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Gelation chemistries for the encapsulation of nanoparticles in composite gel microparticles for lung imaging and drug delivery

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Abstract

The formation of 10 to 40 μm Composite Gel MicroParticles (CGMPs) comprising ~ 100 nm drug containing nanoparticles (NPs) in a poly(ethylene glycol)(PEG) gel matrix is described. The CGMP particles enable targeting to the lung by filtration from the venous circulation. UV radical polymerization and Michael addition polymerization reactions are compared as approaches to form the PEG matrix. A fluorescent dye in the solid core of the NP was used to investigate the effect of reaction chemistry on the integrity of encapsulated species. When formed via UV radical polymerization, the fluorescence signal from the NPs indicated degradation of the encapsulated species by radical attack. The degradation decreased fluorescence by 90% over 15 minutes of UV exposure. When formed via Michael addition polymerization, the fluorescence was maintained. Emulsion processing using controlled shear stress enabled control of droplet size with narrow polydispersities. To allow for emulsion processing, the gelation rate was delayed by adjusting the solution pH. At a pH= 5.4 the gelation occurred at 3.5 hours. The modulus of the gels was tuned over the range of 5 to 50 kPa by changing the polymer concentration between 20 and 70 vol %. NPs aggregation during polymerization, driven by depletion forces, was controlled by the reaction kinetics. The ester bonds in the gel network enabled CGMP degradation. The gel modulus decreased by 50% over 27 days, followed by complete gel degradation after 55 days. This permits ultimate clearance of the CGMPs from the lungs. The demonstration of uniform delivery of $15.8 \pm 2.6 \mu\text{m}$ CGMPs to the lungs of mice, with no deposition in other organs, is shown, and indicates the ability to target therapeutics to the lung while avoiding off-target toxic exposure.

Keywords

microgel particle; venous filtration pathway; drug delivery; lung targeting; hydrogel; nanoparticle

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6. Supporting Information Additional information regarding characterization of the nanoparticles; the absorbance curves of Irgacure 2959 and poly(ethylene glycol) triacrylate (1 kDa); the curvature of gels formed via free-radical polymerization; shifts in solution pH due to the presence of poly(ethylene glycol); the unmodified fluorescent images of composite microgel particles before and after UV exposure; the effect of UV exposure time on bulk gel modulus; the control of composite gel microparticle size; the gel mesh size calculation; additional comments on gel degradation. This material is available free of charge via the Internet at <http://pubs.acs.org>.

1. Introduction

Targeting the lungs via the venous filtration pathway is a promising approach to deliver active pharmaceutical ingredients (APIs) selectively to lung tissue.¹ The lungs receive the entire venous blood supply from the heart. Once in the lungs, the venous blood flows through a branching system of blood vessels until it reaches the intricate capillary beds on the alveoli. Particles larger than red blood cells can be trapped at various branching levels based on their size and modulus, and are effectively filtered out of circulation.²⁻⁴ Previous research has shown that the filtering phenomenon can be used to selectively target particles on the order of 6 to 100 μm to the lungs via IV injection.¹⁻⁴ Traditional nanogels and microgels, which are smaller than a micron,⁵⁻¹² are too small to lodge in the lung and instead circulate in the blood stream. We focus on the creation of 10 to 40 μm hydrogel-based particles, termed Composite Gel MicroParticles (CGMPs), for the delivery to the lungs via this venous filtration pathway. Control of CGMP size, including polydispersity, and CGMP modulus is required for targeted delivery without compromising lung function.^{1, 3, 4, 13} We term the CGMPs as composite particles because the hydrogel particle is a carrier for ~ 100 nm, poly(ethylene glycol)-protected nanoparticles (NPs) encapsulated within the gel matrix. The embedded nanoparticles are loaded with hydrophobic therapeutic agents and/or imaging agents.¹⁴ Drug release is controlled by release from the nanoparticles,^{15, 16} which prevents the burst release of small molecules that is typically observed for hydrogels.¹⁷⁻¹⁹ The longer time-scale degradation of the hydrogel eventually clears the construct from the lungs⁴ with the low molecular weight PEG macromers being renally cleared.⁴

The use of chemically crosslinked hydrogels in biomedical applications has become ubiquitous.²⁰⁻²² Encapsulation of active material during gelation simplifies production and allows for a variety of solutes to be encapsulated.²³⁻²⁵ Encapsulating secondary delivery vehicles, such as nanoparticles, within hydrogels is a recent method by which to control release from hydrogels and prevent burst release of small molecules.^{15, 26, 27} The release is dictated either by the release from the secondary delivery vehicle¹⁵, or in the case of a degradable hydrogel, the degradation of the hydrogel.²⁷

To create a biocompatible CGMP, we use a polyethylene glycol (PEG) based hydrogel. Anseth, Bowman, Hubbell and Metters have done the foundational work on PEG gels and their cross-linking chemistries^{23, 28-39} upon which our work is based. Most of their work is centered on two types of reaction chemistries, radical^{23, 33, 37} and Michael addition^{28, 36, 38} polymerizations. UV radical polymerizations have the advantage of temporal control,³⁴ which is important for decoupling the droplet forming emulsification processes from the initiation of polymerization. However, the effect of UV and radicals on encapsulated material has not been fully appreciated. Only two studies on the stability of proteins during UV polymerization have been reported^{40, 41}. To our knowledge, the effect of radicals produced during gelation on NPs encapsulated in gels, and the API in the NPs cores, has never been investigated. We observed an unexpectedly strong effect of radical-induced degradation of the small molecule agent inside the NP core, which will be described in detail. For drug delivery purposes, the integrity of the API is crucial; degradation of the molecule during gelation is to be avoided.

