January 2009

Factors Affecting Size and Swelling of Poly(ethylene glycol) Hydrogel Microspheres Formed in Aqueous Sodium Sulfate Solutions

Michael Nichols
Washington University in St. Louis

WASHINGTON UNIVERSITY IN ST. LOUIS
School of Engineering and Applied Science
Department of Biomedical Engineering

Thesis Examination Committee:
Dr. Donald Elbert, Chair
Dr. Frank Yin
Dr. Rohit Pappu

FACTORS AFFECTING SIZE AND SWELLING OF POLY(ETHYLENE GLYCOL) HYDROGEL MICROSPHERES FORMED IN AQUEOUS SODIUM SULFATE SOLUTIONS

by

Michael Nichols

A thesis presented to the School of Engineering of Washington University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

August 2009
Saint Louis, Missouri

ABSTRACT

Factors Affecting Size and Swelling of Poly(ethylene glycol) Hydrogel Microspheres Formed in Aqueous Sodium Sulfate Solutions

by

Mike Nichols

Master of Science in Biomedical Engineering

Washington University in St. Louis, 2009

Research Advisor: Professor Donald Elbert

The lower critical solution temperature (LCST) behavior of poly(ethylene glycol) (PEG) in aqueous sodium sulfate solutions was exploited to fabricate hydrogel microspheres under mild conditions without the use of other monomers, polymers, surfactants or organic solvents. Reactive PEG derivatives underwent thermally induced phase separation to produce spherical PEG-rich domains that coarsened pending gelation, resulting in stable hydrogel microspheres that were polydisperse in size. The degree of reaction prior to phase separation, reaction rate within the PEG-rich domains, and duration of the reaction were independently varied to elucidate their effects on final microsphere size and gain insight regarding the mechanism of formation. It was found that both the time required to reach the gel point during coarsening and the extent of crosslinking after gelation impacted the final size of the microspheres. Power law

analyses of microsphere sizes revealed the mean radius of PEG-rich droplets to grow with time to the $1/4^{th}$ power until gelation. Together with dynamic light scattering data, this suggested that a percolation-to-cluster transition occurred soon after phase separation by off-critical spinodal decomposition. This technique of producing PEG microspheres with controlled sizes has considerable potential for an array of applications, including the production of modular scaffolds for tissue engineering.

Acknowledgments

First and foremost I am grateful to Dr. Donald Elbert for giving me the opportunity to work in his laboratory. He has always been there to help guide me while at the same time encouraging my growth as an independent researcher. I am especially thankful to Dr. Frank Yin for encouragement and advisement from my first day at Washington University as an undergraduate student. I will forever be indebted to Evan Scott for his sage advice on both academia and life. His mentoring and friendship during my time in the lab have kept me motivated and passionate about research. Of course, I would also like to thank my other labmates for always taking the time to help me including Casey Donahoe, Peter Nguyen, and Megan Flake. I am also grateful to my family and friends for supporting me during my time in Saint Louis. I would not have made it this far without them. Finally, I must thank my girlfriend of many years Wendy Zhang for having kept my head up when things got tough and having always (at least pretending to have) been interested in hearing about my work. If this work was dedicated to any one person, it would be her.

Michael D. Nichols

Washington University in St. Louis
August 2009

Contents

List of Figures

1. Introduction*

Hydrogel microparticles serve valuable roles in applications spanning from biomedical research to clinical usage. In research, peptides and antibodies can be immobilized on their surfaces for the affinity-based purification of biomolecules and cells (Flodin 1965; Rabel 1980; Margel 1983; Kondo and Fukuda 1997). They have also been employed in the form of a "microparticle-enzyme immunoassay" (MEIA) to quantify small molecules or proteins of interest (Osikwoicz, Fries et al. 1988; Di Serio, Gechtman et al. 2006; Pini, Gallesi et al. 2006). Clinically, microspheres may be useful as both oral and ocular drug delivery systems (Weinstock, Khoobehi et al. 1996; Lopez and Peppas 2004; Bhavsar and Amiji 2007; Liu, Griffith et al. 2008). Microparticles may also be useful as nucleic acid delivery vehicles for use in gene therapy (Cortesi, Esposito et al. 1994; Cavanagh, Dingwall et al. 2001; Bhavsar and Amiji 2008). There is also potential utility for microspheres as bioactive surface coatings (Singh, Bridges et al. 2007). A relatively new and promising application of hydrogel microparticles is their use as building blocks for the assembly of modular scaffolds (McGuigan and Sefton 2006; Yeh, Ling et al. 2006; Rivest, Morrison et al. 2007). As physical and/or chemical characteristics of microspheres may need to be specifically tailored for particular uses

---

* This thesis is an adaptation of the unpublished manuscript:
Nichols MD, Scott EA, Elbert DL. Factors affecting size and swelling of poly(ethylene glycol) microspheres formed in aqueous sodium sulfate solutions without surfactants. *Biomaterials*. (Under review).

within these or upcoming applications, it is advantageous to have several production methods to confer the desired properties.

Presented in this thesis is a novel technique for producing poly(ethylene glycol) (PEG) microspheres in solution by phase separating and then crosslinking functionalized derivatives of the polymer. Specifically, the research focuses on proposing a mechanism for their formation by probing factors influencing their size. Formed microspheres retained residual reactive groups that could potentially be used to conjugate biomolecules or form tissue engineering scaffolds, making them suitable for applications such as those described above.

A brief overview of alternative microparticle production methods is presented below, followed by the motivation for using PEG and pertinent theory of phase separation. The general microsphere fabrication technique is also outlined and objectives of the investigation are clarified.

1.1 Microparticle Production Methods

As noted, hydrogel microparticles can be fashioned using a variety of approaches (Rivest, Morrison et al. 2007). In general, hydrogels are three-dimensional

networks of crosslinked hydrophilic polymer(s) (Peppas, Hilt et al. 2006). These methods describe the production of micron-scale versions of hydrogels, and the strategies fall into two primary categories: serial methods and solution-based methods. In serial production techniques, microparticles are produced successively similarly to parts on a production line. Solution-based methods generate microspheres simultaneously in a suspension. Detailed below are the advantages and disadvantages of these methods along with some examples.

1.1.1 Serial Methods

Serial methods of microparticle fabrication are usually mechanical in nature, which allows precise control over the size, distribution and sometimes geometry of the products. One such method is micromolding (also known as micropatterning), in which gel precursors are cast in patterned molds and then crosslinked (Khademhosseini, Eng et al. 2006; Khademhosseini, Langer et al. 2006; Yeh, Ling et al. 2006; Rivest, Morrison et al. 2007). Khademhosseini et al. demonstrated fibroblast and stem cell encapsulation into both hyaluranic acid and PEG-diacrylate hydrogels of a variety of shapes using this technique (Khademhosseini, Eng et al. 2006). A similar method utilizes photolithography to produce specific microparticle geometries. In this case, gel precursors and photoinitiators are mixed under a photomask and then exposed to light that initiates gelation of unmasked regions, also enabling cell encapsulation (Beebe, Moore et al. 2000; Liu and Bhatia 2002). Another strategy providing a great deal of

control over particle size is to crosslink droplets of gel precursor into microspheres within microfluidic channels (Nisisako, Torii et al. 2002; Xu, Nie et al. 2005; Um, Lee et al. 2008). Yet another technique, atomization, involves mixing gel precursor with a crosslinker immediately prior to spraying the solution out of a nozzle in a fine mist (nebulization) using air or another gas (Kwok, Groves et al. 1991; Del Valle, Herrero et al. 2006; Xie, Marijnissen et al. 2006). The sizes of the resulting microcapsules can be adjusted via the flow rates of solution and/or air during nebulization (Del Valle, Herrero et al. 2006). Despite fine control over some microsphere properties, such methods usually require specialized equipment and their serial character can be time-consuming, hindering scalability required for many applications.

