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Monitoring the Erosion of Hydrolytically-Degradable Nanogels via Multiangle Light Scattering Coupled to Asymmetrical Flow Field-Flow Fractionation

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Abstract

We describe the synthesis and characterization of degradable nanogels that display bulk erosion under physiologic conditions (pH = 7.4, 37 °C). Erodible poly(*N*-isopropylmethacrylamide) nanogels were synthesized by copolymerization with *N,O*-(dimethacryloyl)hydroxylamine, a cross-linker previously used in the preparation of non-toxic and biodegradable bulk hydrogels. To monitor particle degradation, we employed multiangle light scattering and differential refractometry detection following asymmetrical flow field-flow fractionation. This approach allowed the detection of changes in nanogel molar mass and topology as a function of both temperature and pH. Particle erosion was evident from both an increase in nanogel swelling and a decrease in scattering intensity as a function of time. Following these analyses, the samples were recovered for subsequent characterization by direct particle tracking, which yields hydrodynamic size measurements and enables number density determination. Additionally, we confirmed the conservation of nanogel stimuli-responsivity through turbidity measurements. Thus, we have demonstrated the synthesis of degradable nanogels that erode under conditions and on timescales that are relevant for many drug delivery applications. The combined separation and light scattering detection method is demonstrated to be a versatile means to monitor erosion and should also find applicability in the characterization of other degradable particle constructs.

Keywords

Nanogel; Asymmetrical Flow Field-Flow Fractionation; Static light scattering; Multi-Angle Light Scattering; Particle Tracking; Polymer Degradation; pNIPMAm; DMHA

INTRODUCTION

Nano-sized hydrogels (nanogels) have emerged as a unique class of drug delivery vehicles that may be applicable as injectable formulations. These particles share many characteristics with bulk synthetic hydrogels, yet are capable of intravenous administration as colloidal dispersions, making them attractive candidates for targeted drug delivery applications. For example, nanogels are well-hydrated,¹ have demonstrated non-fouling characteristics in serum,^{2,3} and are capable of encapsulating, retaining, and releasing macromolecules from their porous polymer network.^{4–8} As a result, efforts to apply nanogels as drug delivery vehicles have been

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SUPPORTING INFORMATION

Supplementary light scattering and DMHA characterization data (¹H NMR and Elemental Analysis) are available in the Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

increasing in recent years.^{9–11} In the context of cancer treatment, particulate delivery systems are designed to alleviate the pharmacological deficiencies of modern chemotherapeutic drugs, such as poor water solubility, rapid clearance from circulation, low bioavailability, and inefficient cell entry. By assisting in the intravenous transport, accumulation, and uptake by malignant cells, the therapeutic efficacy of drugs may be improved. Furthermore, the toxic side-effects commonly observed in most chemotherapy treatments may be reduced or eliminated by avoiding accumulation in healthy tissues.

Recently, we described the efficient and functional delivery of macromolecular therapeutics (small interfering RNA, siRNA) to ovarian cancer cells.¹¹ Nanogels composed of poly(*N*-isopropylmethacrylamide) (pNIPMAm) were demonstrated in that work to be non-toxic and able to efficiently encapsulate, retain and deliver functional siRNA upon uptake by cancer cells. However, bioaccumulation and nonspecific organ filtration is a concern for colloids designed for intravenous delivery. Degradation into lower molecular weight components may improve clearance by renal filtration, and reduce undesired accumulation within the patient. Renal clearance is considered the most efficient route of nanoparticle excretion, in comparison to liver sequestration and subsequent reticuloendothelial macrophage uptake and hepatobiliary excretion into the intestine.¹² In addition to clearance, erosion may modulate the release of encapsulated drugs through network decomposition.¹³

The design of erodible, responsive nanogels is a challenging task, wherein stimulus-sensitivity and degradability are sought simultaneously in the same particle architecture. Although a variety of erodible polymer colloids have been investigated, including lactic and glycolic-acid based polymers,^{14,15} poly(cyanoacrylate) (PCA),¹⁶ poly(ϵ -caprolactone) (PCL),¹⁷ and poly(alkylcyanoacrylate) (PACA),¹⁸ we and others have found cross-link scission to be a successful strategy for making erodible poly(alkylacrylamide) nanogels.^{19,20} We hypothesized that incorporation of a degradable cross-linker, such as *N,O*-(dimethacryloyl)hydroxylamine (DMHA), would result in degradable nanogels *via* cross-link cleavage. The DMHA cross-linker has been successfully applied in multiple hydrogel biomaterials, including degradable embryonic stem-cell supports,²¹ *in vitro* and *in vivo* drug release devices,^{22,23} and polymer scaffolds in spinal cord regeneration.²⁴ Use of DMHA is attractive since the cross-linker is stable in acidic environments (pH < 5), is easy to synthesize, decomposes under physiologic conditions, and has low *in vivo* toxicity.²⁴