The radical polymerization chemistry is compared to Michael-type addition gelation chemistry, which did not show small molecule degradation. Michael addition gelation chemistry offers a gentle polymerization method, but has not been optimized for control over hour-long periods of time required for controlled emulsion formation. Both temporal control and gentle reaction conditions are required to form CGMPs.

To form our PEG hydrogels, we used a three-armed PEG acrylate macromer that is compatible with both radical and Michael addition polymerizations (Figure 1). For the radical polymerization, we employed UV initiators and study the fate of encapsulated material. For the Michael addition, we used a commercially available di-thiol, DL-dithiothreitol (DTT), as the nucleophile.

As a model of an API-containing NP, we prepared core-shell NPs formed via Flash NanoPrecipitation (FNP) with the hydrophobic dye 2,2,10,10-tetraethyl-6,14-bis(triisopropylsilyl)ethynyl)-1,3,9,11-tetraoxadicyclopenta[b,m]pentacene, (EtTP-5) trapped in the core. FNP is a versatile and scalable method by which core-shell NPs are formed by kinetically controlled self-assembly.^{16, 42-44} The process allows for the stoichiometric encapsulation of hydrophobic active pharmaceutical ingredients (API) and imaging agents.⁴⁵ Additionally, FNP gives control of NP size and surface functionality.⁴⁶ Alone, these NPs will circulate in the blood stream after IV injection;¹⁶ however, as will be shown, when incorporated into CGMPs, the whole construct selectively targeted the lungs. As a second fluorescent model to assess damage during gelation, we chose green fluorescent protein (GFP). The fluorescence of the NPs and GFP was used as a measure of material integrity.

As has been described, production of lung-targeting CGMPs requires that several conditions be met: (1) The NPs and aqueous gel macromers must be processed as a fluid into a uniform water-in-oil emulsion of the required size, with careful control of the polydispersity; (2) The gel chemistry must enable delayed or triggered gelation after formation of the stable emulsion; (3) The gelation process must not compromise the integrity of the therapeutic agent; (4) The gel chemistry must provide degradation for clearance from the lungs on an appropriate time-scale. In this paper we demonstrate a successful system, which achieves these four processing aims, and creates a versatile, lung-targeting platform.

2. Materials and Methods

2.1 Materials

DL-dithiothreitol (DTT) (99.0%), 4,4'-azobis(4-cyanovaleric acid) (ACVA) (98.0%), 2,2'-azobis(2-methylpropanamidine) dihydrochloride (AMPA) (97%), 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (IRG) (98%) (Irgacure® 2959), glycerol ethoxylate 1.0k, silicone oil (10 and 100 cSt) and triethylamine (99%) were purchased from Sigma Aldrich (USA). Tetrahydrofuran (THF) (HPLC grade), sodium acetate (99.0%), acetic acid glacial (99.7%) and phosphate buffered saline powder concentrate (biotech grade) were purchased from Fisher Scientific (USA). Deionized water (18.2 MΩ[cm]) was generated by a NANOpure® Diamond™ UV ultrapure water system (Barnstead International, Germany). Poly(styrene)-b-poly(ethylene glycol) (PS-PEG) 3.8k-b-5.0k was purchased from Polymer Source (Canada). Poly(styrene) homopolymer 1.5k was synthesized by Douglas H. Adamson at the University of Connecticut. Xiameter® 0749 was donated by Dow Corning® (USA). Green fluorescent protein was provided by the A. James Link research group at Princeton University. Ethoxylated (20) trimethylolpropane triacrylate (PEG-TA) 1.2k was donated by Sartomer (USA). 2,2,10,10-tetraethyl-6,14-bis(triisopropylsilyl)ethynyl)-1,3,9,11-tetraoxadicyclopenta[b,m]pentacene, (EtTP-5) was synthesized by protocols previously described.^{47, 48} All materials were used without further purification.

2.2 Formation and Characterization of Nanoparticles

NPs with encapsulated fluorescent dye as a model of an API were created via FNP using a two-inlet vortex mixer as described by Han et al.⁴⁹ A THF stream with molecularly dissolved poly(styrene) (30 mg/mL), stabilizing poly(styrene)-b-poly(ethylene glycol) (40

mg/mL) and EtTP-5 (2.5 wt % of the total poly(styrene)) was rapidly mixed against a deionized water stream in a two-inlet vortex mixer in a 1 to 1 volume ratio and collected in a DI water bath to reduce the final THF concentration to 10 vol%. To remove the THF, the NP solution was dialyzed against a 400-fold larger volume of water in the dark for six hours changing the water every hour. A Spectra/Por® (Spectrum® Labs, USA) regenerated cellulose dialysis bag with a molecular weight cut off of 6-8k was used. Using a Zetasizer® Nano-ZS (Malvern instruments, Malvern, UK), dynamic light scattering (DLS) size measurements were performed on samples after dialysis. Samples were diluted with ultra-pure water and analyzed at 25°C. The intensity weighted nanoparticle diameter was determined to be 199 ± 13 nm. The DLS trace can be found in the supplemental information (Figure S1.)

