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Synthesis and characterization of dual stimuli responsive macromers based on poly(N-isopropylacrylamide) and poly(vinylphosphonic acid)

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Abstract

Stimulus responsive materials hold great promise in biological applications as they can react to changes in physiological stimuli to produce a desired effect. Stimulus responsive macromers designed to respond to temperature changes at or around 37 °C and the presence of divalent cations were synthesized from N-isopropylacrylamide, pentaerythritol diacrylate monostearate, 2-hydroxyethyl acrylate, and vinylphosphonic acid by free radical polymerization. Monomers were incorporated into the macromers in ratios approximating the molar feed ratios, and macromers underwent thermogelation around normal body temperature (36.2 – 40.5 °C) as determined by rheology and differential scanning calorimetry. Macromers containing vinylphosphonic acid interacted with calcium ions in solution, displaying decreased sol-gel transition temperatures (27.6 – 34.4 °C in 100 mM CaCl₂), with decreases of greater magnitude observed for macromers with higher relative vinylphosphonic acid content. Critical micellar concentrations also decreased in a dose-dependent manner with increased vinylphosphonic acid incorporation in solutions with CaCl₂ but not in solutions with NaCl. These dually responsive macromers allow examination of the effect of increasing vinylphosphonic acid content in a macromer which holds promise in biological applications such as drug and cell delivery or tissue engineering due to the macromer responsiveness at physiological temperatures and concentrations of calcium.

Keywords

N-isopropylacrylamide; stimulus responsive; hydrogels; cell delivery

Introduction

The development of injectable biomaterials and similarly the identification of methods through which materials may form *in situ* are popular topics in materials science, particularly in the area of biomaterials. Injectable materials are attractive for biological applications as they can potentially be delivered using minimally invasive techniques, precisely fill complex three-dimensional voids in regenerative medicine applications without the need for extensive

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imaging and custom fabrication, and controllably deliver bioactive molecules and/or cells for therapeutic purposes.

Most research and development in the area of injectable biomaterials focuses on *in situ* formation via self-assembly^{1, 2}, phase separation³, or *in situ* polymerization and cross linking.⁴⁻⁸ Materials that undergo phase separation or self assemble in response to environmental cues or stimuli are promising for biological applications as the stimulus triggering the phase separation or assembly can be one normally encountered upon injection into living tissues, such as a temperature increase or pH change. Such methods for *in situ* formation may reduce or eliminate the need for radical initiators or crosslinking agents that may be cytotoxic at high concentrations,^{9, 10} potentially making these materials ideal for cellular encapsulation and delivery as well as alleviating potential damage to existing host tissues.

Biomaterials have recently been developed that respond to a large number of internal or externally applied environmental stimuli, including temperature,¹¹⁻¹³ pH,¹⁴ ionic strength,¹⁵ the presence of specific biological molecules,¹⁶⁻¹⁸ and magnetic field presence.¹⁹ Recent reviews are available describing the roles of these materials in scaffold fabrication,²⁰ drug delivery,²¹ and as tunable bio-interfaces.²²

In order to impart greater versatility or allow better control over the behavior of these “smart” biomaterials, materials have been developed that respond to multiple stimuli, such as both pH and temperature.²³⁻²⁷ The response to each stimuli may be different²⁸ or additive.²⁹

Materials that undergo tandem gelation via a stimulus responsive, physical mechanism, typically thermogelation, and an additional mechanism such as chemical crosslinking offer comparable versatility to dual stimuli responsive materials. Tandem gelling materials can offer the favorable, nearly instantaneous gelation kinetics of physical gelation coupled with the stability and irreversibility of chemical crosslinking.^{30, 31} These materials may be particularly attractive alternatives for applications where the environmental stimuli that triggers physical gelation is subject to variability, such as pH within a healing wound or fracture, which can be both variable and unpredictable.³²

We have previously synthesized and characterized a series of thermally and chemically gelable macromers based on poly(N-isopropylacrylamide) (pNIPAAm).³³ By copolymerizing N-isopropylacrylamide (NIPAAm) with other monomers to vary the amphiphilic structure of the resulting macromers, we were able to synthesize macromers with variable rheological properties and lower critical solution temperatures (LCSTs) around 37 °C. These macromers displayed less syneresis at body temperature than macromers that thermogelled at lower temperatures; however, chemical modification and subsequent chemical crosslinking was still necessary to produce stable gels.