Polymer degradation is likely to induce changes in colloidal properties. Thus, methods to monitor particle behavior during the erosion process are critical in the design of degradable nanogels. These measurements must be made in complex mixtures (e.g. in the presence of degradation products or in the presence of serum components) while assessing multiple colloidal properties (size, molar mass, number density) in solution. To accomplish these goals, we have developed methods using asymmetrical flow field-flow fractionation separation, coupled with multiangle light scattering detection and differential refractometry (asymmetrical flow FFF/MALS/dRI). Asymmetrical flow FFF is a one-phase, non-destructive technique that permits the separation of a wide range of colloid and polymer sizes and molar masses.^{25–27} The method is particularly enabling when coupled on-line to characterization tools such as MALS and dRI, which allow the determination of the weight-average molar mass (M_w), the root mean square radius (r_{rms}), and the distribution of these values within the sample without the need to calibrate against standards.²⁶ Together, these coupled methods permit the separation of complex colloid/polymer mixtures, followed by direct determination of particle size, molar mass, and when further combined with additional tools such as dynamic light scattering, morphological characterization.

Asymmetrical flow FFF is frequently used in the high-resolution separation of nanoparticles and proteins,^{26,28–32} with the theory of its operation being extensively described elsewhere.

^{25,33} In brief, laminar flow of eluent through a thin separation channel results in a parabolic flow velocity.³⁴ For asymmetrical flow FFF, the field is generated by pumping eluent through a permeable lower plate, which is covered by an ultrafiltration membrane.²⁵ This applied field-flow (cross-flow) is perpendicular to channel flow, driving injected particles towards the membrane (accumulation wall). In a typical separation scheme, particles are constantly driven towards the accumulation wall by the cross-flow while thermal motion of the particles (i.e. Brownian motion) counteracts the field. As elution proceeds, smaller particles assume a higher mean steady-state distance from the wall, and thus elute at a faster rate.²⁶ In contrast, larger particles (with lower diffusion coefficients) occupy regions closer to the accumulation wall and show greater retention.

The eluting particles may be conveniently detected by MALS, which is a powerful means to assess both the size and solution-average molar mass for a variety of macromolecules and nanoparticles. MALS instruments provide for the simultaneous detection of scattered light from several angles. For macromolecules in a size regime greater than 1/20 the wavelength of incident light, the r_{rms} may be derived from the angular dependence of scattering intensity.³⁵ By on-line coupling to a concentration detector (e.g. differential refractometer, dRI), the M_w may be derived from the Debye plot, constructed using the Debye extrapolation method.³⁶

Asymmetrical flow FFF/MALS has been a particularly enabling technique in the measurement of r_{rms} distributions for hydrogel nanoparticles (nanogels).³² This stationary phase-free system allows rapid separations without the application of destructive shear forces observed in more traditional chromatographic systems. Through a separation technique like asymmetrical flow FFF, light scattering analysis on complex colloid/polymer mixtures can be performed since lower molecular weight polymers are separated from the colloid of interest, thereby providing interpretable MALS data. Additionally, eluting particles may be recovered in a fractionated manner for subsequent analysis. In this investigation, we demonstrate an asymmetrical flow FFF/MALS/dRI method to monitor the erosion of pNIPMAm-DMHA nanogels, where multiple colloidal properties can be monitored throughout particle degradation, providing insight into the erosion process.

MATERIALS AND METHODS

Chemicals and Nanogel Synthesis

All reagents were purchased from Sigma-Aldrich (St Louis, MO) and used as received, unless otherwise noted. The monomer *N*-isopropylmethacrylamide (NIPMAm) was twice recrystallized from hexanes (VWR international, West Chester, PA) and dried *in vacuo* prior to use. Reagents sodium dodecyl sulfate (SDS) and ammonium persulfate (APS) were used as received. Water used in all reactions, particle purifications, and buffer preparations was purified to a resistance of 18 M Ω (Barnstead E-Pure system), and filtered through a 0.2 μm filter to remove particulate matter.