2.3 Fluorescence Degradation Measurements of Nanoparticle and GFP Solutions

As a control, NPs were exposed to UV without the PEG macromer, but with free radical initiator. Solutions of NPs (70 µg/mL NPs) were incubated with either no initiator, 3 mM IRG, 3 mM ACVA or 3 mM AMPA. Samples were then exposed to UV light 4 cm from the source (Blak-Ray® B-100A Longwave Ultraviolet Lamp, USA) for 1 to 15 minutes. At these initiator concentrations, previous work has shown that the bulk gel modulus plateaus after ~15 minutes of UV exposure indicating complete consumption of either available acrylate groups or initiator (SI 7). Sample fluorescence was analyzed on a Hitachi F-7000 Fluorescence Spectrophotometer (Japan). EtTP-5 containing NP samples were excited at 470 nm and analyzed at 639 nm. The NP size after UV treatment was analyzed via DLS as previously described. Solutions of GFP (127 µg/mL GFP) were incubated with either no initiator, 3 mM IRG or 3 mM ACVA. GFP samples were excited at 390 nm and analyzed at 507 nm.

2.4 Microgel Particle Formation and Confocal Analysis

CGMP samples for confocal analysis were made via emulsification in 10 cSt silicone oil with 3 vol% of Xiameter® 0749 as the stabilizing surfactant. For CGMPs with encapsulated NPs synthesized via radical polymerization, solutions of 25 vol% PEG-TA and 0.4 wt% NPs in 30 mM sodium acetate buffer (pH 4.32) with no initiator, 3 mM IRG or 3 mM ACVA were prepared. For CGMPs with encapsulated GFP synthesized via radical polymerization, the same solution was prepared, but with 0.004 wt% GFP instead of NPs. A control solution with 25 vol% glycerol ethoxylate and 0.4 wt% NPs in 30 mM sodium acetate buffer (pH 4.32) with no initiator was also prepared. To make the oil in water emulsion, the oil phase was added to the aqueous phase in a 5 to 1 volume ratio and vigorously hand-shaken for 30 seconds. Samples were then pipetted into 50 mm rectangular glass capillaries with inner dimensions of 0.3×3.0 mm (Vitrotubes™, VitroCom USA) and the ends were sealed with molten Parafilm® M (Pechiney Plastic Packaging, USA). Care was taken to avoid air bubbles. To make the fluorescence degradation curve, samples were then exposed to UV light for 1 to 15 minutes as previously described.

For CGMPs with encapsulated NPs synthesized via a delayed Michael addition reaction, a solution of 25 vol% PEG-TA and 0.4 wt% NPs in 30 mM sodium acetate buffer (pH 4.32) was prepared. For CGMPs with encapsulated GFP the same solution was prepared, but with 0.004 wt% GFP instead of the NPs. For CGMPs with encapsulated NPs synthesized via a Michael addition reaction with fast kinetics, a solution of 25 vol% PEG-TA and 0.4 wt% NPs in 10 mM phosphate buffered saline (pH 7.4) was prepared. Solutions were then divided in half. One half was designated the control sample, which underwent no reaction. The other half was designated the reaction sample. DTT was added to the reaction sample such that the molar ratio between DTT and PEG-TA was 3 to 2, which equates to a 1 to 1 molar ratio of reactive end groups. To make the oil in water emulsions, the oil phase was

added to the aqueous phases in a 5 to 1 volume ratio and vigorously hand-shaken for 30 seconds. Samples were then left to react overnight on a rotating wheel (Glas-Col®, USA) spinning at 10 rpm.

Imaging of CGMPs was performed on a Leica DMI6000-B inverted microscope using an argon laser. CGMP samples in oil were pipetted onto the bottom of a polystyrene petri dish (Fisher Scientific, USA) and covered with a layer of water to prevent drying. NP samples were excited at 458 nm and the emission bandwidth was collected from 545 to 690 nm. GFP samples were excited at 488 nm and the emission bandwidth was collected from 500 to 600 nm. Care was taken not to disturb the oil film. An N PLAN 10.0×/0.25 dry objective lens was used for all images for analysis. Images were stored as 8 bit line scans with a resolution of 512×512 pixels representing an area of $387.5 \times 387.5 \mu\text{m}$. An HCX PL FLUOTAR L 40.0×/0.60 dry objective lens was used for all images for the figures. Images were stored as 8 bit line scans with a resolution of 512×512 pixels representing an area of $96.88 \times 96.88 \mu\text{m}$. The CGMP fluorescence was analyzed using ImageJ. For each particle, the fluorescence per unit cross-sectional area was determined by dividing the mean fluorescence for the red or green channel by the measured area determined by the “Measure RGB” plugin.

For *in vivo* lung targeting, the control of particle size and polydispersity is important. To form emulsions of narrow polydispersity, a controlled shear technique developed by Bibette and coworkers was used.⁵⁰⁻⁵² Key variables for the process are (1) the viscosity ratio of the continuous to the discontinuous phase, (2) the applied stress and (3) the uniformity of the shear field.⁵⁰ The control of particle size and size distributions can be found in the supplementary information.

To assess the lung targeting capabilities of the CGMPs, CGMPs loaded with NPs containing the EtTP-5 fluorophore were synthesized. An aqueous solution of 30 vol% PEG TA, 1 wt% NPs and DTT in DI water was emulsified in 100 cSt silicone oil with 3 vol% of Xiameter® 0749 as the stabilizing surfactant. The coarse emulsion was sheared on an Anton Paar MCR 501 rheometer (USA) in a Couette cell under a constant shear stress of 245 Pa for 15 minutes. After shearing, 250 μL of an 8 mg/mL acetic acid in 5 cSt silicone oil solution was added to accelerate the crosslinking reaction. The sample was left to react overnight at room temperature on a rotating wheel (Glas-Col®, USA) spinning at 10 rpm.

