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Simple Radical Polymerization of Poly(Alginate-Graft-N-Isopropylacrylamide) Injectable Thermoresponsive Hydrogel with the Potential for Localized and Sustained Delivery of Stem Cells and Bioactive Molecules

Free radical synthesis of injectable, bio-compatible, and biodegradable thermoresponsive hydrogel that offers tailored viscoelastic properties is investigated. Hydrogel provided enables cell encapsulation without affecting differentiation capability. Technology offers a versatile and improved platform for drug delivery and bone tissue engineering applications.

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Simple Radical Polymerization of Poly(Alginate-Graft-N-Isopropylacrylamide) Injectable Thermoresponsive Hydrogel with the Potential for Localized and Sustained Delivery of Stem Cells and Bioactive Molecules

Sreekanth Pentlavalli, Philip Chambers, Binulal N. Sathy, Michelle O'Doherty, Marine Chalanqui, Daniel J. Kelly, Tammy Haut-Donahue, Helen O. McCarthy, and Nicholas J. Dunne*

In this study, thermoresponsive copolymers that are fully injectable, biocompatible, and biodegradable and are synthesized via graft copolymerization of poly(N-isopropylacrylamide) onto alginate via a free-radical polymerization reaction are presented. This new synthesis method does not involve multisteps or associated toxicity issues, and has the potential to reduce scale-up difficulties. Chemical and physical analyses verify the resultant graft copolymer structure. The lower critical solution temperature, which is a characteristic of sol-gel transition, is observed at 32 °C. The degradation properties indicate suitable degradation kinetics for drug delivery and bone tissue engineering applications. The synthesized P(Alg-g-NIPAAm) hydrogel is noncytotoxic with both human osteosarcoma (MG63) cells and porcine bone marrow derived mesenchymal stem cells (pBMSCs). pBMSCs encapsulated in the P(Alg-g-NIPAAm) hydrogel remain viable, show uniform distribution within the injected hydrogel, and undergo osteogenic and chondrogenic differentiation under appropriate culture conditions. Furthermore, for the first time, this work will explore the influence of alginate viscosity on the viscoelastic properties of the resulting copolymer hydrogels, which influences the rate of medical device formation and subsequent drug release. Together the results of this study indicate that the newly synthesized P(Alg-g-NIPAAm) hydrogel has potential to serve as a versatile and improved injectable platform for drug delivery and bone tissue engineering applications.

1. Introduction

In drug delivery and bone tissue engineering applications, in situ forming (ISF) hydrogels offer significant benefits as the medical device can be directly injected locally at the site of damaged or diseased tissue. In general, ISF hydrogel refers to a hydrophilic polymeric network, constituted in water or physiological fluid, making them biocompatible with a reduced risk of an adverse immune response.^[1,2] Furthermore, ISF hydrogels circumvent invasive surgical implantation thereby enhancing patient compliance and comfort.^[3] Moreover, these biomaterials are designed to protect sensitive active principle ingredients such as proteins/peptides and nucleic acids.^[1,4,5] ISF hydrogels can be synthesized via chemical or physical crosslinking. The major difference between these two systems is the ability to be a reversible system. Chemical reactions create a permanent robust network, whereas physical reactions create a weaker reversible network. Chemical hydrogels

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are typically formed using crosslinkers that are known to be cytotoxic, via the application of ultraviolet (UV) radiation, where the efficiency of crosslinking decreases with increase in the injection depth of the ISF *in vivo*.^[3,6,7] Consequently, physically crosslinked ISF hydrogels have been studied to overcome these limitations.^[1,2] Of many physically crosslinked ISF hydrogel based systems, thermoresponsive ISF hydrogels have gained popularity primarily due to the formation of a medical device at body temperature. Thermoresponsive ISF hydrogels are based on hydrophobic interactions as a result of increasing the polymeric solution temperature above its lower critical solution temperature (LCST). At the LCST, water is expelled from the hydrophilic region of the polymer, increasing hydrophobicity of the polymer.^[1] This induces the polymer to shrink in mass forming a solid opaque hydrogel based medical device at the injection site.^[5] For example, poly(*N*-isopropylacrylamide) (PNIPAAm) polymer is an amphiphilic polymer with a sol–gel transition or LCST at 32 °C. At this temperature, polymerization occurs forming a dehydrated shrunken configuration due to the disruption of hydrogen bonding of water molecules around the amide group of the side polymer chains.^[8] Given that the LCST of PNIPAAm occurs at near normal body temperature, this polymer has potential applications in controlled drug delivery and bone tissue engineering. However, PNIPAAm exhibits poor wet strength^[9] and is nonbiodegradable.^[10,11] Thus, it is essential to modify PNIPAAm to extend its applications and use under physiological conditions. The advantage of thermoresponsive polymers is that the LCST can be tuned via copolymerization and tailored to a particular drug delivery or tissue engineering application. For example, copolymerizing with a more hydrophobic comonomer decreases the LCST and using a more hydrophilic monomer has the opposite effect. Copolymerization also plays an important role in biocompatibility and biodegradability, which are essential in targeted tissue engineering and biomaterials related applications.^[12–16]

Sodium alginate (NaAlg) is a natural polysaccharide that is extracted from brown seaweeds.^[10] Alginic acid is an anionic biopolymer consisting of linear chains of α -L-glucuronic acid and β -D-mannuronic acid with properties such as a high degree of aqueous solubility, a tendency for gelation when reacted with divalent cations—the resulting gels demonstrating high porosity, biocompatibility, and nontoxicity.^[17] Furthermore, NaAlg is a polyelectrolyte polymer where structural changes can be implemented by chemical modifications to enhance its functions through its carboxyl groups.^[10] The biodegradation profile of alginate and its biocompatibility under physiological conditions make it appealing for the fabrication of biomaterials.^[9] Thus, alginate is widely used in biotechnological, medical, and food applications.

The P(Alg-g-NIPAAm) hydrogel can be synthesized using chemical linkers or through the creation of free radicals to graft the pendant group NIPAAm onto the hydrophilic alginate chain. Current literature promotes grafting using intermediary reactions, including the formation of amino functionalized PNIPAAm.^[8] Moreover, grafted PNIPAAm to alginate molecules without previous modifications where the polymerization is mediated by microwave radiation creates a very complex system.^[10] Recently, Soledad Lencina et al. synthesized an Alg-g-PNIPAAm copolymer using low doses of gamma radiation.^[18] These technologies are expensive and the

processing is labor intensive. Therefore, there is a need for the development of a simplified and more cost-effective process.

The overall aim of this study was to synthesize a biocompatible, thermoresponsive P(Alg-g-NIPAAm) copolymer hydrogel using simple free-radical polymerization of NIPAAm onto an alginate backbone in the presence of ammonium persulfate (APS) and *N,N,N',N'*-tetramethylethylenediamine (TEMED). This is a new method of synthesizing this copolymer, which does not involve multistep synthesis and associated toxicity issues and reduces the difficulties in scale-up compared to other reported methods in the literature. Furthermore, for the first time, this work will explore the influence of alginate viscosity on the viscoelastic properties of the resulting copolymer hydrogels, which influences the rate of medical device formation and subsequent drug release. Therefore, the objectives of this study were to prepare P(Alg-g-NIPAAm) copolymer using alginates of differing viscosities and to determine the physical and chemical properties, *in vitro* drug release, and degradation profile. Also, this study further evaluated the optimal P(Alg-g-NIPAAm) hydrogel (in terms of physical and chemical properties, *in vitro* drug release, and degradation profile) with respect to cytotoxic response and the capacity to support osteogenic and chondrogenic differentiation of porcine bone marrow mesenchymal stem cells (pBMSCs) for bone regeneration.