The synthesis of hydrolytically degradable cross-linker was performed as described previously.²³ In brief, *N,O*-dimethacryloylhydroxylamine (DMHA) was produced through the reaction of 0.145 moles (10.1 g) of hydroxylamine hydrochloride with 0.243 moles (25.4 g) of methacryloyl chloride in 50 mL of pyridine at 45 °C. After stirring at ambient temperature for 2 hours, the mixture was diluted in 100 mL of chloroform and 21 mL of concentrated hydrochloric acid. The organic layer was separated, washed four times with distilled, deionized water, and dried over MgSO₄.²³ The product identity was confirmed by ¹H NMR and elemental analysis (see Supporting Information).

The synthesis of pNIPMAm-DMHA nanogels was performed by precipitation polymerization, using a procedure derived from previous illustrations of the method.^{32,37,38} The molar

composition of reagents was 98% NIPMAm and 2% DMHA, with a total monomer concentration of 140 mM in a 50.0 mL total synthesis volume. First, 0.873 g of NIPMAm was dissolved in 48.0 mL of distilled, deionized water. A mass of 115 mg of SDS was added, resulting in a final SDS concentration of 8 mM. This solution was added to a three-neck round-bottom flask, and allowed to equilibrate to 70 °C while purging with N₂ for an hour. Prior to initiation, the DMHA cross-linker was dissolved in DMSO at a concentration of 0.140 M. A 1.0 mL aliquot of the DMHA solution was delivered to the flask by pipette, and allowed to equilibrate for 10 minutes. The polymerization was initiated by delivering a 1.0 mL aliquot of 0.800 M APS solution by pipette. The reaction was allowed to proceed for 7 hours under an N₂ blanket while continuously stirring. Once cooled, the solution was vacuum filtered, followed by repeated ultracentrifugation and resuspension in H₂O. Water was removed from the purified product by lyophilization at -42 °C under 40×10^{-3} mbar for 72 hours. The freeze-dried product was a hygroscopic white powder.

Instruments

The hydrodynamic radii (r_h) of nanogels were measured using dynamic light scattering (DLS). DLS experiments were performed at a 90° scattering angle using a Dynapro DLS (Wyatt Technology Corporation, Santa Barbara, CA). Typical particle concentrations used were ~0.01 wt%. The volume phase transition temperature (VPTT) of degradable nanogels was assessed from turbidity curves collected on a steady-state fluorescence spectrophotometer (Photon Technology International), equipped with a Model 814 PMT photon-counting detector.

Asymmetrical flow FFF was performed using the Eclipse 1 Separation System (Wyatt Technology Corporation, Santa Barbara, CA). The eluent for all studies was composed of distilled and deionized water containing 200 ppm NaN₃ (ionic strength = 3 mM). The Agilent 1100 Series Isocratic pump (Agilent Technologies) was used for eluent flow; Eclipse software Version 2.5.3 (Wyatt Technologies) provided the user interface to program the flow method. An Agilent 1100 Series Vacuum degasser (Agilent Technologies, Santa Clara, CA) was placed between the eluent reservoir and the pump system and an FP Vericel 0.45 µm membrane filter (Gelman Sciences, Ann Arbor, MI) was placed after the eluent pump system to ensure a dust-free mobile phase. Similar to other asymmetrical flow FFF instruments, the channel employed here was trapezoidal in shape.³⁹ The channel had a total length of 26.5 cm, and the channel breadth at the inlet and outlet was 1.5 and 0.5 cm, respectively. The accumulation wall of the channel was equipped with a 10 kDa MWCO regenerated cellulose membrane (Microdyn-Nadir, Germany) with a 350 µm channel spacer. Detection was performed on-line using MALS and dRI detectors.

The MALS photometer was a DAWN-EOS (Wyatt Technology Corporation, Santa Barbara, CA) with a GaAs laser source ($\lambda = 685$ nm). The dRI was a deflection-based Optilab rEX (Wyatt Technology Corporation, Santa Barbara, CA) refractive index detector, operating with an LED light source ($\lambda = 690$ nm). Data collection from both detectors and subsequent light scattering analysis was performed using the Astra software Version 5.3.4.14 (Wyatt Technology Corporation, Santa Barbara, CA). Accurate measurement of nanogel M_w required characterization of the particle refractive increment (dn/dc). Differential refractive index analysis was performed in batch mode using the Optilab rEX system. A series of pNIPMAm-DMHA nanogel dilutions were prepared in dust-free vials, which were rinsed sequentially with distilled deionized water, absolute ethanol, and HPLC-grade acetone. Nanogels were resuspended in the mobile phase over a concentration range from 2.5×10^{-6} g/mL to 3.75×10^{-4} g/mL.