To remove the silicone oil, the sample was first washed with excess 5 cSt silicone oil, followed by a hexane wash. The sample was resuspended in a 1 wt% Tween 80 solution and bath sonicated (Eumax, Ultrasonic Cleaner, USA) for 1 minute. The sample was then washed with 10 mM PBS, filtered through a 50 μm nylon mesh (Small Parts, USA) to remove any aggregates and concentrated on a centrifuge (Sorvall Legend RT, USA) by spinning for 4 minutes at 50 rcf. A Perkin Elmer Pyris 1 TGA (USA) was used to quantify the final solids concentration.

2.5 Bulk Gel Formation

Bulk gel samples were prepared to determine gel modulus dependence on composition of the macromer solution, and degradation kinetics. Samples for the rheological measurements were formed by using Teflon molds resulting in cylindrical gels with a diameter of 25mm and a thickness of 1mm. For radical gelation, solutions of 40 vol% PEGTA in de-ionized water with 3 mM IRG or 3 mM ACVA were made. The solutions were then pipetted into molds and covered with thin glass coverslips to avoid additional exposure to oxygen. The samples were individually exposed to UV light for 15 minutes under the conditions previously described. For Michael addition gelation, solutions of 30 to 70 wt% PEG-TA in a 1 mM triethylamine solution (pH 11.5) or 30 mM sodium acetate buffers ranging in pH from 3.9 to 4.8 were prepared. DTT was added to the solutions at a molar ratio of 3:2 DTT: PEG-

TA. The solutions were then pipetted into molds, covered with thin glass coverslips and allowed to react overnight at room temperature. Prior to rheological measurements, all samples were placed in excess DI water for 24 hours.

2.6 Rheological Characterization of Storage Modulus, Gelation Point and Gel Degradation

The storage moduli of bulk gel samples were measured via dynamic oscillatory shear measurements using an Anton Paar MCR 501 rheometer (USA) in a plate-plate configuration. Using an environmental cell, samples were kept moist during the measurement by adding water to the sample holder. Measurements were performed within the linear viscoelastic regime from 0.01 to 0.1 % strain at 0.75 Hz.

To determine the gel point, solutions of 40 vol% PEG-TA in 30 mM sodium acetate buffers ranging in pH from 3.9 to 4.8 were prepared. Just prior to a measurement, DTT was added to the solution at a molar ratio of 3:2 DTT: PEG-TA. Samples were quickly pipetted onto the rheometer sample plate and the top plate was lowered to a gap height of 1 mm. To prevent samples from drying during gelation, a wet sponge was introduced into the environmental cell. Dynamic oscillatory measurements were conducted using constant strain (0.05 % strain) and frequency (0.75 Hz) until gelation occurred. The gelation point was calculated using Anton Paar RheoPlus analysis software, specifically the Crossover Point Analysis set to calculate the crossover point between the loss and storage moduli.

For degradation measurements, 40 vol% PEG TA bulk gels were formed via Michael addition chemistry in 30 mM sodium acetate buffer (pH 4.3) as previously described. The samples were then incubated in 10 mM phosphate buffered saline (pH 7.4) at 37°C. To monitor the degradation, the gel moduli were measured periodically via dynamic oscillatory shear measurements.

2.7 In Vivo Lung Targeting

CD1 male mice weighing 35 g were purchased from Charles River (Wilmington, MA). The animals were housed in pathogen-limited animal facility with free access to food and water. The light cycle was 12 hours of light followed by 12 hours darkness. The room temperature was maintained between 68 and 74°F. All animal procedures in this study were approved by the Rutgers University Institutional Animal Care and Use Committee.

The mice were injected via a tail vein injection with a 0.1 ml suspension of CGMPs (7 mg/mL in 10 mM buffered saline) or 10 mM buffered saline vehicle, and were sacrificed 5 min later. The lung was inflated with 0.5 ml saline and rinsed in saline to remove the blood. Liver, kidney, spleen and heart were excised and cleaned with saline before the imaging procedure. All organs were immediately imaged. Image acquisition was performed with a Carestream MS FX PRO (Bruker/CareStream, Rochester, NY) and a Carestream Molecular Imaging Software MI (5.3.3). Bright field and fluorescence images were incorporated in the imaging protocol, and the following instrument settings were used: excitation/emission at 460/700 nm, 1×1 binning, aperture 2.8, and 30 sec acquisition time.

After optical imaging, the saline in the inflated lung was replaced with OCT compound/water (1:1) and embedded into OCT compound (Sakura Finetek, Torrance, CA). 10 µm thick sections were fixed in neutral buffered formalin for 5 min and washed twice in PBS. The sections were then examined using an Olympus IX71 microscope equipped with DP30BW camera. The microscopic images were processed with ImageJ 1.47.

3. Results and Discussion

3.1 UV Polymerized Composite Microgel Particles: Effect of Radicals

Upon encapsulation of fluorescent NPs in CGMPs synthesized via radical polymerization using IRG as the initiator, there is a dramatic loss of fluorescence, as shown in Figure 2. Although protected in a solid, hydrophobic core, the dye is still susceptible to radical attack. We hypothesized that the moderately hydrophobic initiator partitioned into the hydrophobic core of the NPs, where it degraded the fluorophore (EtTP-5) upon UV activation. To test the hypothesis, experiments were conducted using initiators with various logP values (i.e. the log of the solute partition coefficient between octanol and water). LogPs were calculated using Molinspiration® software⁵³ taking into account the ionization state of the initiators at pH 5: IRG (logP = 1.1, somewhat hydrophobic), ACVA (logP = -1.3, somewhat hydrophilic) and AMPA (logP = -2.8, hydrophilic). Solutions of NPs with the initiators were exposed to UV light as described in the experimental section, and the fluorescence was monitored over a period of 15 minutes (Figure 2a). In the control solution with no initiator, minor photo-bleaching occurred with a $16 \pm 1\%$ decrease in fluorescence, consistent with previous observations of fluorescent NPs formed via FNP.¹⁴ The fluorophore is stabilized within the core of the NP in a solid state, which reduces photon-induced degradation.¹⁴ The average NP size and polydispersity did not change within experimental error. The NP solution incubated with IRG, the most hydrophobic initiator tested, had a drop in fluorescence of $69 \pm 1\%$. The average NP size increased by $103 \pm 21\%$ and the polydispersity increased by $100 \pm 6\%$. The more hydrophilic initiators, AMPA and ACVA, also caused fluorescence degradation with a decrease of $95 \pm 1\%$ and $48 \pm 2\%$ respectively after 15 minutes of UV exposure. For the particles treated with AMPA, the average NP size increased by $13 \pm 3\%$ and the polydispersity increased by $29 \pm 6\%$. The average NP size and polydispersity of NPs treated with ACVA did not change within experimental error.