2. Experimental Section

2.1. Materials

NIPAAm (731129) was purchased from Sigma-Aldrich, UK and recrystallized in toluene. Sodium alginate (f2, 180947, low viscosity of 1% solution: 15.0–20.0 mPas), APS (248614), and TEMED (411019) were obtained from Sigma-Aldrich, UK. Very low viscosity NaAlg (f1, A18565, viscosity of 1% solution: 4.0–12.0 mPas), medium viscosity NaAlg (f3, B25266, viscosity of 1% solution: 40–90 mPas), and high viscosity NaAlg (f4, J61887, viscosity of 1% solution: 1000–1500 mPas) were obtained from Alfa Aesar, UK and were used as received. Fluorescein sodium salt (NaF, MW 376 Da) and bovine serum albumin (BSA, MW 66 kDa) were obtained from Sigma-Aldrich, UK and were used as received. Bio-Rad Protein Assay Kit (Life Technologies, UK) was used to measure BSA release. Human osteosarcoma cells MG63 (ATCC CRL-1427) were purchased from ATCC, USA. Cells were cultured and maintained in Eagle's minimum essential medium (MEM) (Gibco, Life Technologies, UK) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, UK) in a humidified incubator at 37 °C and 5% CO₂. The pBMSCs were isolated and cultured as previously reported.^[19] These stem cells were used to evaluate the capacity of the grafted poly (Alg-g-NIPAAm) hydrogels to support osteogenic and chondrogenic differentiation as opposed to a cell line as the latter were not the optimal choice for evaluating the viability and potential for supporting tissue formation in tissue engineering applications. Moreover, optimal procedures were established in the laboratory to isolate, characterize, and evaluate pBMSCs.^[19] Prior to use, cells were trypsinized using trypsin/EDTA in phosphate buffer solution (PBS) (2X) (Life Technologies, UK), centrifuged, and resuspended in MEM. **CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS) (Promega, UK).**

2.2. Synthesis of P(Alg-g-NIPAAm) Hydrogel

The synthesis of grafted poly (Alg-g-NIPAAm) was performed via free-radical copolymerization in aqueous solution (**Figure 1, Table 1**). Typically, 1.13 g of NIPAAm (10 mmol) and 113 mg (10 wt% w.r.t. NIPAAm) of NaAlg were dissolved in 20 mL of PBS at pH 7.4.

Figure 1. Schematic illustration of copolymerization of *N*-Isopropylacrylamide (NIPAAm) with sodium alginate (NaAlg).

Dry nitrogen was purged through the solution for 60 min prior to polymerization. Nitrogen bubbling was necessary to remove any oxygen from the solution as to avoid its reaction with the free radical. Then, the initiator, APS, (0.030 g) and the accelerator, TEMED, (30 μ L) were added to the solution and the copolymerization was achieved within 12 h at room temperature (22 ± 2 °C). Thereafter, the polymer solution was dialyzed for 5 d in double distilled water (DDW) at 22 °C (MWCO: 10 kDa) and recovered by freeze-drying at -50 °C. Dialysis was used to purify the reaction mixture by removing any unreacted monomers; thereafter freeze drying was used to finally remove the solvent (water) and also to protect the P(Alg-g-NIPAAm) hydrogel. Grafting efficiency was calculated using Equation (1), shown in Table 1

$$\text{Grafting Efficiency (\%)} = \frac{\text{mass of graft copolymer}}{\text{mass of NIPAAm} + \text{mass of NaAlg}} \times 100 \quad (1)$$

2.3. Chemical Characterization of P(Alg-g-NIPAAm) Hydrogel

P(Alg-g-NIPAAm) hydrogels were initially analyzed using Fourier transform infrared spectroscopy (FTIR) (FTIR 4100, Jasco Ltd., UK) to determine the copolymer formation. For this using KBr pellets

Table 1. Gravimetric and TGA data highlighting the graft copolymer composition of P(Alg-g-NIPAAm) hydrogels of varying viscosities (f1–f4).

Sample Code	NIPAAm monomer [moles]	Alginate viscosity [mPas]	Grafting Efficiency	
			Gravimetric [%]	TGA [%]
f1	0.01	4.0–12	98.36 $\pm$ 1.41	98.29 $\pm$ 0.18
f2	0.01	15–20	97.90 $\pm$ 0.80	97.79 $\pm$ 0.40
f3	0.01	40–90	97.43 $\pm$ 1.63	98.63 $\pm$ 0.005
f4	0.01	1000–1500	96.73 $\pm$ 1.86	98.28 $\pm$ 0.18

of PNIPAAm, NaAlg, and copolymer were prepared and scanned in the wavelength range of 400–4000 cm^{-1} at 256 scans with a resolution of 4 cm^{-1} . Additionally, the samples were subjected to ^1H -NMR (400 plus spectrometer, Bruker Ltd., UK) characterization for both monomers/homopolymers and grafted copolymer in deuterium oxide (D_2O) (as a solvent) and tetramethylsilane (TMS) as internal standard at a frequency of 400 MHz. FTIR and ^1H -NMR characterization was performed at room temperature (22 ± 2 °C).

2.4. Rheological Analysis

Rheological studies were performed on freeze-dried polymer reconstituted—in DDW or PBS. Polymer concentration was expressed in wt%. Rheological properties of the polymers were studied using a stress-controlled AR-2000ex rheometer (TA Instruments, UK) equipped with 40 mm parallel-plate at a constant shear rate (6.283 rad s^{-1}). Temperature sweep experiments from 20 to 50 °C were conducted at a heating rate of 3 °C min^{-1} . The experiments were performed in the linear viscoelastic region, which was established for each sample by a stress sweep at frequencies between 0.1 and 10 Hz. The rheometer was equipped with a Peltier control system that allows accurate control of temperature (± 0.1 °C). A solvent trap was used to minimize changes in concentrations due to water evaporation.

2.5. Thermogravimetric Analysis (TGA)

Thermogravimetric analysis was conducted using a TGA/SDTA851e/LF/1600 machine (TA Instruments, UK). Analysis was performed with 8–10 mg of samples on a platinum pan under nitrogen atmosphere at a heating rate of 10 °C min^{-1} until 900 °C.

2.6. Differential Scanning Calorimetry (DSC)

Thermal analysis was performed using a differential scanning calorimeter (DSC 600, Perkin Elmer, UK). The sample weights ranged from 3.0 to 9.0 mg. The samples were initially heated from 0 to 100 °C at a heating rate of 10 °C min^{-1} , then cooled to 0 °C, and reheated to 400 °C at the same heating rate as before and the second scans were reported. The inflection point method was employed to estimate the glass transition temperature (T_g). The calibration for the temperature and heat flow was conducted using a pure indium standard at the same heating rate of the experiments.

2.7. Equilibrium Swelling Studies

The swelling behavior of the P(Alg-g-NIPAAm) hydrogels was determined by immersing dried samples to swell in DDW or in PBS of certain pH 7.4 at 4 °C. At specific time intervals, the gels were placed at 37 °C for 2 h. The excess water was removed and the progress of swelling process was monitored gravimetrically. The degree of swelling (Q_s) was calculated using Equation (2)

$$Q_s = \frac{W_s - W_d}{W_d} \times 100 \quad (2)$$

where W_s is the weight of swollen test sample at time t of the process and W_d is the weight of the dried test sample.

