproducible, simplistic conditions for growing cells *in vitro*. However, 2D cultures limit important biological behaviors such as matrix remodeling and invasion, which can be observed in even

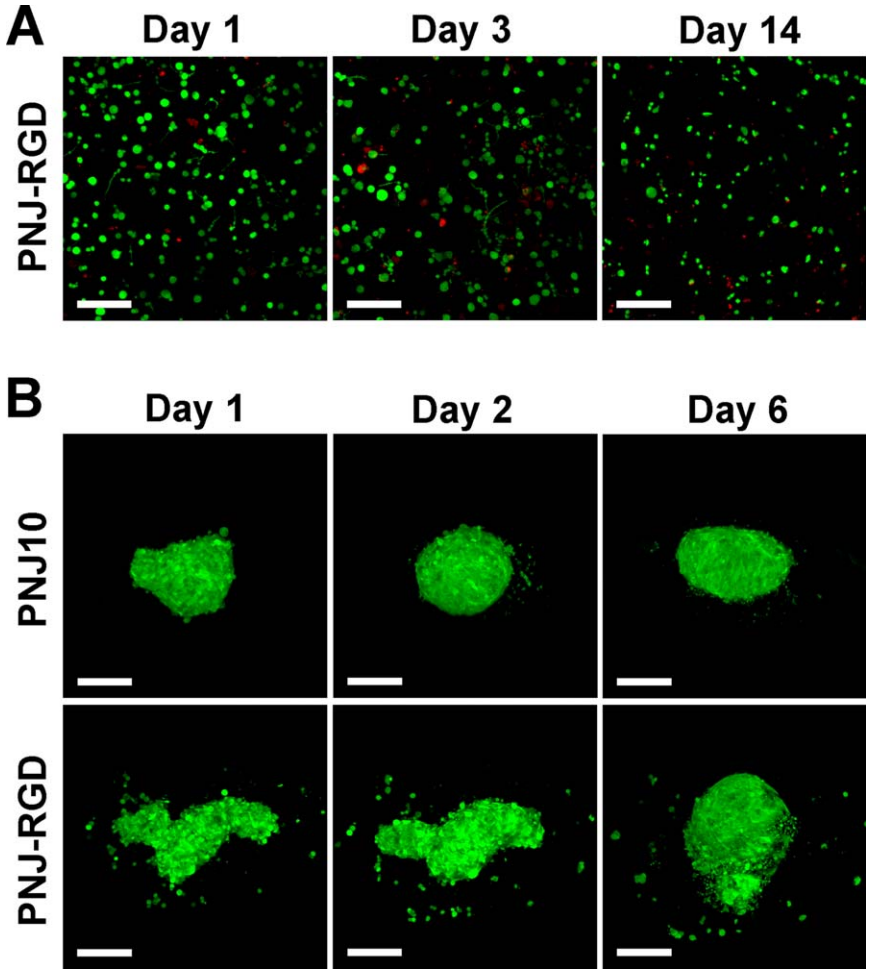

FIGURE 4. Cell viability and behavior assays. A: Live/Dead assay results showing that U118 cells maintain a high viable fraction (green to red) over 14 days in culture. B: U118-GFP proliferation and invasion was imaged over 6 days in PNJ10 and PNJ-RGD hydrogels. The presence of the RGD peptide allowed for cells to both proliferate and invade into the matrix, while cells in PNJ10 did not appreciably exhibit these behaviors. Images of cells are 2D maximum intensity projections of 3D stacks of images (Scale bars = 200 μm).

the most basic 3D substrates such as Matrigel® and collagen.[30] Yet the majority of 3D cultures are designed as endpoint assays that do not allow for cells to be cultured transiently in the scaffold and then recollected. To this end, we aimed to develop a temperature responsive biomaterial that would enable recollection of live cells and improve investigation of GBM cancer cell biology in a controlled microenvironment. PNIPAAm hydrogels are both cytocompatible and temperature responsive, but their slow gel-sol transition and lack of cell adhesion sites limits their utility

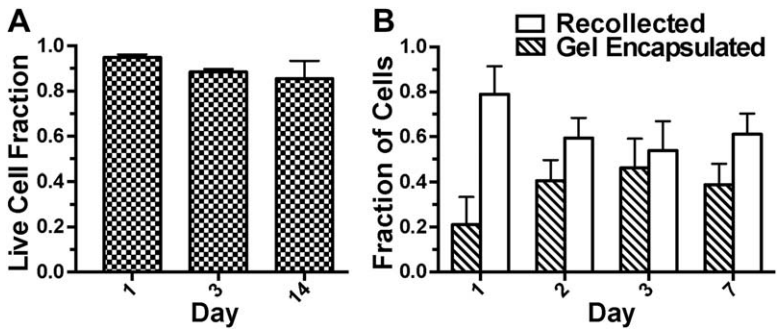

FIGURE 5. Quantification of results from 3D cell culture assays. A: Fraction of live cells compared to total cells counted *via* automated image processing. B: Quantification of cell recollection efficiency. Fraction of cells recollected out of PNJ-RGD hydrogels compared with the fraction remaining encapsulated in the gel after 1–7 days in culture. Error bars represent standard deviation.

in this application. To overcome these limitations, we copolymerized NIPAAm with hydrophilic JAAm at a relatively low molar fraction (<0.9 mol %) of the copolymer to increase the equilibrium water content of the hydrogels while maintaining the LCST within the physiological range.[31–34] HEMA was also incorporated as a comonomer to provide an active site for functionalizing the polymer with the cell adhesion peptide RGD. Our characterization and analysis of the resulting PNJ-RGD copolymer indicates that PNJ-RGD forms a robust temperature responsive scaffold that is well suited as a platform for transient 3D cell culture.