Off-line light scattering measurements were performed using the NanoSight LM20 particle tracking analysis system (Nanosight, Ltd., Amesbury, U.K.). The LM20 is a laser light scattering instrument, which allows the direct visualization of nanoparticle scattering by

microscopy.^{40,41} The LM20 employs a single-mode laser diode (<20 mW, $\lambda = 635$ nm) situated to launch a focused beam through a 500 μ L sample chamber, with a cell depth of 0.9 mm. The light scattering of particles moving in the laser path was visualized using the LM20 CCD camera through a view window above the sample chamber. Particle motion was recorded for a suitable period of time (i.e. 90 seconds) at a rate of 30 frames per second. Video images of particle movements under Brownian motion were analyzed using the Nanosight NTA Software Version 2.0 (Nanosight, Ltd., Amesbury, U.K.). Individual particle trajectories were interpreted by the software, allowing the determination of particle diffusion coefficients. Nanogel r_h values were determined using the Stokes-Einstein equation.

Nanogel Turbidity

The volume phase transition temperature (VPTT) of degradable nanogels was assessed from turbidity curves collected on a steady-state fluorescence spectrophotometer, as described in the *Instruments* section. All turbidity measurements were performed at a particle concentration of 0.016 mg/mL in pH 3.0 formate buffer. Scattering was measured at 600 nm and a temperature ramp was typically set from 25 °C to a maximum of 50 °C for all measurements. A ramp rate of 0.25 °C/min was used, collecting data every 0.1 °C with an integration time of 1 s per data point.

Asymmetrical flow FFF Procedure

Nanogels were separated via asymmetrical flow FFF/MALS/dRI in a two-stage separation scheme at ambient laboratory temperature (22 °C). Through this method, particle size and molar mass-distributions were monitored without extensive scattering interference from degradation products. The stages served to first purify and equilibrate nanogels within the eluent, with the particles being later eluted for subsequent characterization. Sample injection was performed in Focus mode for 1 min using a sample injection flow rate of 0.2 mL/min (injection loop volume = 100 μ L). The flow direction through the channel outlet end is reversed during Focus mode, which serves to concentrate the sample towards a narrow region near the injection point. A bypass flow of 1.0 mL/min was administered through the on-line detectors (MALS, dRI) downstream. Following sample focusing, the flow direction was shifted towards the detectors at a flow rate of 1.0 mL/min with a cross flow of 1.0 mL/min. In this first stage, eluent flow is permitted (containing low molar mass degradation products) while nanogel retention was observed. This stage was maintained for 25 minutes to allow nanogel equilibration in the eluent, and elution of low molar mass components. Nanogels were subsequently eluted in the second stage for detection, using a reduced cross-flow of 0.25 mL/min with a channel flow of 1.0 mL/min. Detected sample peaks were recovered from asymmetrical flow FFF into dust-free glass vials for off-line light scattering characterization with the LM20 particle tracking system. The asymmetrical flow FFF channel and injection loop were flushed extensively (2.0 mL/min flow rate for 2 h) between runs to prevent sample artifacts.

Degradation Study Procedures

To study the degradation process, 4 mg of dry nanogel powder was resuspended in pH or temperature-controlled buffers. In an investigation of the degradation pH-dependence, nanogels were resuspended (0.20 mg/mL particle concentration) in either pH 3.4 formate, pH 5.4 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 7.4 phosphate, or pH 8.4 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer (all buffers were prepared with a buffer concentration of 0.02 M and ionic strength values < 0.05 M). Prior to nanogel resuspension, all buffers were equilibrated to 37 °C. Resuspended nanogels were maintained at this temperature while continuously shaking. At predetermined time intervals, a 1.0 mL aliquot of the solution was removed and analyzed directly via asymmetrical flow FFF/MALS.

Prior to analysis, all aliquots were allowed to equilibrate to ambient laboratory temperature (22 °C) while shaking. In an investigation of the temperature dependence of degradation, nanogels were resuspended in pH 7.4 phosphate buffer and allowed to equilibrate at 4 °C, 22 °C, 37 °C and 50 °C while continuously shaking. Similar to the pH experiments, 1.0 mL of the solution was removed at predetermined time intervals, equilibrated to 22 °C, and analyzed by asymmetrical flow FFF/MALS.