The fluorescence decay and change in NP size does not correlate with initiator hydrophobicity; therefore, it may be related to the absorbance cross section of the initiator, the quantum yield of the initiator,⁵⁴ or the reactivity of the radical produced.⁵⁵⁻⁵⁷ Regardless of the mechanism, the radical degradation of the active material in the NP makes it problematic as a drug delivery platform. Even partial degradation makes FDA approval unlikely, because the extent of degradation and the nature of the degradation products would have to be analyzed and quantified for each formulation. Despite the severity of this problem, the issue does not seem to have been addressed adequately for small molecule delivery from radically polymerized, pre-loaded hydrogel constructs.

Although protein encapsulation and release is not the function of our CGMPs, we tested GFP proteins in the gel matrices because GFP structural integrity can be monitored via fluorescence. The same fluorescence decay experiments as above were performed with GFP using IRG and ACVA (Figure 2a). No bleaching was observed in the control without initiator. In the presence of initiator, the fluorescence intensity of GFP dropped $84 \pm 1\%$ and $57 \pm 6\%$ for IRG and AVCA, respectively, at the end of the 15 min UV exposure.

The experiments above were conducted with the photoinitiator and NPs or GFP, but without the PEG macromer. However, PEG macromer (PEG-TA) concentrations of approximately 25 vol% are required to obtain gels with the desired modulus levels. At these macromer levels, the experiments in Figure 2c show that PEG polymerization itself creates unacceptable NP degradation, independent of the initiator choice. In the absence of an initiator, the 25 vol% PEG-TA exhibits auto-initiation under UV illumination;^{58, 59} the NP fluorescence decreases by $99 \pm 1\%$ at the end of a 15 minute UV exposure. Activation of the acrylate groups is responsible for the degradation, which is shown in the control experiment in which UV exposure of a 25 vol% three-arm hydroxyl-PEG sample with NPs (Figure 2c)

resulted in no degradation above the level observed with the NP and UV light alone. The addition of IRG or ACVA initiator resulted in identical levels of fluorescence decay (Figure 2c) as the auto-initiated case. GFP samples with 25 vol% PEG-TA showed more sensitivity to initiator addition: a decrease in fluorescence of $43 \pm 15\%$ with no initiator, $77 \pm 8\%$ with IRG and $78 \pm 5\%$ with ACVA (Figure 2d.). The fluorescence images of the CGMPs after UV polymerization are shown in Figure 3 for the 25 vol% PEG-TA with IRG, ACVA, and no added initiator. Therefore, at the high PEG-TA concentrations necessary to obtain the desired storage modulus for the CGMP (described below), the auto-initiation of the acrylate groups under UV^{58, 59} or radical initiated polymerization creates unacceptable degradation of the NP contents.

3.2 Michael Addition Polymerized Microgel Particles

Michael addition polymerized gels were developed to overcome the problems inherent in the radically initiated PEG gels. The control of the gelation kinetics to enable emulsification, control of the gel modulus, gel degradation, NP phase behavior during gelation, and the proof of concept of lung delivery by the CGMPs are shown.

3.2.1 Gelation time—For processing of CGMPs by emulsification, control of the gelation reaction is necessary. In most reports on gels for biological applications a noted advantage of the Michael addition is its speed of reaction under mild physiological conditions.^{28, 60} Our interest is in the reverse, slowing the reaction to enable processing. Although the Michael addition reaction does not have the “off-on” control that is afforded by UV radical polymerizations, the reaction rate can be controlled by pH (Figure 4a).⁶⁰ The reactive species in the reaction is the thiolate ion.⁶¹ Acidifying the solution prevents the deprotonation of the thiol and thus the formation of thiolate ion. The acrylate group, however, is activated in acidic conditions.⁶⁰ Thus, an optimum pH exists for slowing the Michael addition reaction rates, and therefore, maximizing the time to reach the gel point.⁶⁰ For solutions of 40 vol% of PEG-TA in 30 mM sodium acetate buffer over the pH range of 5.06 to 5.86, the gelation time was tuned from 25 minutes to 3.5 hours (Figure 4a). For the PEG-TA and DTT system, the maximum delay in gelation for a 40 vol% PEG-TA solution occurred at a solution pH of 5.35 after 3.5 hours.