2.8. Drug Release

A low molecular weight fluorescein sodium (NaF, MW 376 Da) and a high molecular weight BSA (MW 66 kDa) were used as the model drugs to evaluate the release profile from hydrogels of varying viscosities (f1–f4). The different freeze-dried hydrogels (5 wt%) were reconstituted in PBS (pH 7.4) containing one of the model drugs (i.e., 1 mg mL⁻¹ NaF and 2 mg mL⁻¹ BSA). An aliquot of 100 µL of each hydrogel was placed inside a glass vial and incubated at 37 °C for 15 min that resulted in medical device formation. To this 5 mL of PBS was added and the supernatant (1 mL) was collected at predetermined intervals and replaced with the same volume of fresh PBS. NaF fluorescence was then measured using fluorophotometry (FLUOstar Optima, BMG Labtech Ltd., UK) using excitation and emission detection wavelengths of 480 and 520 nm, respectively. For BSA, 5 µL of fraction was added in 250 µL of Bio-Rad Protein Assay Kit (Life Chemical Technologies, UK) and measured the UV absorbance at 595 nm.

2.9. In Vitro Degradation

Degradation of P(Alg-g-NIPAAm) hydrogels was determined using the weight loss method. Each hydrogel (5 wt% of p(Alg-g-NIPAAm)) was initially weighed (W_0) and placed in vials containing 0.1 M PBS, pH 7.4. The vials were incubated at 37 °C in a shaking incubator at 40 rpm over an extended period, with weekly PBS replenishment. At specified time intervals up to 8 weeks, hydrogels were snapped frozen at –80 °C, then lyophilized and weighed (W_t). The weight loss ratio was calculated according to Equation (3)

$$\text{Weight Loss (\%)} = \frac{W_0 - W_t}{W_0} \times 100 \quad (3)$$

where W_0 and W_t are sample weights before and after degradation.

2.10. In Vitro Cytotoxicity

Cytotoxicity of leachable byproducts and degradation products from the f2 derived P(Alg-g-NIPAAm) hydrogel was evaluated by indirect/extraction studies using MG-63 cells. Conversely, cytocompatibility of the 5 wt% of the f2 derived P(Alg-g-NIPAAm) hydrogel in direct contact with the cells was evaluated by encapsulating pBMSCs directly in the hydrogel.

2.10.1. Cytotoxicity of the Degraded f2 Hydrogel Products

Cell culture basal media (MEM without serum) containing degradation products were collected from: (a) hydrogels under static cell culture conditions at 37 °C up to 21 d and (b) polymer solution in accelerated degradation conditions at 4 °C for 21 d. For condition (a), 5 wt% sterile polymer solution was prepared in basal media and hydrogels were prepared in 24 well plate with a volume of 100 µL per well. After hydrogel formation (1 h), 1 mL of basal media was added per hydrogel and was incubated for 21 d at 37 °C/5% CO₂. Basal media alone containing wells were used as the control. Media samples for toxicity analysis were collected at specific time points such as Days 1, 3, 7, 14, and 21 and fresh media was replaced at each time point. For condition (b), sterile freeze-dried polymer was reconstituted in 1 M TRIS buffer (pH 4) at a concentration of 60 mg mL⁻¹. At Day 21, the solution was neutralized to pH 7. Different amount of degradation products were dissolved in the normal culture medium (MEM with 10% FBS) to obtain media containing different concentration of degradation byproducts (1, 5, 10, and 15 mg mL⁻¹).^[20]

MG-63 cells were cultured, expanded in standard cell culture conditions and seeded in a 96 well plate at a seeding density of 1×10^4 cells per well. After overnight incubation at 37 °C/5% CO₂, media from the wells were replaced with media collected from condition (a) and diluted samples from condition (b) were incubated for 48 h. Viability of cells in

the presence of the media containing the byproducts and degradation products from condition (a) and (b) was quantified using the MTS assay. The absorbance of each well was measured at 490 nm with a microplate reader (PowerWave XS, BIO-TEK, USA) and the results were expressed as mean relative absorbance (%) compared with control samples.

2.10.2. Cytocompatibility of the f2 Hydrogel

In brief, the synthesized and characterized f2 derived P(Alg-g-NIPAAm) hydrogel was freeze-dried and sterilized using a table-top ethylene oxide (EtO) sterilizer (Anprolene, Anderson products, UK). The f2 hydrogel was prepared at a concentration of 5 wt% by dissolving sterile polymer in the cell growth media (DMEM-high glucose) containing no serum. Sterile molds for preparing f2 hydrogel specimens (5 mm × 3 mm) was fabricated using sterile agarose (Sigma-Aldrich, Ireland) solution as previously reported.^[19] The agarose molds were covered with prewarmed PBS and maintained at 37 °C using a heating block. pBMSCs were isolated and cryopreserved as previously reported.^[19] Cryopreserved pBMSCs were expanded in the cell growth media containing 10% fetal bovine serum and 1% penicillin (100 U mL⁻¹) and streptomycin (100 mg mL⁻¹) (GIBCO, Ireland). Expanded pBMSCs were trypsinized at 80% confluency and were encapsulated with the f2 derived P(Alg-g-NIPAAm) hydrogel solution at a density of 15×10^6 cells mL⁻¹ and the hydrogel final concentration was adjusted to 5% with cell growth media. PBS was removed from the wells and 50 µL of the cells encapsulated hydrogel solution was injected into each well using a 1 mL syringe with a 16-gauge needle. Hydrogels were maintained in osteogenic and chondrogenic differentiation media at 37 °C and viability of encapsulated pBMSCs were evaluated at Day 0 and Day 14 using LIVE/DEAD assay (Molecular Probes, Life Technologies, Ireland) followed by confocal microscopy (Olympus, FV-1000, UK) as previously described.^[21] The percentage of viable cells were quantified by separating the live and dead cells from the 10x magnification images ($n = 3$) using the software (FV10-ASW Olympus, Country) followed by quantification using the Image J software (National Institute of Health, USA). The DNA content at Day 0 and Day 14 was quantified using Hoechst Bisbenzimidazole assay (Sigma-Aldrich, UK) as previously described^[22] to evaluate the proliferation of encapsulated cells in the hydrogel.

2.10.3. Osteogenic and Chondrogenic Potential of pBMSCs Encapsulated f2 Hydrogel

The osteogenic potential of pBMSCs encapsulated in the f2 derived P(Alg-g-NIPAAm) hydrogel was evaluated by incubating the gels in standard osteogenic media (DMEM-high glucose) containing FBS (10%) (Life Technologies, UK), penicillin (100 U mL⁻¹), streptomycin (100 mg mL⁻¹), dexamethasone (100×10^{-9} M), β-glycerol phosphate (10×10^{-3} M), and ascorbic acid (0.05×10^{-3} M) (Sigma-Aldrich, UK) at 20% oxygen over a three week period. The chondrogenic potential of pBMSCs encapsulated f2 derived P(Alg-g-NIPAAm) hydrogel was evaluated by incubating the gels in standard chondrogenic media (DMEM-high glucose) containing sodium pyruvate (100 µg mL⁻¹), L-proline (40 µg mL⁻¹), L-ascorbic acid-2-phosphate (50 µg mL⁻¹), bovine serum albumin (1.5 mg mL⁻¹), insulin-transferrin-selenium ((1)×), dexamethasone (100×10^{-9} M) (Sigma-Aldrich, UK), and recombinant human transforming growth factor-β3 (10 ng mL⁻¹) (ProSpec-Tany TechnoGene Ltd, Israel) for three weeks at 5% O₂. In both conditions, media change was performed twice weekly and the samples were analyzed for in vitro osteogenesis and chondrogenesis using histological analysis and biochemical assays for sulfated glycosaminoglycans (sGAG), collagen, and calcium in the hydrogel.