1.1.2 Solution-based Methods

Solution-based microsphere production methods are highly scalable but sacrifice control over geometry and usually necessitate the use of organic solvents, surfactants, or other additives that could compromise biocompatibility (Edman, Ekman et al. 1980; Kemp, Meredith et al. 1983). Surfactants act to stabilize phase-separated particles in suspension, inhibiting aggregation or coalescence but are extremely difficult to entirely remove.

In precipitation polymerization, the polymer being formed is insoluble in its monomer/solvent solution upon formation and phase separates into droplets that

continue reacting to form microspheres (Odian 2004). Nolan et al. employed this technique but avoided the use of organic solvents by co-polymerizing PEG-acrylate with *N*-isopropylacrylamide (NIPAm) in water to produce microgels with sub-micron hydrodynamic diameters (Nolan, Reyes et al. 2005). Poly(NIPAm) (pNIPAm) possesses a lower critical solution temperature (LCST) above which it undergoes thermally induced phase separation, but pNIPAm is hydrophobic and thus promotes undesirable non-specific protein adsorption at physiological temperatures (Kawaguchi, Fujimoto et al. 1992; Boutris, Chatzi et al. 1997). For this reason, maximizing the content of hydrophilic polymers such as PEG that resist non-specific protein adsorption is highly desirable.

Emulsion polymerization is performed by mechanically agitating a binary immiscible mixture to create an emulsion followed by polymerizing the resulting droplets to form microspheres (Odian 2004). Agitation by shaking or stirring dictates the size of the resulting phase-separated droplets. This strategy was used by Hennink and colleagues to generate both PEG and dextran microspheres crosslinked using free-radical polymerization in water without using surfactants (Franssen and Hennink 1998; Van Thienen, Demeester et al. 2008; Van Tomme, Mens et al. 2008). The same group also attempted producing PEG microspheres by phase separating PEG in magnesium sulfate solutions and then by vigorously mixing the phases (Franssen and Hennink 1998). However, the method suffered from the formation of large aggregates.

Coacervation to form gelatin microcapsules is achieved slightly differently. Phase separation is achieved by partial desolvation and gradual aggregation of gelatin particles in solution (Arshady 1990). Conventionally, ethanol is slowly added to a well-stirred gelatin solution to partially desolvate the gelatin due to the more thermodynamically favorable water-ethanol interaction. Sodium sulfate can also be used in lieu of ethanol to achieve the same effect (Arshady 1990). Partially desolvated, the gelatin aggregates into droplets which solidify upon cooling and are subsequently crosslinked by formaldehyde to form the microcapsules.

1.2 Poly(ethylene glycol) (PEG)

PEG is a strongly hydrophilic ethylene oxide polymer that is widely utilized in biomaterials because of its robust resistance to non-specific protein adsorption (Jeon, Lee et al. 1991; Elbert and Hubbell 1996; Banerjee, Irvine et al. 2000; Groll, Haubensak et al. 2005; Nolan, Reyes et al. 2005; Lussi, Falconnet et al. 2006; Scott, Nichols et al. 2008). Protein adsorption onto implanted materials may promote adverse biological responses such as thrombosis via activation of platelets, complement, and/or the coagulation cascade (Groth, Derdau et al. 1992; Kao, Sapatnekar et al. 1996; Karlsson, Nygren et al. 1996; Borowiec, Venge et al. 1997; Jenney and Anderson 1999; Janatova 2000; Brodbeck, Colton et al. 2003; Videm 2004). In solution, the high oxygen content of PEG results in extensive hydrogen bonding with water to form a hydration shell that, in conjunction with steric repulsion of the polymer chains, confers substantial resistance

to non-specific protein adsorption (Bailey and Kaleske 1976; Jeon, Lee et al. 1991). Additionally, PEG derivatives possessing reactive moieties can be synthesized with high efficiency from its hydroxyl endgroups, permitting subsequent conjugation of biomolecules or formation of hydrogels (Harris 1997). Importantly and as noted earlier, PEG also exhibits LCST behavior without which our microsphere fabrication method would be impossible (Bailey and Callard 1959; Bailey and Kaleske 1976; Saeki, Kuwahara et al. 1976; Bae, Lambert et al. 1991). These highly desirable and necessary properties drove our interest in fabricating microspheres exclusively from PEG.

1.3 Lower Critical Solution Temperature (LCST)

Generally, a LCST is a critical temperature below which all compositions of a mixture are miscible and thus exist in a single phase (Jenkins, Kratochvil et al. 2009). If the temperature of a mixture is raised above the LCST of one of its components, that component undergoes thermally induced phase separation by either nucleation and growth or by spinodal decomposition (SD) (Crist and Nesarikar 1995; Robeson 2007) (Figure 1.1). By definition, the minimum value occurring at the intersection of the binodal line and spinodal line is the LCST. Whether nucleation and growth or SD occurs depends on how phase boundary is crossed. If only the binodal line is crossed, solvation of the component becomes thermodynamically metastable. Under this scenario, phase separation is favorable but only achieved by nucleation and

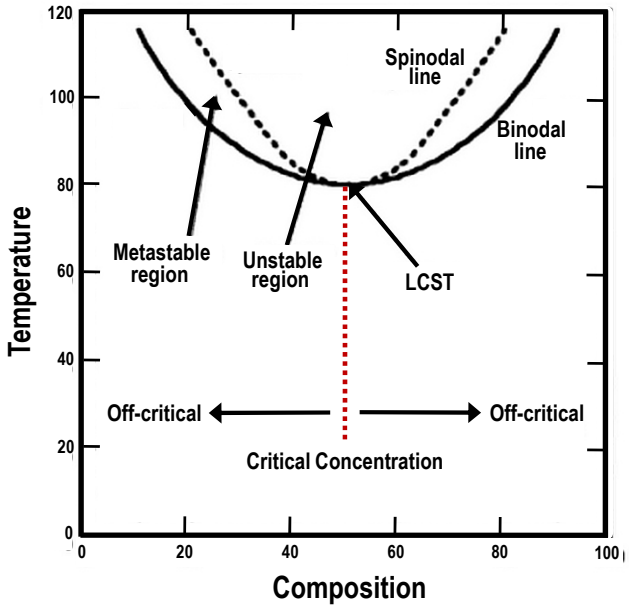

Figure 1.1 – Generic Phase Diagram Illustrating LCST Behavior. Depending on the volume fraction (composition) of polymer, the type of phase separation (binodal or spinodal) and temperature at which it occurs varies. The LCST corresponds to the critical concentration (~50% w/v here) at which the spinodal and binodal lines intersect.

growth (Robeson 2007). If instead the spinodal line is crossed, solvation is unstable and SD will occur (Robeson 2007). However, for SD to occur exclusively the phase boundary must be crossed at the LCST, as only at that temperature does the mixture transition from stable to unstable without passing through a metastable region. Note that phase separation is visually accompanied by an increase in turbidity or cloudiness of a solution corresponding to the cloud point (a temperature), and this value only corresponds to the LCST at the critical composition. For simplicity, an experimentally determined cloud point representing the binodal line is often reported rather than an LCST (Bae, Lambert et al. 1991; Robeson 2007).