3.2.2 Gel Modulus—The gel storage modulus is determined by stoichiometry,⁶¹ polymer concentration²⁸ and gelation rate.⁶² Since this is an addition reaction, chain capping can occur if the ratios of acrylate and thiol groups are non-stoichiometric, or if the network is kinetically trapped such that reactive groups are not accessible. We have used stoichiometric ratios of reactants to maximize the modulus. The moduli as a function of PEG-TA concentration for two pH series corresponding to fast and slow reaction rates are plotted in Figure 5. The moduli varied from about 5 to 50 kPa over the PEG concentration range of 20-60 vol%. Notably, the moduli increased approximately linearly with PEG-TA concentration with a slope of 1.1 for both series until a critical concentration was reached near 60 vol% PEG-TA. Above 60 vol% PEG-TA, the system formed heterogeneous gels with weakly linked domains, resulting in a lower overall modulus. The observation of heterogeneous gels at higher polymer content is consistent with the literature.^{63, 64} The moduli differences between the series correlate with the gelation rate. The faster gelling system, the 1 mM triethylamine, which gelled on the order of 10 minutes, had the lower moduli. A similar decrease in modulus for very fast, high pH gels has been seen by Lutolf and Hubbell.²⁸ They studied gels with gel points ranging from 2 to 25 minutes.²⁸ Our interest is in the slow reactions to allow processing of the emulsion. The slower gelling system, the 30 mM sodium acetate buffer, gelled on the order of three hours, and produced higher moduli gels (Figure 5.).

A more detailed investigation of the effect of reaction rate on modulus was performed with the slower polymerizing series that was also used to optimize the gelation rate. The moduli as a function of pH, which reflects the polymerization rate, for 40 vol% solutions of IPEGTA are plotted in Figure 4b. The gel moduli were essentially constant over the solution pH range of 5.20 to 5.86 at an average modulus of 49 ± 3 kPa. In contrast to these slower polymerizing gels, at a solution pH of 5.06, gelation occurred in 25 minutes and the modulus dropped to 21 ± 1 kPa. A rapid reaction rate created a higher proportion of defects in the matrix, resulting in a lower modulus.

The IPEG TA macromer has a functionality of six for the radical polymerization and three for the Michael addition. However, the values of the gel storage moduli prepared by Michael addition were comparable to the values for the radical polymerized gels at similar concentrations. Figure 6 shows the moduli of ACVA and IRG UV radically polymerized gels and Michael addition gels at 40 vol% PEG-TA; moduli values were between 45 and 58 kPa.

3.2.3 Gel Degradation—To clear from the lungs, the CGMPs should degrade. The PEG acrylate chemistry is inherently degradable because of the hydrolysable ester between the PEG backbone and acrylate end-group. The degradation of PEG hydrogels has been extensively studied and shown to be tunable by changing the gel architecture or increasing the number of hydrolysable groups flanking the central PEG chain.^{33, 34, 65} We were interested in the degradation rate of 40 vol% PEG TA gels formed via Michael addition under physiological conditions. By virtue of the thiol-based Michael-type addition, the esters along the polymer backbone had a sulfide at the β position, which is known to accelerate ester hydrolysis (Figure 1).^{66, 67} As is shown in Figure 7., the modulus decreases by 50% over 27 days. By day 55, the gels had completely degraded. The slow degradation would allow for long retention in the lung for sustained delivery of APIs. More rapid clearance could be obtained using PEG-lactide hybrids as has been demonstrated previously.^{32, 33, 65}

3.2.1 Gel-Nanoparticle Interactions—We observed a phenomenon not addressed in the literature on nanoparticles encapsulated in hydrogels. During slow gelation reactions the initially uniformly distributed fluorescent NPs (Figure 8a) clustered and phase separated (Figure 8d). This phenomena is well known in the colloid field and is termed depletion flocculation.^{68, 69} The depletion force arises from the exclusion of polymer chains between two colloids when they are at a separation less than the radius of gyration of the chain.⁷⁰ This “depleted” area creates an unbalanced osmotic force on the colloids causing them to aggregate and phase separate.⁷⁰ The magnitude of the force depends on the radius of gyration of the polymer relative to the radius of the colloid and the relative concentration of both.^{69, 71} The size of the 1k PEG relative to the 200 nm NP was too small to induce depletion flocculation of the initial mixture, and confocal images always showed homogeneous fluorescence in a liquid droplet (Figure 8a-c).

As the 1k PEG polymer reacted, its molecular weight increased. Once the growing polymer reached a critical molecular weight, a phase boundary was crossed and phase separation occurred. NPs were aggregated into a NP rich phase and the gelling polymer was found in a polymer rich phase. Figure 8a and 8d show the uniform initial fluorescence and the concentration of NPs at the CGMP surface after slow polymerization. Depletion flocculation is itself slow in these viscous solutions, so it is possible to kinetically form the gel rapidly enough to prevent observable phase separation. A fast gelling formulation with a gel point of 5 minutes was used resulting in CGMPs with homogenous fluorescence (Figure 8f).

The requirement of a delayed gelation to enable processing, and fast polymerization to kinetically trap the NPs with a uniform distribution on the gels, led us to develop a hybrid

approach to gel processing. To kinetically trap the NPs in the CGMP, the Michael addition reaction was acid catalyzed after uniform droplet formation. After emulsification, a solution of acetic acid in silicone oil was mixed with the emulsion, lowering the pH and catalyzing the reaction.

In the slow gelling formulation with GFP, phase separation was not observed (Figure 8e). GFP, with a 2.4 nm diameter,⁷² is small enough relative to the polymer chains such that depletion forces do not arise.^{69, 71} In fact, based on the bulk gel modulus, an approximate mesh size was calculated to be 4 nm indicating that the protein can diffuse through the gel matrix (Supplementary Information).