Biochemical analysis of the gels for sGAG, collagen content, and calcium amount were performed as previously described.^[21–23] In brief, overnight enzymatic digestions of the hydrogels were performed using papain buffer extract (100×10^{-3} M) sodium phosphate buffer containing sodium acetate (0.1 M), ethylenediaminetetraacetic acid (0.05 M), pH 6.0, containing 125 µg mL⁻¹ of papain activated with 10×10^{-3} M

cysteine hydrochloride (10×10^{-3} M) (Sigma-Aldrich, UK) at 60 °C with agitation. Dimethylmethylene blue dye-binding assay (Blyscan, Biocolor Ltd., UK) was used to estimate the sGAG levels in the constructs. Papain digested samples, which were treated with HCl (37%) for acid hydrolysis and collagen content in the construct, were quantified by measuring the hydroxyproline content using the Chloramine-T assay as previously described.^[22,24] For the calcium assay, standard calcium assay was performed on the acid digested samples (1 M HCl) using standard calcium assay kit (Sentinel Diagnostics, Italy). The collagen and sGAG content in the hydrogels were normalized with the DNA content and the calcium amount was normalized with the wet weight of the samples.

For histological analysis, gels were fixed in paraformaldehyde, dehydrated, and wax embedded as previously described.^[19] 7 μ m thick sections were made from the wax embedded samples using a microtome and aldehyde fuchsin/alcan blue staining was performed for sGAG. Standard picro sirius red staining was performed for evaluating the collagen deposition and alizarin red staining was performed for evaluating the calcium deposition as previously described.^[19]

2.11. Statistical Analysis

All data were analyzed using *t*-test and ANOVA statistical methods. The results were reported as mean $\pm$ standard error deviation (SEM) with *n* = 3 (unless stated otherwise). To determine a statistically significance difference between the groups with a *p*-value < 0.05 considered to be significant. All the statistical analyses were performed by GraphPad Prism software (v.6, GraphPad, USA).

3. Results

Graft copolymerization of different types of NaAlg with NIPAAm was synthesized by free-radical polymerization. The successful synthesis of the copolymer was confirmed by FTIR and ¹H-NMR. Figure 2a and Table S1 (Supporting Information) show FTIR spectra and associated assignments of NaAlg, PNIPAAm homopolymer, and the synthesized graft copolymer, f2. Similar spectra were also observed for each of the grafted copolymers (f1, f3, and f4). In order to show the common absorption bands between the homopolymers and copolymer, spectra in Figure 2a were shifted in the transmittance axis.

Alginate characteristics bands have been widely described in the literature; the principle bands were highlighted in the spectrum of Figure 2a and Table S1 (Supporting Information).^[25–27] A broad band ranging from 3000 to 3600 cm^{-1} , which corresponded to O–H stretching vibrations was assigned to the NaAlg. The vibration of aliphatic C–H bond appears at 2884 cm^{-1} and two strong peaks at 1595 and 1403 cm^{-1} were attributed to the asymmetric and symmetric stretching of carboxyl groups, respectively. At 1294 cm^{-1} a weak band was evident, which represented the vibration of C–O bond. The bands observed in the range of ≈ 1170 –1030 cm^{-1} corresponded to symmetric and asymmetric vibration bands of C–O–C bonds typical polysaccharide rings. PNIPAAm spectrum displayed absorption bands at 2971 and 2869 cm^{-1} corresponding to the symmetric and asymmetric vibration bands of methyl groups. The amide bonds at 1637 and 1534 cm^{-1} were clearly evident and are representative of the C=O stretching and the N–H vibration. Asymmetric and symmetric vibration bands of the germinal methyl groups were observed as weak signals at 1458 cm^{-1} and as a double peak band at 1386 and 1366 cm^{-1} .^[28,29]

P(Alg-g-NIPAAm) spectra presented some common characteristic absorption bands, e.g., the symmetric and asymmetric vibrations of methyl groups of PNIPAAm at 2970 and 2869 cm^{-1} , and the vibration of C–H bonds at 2928 cm^{-1} that were observed in both PNIPAAm and alginate molecule. The characteristic bands at 1639 and 1528 cm^{-1} , corresponded to C=O stretching and –NH bending due to presence of PNIPAAm within the copolymer. Furthermore, the bands between ≈ 1030 cm^{-1} and ≈ 1170 cm^{-1} represented the symmetric and asymmetric vibrations of the C–O–C bonds typical from alginate rings. Additionally asymmetric stretching associated with the carboxylic groups of the alginate was observed at 1456 cm^{-1} . These different peaks are indicative that PNIPAAm polymerization in the copolymer had successfully occurred on the alginate backbone. Prolonged dialysis of the copolymer helped remove unreacted monomers and unattached homopolymers, thus the bands represented in the final product post freeze-drying corresponded to the copolymer.

Figure 2. a) FTIR spectra; b) ¹H-NMR spectra of NIPAAm monomer, NaAlg, and (Alg-g-NIPAAm) hydrogel (f2).

The successful formation of a copolymer was also confirmed by the $^1\text{H-NMR}$ analysis (Figure 2b and Figure S1, Supporting Information). Two broad peaks at 1.5–2.2 ppm represented the $(-\text{CH}-\text{CH}_2-)$ groups and the strong singlet at 1 ppm corresponded to methyl group of PNIPAAm. In addition, the characteristic peak of PNIPAAm at approximately 3.7 ppm corresponded to the methine of isopropyl groups. NaAlg spectrum showed peaks of protons on the carbon (ring protons) between 3–4 ppm. Synthesized P(Alg-g-NIPAAm) copolymer showed peaks at 3.7, 1.9, 1.4, and 1.03 ppm and were assigned to the $-\text{CH}$, $-\text{CH}_2-$, $-\text{CH}-$, and $-\text{CH}_3$ groups, which showed existence of NIPAAm. The methine of isopropyl groups of PNIPAAm peaked at 3.7 ppm and overlapped with the characteristic peak of NaAlg. These peaks are characteristic to the alginate and P(NIPAAm) structure proving that both elements are present within the copolymer. Moreover, the peak at 4.6 ppm in the NaAlg, NIPAAm, and P(Alg-g-NIPAAm) was indicative of the D_2O solvent. The same characteristic signals were observed for all graft copolymers in both the FTIR and $^1\text{H-NMR}$ spectra (figures are not presented). Grafting efficiency was determined by gravimetric analysis using Equation 1 and by TGA (Table 1). The grafting efficiency decreased slightly as the viscosity of alginates increases.

Synthesized copolymers are expected to present thermoresponsive behavior, since intermolecular and intramolecular associations are favored with an increase in temperature. This particular characteristic is due to the presence of the PNIPAAm.^[30] From the macroscopic perspective, below LCST polymer chains dissolve in water and above the critical temperature a collapsed state is achieved producing phase separation.^[31,32] The change in the copolymer structure can be easily observed as turbidity of the solution, therefore the LCST can be estimated via simple visual inspection. However, to determine the LCST accurately, the rheological properties of P(Alg-g-NIPAAm) copolymer hydrogel were measured by

monitoring the storage modulus (G') and loss modulus (G'') as a function of temperature (Figure 3). The LCST of P(Alg-g-NIPAAm) copolymer was noted at 32 °C. The G' and G'' moduli values for the copolymer remained unchanged as the temperature increased above 37 °C, which indicated the formation of a stable hydrogel network at 37 °C (Figure 3a). The viscoelastic properties of the resultant hydrogels indicated that the LCST of the various copolymers (f1–f4) remained stable over the range of the alginate viscosity investigated. The LCST remained unchanged at 32 °C.