Evaluation of Multiangle Light Scattering Data—Multiple reviews elegantly describe the relationships employed to derive the polymer r_{rms} and M_w from scattering data.^{35,36} In brief, MALS is used to measure scattering at multiple angles in order to extrapolate the Rayleigh ratio ($R(\theta)$) at $\theta = 0^\circ$, where the relationships between molar mass, concentration, and scattering intensity may be predicted.^{35,42} The Debye extrapolation method to construct the Debye plot was used for all data presented, using scattering data from 15 angles ($\theta = 25.9\text{--}163.3$). The intercept of this plot is a function of the polymer molar mass, the slope at the ordinate ($\sin^2(\theta/2) = 0$) is a function of molar mass and r_{rms} .³⁶ In comparison to the Zimm and Berry extrapolation methods for constructing the Debye plot, the Debye method has been shown to be superior for spherical particles with an $r_{\text{rms}} > 50$ nm.³⁶ The second virial coefficient for all analyses was set to 0, which may be assumed at the limit of low nanogel concentration. As described in the *Instruments* section, all Debye extrapolations were performed using the Astra software.

RESULTS AND DISCUSSION

Macroscopic hydrogels cross-linked with DMHA have been investigated by others as copolymers with *N*-(2-hydroxypropyl)methacrylamide (HPMA).²³ Polymers containing DMHA demonstrated degradation *in vitro* and *in vivo*, depending on the pH of the microenvironment and other factors. Specifically, these hydrogels degraded more rapidly at pH > 5, with little erosion being observed at lower pH. Additionally, when lower DMHA concentrations were used in synthesis, the erosion rate was shown to increase. In the pursuit of a degradable nanogel construct, we considered this a promising cross-linker due to its ease of copolymerization with acrylamide-based polymers, its stability under the acidic conditions commonly observed in persulfate-initiated precipitation polymerization, and its degradation under physiologic conditions.

A precipitation polymerization method was developed for production of pNIPMAm-DMHA nanogels, as described in the *Materials and Methods* section. Through DLS, the average hydrodynamic radius (r_h) of pNIPMAm-DMHA particles was determined to be 66.4 ± 0.5 nm. The dimensions of purified pNIPMAm-DMHA nanogels were further investigated by asymmetrical flow FFF/MALS analysis. Through this method, the average root mean square radii (r_{rms}) of particles were determined to be 40 ± 0.4 nm. Polydispersity values determined by both methods were low ($P_d < 15\%$).

The combination of data from dynamic and static light scattering enables the characterization of nanoparticle topology. The r_{rms} (also referred to as a radius of gyration, r_g) is a particle dimension, weighted by the mass distribution about the center of particle mass. When this value is compared to the hydrodynamic radius, structure-sensitive information is obtained;⁴³ this comparison is illustrated schematically in the Supporting Information. Specifically, nanoparticles that occupy a similar volume in solution may differ drastically in the value of a dimensionless parameter $\rho = r_{\text{rms}}/r_h$. Using this ratio, the phase behavior of thermoresponsive poly(*N*-isopropylacrylamide) (pNIPAm) polymers above and below their lower critical solution temperature (LCST) has been monitored in terms of chain topology.⁴⁴ For chains below the LCST, $\rho = \sim 1.4$. This value of ρ is representative of low cross-link density, slightly branched, or linear chains in solution. However, after undergoing chain collapse at elevated

temperature ($T > \text{LCST}$), ρ decreased sharply from 1.4 to 0.54. This value of ρ was indicative of the surface of the globule having a lower density than the center.⁴⁴ In contrast, nanogel spheres in the swollen state typically have ρ values of ~ 0.6 – 0.85 .⁴⁵ The pNIPMAm-DMHA nanogels under study here were found to have a ρ value of 0.60 at 20 °C.

Stimuli-responsivity

To verify conservation of temperature responsivity for pNIPMAm-DMHA nanogels, the volume phase transition temperature (VPTT) value was measured from turbidity curves collected on a steady-state fluorescence spectrophotometer, as described in the *Materials and Methods* section. When the temperature-dependent scattering intensity from a dispersion of pNIPMAm-DMHA particles was measured pH 3.4, a sharp increase in scattering intensity was observed as the solution temperature was raised above 43 °C (see Supporting Information). This scattering intensity increase reflects a change in the hydration state of the polymer. Elevated temperatures above the lower critical solution temperature (LCST) caused expulsion of solvent from the polymer network, resulting in a deswollen, hydrophobic particle state.^{46–48} Particle size above and below the LCST was determined by DLS. At temperatures above the polymer LCST ($T > 44$ °C for pNIPMAm), the particle size decreased as the particles assumed a deswollen state (r_h of 45 ± 0.4 nm at 45 °C, pH 3.4).⁴⁸ As described earlier, particles equilibrated below this temperature were in a solvent-swollen state, showing a larger hydrodynamic size (r_h of 66.4 ± 0.5 nm at 22 °C, pH 3.4). This VPTT value is in agreement with previously synthesized pNIPMAm-based nanogels,³² and demonstrates conservation of stimuli-responsive behavior upon DMHA incorporation.