Considering only binary aqueous polymer solutions, the LCST of a polymer is dependent upon an array of factors affecting solubility (Bailey and Callard 1959; Saeki, Kuwahara et al. 1976; Yen, Raghavan et al. 1996; Sun and King 1998; Zhang and Cremer 2006; Robeson 2007; Cho, Zhang et al. 2008). Under identical solution conditions, polymers with higher hydrophobicity usually possess lower LCSTs due to their higher thermodynamic cost of solvation (Bailey and Callard 1959; Zhang and Cremer 2006; Cho, Zhang et al. 2008). Increasing molecular weight (MW) also tends to depress the LCST and broadens the spinodal and binodal lines, but these effects are increasingly dampened as the MW increases (Bailey and Callard 1959; Saeki, Kuwahara et al. 1976; Bae, Lambert et al. 1991; Yen, Raghavan et al. 1996). Higher pressures also lower the LCST by weakening hydrogen bonding and increasing hydrophobic interactions (Sun and King 1998). Of all factors used to alter the LCST, perhaps the most well-known and exploited is changing the salt content of the solution. The effect of salt on the LCST follows the Hofmeister series, which is typically used to increase or decrease the solubility of proteins (or the LCST of polymers) with chaotropic and kosmotropic salts, respectively (Hofmeister 1888; Bailey and Callard 1959; Collins and Washabaugh 1985; Yen, Raghavan et al. 1996; Kunz, Lo Nostro et al. 2004; Zhang and Cremer 2006; Cho, Zhang et al. 2008). More specifically, kosmotropic salts have been shown to linearly decrease the LCST of PEG in a concentration-dependent manner, with salts of more potent Hofmeister anions (e.g. CO_3^{2-}, SO_4^{2-}, etc.) inducing stronger effects (Bailey and Callard 1959; Yen, Raghavan et al. 1996; Cho, Zhang et al. 2008).

Using this technique, the LCST of PEG can be reduced from near the boiling point of water to below room temperature (Bailey and Callard 1959; Yen, Raghavan et al. 1996).

The molecular mechanism by which Hofmeister salts operate has been controversial up until recently. It was long believed that a major contributor to the phenomenon was the ability of the series to affect the structure of bulk water (its hydrogen bond network), which in turn altered solubility (Franks 1973; Schuster, Zundel et al. 1976; Zaslavsky, Mestechkina et al. 1983; Zaslavsky, Bagirov et al. 1989; Gupta, Nath et al. 2002). However, recent evidence has emerged that largely dismisses the notion that the ions extensively alter bulk water structure beyond their immediate few hydration shells (Omta, Kropman et al. 2003; Gurau, Lim et al. 2004; Koga, Westh et al. 2004; Kunz, Lo Nostro et al. 2004; Collins, Neilson et al. 2007; Smith, Saykally et al. 2007). Instead, direct ion-macromolecule interactions have been implicated as the key players in the phenomenon (Song, Ryoo et al. 1991; Bostrom, Williams et al. 2001; Schellman 2003; Pegram and Record 2006). Specifically concerning the ability of kosmotropic salts to lower the LCST of polymers, the ions are thought to operate by both increasing the cost of hydrophobic hydration and weakening hydrogen bonds. It becomes more thermodynamically unfavorable to solvate macromolecules in the presence of the ions due to increased surface tension at the polymer-water interface (Zhang, Furyk et al. 2005; Cho, Zhang et al. 2008). Hydrogen bonds formed directly between water and polymer were found to be weakened due to polarization by the anions, which would likely disrupt the unique hydration shell of PEG and be the

primary cause of the ions reducing its LCST (Zhang, Furyk et al. 2005; Cho, Zhang et al. 2008).

1.4 PEG Microsphere Fabrication Technique

We developed a method of engineering 100% PEG microspheres in aqueous solution without surfactants by thermally phase separating and then crosslinking reactive PEG derivatives. Upon phase separation, spherical PEG-rich domains formed and grew in size over time by a process called coarsening. A crosslinking reaction occurred between the PEG derivatives within the phase-separated domains during coarsening until the gel point was reached, forming hydrogel microspheres. The sizes of the formed microspheres were affected by the rate of gelation relative to the rate of coarsening as well as the extent of reaction both before and after phase separation.

1.4.1 Phase Separation and Coarsening

Phase separation of PEG in solution was promoted by the addition of the kosmotropic salt sodium sulfate to lower the cloud point to just above room temperature. If phase separation occurs by SD, an initially percolated web-like structure results that eventually breaks down into spherical polymer-rich domains (McMaster 1975; Siggia 1979; Lauger, Lay et al. 1994). This occurs in off-critical polymer solutions,

in which the volume fraction of the polymer substantially deviates from that corresponding to the LCST. Nucleation and growth on the other hand simply results in the formation of spherical domains (Friedlander 1977; Gunton, Miguel et al. 1983; Ratke and Voorhees 2002). Whether by nucleation or SD, the manifested spherical domains grow in size (coarsen) by coalescence and/or Ostwald ripening (Friedlander 1977; Gunton, Miguel et al. 1983; Ratke and Voorhees 2002). Coalescence results from collision and subsequent fusion of phase-separated domains caused by Brownian motion, fluid flow or buoyancy effects (Friedlander and Wang 1966; Siggia 1979; Gunton, Miguel et al. 1983). Ostwald ripening results from mass transfer from smaller domains to larger domains by diffusion of minority phase molecules through the majority phase (Lifshitz and Slyozov 1961; Wagner 1961; Gunton, Miguel et al. 1983). In classical coarsening by coalescence and/or Ostwald ripening, the mean sizes of the phase-separated domains evolve according to the power law $R \propto time^{1/3}$, where R is a characteristic length scale of the phase-separated domains (Lifshitz and Slyozov 1961; Friedlander and Wang 1966; Crist and Nesarikar 1995). Unabated, coarsening ultimately results in the formation of two distinct phase-separated layers due to differences in the densities of the phases (Figure 1.2).

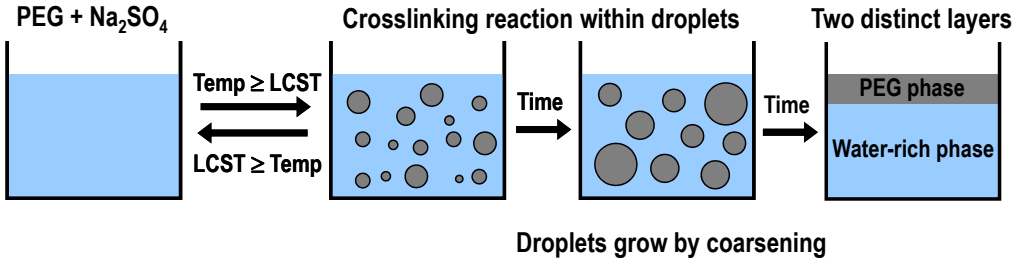

Figure 1.2 – Uninterrupted Coarsening of Unreactive PEG. Following phase separation of PEG-OH, resultant spherical domains will coarsen over time until two distinct layers of the phases are formed due to differences in the densities of the phases.

1.4.2 Gelation and Buffer Exchange

To form hydrogel microspheres rather than two distinctly layered phases, functionalized PEG was phase-separated into spherical domains and allowed to react during coarsening (Figure 1.3). We utilized eight-arm PEG derivatized either with vinylsulfone groups (PEG_8-VS) or amine groups (PEG_8-amine), which over time react in aqueous solution to form a bulk hydrogel (Scott, Nichols et al. 2008). This same reaction occured within the PEG-rich domains during their growth by coarsening until the gel point was reached. Upon gelation, coarsening was arrested and a microsphere version of the bulk hydrogel was formed. The extent of coarsening that occured prior to gelation affected the mean size of the PEG-rich domains and thus the mean size of the resulting microspheres. After fabrication, microspheres were simply buffer exchanged into PBS to remove the sodium sulfate.