As observed in Figure 3b, the P(Alg-g-NIPAAm) hydrogel was dissolved in different dissolution media (DDW, PBS at pH 5.4 and PBS at pH 7.4) at 5 wt% concentration and analyzed for rheology to study the effect on mechanical properties. The LCST of the hydrogel increased from 32 °C to 35 °C in DDW whereas the G' and G'' moduli values were very low compared to PBS solutions. The G' moduli of the hydrogels were recorded as 126.2, 547.9, and 857.4 Pa at 37 °C when tested in DDW, PBS pH 5.4 and PBS pH 7.4, respectively. These results are conceptually similar to the Young's modulus of the hydrogel.

TGA degradation curves and associated analysis for the NaAlg, PNIPAAm, and P(Alg-g-NIPAAm) are shown in Figure 4a, Figures S2 and S3 (Supporting Information) and Table 2. The PNIPAAm polymer showed a two-stage degradation profile at 98 and 408 °C.^[14,15] The reduction in mass until 98 °C corresponded to the evaporation of the water molecules and beyond 408 °C was indicative of decomposition. The NaAlg also exhibited multistage degradation. For example, the percentage mass loss observed until 87 °C, was due to the dehydration of water molecules present on the alginate backbone and the second mass loss at 250 °C can be attributed to the decomposition of the saccharide rings.^[11,16] The temperature of 50% mass loss (T_{50}) was found to be 291 °C for NaAlg and 401 °C for PNIPAAm. Finally, from TGA curves, the P(Alg-g-NIPAAm) copolymers with varying alginate

Figure 3. Typical plot showing variation in tan delta ($\tan \delta$) as a function of temperature a) 5 wt% P(Alg-g-NIPAAm) copolymers of differing alginate viscosity (f1–f4) in PBS at pH 7.4 and b) $\tan \delta$ of 5 wt% P(Alg-g-NIPAAm) hydrogel (f2) in different solutions (DDW and PBS) at various pH levels. Dotted line represents equilibrium state of the gel when liquid and solid characters of the gel are equal ($G' = G''$). Plots represent average of three independent samples.

Figure 4. a) Typical thermogravimetric curves (solid line) and first derivative curves (dotted line) for the PNIPAAm, NaAlg, and P(Alg-g-NIPAAm) hydrogel (f2). b) DSC thermograms for the PNIPAAm, NaAlg, and P(Alg-g-NIPAAm) hydrogel (f2). Values represent average of three independent samples ($n = 3$).

viscosity (f1–f4) presented a percentage mass loss below 100 °C due to the water evaporation. Moreover, a small variation in mass (10–15%) was observed at ≈ 250 °C. This temperature correlated with the degradation of the alginate chains. However,

the lower influence on the structure seemed to be due to the protection of the alginate by PNIPAAm chains that stabilized the structure. Finally, complete degradation of the copolymer was achieved at near 400 °C similar to the PNIPAAm

Table 2. Thermogravimetric analysis (TGA) highlighting the degradation profiles for NaAlg, PNIPAAm, and P(Alg-g-NIPAAm) hydrogels of varying viscosities (f1–f4).

Sample code	First depression		Second depression		Third depression		50% Mass loss (T_{50})	Residue at 800 °C
	Temperature [°C]	Mass loss [%]	Temperature [°C]	Mass loss [%]	Temperature [°C]	Mass loss [%]		
NaAlg	87	5.79	250	39.01	–	–	291	28.58
PNIPAAm	98	7.70	–	–	408	62.72	401	0.00
f1	90	7.27	272	13.20	382	74.96	372	0.00
f2	94	2.94	271	7.20	386	60.06	378	5.17
f3	93	6.11	265	10.42	391	70.75	383	4.41
f4	98	5.63	264	10.74	391	69.69	383	1.36

degradation. Copolymer composition was determined using first derivative (dotted line, Figure 4a) of TGA curves in order to obtain the mass associated to weight loss of both components. Two major weight losses were observed from the first derivative TGA plots, the area under the curve of the first peak (m_1) was attributed to the alginate mass content into the copolymer; in the same way, the area under the second peak (m_2) was considered as the PNIPAAm mass loss. The total mass of the graft copolymer was the sum of the both values ($m_1 + m_2$) and the NIPAAm molar percentage was determined using following Equation 4

$$\text{NIPAAm (\%)} = \frac{\frac{m_2}{113.16}}{\frac{m_1}{198} + \frac{m_2}{113.16}} \times 100 \quad (4)$$

DSC analyses were performed in order to understand the thermal behavior of the grafted copolymers. As shown in Figure 4b and Figure S4 (Supporting Information), the NaAlg polymer had a glass transition temperature (T_g) of 121 °C. Furthermore, the NaAlg alginate polymer presented an endothermic peak at 250 °C that was characteristic to absorption of energy during structural reorganization (e.g., after dehydration of the saccharide ring). Moreover, PNIPAAm showed a lower T_g than the NaAlg at 110 °C. This observation could be due to the thermoresponsive nature of PNIPAAm. However, the PNIPAAm polymer showed an exothermic peak at 398 °C corresponding to the degradation to the polymer. Finally, the P(Alg-g-NIPAAm) copolymers presented a combination of the characteristic of copolymers. For example, P(Alg-g-NIPAAm) presented a T_g at 105.3 °C that was similar to the PNIPAAm homopolymer and a slight endothermic peak between 245–252 °C was observed due to the crystallization of the alginate. Also these copolymers showed an exothermic peak of between 373 and 396 °C, which was in the range of PNIPAAm degradation.

The degree of swelling over time of P(Alg-g-NIPAAm) hydrogel in PBS and DDW at 37 °C is shown in Figure 5a,b. The highest degree of swelling of the hydrogel occurred in DDW compared to PBS. The swelling percentage increased exponentially in DDW, whereas in PBS it remained relatively constant over time. The degree of swelling (Q_s) ranged from 239 ± 30% and 986 ± 27% for hydrogels in DDW and from 572 ± 14% to 629 ± 29% in PBS, over a period of two weeks.

The release profiles of the model small molecule (NaF) and protein molecule (BSA) from P(Alg-g-NIPAAm) at temperatures above LCST are shown in Figure 5c. The efflux of drugs from the hydrogels was mainly through a diffusion process, which is typically size-dependent. For example, after 1 h, the percentage release of NaF was ≈30%, whereas BSA was ≈7%. Over a four week period, the release of NaF and BSA was approximately 70% and 22% for all four hydrogels (f1–f4).

The degradation profile of a biomaterial or medical device is a key factor for the tissue formation or drug release profile. In vitro degradation study of the copolymer at 37 °C showed that P(Alg-g-NIPAAm) hydrogels demonstrated a controlled degradation (Figure 5d) with a maximal weight loss of ≈70% after 8 weeks. The degradation profile of the P(Alg-g-NIPAAm)

hydrogels exhibited was characteristic of surface degradation, which involves the random cutting of the chain length by hydrolysis. Such a mechanism of degradation is advantageous for long-term delivery of drugs in a controlled manner whilst maintaining the mechanical properties of the hydrogel.