The goal of the present study is to examine the effect of ionic interactions on the rheological and micellar behavior of pNIPAAm-based macromers similar to those previously synthesized and characterized in our laboratory by incorporating phosphonic acid moieties through copolymerization of NIPAAm, vinylphosphonic acid (VPA), and other monomers to modulate the amphiphilic structure of the macromers. A hydrophobic chain was incorporated through copolymerization with pentaerythritol diacrylate monostearate (PEDAS), a bifunctional monomer incorporating a stearic acid chain and two acrylic acids esterified to the biocompatible alcohol pentaerythritol. Hydroxyethylacrylate (HEA) was incorporated to allow for possible chemical modifications of the accessible hydroxyl groups and also to further balance the hydrophobicity of the macromers. The synthesis and characterization of macromers that thermogel near normal physiological temperature, as well as the interactions of these macromers with divalent ions in solution towards gel and micelle formation, is described.

Materials and Methods

Materials

Pentaerythritol diacrylate monostearate (PEDAS), N-isopropylacrylamide (NIPAAm), acrylamide (AAm), 2-hydroxyethyl acrylate (HEA), vinylphosphonic acid (VPA), and 2,2'-azobis(2-methylpropionitrile) (azobisisobutyronitrile, AIBN) were purchased from Sigma-Aldrich (Sigma, St. Louis, MO) and used as received. Analytical grade tetrahydrofuran (THF), diethyl ether, isopropyl alcohol, and acetone were purchased from Fisher Scientific (Pittsburgh, PA) and used as received.

Methods

Macromer synthesis—Macromers were synthesized using free radical polymerization (Scheme 1) as previously described.³³ Briefly, 3 g of PEDAS and amounts corresponding to the desired molar ratios of comonomers (NIPAAm, VPA, HEA, AAm) were dissolved in 250 mL THF at 60 °C under nitrogen and initiated with 1.5 mol % AIBN. Reactions proceeded under nitrogen for 18 h at 60 °C followed by an additional 1 h of reflux. Products were isolated by rotoevaporation, precipitation in ice cold diethyl ether followed by drying and a second dissolution in THF and precipitation in ice cold diethyl ether. Products were then vacuum dried at ambient temperature for 7 days before being dissolved in 200 mL isopropanol and dialyzed against isopropanol for 4 days in a 1,000 MWCO membrane (Spectra-Pore, Spectrum Laboratories, Rancho Dominguez, CA). Following dialysis, purified products were isolated through azeotropic distillation with acetone followed by precipitation in ice cold diethyl ether and finally followed by filtration and vacuum drying at ambient temperature for 7 days. A summary of the synthesized macromers is provided in Table 1.

Nuclear magnetic resonance—¹H and ³¹P NMR spectra were obtained using a 500 MHz spectrometer (Inova, Varian, Inc., Palo Alto, CA). Samples were dissolved at a concentration of 20 mg/mL in equal parts CDCl₃ and deuterated DMSO. Post-acquisition data analysis was performed using MestRe Nova software (Mestrelab Research S.L., Spain).

Osmometry—Freezing point osmometry was used to determine the number average molecular weight of the synthesized macromers. Macromers were dissolved at varying concentrations (0.5%-15% w/v) in ultrapure (Type I) water (Super Q, Millipore Corporation, Billerica, MA) and left overnight on an orbitally rotating platform. Samples were tested to determine which samples fell within the calibrated detection range of the osmometer (Osmette A, Precision Systems, Inc., Natick, MA), and osmometry measurements were performed on 1% w/v samples in triplicate.

Thermogravimetric analysis (TGA)—Thermal stability of the synthesized macromers was analyzed under a nitrogen atmosphere (80 mL/min) using a TA Instruments TGA Q50. Approximately 5 mg of vacuum dried sample was ground into a fine powder and evenly distributed over the surface of a platinum sampling pan (TA Instruments). Samples were heated to 100 °C and held at that temperature for 5 minutes to remove residual moisture, after which the samples were heated from 100 °C – 800 °C at a rate of 10 °C/min.

Rheology—For rheological characterization of the macromers, samples were dissolved in ultrapure water, and pH was adjusted to 7.4 using 0.5 N NaOH. CaCl₂, NaCl, MgCl₂, were added to samples at varying (CaCl₂ and MgCl₂: 2.5 mM, 100 mM, NaCl: 5 mM, 200 mM) concentrations. Sample concentration was finally adjusted to 7.5% w/v. All samples were left overnight on an orbitally rotating platform to ensure complete dissolution.

Measurements were performed using a thermostatted, oscillating rheometer (Rheolyst AR1000, TA Instruments, New Castle, DE) equipped with a 6 cm steel cone geometry (1°). Gelation properties and transition temperatures were determined as previously described.³³ Briefly, samples were loaded onto the rheometer and cooled to 5 °C, presheared for 1 min (rate 1 s⁻¹), and equilibrated for 15 minutes before a temperature sweep was performed from 5-60 °C at a rate of 1 °C/minute and a displacement of 1 × 10⁻⁴ rad. All formulations were tested in triplicate, and analysis was performed using the manufacturer's supplied software (TA Data Analysis, TA Instruments).