pH-dependent erosion

The pH-dependent erosion behavior of nanogels was observed through the asymmetrical flow FFF/MALS method, monitoring scattering intensity and the angular dependence of scattering as a function of time. Through this approach, the average r_{rms} of particles was determined using the Debye extrapolation method for the construction of the Debye plot.³⁵ Specifically, the r_{rms} was determined from the slope of the first-order polynomial (linear) fit of the Debye plot. Under conditions where the concentration of soluble polymer is constant, the intensity of scattered light is proportional to polymer molar mass. However, initial experiments to monitor erosion were challenging, as angle-dependent fitting of the light scattering is error-prone in the presence of low molecular weight degradation products. To resolve these issues, we employed an asymmetrical flow FFF separation technique to first purify and subsequently elute particles for size distribution and scattering intensity detection. Furthermore, this two-stage separation method was employed to minimize the effects of variable carrier composition on nanogel fractionation, where slight variance in sample pH and osmotic strength may adversely affect polymer elution by altering the eluent environment.³³ As described in the *Materials and Methods* section, the first stage of the asymmetrical flow FFF retained nanogels for 25 minutes at a cross-flow of 1.0 mL/min prior to elution. Equilibration of samples to the eluent environment was evident since variance in peak retention and symmetry did not occur between all nanogels at initial time-points (prior to erosion, $t < 1$ hr). The high precision of the separation method suggests that nanogels were equilibrated to the carrier composition, and that variation in buffer properties (pH, osmotic strength) did not significantly affect nanogel characterization in these experiments.

To initiate the erosion, pNIPMAm-DMHA nanogels were resuspended in pH-controlled buffers, with pH values ranging from 5.4 – 8.4. Nanogel suspensions displayed dramatic changes in scattering intensity over time when equilibrated at elevated pH values. This behavior was observed visually, where samples would become significantly less turbid with erosion time. To more quantitatively investigate the erosion, solutions were monitored by asymmetrical flow FFF/MALS immediately upon particle resuspension and equilibration. Typical

asymmetrical flow FFF peak heights observed throughout the experiments are summarized in Figure 1, where a decrease in the normalized scattering intensity is evident when the pNIPMAm-DMHA nanogels were incubated at 37 °C and at pH values greater than 3.4. The decrease in the scattered light intensity is likely due to a combination of a decrease in the average particle molar mass and a decrease in the particle number density, both of which should accompany particle erosion.

Scission of the DMHA cross-linker was expected to induce changes in nanogel dimensions, as the cross-linker content influences polymer chain density within the nanogel and thus the equilibrium swelling capacity.⁴⁹ Using the angular dependence of light scattering, particle r_{rms} values were monitored over the course of the degradation reaction. As shown in Figure 2, the r_{rms} of particles increased as a function of incubation time at pH ≥ 5.4 . In contrast, particles incubated under more acidic conditions did not display time-dependent changes in swelling. The stability in size and scattering intensity observed for particles at pH 3.4 suggests limited cross-link scission, whereas higher pH values significantly affect the nanogel structure as a function of time.

Temperature-dependent erosion

Whereas pH-dependent hydrolytic erosion was an expected characteristic for this cross-linker, 21–24,50 we also found temperature-dependent behavior for the scattering and swelling. The temperature effects were characterized through the asymmetrical flow FFF/MALS method, assessing size and scattering for particles incubated at several temperatures. Similar to the pH-studies, nanogels were analyzed at predetermined time intervals for scattering intensities and the r_{rms} throughout the reaction. Figure 3 shows a series of asymmetrical flow FFF/MALS elutions, with normalized light scattering signal at 90°. As shown in Figure 3a, the scattering intensity of particle elutions remained stable when nanogels were maintained at room temperature (22 °C). However, when incubated at elevated temperatures, the particles showed significant decay in scattering signal, as observed in Figure 3b. Whereas particles at 22 °C showed minimal erosion, there was no observable difference in scattering intensity or size for nanogels maintained under refrigeration (4 °C, data not shown). Nanogel swelling and scattering decay progressed in a similar fashion as observed in the pH-dependent erosion experiments (all pH-dependent erosion experiment chromatograms are represented in Supporting Information). As shown in Figure 4, the nanogels expanded in size, presumably due to cross-link scission, when incubated at elevated temperatures (e.g. 37–50 °C). This phenomenon was not observed for particles maintained at lower temperatures (e.g. 4 °C), where the particles maintained their original r_{rms} values.