The viability of encapsulated pBMSCs in the f2 derived hydrogel is shown in Figure 6A. Approximately 60% of cells were found to be viable immediately after making the hydrogels, which increased to more than 80% after a 2 week culture period in both osteogenic and chondrogenic conditions. The DNA concentration at the 3 week time point in chondrogenic condition showed significant increase ($p < 0.001$) compared to that of osteogenic conditions and Day 0. Biochemical analysis of sGAG and collagen deposition after 3 weeks of culture were found to be significantly higher in chondrogenic conditions compared to that of osteogenic conditions (Figure 6B–h). The calcium content in the osteogenic conditions showed a statistically significant increase compared to Day 1 ($p < 0.0001$). Corroborating with the biochemical data, the histological data showed intense collagen and sGAG staining for hydrogels maintained in chondrogenic conditions compared to osteogenic conditions (Figure 6B). In contrast, hydrogels in osteogenic conditions showed very intense mineralization after 3 weeks (Figure 6B–i). Studying the cytotoxicity of f2 derived hydrogel degraded byproducts revealed no significant alteration of MG63 cell viability when compared to the control. In fact, cell viability remained above 80% across all treatment groups (Figure 6C–a). Furthermore, cells incubated with hydrogel supernatants, conditioned with degraded hydrogel byproducts up to 21 d, exhibited viability >80% (Figure 6C). No statistically significant difference in cell viability was noted after 48 h incubation in 1–21 d hydrogel-conditioned supernatant (Figure 6C–b).

4. Discussion

The development of localized drug delivery systems represents a valuable option to overcome the typical drawbacks associated with conventional systemic mode of administration, which is not directed to the target tissue.^[16] In this context, injectable implant forming (e.g., ISF hydrogels) systems have emerged as a superior, nonsurgical alternative for tissue regeneration or localized drug delivery.^[17] In this work, we have described the development and characterization of a thermoresponsive based hydrogel system for localized cell and/or drug delivery for bone tissue engineering applications.

P(Alg-g-NIPAAm) copolymers were synthesized via a simple free-radical polymerization route. Alginate is a natural biodegradable and biocompatible polymer, which is a promising material for biomedical applications. In order to make the alginate thermoresponsive as well as to improve its solubility at physiological pH 7.4, we introduced the thermoresponsive moieties of Poly(NIPAAm) to alginate backbone through free radical polymerization. The success of copolymerization was confirmed by FTIR and ¹H-NMR. These results are similar to that of microwave induced alginate-grafted PNIPAAm copolymer.^[10]

It is extremely important to control the viscoelastic properties when designing a biomaterial to mimic the physiological

Figure 5. Percentage of swelling of 5 wt% P(Alg-g-NIPAAm) hydrogels of varying viscosities (f1–f4) in a) PBS and b) DDW. Values represent mean ± SEM ($n = 6$). The asterisk denotes significant difference at Days 3, 7, and 14 when compared to Day 1 (* $p \leq 0.05$; ** $p \leq 0.001$, and *** $p \leq 0.0001$ using an unpaired t -test). c) Cumulative release profile of fluorescein sodium (NaF, MW 376 Da) and bovine serum albumin (BSA, MW 66 kDa) from 5 wt% P(Alg-g-NIPAAm) hydrogel of varying viscosities (f1–f4) in PBS at 37 °C (pH 7.4). Values represent mean ± SEM ($n = 3$). d) Degradation plots of 5 wt% P(Alg-g-NIPAAm) hydrogel of varying viscosities (f1–f4) in 0.1 M PBS at 37 °C (pH 7.4). Values represent mean ± SEM ($n = 3$).

microenvironment. Therefore, to investigate the viscoelastic characteristics of P(Alg-g-NIPAAm) copolymer solution at different temperatures before and after gelation. At low temperatures (20–30 °C), the shear loss modulus (G'') dominated the flow property and the shear storage modulus (G') was too low to be accurately measured. This corresponded to the solution state of the samples. During this temperature range, the G'' decreased marginally with an increase in the temperature due to thermal movement of polymer chains leading to a lower viscosity. Beyond this temperature range, a sharp increase in both G' and G'' was observed and after crossover between two lines, G' starts to become higher than G'' , which indicates hydrogel formation and is further evident from the tan delta ($\tan \delta = G''/G'$) (Figure 3). The crossover between G' and G'' lines was considered as the gelation point and the corresponding temperature was termed as the gelation temperature (T_{gel}), which is close to the LCST of the copolymer. The results of rheological properties

such as G' and G'' showed significant improvement compared with previously reported alginate-PNIPAAm based hydrogels.^[9,16,19] The G' and G'' of 5 wt% P(Alg-g-NIPAAm) solutions increased from 32 to 35 °C. P(Alg-g-NIPAAm) solutions transformed into stable hydrogels in situ at 37 °C, a phenomenon reflected by the levelling off of G' and G'' . The sol-gel phase transition behavior is beneficial for cell entrapment to give uniform distribution of cells within the gelated hydrogels.^[17,18] It is also favorable for drug delivery, especially for proteins.^[25]

To study the effect of the dissolution medium, f2 hydrogel (5 wt%) was dissolved in different media. As observed in Figure 3a, the rheological analysis showed a decrease in the LCST from 35 to 32 °C in PBS. This could be attributed to the presence of ions such as sodium chloride, sodium phosphate, in the PBS solution, which influenced the precipitation of the PNIPAAm chains with temperature. Thus, the interaction of ions in the solution with the copolymer needs a lower amount

Figure 6. A) In vitro cytocompatibility of pBMSCs encapsulated in the 5 wt% P(Alg-g-NIPAAm) hydrogel (f2). Representative images of hydrogel using Live/Dead assay: a) at day 0; b) at Day 14 in osteogenic and c) chondrogenic conditions. d) Viability percentage quantified from the live/dead images. e) DNA amount at 3 weeks in osteogenic and chondrogenic conditions. Values represent mean $\pm$ SEM ($n = 3$). The asterisk denotes significant difference at Day 14 when compared to Day 0 (* $p \leq 0.05$, ** $p \leq 0.001$, and *** $p \leq 0.0001$ using an unpaired t -test). B) Osteogenic and chondrogenic potential of 5 wt% f2 derived P(Alg-g-NIPAAm) hydrogel. a, c) Picro sirius red, c, d) aldehyde fuchsin-alcian blue, and e, f) alizarin red staining of hydrogel from osteogenic and chondrogenic conditions at 21 d of culture. Scale bar 100 μ m for higher magnification and 500 μ m for lower magnification images. Quantitative results are shown as g) collagen/DNA content, h) GAG/DNA content, and i) calcium content (for constructs maintained in osteogenic conditions only). C) In vitro cytocompatibility studies of 5 wt% P(Alg-g-NIPAAm) hydrogel (f2): a) viability of MG63 cells after culture (24 h) in varying concentrations of degradation products; b) metabolic activity of MG63 cells cultured in media conditioned in f2 hydrogel from 1 to 21 d. Dotted line represents acceptable cell viability percentage. Values represent mean $\pm$ SEM ($n = 3$).

of thermal energy to induce the PNIPAAm chains to shrink. Whereas, the G' was recorded as 126.2, 547.9, and 857.4 Pa at 37 $^{\circ}$ C for solutions in water, PBS pH 5.4 and PBS pH 7.4. The increase in stiffness could be explained by pH responsive nature of the alginate. Moreover, the PBS solution increased the mechanical properties of the P(Alg-g-NIPAAm) hydrogel with an increase in the $\tan \delta$.