Differential scanning calorimetry (DSC)—Transition temperatures were also determined by DSC. Samples were prepared in the same manner as performed for rheological characterization (7.5% w/v, pH 7.4), and 20 µL of each sample were pipetted into an aluminum sample pan (TA Instruments, Newcastle, DE) that was then hermetically sealed. Experiments were performed in triplicate on a TA Instruments DSC 2920 with a refrigerated cooling system. Samples were equilibrated at 5 °C for 10 minutes and then heated to 60 °C at a rate of 5 °C/minute.

Calcium-binding assay—Samples were prepared in triplicate in ultrapure water (7.5% w/v, pH 7.4) augmented with CaCl₂ at concentrations of 0.01 mM, 0.1 mM, 1 mM, 2.5 mM, 4.0 mM, 10 mM, 25 mM, 100 mM, and 1000 mM and stored for 24h at 37 °C on a shaker table. After 24 h, the supernatant was removed, cooled to room temperature, and analyzed for residual calcium ions using a colorimetric calcium assay (Arsenazo III, Genzyme Diagnostics, Framingham, MA). Samples were diluted to within the assay's linear range (≤ 5.0 mM), and sample concentration was determined by comparison to a standard curve using an absorbance microplate reader (650 nm, FLx800, Bio-Tek Instruments, Winooski, VT).

Dynamic light scattering (DLS)—Dynamic light scattering (DLS) characterization of macromers in aqueous solution was performed using a 90PLUS particle size analyzer (Brookhaven Instruments Corp, Holtsville, NY) operating at 659 nm wavelength. Dilute macromer samples (0.01%, 0.10%, 0.25%, 0.5%, 1.0%, 2.5% w/v) were prepared in freshly filtered (0.22 µm, Millipore Corporation) ultrapure water with 0, 100 µM, 500 µM, 2.5 mM, 4.0 mM, 10 mM, or 100 mM of CaCl₂ or control solutions with double the CaCl₂ concentration of NaCl (200 µM, 1 mM, 5 mM, etc.) to better elucidate effects due to calcium ions rather than chloride ions in solution. Prepared samples were left on an orbitally rotating platform overnight before sampling. DLS was performed on samples at 25 °C and 37 °C in triplicate; each sample was allowed to temperature equilibrate for 15 min prior to measurement. Intensity weighted size (R_h) distributions were determined from the autocorrelation function through the inverse Laplace transform non-negatively constrained least squares (CONTIN, regularized) algorithm.³⁴

Statistical analyses—Statistical analysis was performed between groups for rheological and DSC characterization of transition temperature, TGA of residual sample weight percentages, and calcium binding assays. Analyses of variance were performed using Minitab® statistical software (Minitab, Inc., State College, PA) and an *a priori* significance level of 0.05. Pairwise comparisons were performed using Tukey's HSD test. Where applicable, data are presented as mean ± standard deviation.

Results and Discussion

Cell and drug delivery strategies may be central to the advancement of many areas of medicine, and, in particular for tissue engineering applications. Previously, we synthesized and characterized amphiphilic thermogelling macromers based on pNIPAAm and PEDAS for potential applications in cellular delivery for bone tissue engineering.³³ The copolymerization

of NIPAAm with hydrophobic (PEDAS) and hydrophilic (AAm, HEA) monomers allowed tuning of the transition temperature to 37 °C and it was thought that the inclusion of an aliphatic pendant chain might increase cohesive interactions and thus the mechanical strength of the macromer. In the present study, VPA was used to modify the macromers to potentially allow further mechanical stabilization due to ionic crosslinking, making them more suitable matrices for progenitor cell delivery in bone tissue engineering applications.³⁵ Furthermore, the interaction of VPA with calcium was thought to potentially increase local calcium concentrations, which can be beneficial for the differentiation and mineralization of osteoblasts.^{36, 37}

Macromer synthesis and structural characterization

Macromers were synthesized as shown in Table 1. Relative molar contents of the macromers were calculated from ¹H NMR spectra (Figure 1A) and correlated well to the theoretical and actual comonomer feed ratios. The peak at 0.85 ppm was assigned to the methyl protons of PEDAS, peaks at 1.1 and 1.2 ppm were assigned to the methyl protons contributed by NIPAAm and 28 of the 32 methylene protons of the stearate chain of PEDAS, respectively, and the peak at 3.7 ppm was assigned to the backbone methine proton of VPA. A solvent (CD₃(SO)) peak is visible at 2.5 ppm in all spectra. ³¹P NMR (Figure 1B) also confirms the presence of the phosphorous in polymerized VPA at 30 ppm, close to previously reported values.³⁸ VPA monomer impurities, previously reported to appear between 16.0-18.6 ppm on ³¹P NMR,^{38, 39} were not observed after dialysis.