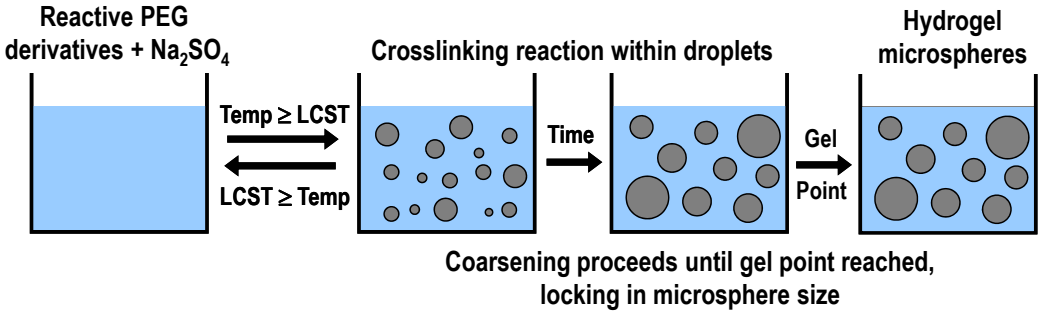

Figure 1.3 – Gelation of Phase-Separated PEG Derivatives. Following phase separation of reactive PEG derivatives, crosslinking will occur within the PEG-rich domains during coarsening until the gel point is reached. At the gel point, coarsening is halted and hydrogel microspheres are formed.

1.5 Objectives

The primary goal of this investigation was to gain insight behind the mechanism of microsphere formation by the described method. This was achieved by specifically probing the effects on microsphere size of various factors affecting the reaction conditions. Observed influences of these factors on size were analyzed quantitatively and qualitatively to generate a probable mechanism of formation. Though this investigation tested a range of conditions for microsphere formation, it was conducted within the context of a separate goal to make the microspheres under mild conditions and thus harsh reaction conditions (e.g. extreme pHs or temperatures) were generally avoided.

Preparation of the microspheres under mild conditions was desirable due to their intended use in biological applications. Because of their anticipated usage, the protocol by which they are produced needed to (1) allow conjugation of biomolecules (e.g. proteins or peptides) during fabrication and (2) minimize the use of additives such as organic solvents and surfactants, which can be difficult to remove and can compromise cell viability (Edman, Ekman et al. 1980). The first requirement in part motivated the use of vinylsulfone-derivatized PEG, as vinylsulfone is able to undergo nucleophilic conjugate addition reactions at useful rates under physiological pH and temperature (Masri and Friedman 1988; Wacker, Scott et al. 2006). The second requirement was fulfilled by reducing the LCST of PEG with salt, permitting phase separation below temperatures that would accelerate biomolecule degradation without employing additives. As a bonus, the method used is relatively simple, requires no specialized equipment and is highly scalable so that mass quantities of microspheres can be produced with ease.

2. Methods

2.1 Pre-reaction of PEG Derivatives

All reagents were purchased from Sigma-Aldrich unless otherwise noted. Eight-arm PEG-vinylsulfone (PEG_8-VS), PEG-amine (PEG_8-amine), and PEG-acrylate (PEG_8-acrylate) were prepared from eight-arm PEG-OH (PEG_8-OH; mol. wt. 10,000; Shearwater Polymers, Huntsville, AL) as previously described[†] (Elbert and Hubbell 2001; Wacker, Scott et al. 2006). PEG_8-VS and PEG_8-amine solutions were prepared at 20% (w/v) in Dulbecco's phosphate buffered saline (Pierce) and sterile filtered with 0.22 μm syringe filters (Millipore). PEG_8-VS was 'pre-reacted' with PEG_8-amine below the cloud point by combining the solutions at a 1:1 ratio of vinylsulfone to amine groups for a total volume of 1 mL in 1.5 mL centrifuge tubes. Once mixed, these solutions were reacted by incubation at 37°C with rotation at 40 rpm. The progress of the reactions was followed by dynamic light scattering (DLS) until the desired mean effective diameter (d_{PCS}) was reached.

2.2 Cloud Point Determination

Solutions of PEG_8-VS, PEG_8-acrylate or PEG_8-amine were diluted to 2% (w/v) from the 20% (w/v) stock solutions with PBS and PBS + 1.5 M sodium sulfate to

[†] Evan A. Scott performed the synthesis of the PEG derivatives.

achieve the desired sodium sulfate concentration. Cloud points of the polymers were determined by increasing solution temperatures in a thermal cycler in 2°C steps (PCR Sprint Thermal Cycler, Thermo Electron Corp.) and visually observing the temperature at which the cloud point was reached.

2.3 Dynamic Light Scattering (DLS)

Mean effective hydrodynamic diameters (d_{PCS}) of pre-reacted solutions were monitored by dynamic light scattering/photon correlation spectroscopy (DLS/PCS; 90Plus Particle Size Analyzer, Brookhaven Instruments, Holtsville, NY) at a scattering angle of 90° and wavelength of 658 nm. Values of d_{PCS} and statistics for the gathered data were performed with Brookhaven Instruments Particle Sizing Software (version 2.34, Brookhaven Instruments). Disposable polystyrene cuvettes (Brookhaven Instruments) were cleaned 1x with 95% ethanol and 2x with DI water prior to use.

2.4 Microsphere Fabrication

PEG_8-VS/PEG_8-amine microspheres were fabricated from pre-reacted solutions of PEG_8-VS and PEG_8-amine ($d_{PCS} \cong 100$ unless otherwise stated). The 20% (w/v) PEG pre-reacted solutions were diluted to 2% (w/v) PEG with PBS and PBS + 1.5 M sodium sulfate to a final sodium sulfate concentration of 0.6 M and volume of 50 μL. The PEG_8-VS/PEG_8-amine solutions were then incubated above the cloud point

at 37°C for 45 min unless otherwise stated. Suspensions of microspheres were subsequently buffer exchanged into PBS 2x to remove the sodium sulfate by: (1) diluting the microsphere solution 3:1 with PBS and titurating, (2) centrifuging at 14,100*g* for 2 min, (3) removing the supernatant. PEG_8-acrylate/PEG_8-amine microspheres were produced as above but in PBS + 0.8 M sodium sulfate incubated 5 min at room temperature and then 5 min at 95°C.

2.5 Microsphere Sizing

Phase-contrast photomicrographs of microspheres were analyzed to determine microsphere diameters. Three separately fabricated samples of microspheres at each condition were prepared and then diluted 3x either during buffer exchange into PBS or with the original reaction buffer. Each sample was titurated to obtain a well-mixed suspension of microspheres, pipetted onto a cleaned glass microscope slide (Corning Inc.), covered with a cleaned glass coverslip (12 mm diameter, Ted Pella Inc.), and immediately imaged at 20X with an Olympus IX70 microscope (Olympus America, Melville, NY) to obtain several representative photomicrographs. ImageJ software (NIH) was used to manually threshold and quantify >500 microsphere areas that were later converted to diameters for each reaction condition. However, microspheres smaller than about 1.5 μm in diameter were unable to be counted due to the lower fidelity of sizing the particles at 20X below that size. In MATLAB (The Mathworks,

Inc.) 500 diameters were randomly selected for each condition and compared by a one-factor ANOVA with a Scheffe *post-hoc* test.