3.4 *In Vivo* Lung Targeting with Nanoparticle Loaded Composite Gel Microparticles

As a proof of concept, a suspension of CGMPs was prepared and administered to a mouse to demonstrate localization of the CGMPs in the lungs. CGMPs were prepared using the controlled shear emulsification technique and Michael addition chemistry. Nearly monodispersed, $15.8 \pm 2.6 \mu\text{m}$ particles (Figure 9a.) were synthesized. The CGMPs were loaded with NPs to allow for fluorescence imaging and to mimic the loading of the therapeutic NPs. To kinetically trap the NPs in the CGMP, the reaction was acid catalyzed after shearing with the addition of acetic acid as previously described.

Mice were dosed intravenously with either a suspension of CGMPs (1.3×10^5 CGMPs per gram body weight) or a buffered saline vehicle. After dosing, the mice were sacrificed, dissected and analyzed using a whole animal fluorescence imager. As shown in Figure 9c., the lungs of the mouse treated with CGMPs were fluorescent, while the lungs of the mouse treated with saline remained dark, indicating successful CGMP accumulation in the lungs. The other organs were also imaged (Figure 9c.). The hearts, kidneys and spleens exhibited no fluorescence in both the treated and control mice. Liver auto-fluorescence was observed in both mice; therefore, the fluorescence does not indicate that CGMPs were delivered to the liver. The treated lungs were further analyzed via fluorescence microscopy. In lung cryosections, CGMPs were distributed uniformly throughout the lung tissue. As shown in Figure 9b., the fluorescent CGMPs were trapped within lung alveolar capillaries. Hence, the CGMPs are successful in specifically targeting the lungs after IV injection.

4. Conclusion

To create lung-targeting gel microparticles (with sizes ranging between 10 and 40 μm) that are carriers for therapeutic drug nanoparticles (sizes ~ 100 nm), a polymerization reaction is required that does not compromise the integrity of drug. We compared two reaction chemistries, UV radical polymerization and Michael addition, for encapsulation of NPs in PEG gels. A hydrophobic fluorescent dye (EtTP-5) was encapsulated in the solid core NP as a model and reporter of damage during polymerization. In addition, GFP was encapsulated as a reporter, since its fluorescence is known to be sensitive to damage to the protein structure. Independent of initiator, the radical polymerization was detrimental to encapsulated material with a severe loss of fluorescence observed in all cases. The degradation of encapsulated material is unacceptable for drug delivery purposes. The Michael addition-based chemistry did not interfere with the NP or GFP as demonstrated by constant fluorescence signal before and after polymerization. A controlled shear procedure at constant stress with control of the inner and outer fluid viscosities enabled the rapid production of emulsion drops of narrow size distribution (e.g. $15.8 \pm 2.6 \mu\text{m}$) over the desired size range. Changing the PEG macromer concentration in the aqueous internal phase enabled control the modulus of the gels from 5 kPa to 50 kPa. For processing purposes, delaying the gelation of the system is important. By changing the reaction pH, the Michael addition reaction was slowed to reach the gel point in 3.5 hours, which provided adequate

time to allow for formation of CGMPs. Depletion flocculation was observed in the slow gelling Michael addition system with NPs. Since release of the drug is controlled by the NP, and not the release of NPs from the gel,^{15, 16} some level of aggregation is acceptable. However, to prepare more homogeneous CGMPs a triggered gelation process was developed; emulsion preparation was done at a pH of 5.4 to minimize the reaction, followed by rapid shifting of the pH by adding acetic acid to the oil phase. The pH shift initiated rapid gelation that kinetically trapped the NPs in the gel matrix. The CGMP samples can be lyophilized for long-term stability.

As a demonstration of lung-targeting, a tail vein injection of a CGMP dispersion encapsulating the fluorescent NPs showed selective delivery to the lungs. No off-site deposition of CGMPs in the heart, kidney, spleen or liver was observed. The mouse displayed no apparent adverse reaction to the injection.

The promise of these results opens further avenues of investigation. Our ongoing studies are with the delivery of camptothecin from NPs with the CGMP for non-small cell lung cancer. The applicability of the approach for other lung disease such as TB would be of interest. The number density of particles that can administered to mice,^{13, 73, 74} rats,^{13, 73, 74} and man³ without adverse affects on lung function are known. For mice 2.0×10^4 particles per gram of body weight are tolerated. However, those studies were done with relatively poorly characterized samples (i.e. albumin particles with an average size of 28 μm but a standard deviation of $\pm 12 \mu\text{m}$).⁷³ Larger particles block blood flow at larger vascular branching points, and affect blood flow over a larger volume of tissue. Studies of the short and longer-term effects of these more monodisperse, soft, and permeable CGMPs is of interest. Our ongoing studies are on the efficiency of capture as a function of size and gel modulus. In this study, we have presented modulus measurements on bulk gels of the same chemistry as the CGMPs. A description of techniques involving AFM studies of individual beads to relate CMPG gel rheology to bulk gel rheology will be reported elsewhere. Studies of the clearance rate from the lungs are being pursued with CMPGs with long wavelength dyes to enable longer-term studies of single animals. Finally, the delivery of camptothecin-loaded NPs and its efficacy on an orthotopic NSCLC model is being pursued.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACVA	4,4'-azobis(4-cyanovaleric acid) (98.0%)
AMPA	2,2'-azobis(2-methylpropionamidine) dihydrochloride (97%)
API	active pharmaceutical ingredient

CGMP	composite gel microparticle
DTT	DL-dithiothreitol (99.0%)
EtTP-5	2,2,10,10-tetraethyl-6,14-bis(triisopropylsilylethynyl)-1,3,9,11-tetraoxadicyclopenta[b,m]pentacene
FNP	Flash NanoPrecipitation
GFP	green fluorescent protein
IRG	2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone 98% (Irgacure®2959)
NP	nanoparticle
PEG	poly(ethylene glycol)
PEG-TA	Ethoxylated(20) trimethylolpropane triacrylate

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Figure 1.