Number average molecular weights as determined by freezing point depression osmometry are reported in Table 1. Consistent decreases in Mn were observed with increasing VPA in the monomer feed and subsequently in the synthesized macromers; however, the comonomer ratios of the synthesized macromers approximated the feed ratios well despite the slight variability in Mn. These values are within the range of molecular weights for similarly synthesized macromers containing PEDAS and NIPAAm as reported by Hacker et al.³³

TGA curves (Figure 2A) show 60-80% thermal decomposition of all macromers by 500 °C. Differential TGA curves (Figure 2B) show a left shoulder followed by a peak at around 430 °C for the low and medium VPA containing macromers while the high VPA containing macromer shows two distinct peaks at 310 °C and 380 °C along with a broad right shoulder extending from 410 °C to 500 °C. Pure pNIPAAm has previously been shown to thermally decompose at 422 °C; however, when copolymerized at high molar ratios with other monomers, the decomposition temperature of pNIPAAm falls.⁴⁰ The TGA curves for the control macromer demonstrate this effect. The different character of the differential TGA curve for the high VPA containing macromer is likely due to a similar decrease in the decomposition temperature of pNIPAAm due to the relatively high NIPAAm composition of this formulation combined with cleavage of the more abundant carbon-phosphorus bond of polymerized VPA.⁴¹ The left peak is likely due to the loss of water evolved during the condensation of phosphonic acid moieties and potentially ethylene evolution from any residual monomer.⁴²

Figure 3 shows the average residual weight percentages at 800 °C for all VPA-containing macromers and the control macromer. Poly(VPA) or copolymers containing VPA or other pendant phosphorous moieties typically leave a nonvolatile residue of approaching 40 wt% when degraded in nitrogen.^{42, 43} The residual weight percentages for each synthesized macromer was significantly different from that of all the other synthesized macromers (*p* < 0.05) and the relative differences between the groups correlated well to the relative differences in VPA molar ratios between the macromers, further verifying the incorporation of the VPA and its effect on macromer functional character despite the relatively low amounts of VPA present. The residue most likely results from the formation of a carbon-based radical upon the cleavage of the carbon-phosphorous bond, which then degrades to form polyacetylene and later

a graphitic char that, together with phosphorous oxide likely forms the residue seen in increasing amounts with increasing VPA.⁴²

Functional characterization

Thermogelation by rheology and DSC—Rheology and DSC were used to characterize the transition temperatures of the synthesized macromers. For rheological characterization, the gelation temperature, denoted T_δ , was considered to be the inflection point of the phase angle (δ) curve. In general, below this inflection point the complex viscosity for each macromer remained below 0.1 Pa·s and then increased rapidly by at around 2 orders of magnitude at temperatures above T_δ . Figure 4 shows representative rheological traces of a 7.5% w/v aqueous macromer solution of VPA_H, as well as the effect of the presence of both divalent (Ca^{++}) and monovalent (Na^+) ions on the complex viscosity and phase angle during temperature sweeps.

Using DSC, the transition temperature (T_{DSC}) of the macromers was defined as the onset at inflection of the endothermic peak in the DSC thermogram. Transition temperatures for the synthesized macromers from both DSC (in water only) and rheological experiments are shown in Figure 5. Statistically significant differences ($p < 0.05$) were found for the T_δ of all three VPA-containing macromers in water when pairwise comparisons were performed.