The $\tan \delta$ of the copolymer in PBS solution at pH 7.4 has a value 0.43, which decreased to 0.24 when the P(Alg-g-NIPAAm) was in the DDW solution. This observation can lead to the supposition that the positive ions contained in PBS such as sodium induced electrostatic interactions between the alginate chain to form an egg-box configuration and reinforce the hydrogel strength. However, when the pH of the PBS solution decreased from 7.4 to 5.4, the $\tan \delta$ decreased from 0.43 to 0.26. Alginate chain contains carboxylic acid groups

whose acid dissociation constant (pK_a) is about 3.2. Thus, at a pH superior to 3.2, the $-\text{COOH}$ groups are negatively ionized forming $-\text{COO}^-$ groups. Consequently, in decreasing the pH of the solution, the hydrogen ions contained in the solution can interact with the $-\text{COO}^-$ groups to neutralize their charge and reduce the repulsion of chains that are responsible for the reduction of interaction inside the hydrogel and the reduction of stiffness.

The thermal stability was determined by TGA of the copolymers (f1–f4) as well as the composition, based on the degradation of pure NaAlg and PNIPAAm homopolymers as shown in Figure 4a. The major degradation of NaAlg observed between 200 and 300 $^{\circ}$ C was 36%. The final residue of initial mass after 800 $^{\circ}$ C was approximately 28%. For PNIPAAm, the major mass loss observed between 350–450 $^{\circ}$ C (≈ 408 $^{\circ}$ C) that involved almost 90% of the total mass. The thermograms

obtained for all copolymers showed three stage degradation or depressions. As shown in Table 2, the loss representing alginate backbone is higher in copolymer (264–272 °C) than the pure alginate (250 °C) due to protection of PNIPAAm chain whereas the degradation of PNIPAAm chains is less in copolymer (382–391 °C) than the pure PNIPAAm (408 °C). The TGA confirmed the grafting of the PNIPAAm chains on the alginate backbone and its enhancement in terms of stability (Table 2).

Furthermore, the thermal behavior of copolymers was studied using DSC (Figure 4b and Figure S4, Supporting Information). The endothermic peak was slightly shifted as viscosity of alginates varied. Also the exothermic peak decreased as viscosity decreased (373 °C for f1 and 396 °C for f4). The T_g value for pure NaAlg was 121 °C, whereas the T_g values of copolymers were approximately 135 °C. The T_g of the copolymers increased due to PNIPAAm side chains protecting the alginate backbone.^[13]

Swelling is directly related to the structure of grafted polymer network.^[33] The water uptake capacity of the samples was clearly influenced by the viscosity of NaAlg and medium used for swelling (Figure 5a,b). In the first 24 h, the samples having a very low viscosity NaAlg (f1) presented a slower swelling, with smaller Qs values than samples with high viscosity alginate (f4). After 3 d, the samples reach equilibrium swelling state with a marked increase in Qs. The hydrogel with the high viscosity NaAlg absorbed faster at the beginning of the swelling process because the water molecules interacted first with the most polar hydrophilic groups on the polymer backbone (COO[−] groups of alginate).^[34] In addition, electrostatic repulsive forces between the charged sites (COO[−]) also contributed to an increase in swelling. As a result the network expanded, with a faster increase in Qs, followed by a slower and less significant increase until reaching equilibrium, due to high polymer chain density, which hindered further expansion of the network by absorption of water. Where as in PBS, the samples reached equilibrium swelling within 24 h. The ions in PBS neutralized the charged sites (COO[−]) of alginate. The swelling kinetic measurements indicated that the hydrophilic character of alginate influenced the water uptake capacity of the hydrogel samples, especially when equilibrium was established. Therefore the maximum swelling determined at equilibrium was directly proportional to NaAlg viscosity. This feature can be attributed to the increased number of free carboxylic acid groups in ionized alginate, which can break hydrogen bonding and generate electrostatic repulsion among alginate macromolecules attracting more water into the hydrogel, which results in higher swelling ratio.

When the thermoresponsive gel solidified at 37 °C, a temperature gradient was generated across the polymeric matrix. This resulted in the collapse of the gels surface faster than the interior of gel matrix, forming a dense polymeric “skin” layer. The “skin” layer hindered the outward diffusion of drug molecule, causing a build-up of hydrostatic pressure inside the gel. This pressure would eventually “squeeze out” the water containing the drug as time passes. For NaF release at 37 °C, there was an initial burst release of surface encapsulated NaF accompanying the formation of the dense skin layer. Subsequently, the hydrostatic pressure generated drove NaF release. However,

the skin layer formation hindered the drug diffusion process causing controlled release of NaF. Whereas, BSA is a larger protein molecule, can be analogous to a polymer chain. Therefore, when the protein molecules interacted with the polymer network, it is possible that physical crosslinks formed during the process, hindering the diffusion of free proteins out of the gel matrix. Conversely, it has been well documented that BSA can form disulfide-linked covalent aggregates in solution.^[35–37] BSA aggregates form more easily and thus have the potential to be entrapped within the gel network causing a lower release or controlled release over a long period. The drug release profile of this polymer is passive diffusion, which is dependent of the drug size. Macromolecules will be released slowly influenced by the degradation of the hydrogel after the burst release (Figure 5c).

Biodegradable hydrogels have attracted considerable attention for biomedical uses because they do not require any removal process after the system fulfils its goal. In addition, the degradation can tailor to control the physicochemical properties of the hydrogel systems, and thus to provide flexibility in the design of biomedical devices.^[27] The weight loss could be attributed to the electrostatic interaction of ions with polymeric network helps to hydrolyze the alginate backbone.

Biocompatibility of the developed injectable hydrogel is equally important to that of the physicochemical and mechanical properties. It determines the potential use of the hydrogel in the area of drug delivery and tissue engineering. The degraded products from the hydrogel had minimal cytotoxic effect. In addition, pBMSCs directly encapsulated in the hydrogel remained viable and maintained their differentiation potential. In vitro studies demonstrated the pBMSCs encapsulated in the hydrogels had the capacity to differentiate down both the osteogenic and chondrogenic pathway in response to soluble cues. The increase in cell number or DNA content in the hydrogels incubated in the chondrogenic conditions compared to Day 0 and osteogenic conditions (Figure 6A) could be due to the proliferative effect of TGF- β 3 in the chondrogenic conditions.^[38] Gels incubated in osteogenic conditions showed extensive mineralization at the 3 week time point, which indicated the capability of the hydrogel to facilitate the development of mineralized matrix. On the other hand, gels incubated in chondrogenic conditions demonstrated robust total collagen and sGAG deposition with no evidence of mineralization (a marker of chondrocyte hypertrophy) at the 3 week time point. These results demonstrate the ability of the developed hydrogel to encapsulate stem cells without affecting their functionality for drug delivery and tissue engineering applications. To build on these results, in vivo studies will be required to fully the evaluate osteogenic and chondrogenic potential of pBMSCs encapsulated f2 hydrogel and the mRNA expression of phenotypic gene (e.g., ALP, Col1A1, Aggrecan, Col2a1, COMP, RUNX2, SOX9) and will be tested accordingly using immuno-histochemical analysis.