Characterization of nanogel molar mass, number density, and topology

The decrease in scattering signal and enhanced swelling observed in Figures 1–4 are indicative of nanogel erosion. We hypothesized that cross-link scission caused mass loss in the particle along with swelling due to a decrease in network connectivity. Additional experiments were performed using asymmetrical flow FFF/MALS/dRI, where on-line refractometry permitted the measurement of nanogel concentration in eluting fractions, and thus the derivation of molar mass.³⁵ Dry pNIPMAm-DMHA nanogels were resuspended under erosion-inducing conditions (pH 8.4, 37 °C) and analyzed at predetermined time intervals by asymmetrical flow FFF/MALS/dRI. The scattering intensity of the eluting nanogels decreased throughout the erosion reaction, in agreement with the pH and temperature-dependent erosion experiments (see Supporting Information).

The observed decay in scattering provided qualitative evidence of mass loss in the nanogel construct, as described above. Cross-linker scission likely permitted polymer dissociation from the nanogels, since cross-linking agents are what impart dimensional stability to these

constructs below the VPTT.¹ Thus, it is likely that polymer loss occurred upon DMHA hydrolysis, resulting in a decrease in nanogel molar mass. Using asymmetrical flow FFF/MALS/dRI, the weight-average molar mass of nanogels was monitored for each eluting fraction. As shown in Figure 5, the molar mass of pNIPMAm-DMHA nanogels indeed decreased substantially during the erosion process. However, after 120 hours of incubation a significant portion of nanogel mass remained ($\sim 1.9 \times 10^7$ g/mol).

An attractive feature of the asymmetrical flow FFF/MALS/dRI method is the ability to recover eluted fractions for subsequent analysis. Using off-line particle tracking analysis via the NanoSight LM20, the r_h of fractionated particles was determined. The LM20 is capable of tracking nanogel particles at remarkably low concentrations (~ 0.008 mg/mL for particle fractions), which is below the concentration threshold for conventional DLS analysis of this nanoparticle type. Through characterization of r_h and r_{rms} for each particle fraction, nanoparticle topology was monitored throughout erosion. From these analyses, we found that the pNIPMAm-DMHA nanogels do not demonstrate a statistically significant change in r_{rms}/r_h throughout the reaction (see Supporting Information). Since the topology remains relatively stable throughout erosion, it is evident that the degradation-induced swelling observed occurs homogeneously throughout the particle.

The abundance of particles available for tracking decreased throughout erosion. Using the LM20 particle tracking system, the average observed number density of particles in the elution fractions were estimated (see Supporting Information). It is important to note that this number density value represents the remnant particles detectable by the particle tracking system, and is not necessarily representative of the absolute number density of particles remaining. Nanogels were undetectable by particle tracking after ~ 128 hours of incubation in erosion-inducing conditions (pH 8.4, 37 °C). However, from MALS analysis it is clear that a significant portion of particles remained after extensive erosion times, as shown in Figures 2 and 4.

From the data presented above, we have found that pNIPMAm-DMHA nanogels undergo erosion at neutral pH and elevated temperatures. Originally we anticipated that cross-link scission would result in complete dissolution of polymer from the nanogel network. Without cross-linking agents in the network, the particles would lose dimensional stability and dissociate completely into linear chains. In this scheme, nanogels would not be detectable after DMHA hydrolysis. However, a detectable population of particles remains despite extensive erosion times (2 months in erosion-inducing buffers). This population of particles was undetectable by our dRI configuration and nanoparticle tracking system, which makes molar mass and r_h determination not possible. However, asymmetrical flow FFF/MALS analysis was an effective means for characterizing the size and scattering profile of the remaining nanogels. After 4 weeks of erosion (pH 8.4, 37 °C), a stable population of highly swollen ($r_{rms} = 55\text{--}60$ nm) particles of very low scattering intensity ($< 10\%$ of the original $R(\theta)$ value) was present, as observed by the peak intensity at $\theta = 90^\circ$.