Furthermore, for the VPA_H macromer, increasing concentrations of CaCl_2 led to significant decreases in T_δ when compared to the same macromer (VPA_H) in water alone and also when compared to similar solutions of VPA_M. (i.e. T_δ for VPA_H in 2.5 mM and 100 mM CaCl_2 were significantly different from T_δ for VPA_M in 2.5 mM and 100 mM CaCl_2 when compared at the same CaCl_2 concentration). As seen in Figure 4, increasing concentrations of CaCl_2 changed the shape of rheograms for macromers containing VPA. With 2.5 mM CaCl_2 , the shape of the complex viscosity curve remained the same as the curves generated in control and NaCl augmented solutions with primarily a left shift of the curve to lower temperatures. In 100 mM CaCl_2 solution, however, the shape of the curve changed markedly at temperatures above T_δ . It is likely that the high amount of free calcium relative to the VPA content of the macromer allows for more complete ionic crosslinking of the macromer with faster dynamic exchange of the free and bound calcium (due to both the higher salt concentration and high amount of VPA in the macromer) such that when the NIPAAm domains collapse, the cohesive interactions between the now hydrophobic moieties are increased due to the formation of a more tightly bound network from ionic interactions. These data correlate with Figure 5 as well, where for all VPA containing macromers there is a decrease in T_δ with increasing concentrations of CaCl_2 . T_{DSC} values for the VPA_H macromer were significantly different from the low and medium VPA-containing macromers in water, and all three VPA-containing macromers had significantly lower T_{DSC} than the control macromer, which was synthesized using AAm instead of VPA. Pendant phosphonic acid moieties likely had an incomplete interaction with water due to intra- or intermolecular hydrogen bond formation among the charged residues, resulting in a decrease in T_{DSC} relative to the expected increase due to more complete interaction of the macromer with water and thus delayed hydrophobic collapse of NIPAAm groups seen with most hydrophilic monomer incorporation, including AAm in a similar system.³³ Significant differences for T_δ of the Control_AAm macromer were also found in the presence of CaCl_2 when compared to all of the VPA-containing macromers. At high concentrations (100 mM) of CaCl_2 , there were significant differences in the T_δ of the Control_AAm compared to the same Control_AAm macromer at lower (2.5 mM) salt concentrations. There were significant differences in the T_δ of all VPA-containing macromers in 100 mM CaCl_2 when compared to the T_δ of the same macromers in 200 mM NaCl. There was, however, no significant difference between T_δ in the presence of 100 mM CaCl_2 or 200 mM NaCl for the control macromer. Changes due to the presence of MgCl_2 compared to equimolar concentrations to CaCl_2 were not significant (not shown). Additionally, no differences in T_{DSC} were found in the presence of any salt solutions when compared to the

macromer T_{DSC} in water alone (not shown). It is important to note that due to the design of these macromers for biological and specifically cell delivery applications, all macromer solutions were characterized at pH 7.4. Adjustment of the pH with NaOH thus added slight variability in solution osmolarity between the VPA-containing macromers; however, the amount of salt contained in the tested solutions was greater than that used for pH adjustment.

Previous studies have shown that salts tend to decrease the LCST of pNIPAAm-based materials based on the concentration of the salt and following the trend of the Hofmeister series.^{44, 45} While these and other studies have shown that the DSC-determined transition temperature of the materials does change due to a salting out phenomenon where salts tend to affect hydrogen bonding of the material as well as hydrophobic and ionic interactions between the material and surrounding water, altering the solubility of the amide groups and making the hydrophobic collapse of these groups entropically favorable at lower temperatures, the concentrations of salt used to elicit these changes (0.5 – 2.5 M NaCl) are far higher than those used in the current study.⁴⁶ Furthermore, the changes in transition temperature observed with DSC often differ with those reported using other methods.^{47, 48} In the present study, decreases in T_{DSC} were not found in the presence of relatively low concentrations of salt; however, similar concentrations of salts containing divalent cations resulted in changes in sol-gel transition temperature (T_g) when detected using rheology. Considered along with the lack of change in T_{DSC} , this more functional measurement of material gelation and the fact that equivalent salt concentrations with monovalent cations did not change the gelation temperature indicates that ionic crosslinking of macromer strands is likely responsible for this behavior rather than a salting out phenomenon. The transition is still primarily due to the hydrophobic collapse of the NIPAAm moieties once enough entropy is introduced into the system such that aggregation of the NIPAAm domains is more favorable than hydration, but ionic crosslinking likely gives the material the stability to meet the criteria for rheological gelation at a lower temperature than that which is detected calorimetrically. Fine-tuning of the LCST via the addition of salts or through the fabrication of materials that will interact with salts *in situ* could prove to be a useful technique for biomaterial applications. Gan *et al.* recently characterized microgel system of copolymerized NIPAAm and 2-hydroxyethyl methacrylate in a 7:3 ratio that required the presence of $CaCl_2$ or sufficiently high concentrations of NaCl for the formation of a gel with increasing temperatures.⁴⁹ This is likely due to the purely hydrophilic nature of the 2-hydroxyethyl methacrylate comonomer, which allows rearrangement of the colloidal dispersion at high temperatures such that the collapsed NIPAAm domains arrange in the core of microgel particles, minimizing cohesive interactions between particles that would lead to gel formation.

Previous work investigating the sol-gel transition of thermogelling copolymers of VPA and NIPAAm focused on copolymers synthesized from monomer feeds with a NIPAAm:VPA ratio of at least 9:1.^{50, 51} Comparing the sol-gel transition temperatures reported in the present study to those previously reported, much greater amounts of calcium ions were needed to elicit similar changes in the sol-gel transition temperature than those found using the current formulations, which have a much lower ratio of NIPAAm to other monomers. Although the magnitude of the sol-gel transition, with respect to viscosity increase, may be diminished due to the lower relative amount of NIPAAm, the proportionally higher amount of VPA enables larger changes in the LCST to occur at physiologically relevant calcium concentrations that should not be toxic to encapsulated cells or cells present at a delivery site. Although long-term studies on gel stability were not performed, the increases in complex viscosity observed with increasing temperature in the presence of levels of free calcium expected to be encountered *in vivo* would be appropriate for localized, cell delivery applications; however, the mechanical properties and long term stability conferred through physical crosslinking alone may not be suitable for structural support of cells or an extracellular matrix under load bearing conditions.