Microsphere diameters were converted to volumes to generate polydispersity indices (PDIs). Volume-based polydispersity indices (PDIs) were calculated as the volume average volume $\overline{V}_v$ over the number average volume $\overline{V}_n$.

$$\overline{V}_v = \frac{\sum_{i=1}^{N} V_i^2}{\sum_{i=1}^{N} V_i} \qquad (2.1)$$

$$\overline{V}_n = \frac{\sum_{i=1}^{N} V_i}{N} \qquad (2.2)$$

$$PDI = \frac{\overline{V}_v}{\overline{V}_n} \qquad (2.3)$$

2.6 Power Law Models‡

2.6.1 Microsphere Size as a Function of Reaction Rate

A power law for microsphere size as a function of pH-based reaction rate changes was derived for the second-order reaction between functional groups on the

‡Dr. Donald Elbert helped develop this model.

derivatized PEG. The reaction of vinylsulfone ($-VS$) groups on PEG_8-VS with amines on PEG_8-amine is primarily due to the more nucleophilic $-NH_2$ groups instead of the $-NH_3^+$ groups.

$$-NH_2 + -VS \xrightarrow{k_f} NH-VS \qquad (2.4)$$

The rate of the irreversible reaction is dominated by:

$$\frac{d[NH-VS]}{dt} = k_f[-NH_2][-VS] \qquad (2.5)$$

The Henderson-Hasselbach equation describes the relationship between pH thus proportion of nucleophilic amines:

$$pH = pK_a + \log_{10}\left(\frac{[\text{conjugate base}]}{[\text{acid}]}\right) = pK_a + \log_{10}\left(\frac{[\text{-}NH_2]}{[-NH_3^+]}\right) \qquad (2.6)$$

By conservation we have of the total number of free amines:

$$[\text{-}NH_3^+] = [\text{-}NH]_{tot} - [\text{-}NH_2] \qquad (2.7)$$

Plugging (2.7) into (2.6) and rearranging for $[-NH_2]$:

$$[-NH_2] = \frac{10^{pH-pKa}}{1+10^{pH-pKa}}[-NH]_{tot} = \frac{1}{1+10^{pKa-pH}}[-NH]_{tot} \qquad (2.8)$$

pH is held constant by buffering and thus the fraction of total amines as $-NH_2$ groups is constant throughout the reaction. Note that $[-NH]_{tot}$ is not $[-NH]_{tot,0}$, which is the initial concentration of amine groups. Substituting (2.8) into (2.5) gives reaction rate as:

$$\frac{d[NH-VS]}{dt} = \frac{k_f}{1+10^{pKa-pH}}[-NH]_{tot}[-VS] \qquad (2.9)$$

The reaction mixture always contains a 1:1 molar ratio of amine to vinylsulfone groups due to the initial concentrations of the reagents and the reaction stoichiometry:

$$[-VS] = [-NH]_{tot} \qquad (2.10)$$

Expressing $[-NH]_{tot}$ (and $\therefore [-VS]$) as a function of the concentrations of bonds formed, the rate of reaction can be described solely in terms of $[NH-VS]$:

$$[-NH]_{tot,0} = [-NH]_{tot} + [NH-VS] \qquad (2.11)$$

$$\frac{d[NH-VS]}{dt} = \frac{k_f}{1+10^{pKa-pH}}([-NH]_{tot,0} - [NH-VS])^2 \qquad (2.12)$$

Separating variables and integrating:

$$\frac{1}{[-NH]_{tot,0} - [NH-VS]} = \frac{k_f t}{1+10^{pKa-pH}} + C \qquad (2.13)$$

Applying the initial condition $[NH-VS] = 0$ at $t = 0$ and rearranging:

$$\frac{[NH-VS]}{([-NH]_{tot,0})([-NH]_{tot,0}-[NH-VS])}=\frac{k_f t}{1+10^{pKa-pH}} \quad (2.14)$$

The degree of conversion χ is defined as:

$$\chi=\frac{[NH-VS]}{[-NH]_{tot,0}} \quad (2.15)$$

Substituting (2.15) into (2.14):

$$\frac{\chi}{(1-\chi)}=\frac{[-NH]_{tot,0}k_f t_\chi}{1+10^{pKa-pH}} \quad (2.16)$$

The time to reach a certain extent of reaction is then:

$$t_\chi=\frac{\chi}{(1-\chi)}\frac{1+10^{pKa-pH}}{[-NH]_{tot,0}k_f}\propto 1+10^{pKa-pH} \quad (2.17)$$

Gelation should occur at the same degree of conversion (the gel point) regardless of pH, although this degree of conversion at which coarsening stops is unknown.

As PEG-rich domains grow by coarsening, their mean diameters $\bar{d}$ scale with time by some scaling exponent α. From (2.17), we can represent this scaling as in Equation (2.18):

$$\bar{d}\propto {t_\chi}^{\alpha}\propto(1+10^{pKa-pH})^{\alpha} \quad (2.18)$$

The scaling exponent α was determined by constructing a log-log plot of experimentally determined mean microsphere diameters fabricated at different pHs

against $1+10^{pKa-pH}$ evaluated at corresponding pH values. A *pKa* value of 9.8 corresponding to primary amines was used. The slope of the plot was α.

2.6.2 Microsphere Size as a Function of Fractional Time to the Gel Point

A power law for microsphere size as a function of fractional time remaining to the gel point was derived. Fractional times remaining until PEG solutions pre-reacted to various degrees reached the gel point were calculated as in Equation (2.19). The time

$$t_{frac} = \frac{t_{gel} - t_{pre-reaction}}{t_{gel}} \qquad (2.19)$$

at which pre-reacting solutions (20% w/v PEG, 37°C, pH 7.4) reached the gel point, t_{gel}, was approximately 7 h. The duration a PEG solution had been pre-reacted, $t_{pre-reaction}$, was used to express a normalized fractional time remaining to the gel point t_{frac}. Since the time to reach the gel point t_{χ} is linearly proportional to the time remaining to the gel point (considered in section 2.6.1), mean PEG-rich domain diameter growth should scale with time during coarsening as:

$$\bar{d} \propto t_{\chi}{}^{\alpha} \propto t_{frac}{}^{\alpha} \qquad (2.20)$$

The scaling exponent α was determined by constructing a log-log plot of experimentally determined mean microsphere diameters fabricated PEG solutions pre-

reacted various extents against t_{frac} for corresponding pre-reacted solutions. The slope of the plot was α.

2.7 Statistics

A p-value < 0.05 was considered significant. Data were mean ± standard deviation unless otherwise indicated. Error in the power law plots was calculated by propagation of error.

3. Results

3.1 Microsphere Fabrication

Hydrogel microspheres were formed by gelation of reactive PEG derivatives that had been phase separated into spherical PEG-rich domains above the cloud point. Within these domains, a conjugate addition reaction (crosslinking) occurred between electrophilic vinylsulfone groups on eight-arm PEG-vinylsulfone (PEG_8-VS, 10 kDa) and nucleophilc amines on eight-arm PEG-amine (PEG_8-amine, 10 kDa) (Figure 3.1). Microsphere fabrication under mild conditions (pH 7.4, 37°C) was only achieved by utilizing solutions containing PEG_8-VS and PEG_8-amine that were initially reacted prior to phase separation ('pre-reacted').