Reaction scheme for (a.) radical gelation and (b.) Michael addition gelation. (a.) Aradical (1) attacks the acrylate groups on the PEG triacrylate (2) to form a gel with a polyacrylic acid backbone (3). (b.) The acrylate groups on the PEG triacrylate (2) undergo nucleophilic attack from deprotonated thiols on DL-dithiothreitol (5) to form a sulfur carbon bond (6) and subsequently form a gel (6'). The PEG backbone (4) is denoted as a wavy line.

Figure 2.

Fluorescence of EtTP-5 nanoparticles and GFP in sodium acetate buffer or in a solution of 25 vol% PEG in sodium acetate buffer as a function of UV exposure time. (a) EtTP-5 nanoparticles in sodium acetate buffer incubated with no initiator (■), IRG (◆), ACVA (▲), and AMPA (●). (For each point, $n=3$.) (b) GFP in sodium acetate buffer incubated with no initiator (■), IRG (◆), and ACVA (▲). (For each point, $n=3$.) (c) EtTP-5 nanoparticles in a solution of 25 vol% PEG-TA in sodium acetate buffer no initiator (■), IRG (◆), and ACVA (▲). EtTP-5 nanoparticles in a solution of 25 vol% glycerol ethoxylate in sodium acetate buffer no initiator (■). (For each point, $n>3$.) (d) GFP in a solution of 25 vol% PEG-TA in sodium acetate buffer incubated with no initiator (■), IRG (◆), and ACVA (▲). (For each point, $n>3$.)

Figure 3.

Images of composite gel microparticles (CGMPs) with 25 vol% PEG triacrylate before and after UV exposure. (a) A droplet containing EtTP-5 nanoparticles (NPs) prior to UV exposure. (b) An CGMP with encapsulated NPs after exposure to 15 minutes of UV using ACVA as the initiator. (c) An CGMP with encapsulated NPs after exposure to 15 minutes of UV using Irgacure as the initiator. (d) An CGMP with encapsulated NPs after exposure to 15 minutes of UV with no initiator. (e) A droplet containing GFP prior to UV exposure. (f) An CGMP with encapsulated GFP after exposure to 15 minutes of UV using ACVA as the initiator. (g) An CGMP with encapsulated GFP after exposure to 15 minutes of UV using Irgacure as the initiator. (h) An CGMP with encapsulated GFP after exposure to 15 minutes of UV with no initiator. (e.-h. To improve contrast for printing purposes, the GFP fluorescence image was enhanced prior to creating the overlaid image by converting all pixels above a desired threshold to red. The unenhanced overlaid images can be found in the supplemental information.)

Figure 4.

Control of the gel point and modulus via pH. (a) The gel point of a 40 vol% PEG triacrylate in 30 mM sodium acetate buffer as a function of the solution pH. The longest delay in gelation is observed at a solution pH of 5.40. (For each formulation, $n=3$.) (b) The modulus of swollen gels made from 40 vol% PEG triacrylate in 30 mM sodium acetate buffer at different pHs. Gels were reacted overnight and then swollen for 24 hours prior to the oscillatory shear measurement. (For each formulation, $n=3$.)

Figure 5.

Gel storage modulus of gels formed via Michael addition under slow gelling conditions in 30 mM sodium acetate buffer solution, pH 4.3, (■) and under fast gelling conditions in 1 mM triethylamine, pH 11.5, (○) as a function of PEG triacrylate concentration. (For each formulation, $n=1$.)

Figure 6.

Comparison of storage moduli between swollen gels formed with ACVA, Irgacure and via Michael addition. All gels were formulated with 40 vol% PEG triacrylate either in water for the UV exposed gels or in 30 mM sodium acetate buffer (pH 4.32) for the Michael addition gels. The ACVA and Irgacure samples were exposed to UV for 15 minutes. The Michael addition samples were allowed to react overnight. All samples were swollen in excess DI water for 24 hours prior to the oscillatory shear measurement. (For each formulation, n=3.)

Figure 7.

The degradation of a bulk 40 vol% PEG triacrylate gel formed via Michael addition. The modulus drops to 50% of its original value by day 27. (For each point, n=3.)

Figure 8.

Images of composite gel microparticles (CGMPs) formed via a Michael addition reaction before and after the reaction. (a) A droplet with EtTP-5 nanoparticles (NPs) prior to a reaction with slow kinetics. (b) A droplet with GFP prior to reaction with slow kinetics. (c) A droplet with NPs prior to a reaction with fast kinetics. (d) An CGMP with NPs post reaction with slow kinetics. Flocculation depletion is observed as indicated by the increase in fluorescence at the surface of the particle. (e) An CGMP with GFP post reaction with slow kinetics. (f) An CGMP with NPs post reaction with fast kinetics. Flocculation depletion is not observed as indicated by the homogenous fluorescence of the particle.

Figure 9.

In Vivo Lung Targeting with Nanoparticle Loaded Composite Gel Microparticles (CGMP)
 (a) An image of the nearly monodispersed CGMPs with embedded fluorescent EtTP-5 nanoparticles dispersed in phosphate buffered saline. (b) A lung cryosection of a CGMP treated mouse. The fluorescent CGMPs (red) are trapped in a lung capillary. (c) The fluorescence images of organs from a control mouse (top) and from a CGMP treated mouse (bottom). Fluorescence is observed in the treated lung, indicating the lodging of fluorescent CGMPs in the lung. No increase in fluorescence was observed between the heart, kidneys, spleen and liver of a control mouse (top) and a CGMP treated mouse (bottom). The livers exhibited auto-fluorescence.