The pendant stearic acid chain of PEDAS likely contributes to this effect as well. Previous work has shown that for hydrogels with long pendant hydrophobic chains ($R > C_8$) binding and mixed micelle formation occurs with surfactant molecules in a relatively concentration independent manner.⁵² Additionally, cohesive associations between hydrophobic pendant groups have been shown to increase the mechanical toughness of hydrogels.⁵³ From a design standpoint, PEDAS was included in the macromers for this reason, as for potentially mineralizable hydrogels to have a role in hard tissue applications such as bone tissue engineering, they will need to have a greater mechanical toughness and compressive properties than typically achieved with hydrogels. Thus a combination of the presence of the stearic acid chain of PEDAS and the relatively high VPA content of the macromers may explain the observed rheological changes at low salt concentrations relative to previous studies. Furthermore, the presence of additional functional moieties such as the hydroxyl group of HEA potentially allow for these macromers to be acrylated or methacrylated and then chemically crosslinked with increasing temperature. Such reactions have been carried out on similar macromers while not compromising the cytocompatibility of the macromers.⁵⁴ Such covalent crosslinking could strengthen the gels at temperatures above the sol-gel transition temperature beyond what might be achieved by increasing the relative content of NIPAAm.

Calcium-binding assay—Macromer binding or complexation of free calcium ions in solution was measured by determining the concentration of free calcium ions in the supernatant of 7.5% w/v samples of each macromer after 24 h incubation. Data are presented (Figure 6) as the absolute millimoles of calcium ion bound per 1 gram of macromer in solution. Statistically significant differences in calcium binding were present for all VPA-containing macromers at and above concentrations of 10 mM CaCl_2 . Calcium binding for the Control_AAm macromer was significantly different from all macromers at concentrations of CaCl_2 greater than and equal to 1 mM, except for formulations in 1000 mM CaCl_2 solutions, where the binding was not significantly different from the VPA_M group. In general, the relative calcium bound by the VPA-containing macromers at higher concentrations of calcium, possibly indicating saturation, was similar to the relative VPA-content of each macromer. Increases in the bound Ca^{++} were observed for the Control_AAm macromers with increasing CaCl_2 concentrations; however, given the rheological behavior of these macromers, these are thought to be nonspecific interactions such as entrapment in micelles or other salted out colloids, which may similarly be contributing to the interaction of VPA-containing macromers with calcium.

The ability of these materials to bind or complex calcium as described in this assay could potentially prove useful in a number of biological applications. Surface induced mineralization of a number of substrates has been demonstrated previously through surface modification with nucleating proteins or moieties that mimic such proteins has been demonstrated as a way to potentially increase orthopaedic implant adhesion to bone or to create a synthetic bone analogue.⁵⁵⁻⁵⁷ Many of these natural nucleators contain phosphoserines, and, when dephosphorylated or degraded lose their ability to interact with apatite mineral, leading to decreased calcification of the organic matrices to which they are associated and indicating the key role of these moieties in mineralization.⁵⁸ A number of strategies can be used to mineralize a synthetic matrix,⁵⁹ including modification with phosphoserine itself.⁶⁰ Schneiders *et al.* modified hydroxyapatite/collagen composite bone cements with phosphoserine and after implanting the composites into a bone defect within a rat tibia found that new bone formation, particularly in the early phases of healing, was significantly greater in defects implanted with the phosphoserine modified composites compared to unmodified composites.⁶¹

Phosphonic acid, the structure of which is similar to that of the phosphoserines found in many nucleators, has previously been used in biomaterials applications to improve the adhesion of dental cements to the mineralized components of teeth.^{38, 62, 63} Also used as in chelation applications for divalent metal ions, including calcium, the mechanism by which phosphonic

acid binds divalent metal ions is not known; however, all likely mechanisms involve ionic binding of the cation by a charged P-O^- group of the deprotonated phosphonic acid.⁶⁴ Copolymers of VPA have been shown to mineralize *in vitro* over 1 month in a solution super saturated with calcium phosphate.⁶⁵ Copolymers of VPA and AAm have also been shown to increase attachment and proliferation of fibroblasts and osteoblast like cells.^{66, 67} The interactions shown between the macromers synthesized in the present study and calcium ions similarly reflect the ability of phosphonic acid containing materials to interact with cations such as those found in the inorganic matrix of bone. In the present study, the presence of phosphonic acid along with calcium ions at concentrations similar to those found in plasma significantly affect the sol-gel transition of the material in a dose dependent manner, thereby facilitating additional control over the handling and delivery of such macromers in addition to the previously shown effect on mineralization and cell-material interactions. Long-term mineralization and the stability of the matrix due to ionic crosslinking will, however, likely be affected by the dynamic equilibrium between free and bound calcium ions.