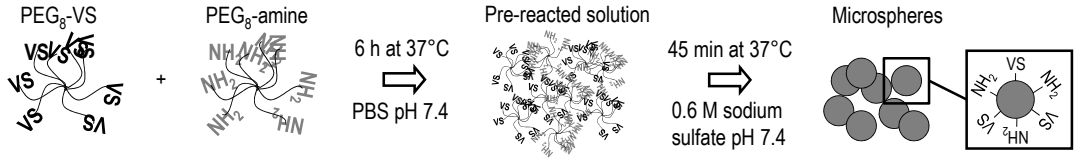

Figure 3.1 – Overview of Microsphere Production. The conjugate addition reaction between eight-arm PEG-vinylsulfone (PEG_8-VS) and eight-arm PEG-amine (PEG_8-amine) was followed by dynamic light scattering (DLS) to detect the formation of PEG oligomers/microgels during crosslinking prior to phase separation ('pre-reaction'). At a certain mean microgel diameter, the pre-reacted solution was diluted in PBS + 0.6 M sodium sulfate and then heated above the cloud point to produce microspheres.

The progress of pre-reaction toward the gel point was followed by dynamic light scattering/photon correlation spectroscopy (DLS/PCS), which provided an intensity-weighted (i.e. z-average) measure of the mean effective diameter (d_{PCS}) of polymers in the reacting solution. A d_{PCS} of about 10 nm was observed after a few hours of reaction, while a d_{PCS} of about 180 nm was found just prior to gelation. Our lab has found via gel permeation chromatography of pre-reacted solutions that the intensity-weighted signal of right angle light scattering is much more sensitive to the presence of larger reacted PEG species (microgels) than monomers and oligomers that form[§]. Thus, the d_{PCS} from DLS was a measure of the average size of only the largest PEG oligomers/microgels but was a useful metric to monitor the progress of pre-reaction.

The extent of pre-reaction in PEG solutions used to form microspheres under mild conditions influenced their final sizes. Microspheres were initially formed from solutions that were pre-reacted to $d_{PCS} \cong 100$ nm and diluted to 2% (w/v) PEG in PBS + 0.6 M sodium sulfate at room temperature. Dilution was important in slowing the pre-reaction rate, as a solution with $d_{PCS} \cong 100$ nm was about 30 min from the gel point at 20% (w/v) PEG and 37°C. Diluted to 2% (w/v), gelation was not observed within 24 h, consistent with a 100-fold decrease in the rate of the second-order reaction expected for the 10-fold dilution. The solutions were diluted in PBS + 0.6 M sodium sulfate, which was below the cloud point of the solution at room temperature. To cause

---

[§] Evan A. Scott prepared the samples for GPC analysis and Dr. Donald Elbert analyzed the raw data.

phase separation, the temperature of the diluted solution was raised above its cloud point to 37°C, producing spherical, PEG-rich domains. Within 45 min, stable PEG_8-VS/PEG_8-amine microspheres formed that were stable following buffer exchange into PBS (Figure 3.2). As the length of the pre-reaction step was increased, i.e. at larger d_{PCS} values, the sizes of the formed microspheres decreased (Figure 3.3). Generally, microspheres formed aggregates during production that were easily dispersed via tituration. No stable microspheres were observed to form under these conditions if the PEG was not pre-reacted.

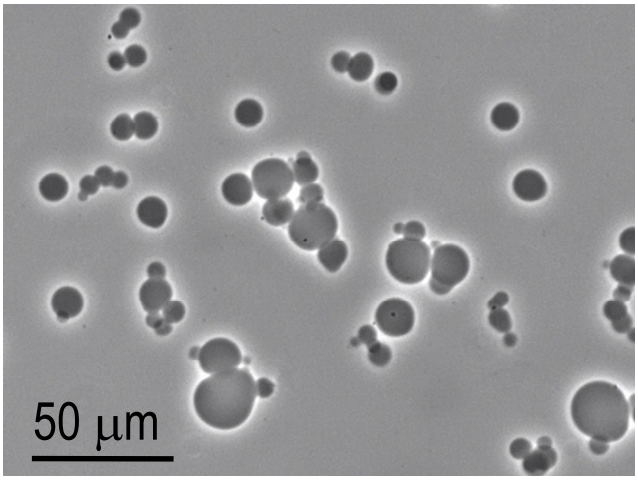

Figure 3.2 – Representative Photomicrograph of Microspheres. Phase-contrast photomicrograph at 20X magnification of PEG_8-VS/PEG_8-amine microspheres formed from a pre-reacted PEG solution. Image was captured following buffer exchange into PBS.

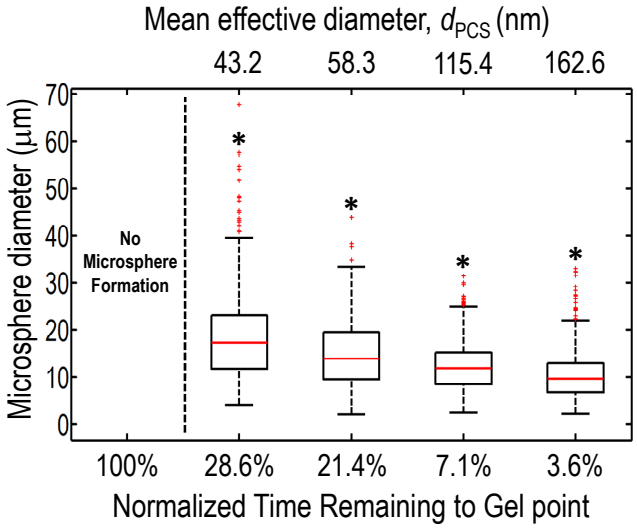

Figure 3.3 – Influence of Pre-reaction Extent on Microsphere Size. PEG_8-VS and PEG_8-amine were pre-reacted to various degrees. Solutions were diluted to 2% (w/v) PEG in PBS + 0.6 M sodium sulfate, incubated for 45 min at 37°C and then buffer exchanged into PBS. Under these conditions, stable microspheres were only formed using pre-reacted PEG. Increased pre-reaction (less time to gel point or larger d_{PCS} values) significantly decreased the sizes of mean microsphere diameters. Data represent n = 500 microspheres. * $p < 0.05$ versus all other conditions.

3.2 Cloud Point Measurements

Pre-reaction of the PEG derivatives may have been critical in obtaining stable microspheres due to the relatively high cloud point of PEG_8-amine. As observed in the literature, cloud points of the PEG derivatives decreased linearly with increasing sodium sulfate concentration. However, the endgroups of the derivatives dramatically impacted the cloud point. While the 2% (w/v) PEG_8-VS in PBS + 0.6 M sodium sulfate possessed a cloud point near room temperature, PEG_8-amine exhibited a cloud point >100°C under the same conditions (Figure 3.4). Though not directly utilized in

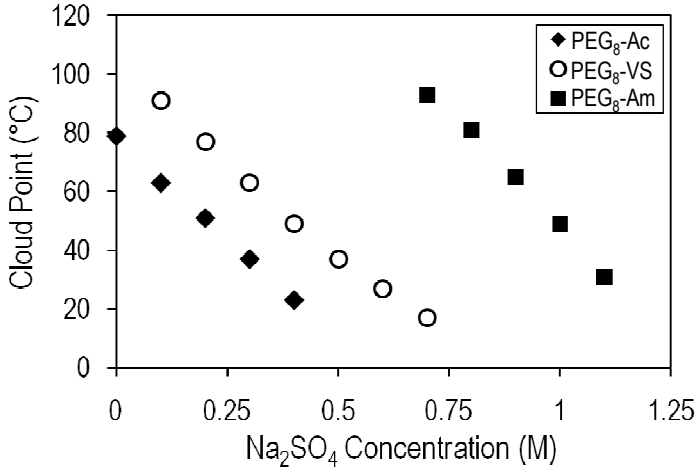

Figure 3.4 – Cloud Points of PEG Derivatives. Cloud point data for 2% (w/v) PEG_8-VS, PEG_8-amine (PEG_8-Am) and PEG_8-acrylate (PEG_8-Ac) in PBS + sodium sulfate at pH 7.4. As reported elsewhere, a linear decrease in LCST with increasing salt concentrations was observed. Data represent $n = 3$ and standard deviations were ≤ 1ºC for all points.

this study, the cloud point of eight-arm PEG-acrylate (PEG_8-acrylate, 10 kDa) was also investigated and found to be substantially lower than that for PEG_8-VS.