All of the synthesized polymers exhibited a favorable LCST for 3D culture in that polymers were soluble at room temperature and gelled at body temperature. However, the transition temperature measured by cloud point [Fig. 2(A)] was consistently higher than when measured by rheology [Fig. 2(B)] for each material. Cloud point measurements were taken at low concentrations (0.1 wt %) to observe the phase transition (that is, collapse of polymer chains) that produces precipitation. Rheology measurements were made at higher concentrations (16.7 wt %) in parallel to observe subtle changes in mechanical properties during physical crosslinking (that is, increase in gel strength) that do not require complete phase transition. Thus, these data highlight that increased strength precedes complete polymer precipitation, as expected. Ultimately, both measures demonstrate that hydrophobic HEMA-acrylate decreased the LCST and hydrophilic JAAm had a small increasing effect on LCST, which is consistent with our previous reports.[11,25] The inclusion of both monomers along with the hydrophilic RGD peptide caused a modest LCST increase over PNIPAAm homopolymer. Therefore, the measured LCST was consistently a function of the hydrophobic versus hydrophilic content of NIPAAm copolymers.

One of the primary obstacles with using PNIPAAm hydrogels for transient cell culture is the extended time required to solubilize the gel when cooled below the LCST. This is primarily caused by the phase separation of the precipitated polymer and can be mitigated by the addition of hydrophilic comonomers. This is supported by rheological measurements of hydrogel dissolution [Fig. 3(A)] which indicate that PNJ-RGD is capable of rapid and complete phase reversal following physical crosslinking. The remaining formulations displayed incomplete phase reversal that was proportional to hydrophilic monomer content. Moreover, measurements of the mechanical properties of PNJ-RGD [Fig. 3(B)] showed that the physical gelation was tunable over almost two orders of magnitude (40 Pa–3 kPa) by simply varying polymer−solution concentration.

Hydrogels formed with PNJ10 and PNJ-RGD copolymers displayed suitable properties for 3D cell culture, including LCST between room and body temperature, high equilibrium water content, and high gel-state viscoelasticity. We compared these two gels as scaffolds for transiently culturing a GBM cell line (U118) that spreads and proliferates rapidly in adherent conditions.[29,35] In 3D cultures, PNJ-RGD hydrogels facilitated U118 cell viability, attachment, and invasion (Fig. 4) which are all behaviors that are responsible for the overwhelming morbidity associated with GBM. More importantly however, PNJ-RGD enabled rapid recovery of live cells from 3D culture under mild conditions [Fig. 5(B)]. From 1 to 7 days in culture, a high fraction (>50%) of viable cells were extracted from the hydrogel scaffolds with the potential to be used in subsequent assays.

Many conventional biomaterials available for 3D cell culture (for example, Matrigel®, collagen, hyaluronic acid) may require harsh conditions, such as prolonged enzymatic digestion, to dissolve the substrate. One of the primary goals of this work was to engineer a synthetic cell culture scaffold that presented a bioactive ligand and could be dissolved without the use of enzymes; that is, by a mild drop in temperature. The developed PNJ-RGD material is thermally reversible and liquefies completely in response to reduced temperature (Fig. 3). However, in cell culture we observed that dissolution of the synthetic hydrogel did not enable complete release of cells from PNJ-RGD scaffolds. Additionally, longer cultures and increased cell densities correlated negatively with cell recollection efficiency (Supporting Information Fig. 3). We hypothesized that this incomplete recollection was due to cellular attachment to RGD binding sites and protein deposition. This hypothesis was supported by the observation that brief (5 min) treatment of PNJ-RGD gels with trypsin enabled release of the cells albeit incomplete during extended cultures. Importantly, this enzymatic treatment does not affect the synthetic hydrogel, and was used only to release cells from RGD binding sites and to degrade proteins deposited by cells within the gel.

To our knowledge, this is the first report of a 3D PNIPAAm-RGD-based platform that enables transient 3D culture through rapid recovery of live cells under mild conditions. This material offers several distinct advantages as a cell culture biomaterial. First, the 3D format allows for observation and measurement of interactions between cells and their surrounding microenvironment, an approach that is becoming increasingly valuable in the fields of cancer biology and regenerative medicine. Second, by engineering the scaffold to enable easy recovery of live cells, this platform could be used to study a variety of relevant cell behaviors and to perform analysis of transient biomarkers [for example, measurement of protein expression in live cells *via* fluorescence activated cell sorting (FACS)] that could not be studied using other scaffolds. Finally, the thiol-reactivity of the PNJHAc copolymer intermediate allows for other thiol-containing molecules of interest to be interchangeably immobilized to the scaffold. In summary, this work shows that PNJ-RGD is a suitable material for maintaining and passaging malignant cells in 3D culture, and it presents an opportunity for studying long-term, progressive responses of cells to their microenvironment in future studies.

CONCLUSIONS

Temperature responsive PNIPAAm copolymers were successfully synthesized with and without RGD for cell adhesion as a platform for transient 3D culture. PNJ-RGD

solutions formed mechanically tunable viscoelastic gels when heated above 29°C and displayed rapid phase reversal when cooled. In cell culture assays, the hydrophilicity of the hydrogel structure and presence of the RGD peptide promoted viability of an adherent cancer cell line over 14 days in culture. Additionally, invasive phenotypes were observed in 3D cultures, indicating that PNJ-RGD gels were capable of elucidating physiological phenotypes important to cancer research. Finally, recollection of live cells was achieved from the hydrogels from 1 to 7 days in culture. In all, these findings indicate that this hydrogel is an effective *in vitro* biomaterial platform for maintaining and affecting the biology of adherent tumor cells in a transient 3D culture.

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