The presence of an intact polymer network with dimensional stability suggests that cross-linking remains in the nanogels, despite hydrolysis of DMHA. We attribute the presence of stable nanoparticles (despite cross-link scission) to the self-cross-linking of pNIPMAm chains via a chain transfer reaction. This phenomenon has been observed for similarly structured pNIPAm chains, where particles are formed in precipitation polymerization despite a lack of added cross-linking agents.⁵¹ The pNIPMAm chains are likely able to undergo a similar reaction, involving free radical formation on the tertiary radical of the pendent isopropyl group on the side chain. A result of chain transfer is the formation of intrinsic cross-links in the polymer network, which would prevent complete polymer dissociation upon scission of DMHA. However, additional experiments are necessary to validate these claims.

CONCLUSIONS

We have described the synthesis of hydrolytically degradable, thermoresponsive nanogels via persulfate-initiated precipitation polymerization. These particles were produced through the copolymerization of NIPMAm and DMHA, yielding monodisperse, thermoresponsive nanogels of appropriate size for intravenous drug delivery applications. To fully characterize nanogel erosion, asymmetrical flow FFF/MALS was used to monitor pNIPMAm-DMHA nanogel behavior in various media. Through asymmetrical flow FFF, particles were separated from degradation products to improve detection by light scattering. Nanogels showed pH and temperature dependent erosion, where particles degraded faster at neutral to basic pH and at elevated temperatures. Under these conditions, DMHA scission was evident from a dramatic increase in particle swelling, and a decrease in scattering intensity for eluting peaks. The loss in light scattering is indicative of mass loss within the nanogel construct and an overall decrease in particle number density, which is an attractive feature from the standpoint for intravenous drug delivery and renal clearance. By adding on-line differential refractometry detection and off-line particle tracking analysis to our method, nanogel size (r_h and r_{rms}), molar mass, and number densities were monitored for sample fractions. Using this approach, we confirmed a significant loss of molar mass for nanogels maintained in erosion-inducing buffers. Using the ratio of r_{rms}/r_h (ρ), the topology of nanogels was found to remain stable throughout degradation. The ρ value is indicative of the distribution of polymer mass throughout the nanogel architecture, which was conserved despite scission of DMHA and polymer dissociation from the nanogels.

The presence of remaining nanogels despite extensive erosion was a surprising result. After erosion, a highly swollen fraction of particles persisted whose low scattering profile suggested a decreased molar mass. We attribute the presence of remaining particles to possible self-cross-linking behavior of the pNIPMAm chains; this phenomenon is under study at this time. The analytical method described herein is a highly versatile means to assess the size, molar mass, topology, and number density of degradable nanogels during the erosion process. We anticipate that this technique should find applicability in the characterization of other degradable particle constructs, and will enable the future development of improved architectures for drug delivery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Particle erosion results in decreased scattering intensities. Microgels were incubated (37 °C) at pH 3.4 (open circles), pH 5.4 (filled circles), pH 7.4 (open triangles), and pH 8.4 (filled triangles) and the maximum scattering intensity was observed at a scattering angle of 90° as a function of reaction time. All chromatograms are shown in Figure S2 (Supporting Information).

Figure 2.

DMHA scission results in particle swelling. All r_{rms} data were weight-averaged across all slices for nanogel peaks, where the size is reported versus reaction time at pH 3.4 (open circles), 5.4 (filled circles), 7.4 (open triangles), and 8.4 (filled triangles). All reactions occurred at 37 °C. Error bars represent the error of the Debye plot polynomial fit of the scattering data, reported across all slices and detectors.

Figure 3. Nanogel erosion rates are temperature-dependent. Asymmetrical flow FFF/MALS elution profiles for temperature-induced erosion experiments at pH 7.4. Data were collected for samples incubated at (a) 22 °C and (b) 37 °C. All analyses were performed at 22 °C.

Figure 4.

Erosion-induced particle swelling is temperature dependent. Nanogel weight-average r_{rms} values were determined by asymmetrical flow FFF/MALS as a function of incubation time and temperature at 4 °C (open circles), 22 °C (filled circles), 37 °C (open triangles), and 50 °C (filled triangles) at pH 7.4. Error bars represent the error of the Debye plot polynomial fit of the scattering data, reported across all slices and detectors.

Figure 5.

Nanogel weight-averaged molar mass (M_w) decay for particles incubated in erosion-inducing buffers (pH 8.4, 37 °C incubation), as measured through the asymmetrical flow FFF/MALS/dRI method. Particle M_w were determined through a Debye plot of light scattering data. Error bars represent the error of the Debye plot polynomial fitting, reported across all slices and detectors.