3.3 Effects of Reaction Conditions on Microsphere Size

During buffer exchange into PBS to remove the sodium sulfate, microspheres swelled to much greater sizes (Figure 3.5). Both the rate and duration of the crosslinking reaction were found to affect swollen microsphere size (following buffer exchange), but only the kinetics influenced unswollen size (prior to buffer exchange).

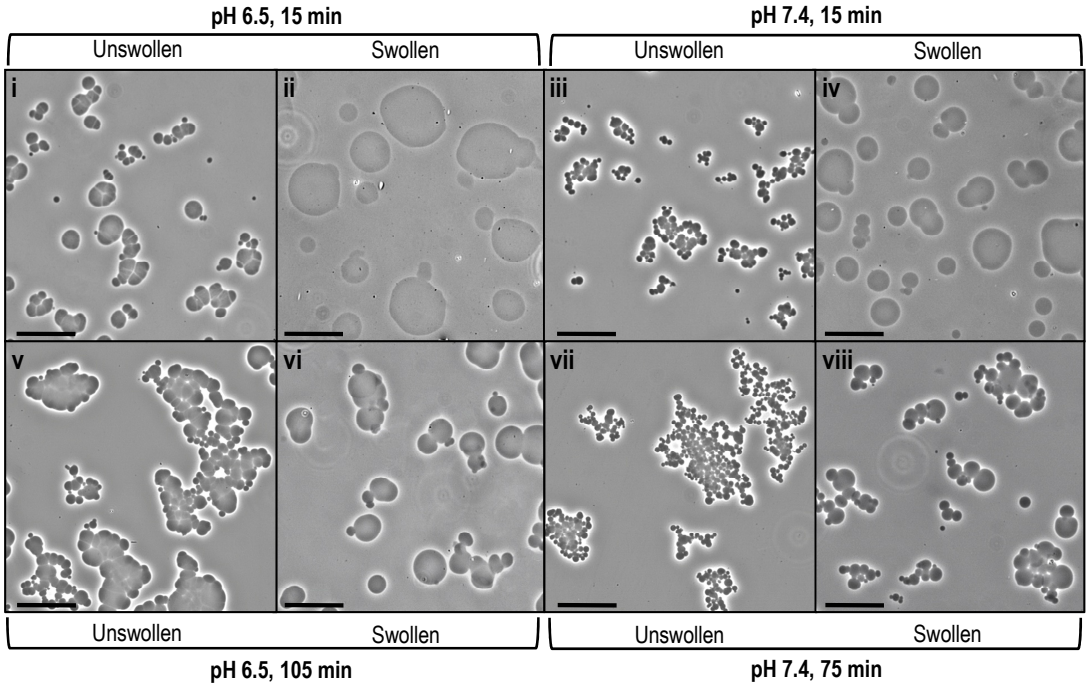

Figure 3.5 – Swelling of Microspheres upon Buffer Exchange. Representative phase-contrast photomicrographs at 20X magnification demonstrating swelling of PEG_8-VS/PEG_8-amine microspheres following buffer exchange into PBS. All microspheres were formed from pre-reacted solutions ($d_{PCS} \cong 100$ nm) diluted to 2% (w/v) in PBS + 0.6 M sodium sulfate and incubated at 37ºC for: (i) 15 min at pH 6.5 without buffer exchange (ii) 15 min at pH 6.5 with buffer exchange (iii) 15 min at pH 7.4 without buffer exchange (iv) 15 min at pH 7.4 with buffer exchange (v) 105 min at pH 6.5 without buffer exchange (vi) 105 min at pH 6.5 with buffer exchange (vii) 75 min at pH 7.4 without buffer exchange (viii) 75 min at pH 7.4 with buffer exchange. Scale bars represent 25 μm.

3.3.1 Influence of Reaction Length

Increased lengths of reaction of the PEG-rich domains resulted in decreased swollen microsphere sizes, but after 45 min at pH 7.4 or 75 min at pH 6.5, the swollen sizes did not decrease further (Figure 3.6). These asymptotic swollen sizes were reached

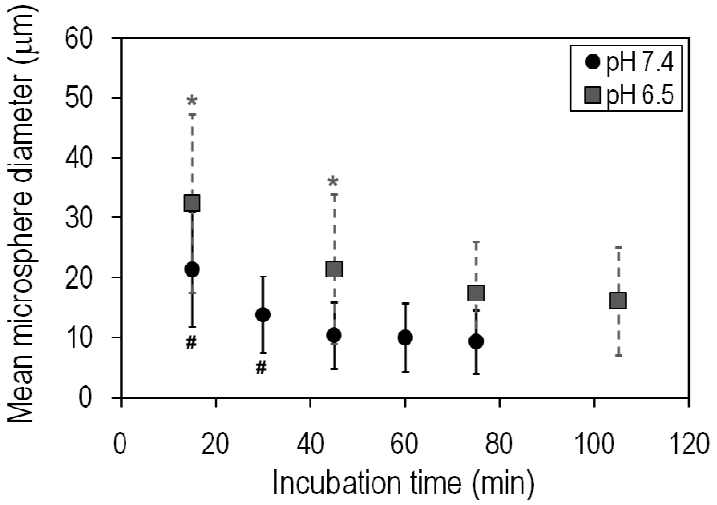

Figure 3.6 – Influence of Reaction Time on Microsphere Size. Microsphere diameters decreased with increasing reaction (incubation) time, with pH 6.5 microspheres approaching but not matching pH 7.4 sizes even at extended timepoints. Microspheres were formed from pre-reacted solutions of PEG_8-VS and PEG_8-amine ($d_{PCS} \cong 100$ nm), diluted to 2% (w/v) in PBS + 0.6 M sodium sulfate, incubated at 37ºC for various durations, and buffer exchanged into PBS. For pH 6.5 reactions, PEG solutions were pre-reacted to $d_{PCS} \cong 150$ nm to allow multiple observations prior to microsphere aggregation/bulk gel formation. Data represent $n = 500$ microspheres at each timepoint. $^{*}\ p < 0.05$ versus the 105 min timepoint for pH 6.5 and $^{\#}\ p < 0.05$ versus the 75 min timepoint for pH 7.4 (no significant changes in size were observed after 75 min at pH 6.5 or after 45 min at pH 7.4).

more quickly at the higher reaction pH. Despite the large differences in the sizes of swollen microspheres, unswollen sizes were unaffected by the duration of the reaction. Thus, the swelling ratio (Q, the increase in volume after buffer exchange) decreased with increasing reaction time above the cloud point (Figure 3.7).

	pH 6.5		pH 7.4	
Incubation time (min)	15	105	15	75
Mean unswollen diameter (mm)	8.78±3.83	8.37±3.65	5.51±1.91	4.90±1.66
Mean swollen diameter (mm)	32.41±14.92	16.10±8.99	21.44±9.65	9.36±5.29
Q (swelling ratio)	50.32	7.13	58.98	6.99

Figure 3.7 – Swelling Ratios of Microspheres. Q was the ratio of the unswollen mean volume to the swollen mean volume, with volumes calculated from mean diameters. Differences in mean microsphere diameters were statistically significant except for unswollen microspheres at pH 6.5 (15 vs. 105 min) and pH 7.4 (15 vs. 75 min).