Taken together, the findings of this study show the potential of P(Alg-g-NIPAAm) for use as an injectable, thermoresponsive, and biocompatible copolymer due to the ease of manufacture compared to previously described systems, and also provide stable environment for sustained release of bioactive

molecules. Further improvement to the system will be considered in future studies including use of growth factors, which is an important step in the right direction for moving P(Alg-g-NIPAAm) into clinical applications.

5. Conclusion

This study has demonstrated that graft copolymers of sodium alginate with *N*-isopropyl acrylamide can be synthesized by simple free-radical polymerization using APS and TEMED at room temperature. The copolymers demonstrating thermoresponsive properties and a controlled release of model drug molecules were successfully prepared by varying alginate viscosity. All copolymers exhibited thermoresponsive behavior at 32 °C. Equilibrium swelling studies of the copolymers also clearly demonstrated the temperature responsive nature of the hydrogels. The degradation profile of the P(Alg-g-NIPAAm) hydrogels investigated that ≈70% was degraded after 8 weeks via surface degradation. The release of NaF was observed to be much higher compared to large protein molecule BSA at pH 7.4 and 37 °C. These results are complimenting each other. The viscosity of alginate played an important role with respect to the mechanical and structural characteristics of P(Alg-g-NIPAAm) hydrogel. In addition, the hydrogel systems investigated showed very good biocompatibility and ability to encapsulate pBMSCs without affecting their differentiation capability. Therefore the synthesized thermoresponsive biocompatible and biodegradable hydrogel has the potential to be used as an injectable hydrogel for drug delivery and bone tissue engineering applications.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

alginate, bone tissue engineering, free radical polymerization, hydrogel, PNIPAAm, thermoresponsive

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- [1] R. Jin, in *Biomedicine*, Vol. 2 (Ed: C. Lin), In Tech, Croatia **2012**, pp. 35–59.
- [2] N. K. Sahu, P. S. Gils, D. Ray, P. K. Sahoo, *Adv. Polym. Sci. Technol.* **2013**, 3, 22.
- [3] R. Jin, L. S. Moreira Teixeira, A. Krouwels, P. J. Dijkstra, C. A. van Blitterswijk, M. Karperien, J. Feijen, *Acta Biomater.* **2010**, 6, 1968.
- [4] M. K. Nguyen, E. Alsberg, *Prog. Polym. Sci.* **2014**, 39, 1235.
- [5] S. Kempe, K. Mäder, *J. Controlled Release* **2012**, 161, 668.
- [6] D. S. Bag, K. U. B. Rao, *J. Polym. Mater.* **2006**, 23, 225.
- [7] J. Berger, M. Reist, J. M. Mayer, O. Felt, N. A. Peppas, R. Gurny, *Eur. J. Pharm. Biopharm.* **2004**, 57, 19.
- [8] S. Chen, H. Zhong, B. Gu, Y. Wang, X. Li, Z. Cheng, L. Zhang, C. Yao, *Mater. Sci. Eng., C* **2012**, 32, 2199.
- [9] N. Isiklan, G. Küçükbalci, *Eur. J. Pharm. Biopharm.* **2012**, 82, 316.
- [10] C. N. Cheaburu, O. N. Ciocoiu, G. Staikos, C. Vasile, *J. Appl. Polym. Sci.* **2013**, 127, 3340.
- [11] M. Hamidi, A. Azadi, P. Rafiei, *Adv. Drug Delivery Rev.* **2008**, 60, 1638.
- [12] K. Kimura, H. Sakamoto, T. Nakamura, *J. Nanosci. Nanotechnol.* **2006**, 6, 1741.
- [13] T. M. Don, H. R. Chen, *Carbohydr. Polym.* **2005**, 61, 334.
- [14] N. Isiklan, F. Kursun, M. Inal, *J. Appl. Polym. Sci.* **2009**, 114, 40.
- [15] D. E. Discher, D. J. Mooney, P. W. Zandstra, *Science* **2009**, 324, 1673.
- [16] H. P. Tan, C. M. Ramirez, N. Miljkovic, H. Li, J. P. Rubin, K. G. Marra, *Biomaterials* **2009**, 30, 6844.
- [17] G. Yi, Y. Huang, F. Xiong, B. Liao, J. Yang, X. Chen, *J. Wuhan Univ. Technol., Mater. Sci. Ed.* **2011**, 26, 1073.
- [18] M. M. Soledad Lencina, A. E. Ciolino, N. A. Andreucetti, M. A. Villar, *Eur. Polym. J.* **2015**, 68, 641.
- [19] E. J. Sheehy, T. Mesallati, L. Kelly, T. Vinardell, C. T. Buckley, D. J. Kelly, *BioRes. Open Access* **2015**, 4, 229.
- [20] F. Wang, Z. Li, M. Khan, K. Tamama, P. Kuppusamy, W. R. Wagner, C. K. Sen, J. Guan, *Acta Biomater.* **2010**, 6, 1978.
- [21] H. V. Almeida, G. M. Cuniffe, T. Vinardell, C. T. Buckley, F. T. O'Brien, D. J. Kelly, *Adv. Healthcare Mater.* **2015**, 4, 1043.
- [22] O. Guillaume, A. Daly, K. Lennon, J. Gansau, S. F. Buckley, C. T. Buckley, *Acta Biomater.* **2014**, 10, 1985.
- [23] S. F. Carroll, C. T. Buckley, D. J. Kelly, *J. Biomech.* **2014**, 47, 2115.
- [24] N. Y. Ignateva, N. A. Danilov, S. V. Averkiev, M. V. Obrezkova, V. V. Lunin, E. N. Sobol, *J. Anal. Chem.* **2007**, 62, 51.
- [25] G. Sen, R. P. Singh, S. Pal, *J. Appl. Polym. Sci.* **2010**, 115, 63.
- [26] G. Lawrie, I. Keen, B. Drew, A. Chandler-Temple, L. Rintoul, P. Fredericks, *Biomacromolecules* **2007**, 8, 2533.
- [27] W. Yang, L. Zhang, L. Wu, J. Li, J. Wang, H. Jiang, Y. Li, *Carbohydr. Polym.* **2009**, 77, 331.
- [28] S. B. Lee, E. K. Park, Y. M. Lim, S. K. Cho, S. Y. Kim, Y. M. Lee, Y. C. Nho, *J. Appl. Polym. Sci.* **2006**, 100, 4439.
- [29] Z. Zhao, Z. Li, Q. Xia, H. Xi, Y. J. Lin, *Eur. Polym. J.* **2008**, 44, 1217.
- [30] C. Vasile, L. E. Nita, *Carbohydr. Polym.* **2011**, 86, 77.
- [31] V. Ozturk, O. Okay, *Polymer* **2002**, 43, 5017.
- [32] C. Karakasyan, S. Lack, F. Brunel, P. Maingault, D. Hourdet, *Biomacromolecules* **2008**, 9, 2419.
- [33] J. N. Hiremath, B. Vishalakshi, *Der. Pharma Chem.* **2012**, 4, 946.
- [34] J. M. Rosiak, F. Yoshii, *Nucl. Instrum. Methods Phys. Res., Sect. B* **1999**, 151, 56.
- [35] G. Crotts, T. G. Park, *J. Microencapsulation* **1998**, 15, 699.
- [36] K. Y. Lee, D. J. Mooney, *Prog. Polym. Sci.* **2012**, 37, 106.
- [37] A. A. Naddaf, I. Tsibranska, H.-J. Bart, *Chem. Eng. Process.* **2010**, 49, 581.
- [38] S. S. Huang, J. S. Huang, *J. Cell. Biochem.* **2005**, 96, 447.

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