Dynamic light scattering—DLS experiments were used to characterize the behavior of dilute concentrations of macromers in aqueous solutions. The approximate critical micellar concentration (CMC), reported in Table 1, was determined by analyzing the size distributions of macromers over a range of concentrations and identifying the concentration at which there was a consistent increase in the peak particle size. Additionally, the effect of calcium ions on macromer conformation was analyzed through the addition of CaCl_2 . Representative size (R_h) distributions are shown in Figure 7. Control solutions containing NaCl were also examined at twice the molar concentration as the tested CaCl_2 solutions to clearly elucidate the effect of divalent ions versus monovalent (Na^+ , Cl^-) ions.

In general, the CMC decreased with increasing VPA content. At 25 °C, the presence of calcium ions led to the disruption or prevented the formation of micelles for the experimental macromers at concentrations above the CMC but did not affect micelle formation of a control macromer that did not contain VPA (Figure 7). Increasing VPA content also led to decreased calcium sensitivity with respect to micelle formation, evidenced by lower concentrations of calcium eliciting a greater effect on the size distribution of macromers with low VPA content (not shown). For example, at 25 °C a 500 μM CaCl_2 solution consistently shifted the R_h peak intensity of the macromer containing the lowest relative VPA (VPA_L) content back to the radius observed at concentrations below the CMC, while for the macromer containing the highest relative amount of VPA (VPA_H), similar changes were only observed in solutions containing greater than 500 μM CaCl_2 . This is potentially due in part to the larger size of macromers containing lower VPA content, as intramolecular salt bridging may be more likely in these macromers than for smaller VPA_H chains. Additionally, in the VPA_H chains, interactions of hydrophilic charged moieties with surrounding water are not stabilized in the presence of CaCl_2 relative to VPA_L or VPA_M macromers, resulting in a greater propensity to form micelles to shield hydrophobic moieties from water, as reflected in the general trend for the CMC.

Similar changes in the presence of calcium salts were previously observed for dispersions of poly(1,3-butadiene-*co*-methacrylic acid).⁶⁸ In solutions with high enough pH that RCOO^- groups were present, ionic crosslinking of with calcium ions prevented particle aggregation and swelling. In the present study, calcium ions were needed to prevent or disrupt micelle formation; however, NaCl did not have the same effect (not shown). NaCl decreased the CMC of all tested formulations, probably due to charge stabilization and reduced repulsion within or between macromers,⁶⁹ whereas calcium ions may have facilitated intramacromer or intermacromer ionic crosslinking or, as a divalent ion in excess, may not have resulted in charge stabilization in binding to single phosphonic acid moieties.

At 37 °C, all macromer formulations showed peak R_h greater than three times those observed above the CMC at 25 °C (Figure 7C). This is likely due to micellar aggregation as the stearate chains of PEDAS probably still reside in the core of the micelles, thus as NIPAAm undergoes hydrophobic collapse, stabilization of the hydrophobic moieties located on the shell of the micelles is likely achieved through aggregation in a manner similar to that observed in previously studied PNIPAAm-based polymers.⁷⁰⁻⁷² Below the CMC as determined at 25 °C, macromers tended to form micelles or aggregates upon temperature increases above 37 °C. The addition of NaCl and CaCl₂ did not have a noticeable effect on the peak R_h for macromers at 37 °C, as aggregation following hydrophobic collapse of the NIPAAm moieties likely drives micelle formation or chain aggregation rather than charge stabilization or ionic crosslinking, although when taken over a series of temperatures it is likely that the addition of salts would lead to a more discrete point at which micelles aggregate.⁷³ Similarly, it is important to note that while 37 °C is below the LCST reported for some of the macromer formulations and conditions tested, R_h representing micellar aggregates were uniformly seen as the dominant species; however, the intensity weighted CONTIN method used to analyze the DLS exaggerates the proportion of large particles, thus for these samples, micellar aggregates may not have been the dominant species.⁷⁴

Polymeric micelles that can serve as nanocarriers for drug delivery are currently being investigated for a number of applications, including intracellular drug delivery.⁷⁵⁻⁷⁷ Micelles that respond to environmental stimuli present within pathological cell populations, such as decreased pH within cancerous tissue, or within specific intracellular domains, have been proposed as potential delivery agents whereby disruption or rearrangement of the micelle conformation could result in environmentally-cued drug release.⁷⁸ In the current study, VPA-containing thermosensitive macromers did not form micelles in the presence of calcium ions below the LCST of the materials. Modifications such that the materials gel above body temperature or do not gel could enhance the potential for use of these materials in drug delivery applications to specific sites with higher local calcium content than surrounding environments, such as the lysosome.⁷⁹

Conclusions

In conclusion, thermosensitive macromers based on PNIPAAm and other functional monomers, including VPA, were synthesized and characterized with an end goal of creating functional, environmentally responsive materials for biological applications. Because of this, macromers were characterized over temperatures ranging from room temperature to body temperature at physiological pH. The synthesized macromers incorporated the monomers in ratios close to those of the molar feed ratios and underwent a sol-gel transition close to body temperature. Due to the addition of vinylphosphonic acid, the macromers interacted with calcium ions in solution and underwent ionic crosslinking and subsequent decreases in the sol-gel transition temperatures at physiologically relevant calcium ion concentrations. This effect was dependent on the relative amount of vinylphosphonic acid incorporated in the macromer. At temperatures below the transition temperature, calcium ions prevented or disrupted micelle formation while at temperatures above the transition temperature, hydrophobic interactions promoted aggregation of macromer micelles. Based on previous efforts in this area and the results presented, these macromers hold potential in drug and cell delivery applications as well as bone tissue engineering applications.

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Figure 1.

(A) ^1H -NMR of the three experimental macromers of the form poly(PEDAS₁-*stat*-VPA _{n} -*stat*-HEA_{4.5- n} -*stat*-NIPAAm_{10.5}) where the spectra represent: (1) VPA_H ($n=3.375$), (2) VPA_M ($n=2.25$), and (3) VPA_L ($n=1.125$).

(B) Representative ^{31}P -NMR of VPA_M verifying the presence of polymerized VPA in the macromer.

Figure 2.
(A) TGA curve showing the degradation of the three experimental macromers (--- VPA_H, - · - VPA_M, - VPA_L, ... Control_AAm) thermally degraded at a rate of 10 °C/min in a nitrogen atmosphere. (B) Differential % weight loss as a function of increasing temperature.

Figure 3. Residual weight % (n=3, means $\pm$ SD) remaining after thermal degradation of macromer samples from 100 °C – 800 °C.

Figure 4.

(A) Representative complex viscosity ($|\eta^*|$) rheograms of VPA_H. (B) Representative phase angle changes of VPA_H in aqueous solutions with and without added salts. Rheograms were obtained by increasing the temperature at a rate of 1 °C/minute and with a constant displacement of 1×10^{-4} rad.

Figure 5. Gelation temperatures ($n=3$, means $\pm$ SD) or LCST as determined by rheology or DSC for all VPA-containing macromers and the control macromer.

Figure 6.

Calcium binding or complexation for macromers was measured spectrophotometrically using a colorimetric calcium assay. The millimoles Ca^{++} bound per gram of macromers (inset depicts binding at low CaCl_2 concentrations) is shown for macromers dissolved at 7.5 % w/v in H_2O . Data points and error bars represent means $\pm$ SD (n=3).

A

B

C

Figure 7.

Representative hydrodynamic radius plots of macromers (A) VPA_M at 25 °C, (B) Control_AAm at 25 °C, and (C) VPA_M at 37 °C at different concentrations in water and in aqueous solutions with 2.5 mM CaCl₂.

Scheme 1.

Simplified schematic of the radical copolymerization of experimental macromers from pentaerthritol diacrylate monostearate (PEDAS), N-isopropylacrylamide (NIPAAm), 2-hydroxyethyl acrylate (HEA), and vinylphosphonic acid (VPA). Δ indicates possible branching sites in the highly simplified schematic of the branched statistical copolymer structure.

Table 1

Compositions, Molecular Weights, and Characteristics of Synthesized Macromers

designation	theoretical composition	actual comonomer feed [mol] (based on 1 mol PEDAS)	¹ H-NMR results (based on 1 mol PEDAS)	molecular weight	critical micellar concentration
	PEDAS/VPA/HEA/NIPAAm/AAm	VPA/HEA/NIPAAm/AAm	VPA/HEA/NIPAAm/AAm	M _n , [Da]	wt. %, log ₁₀
VPA_L	1/1.125/3.375/10.5/0	1.14/3.38/10.50/0	0.9/4.6/11.4/0	1810	-0.3
VPA_M	1/2.25/2.25/10.5/0	2.25/2.25/10.51/0	1.8/2.7/10.3/0	1790	-0.6
VPA_H	1/3.375/1.125/10.5/0	3.40/1.14/10.51	2.9/1.6/12.1/0	1340	-1
Control_Aam	1/0/3/14/3	0/3.07/14.03/3.01	0/3.2/15.3/1.9	2410	0