3.3.2 Influence of Reaction Rate

Unlike the reaction length, a lower pH during the crosslinking reaction increased the sizes of both swollen and unswollen microspheres, which is contrasted with the length of reaction time that only influenced the sizes of swollen microspheres (Figure 3.7; Figure 3.8). The swelling ratio was relatively unaffected by the pH of the reacting solution (Figure 3.7). Temperature was also observed to impact microsphere size, with higher reaction temperatures resulting in smaller microspheres (Figure 3.9).

3.3.3 Microsphere Size Distributions

Polydispersity indices were calculated for microspheres formed at various pHs and generally decreased with increasing pH (Figure 3.8, inset). Representative observed distributions of the microspheres more closely resembled that expected for coalescence than for Ostwald ripening (Figure 3.10).

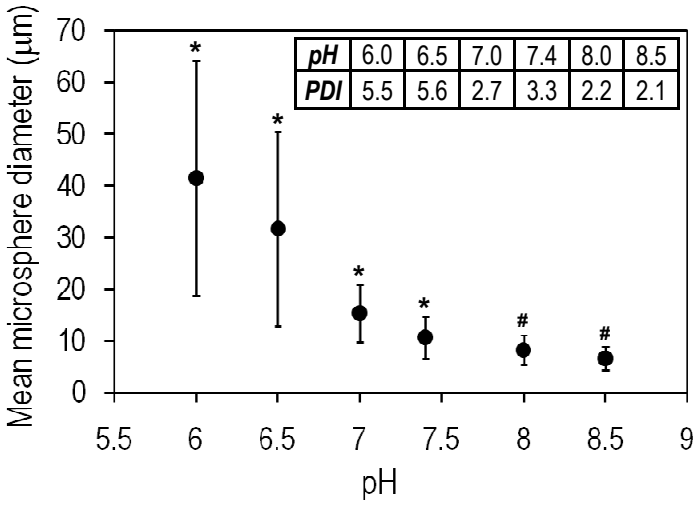

Figure 3.8 – Influence of Reaction pH on Microsphere Size. Microsphere diameters and polydispersity indices (PDIs) were observed to decrease with increasing pH. Microspheres were formed from pre-reacted solutions of PEG_8-VS and PEG_8-amine ($d_{PCS} \cong 100$ nm) diluted to 2% (w/v) in PBS + 0.6 M sodium sulfate at various pHs, incubated at 37ºC for 45 min, and buffer exchanged into PBS. Data represent n = 500 microspheres at each pH. $^{*}\ p < 0.05$ versus all other pHs and $^{\#}\ p < 0.05$ versus pH 6.0 -7.4.

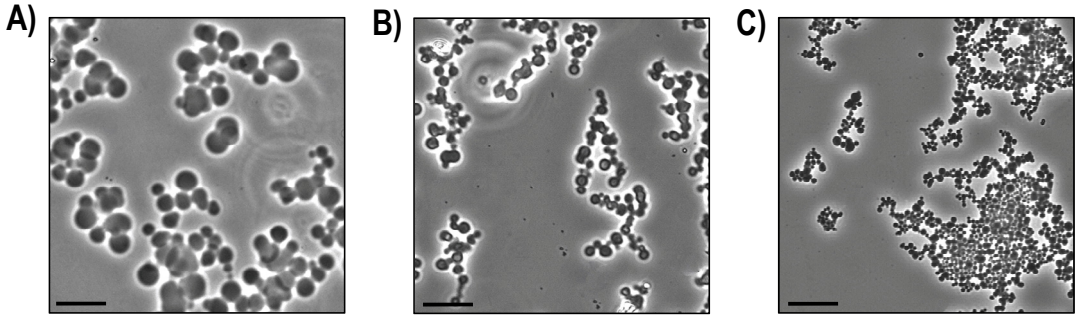

Figure 3.9 – Influence of Reaction Temperature on Microsphere Size. Phase-contrast photomicrographs at 20X magnification of microspheres produced from pre-reacted solutions of PEG_8-VS and PEG_8-amine ($d_{PCS} \cong 100$ nm) diluted to 2% (w/v) in PBS + 0.6 M sodium sulfate and incubated at pH 7.4 for: (i) 45 min at 37°C (ii) 10 min at 65°C (iii) 5 min at 95°C. Scale bars represent 25 μm.

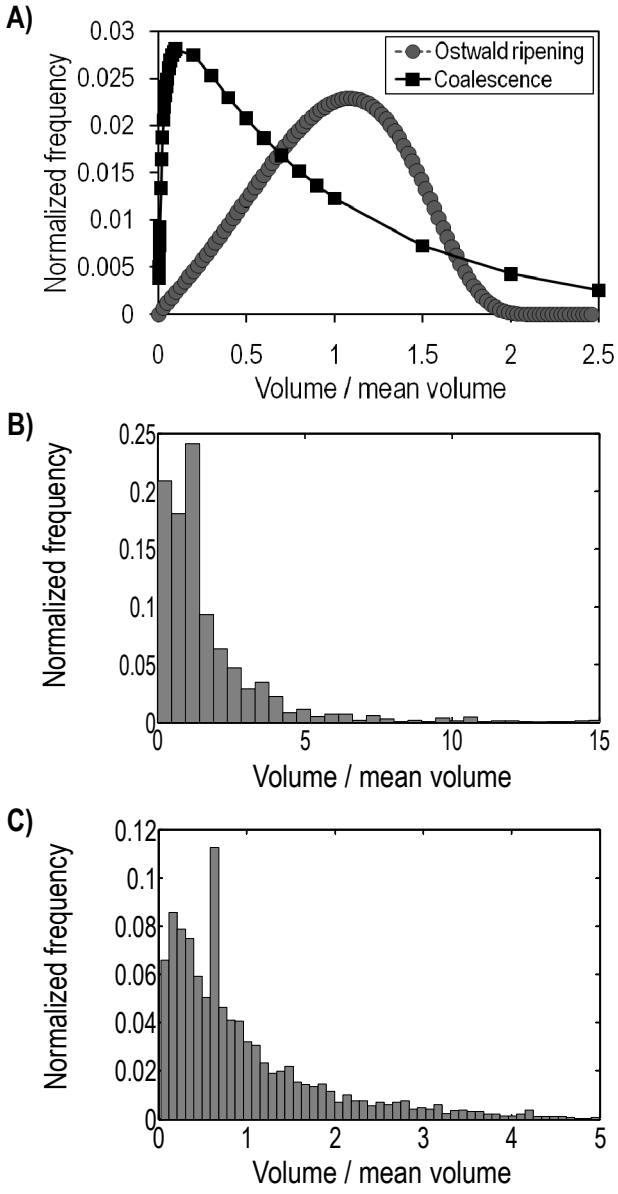

Figure 3.10 – Microsphere Size Distributions. Distributions of formed microspheres were similar to those expected for coalescence. A) Theoretical size distributions expected to result from Ostwald ripening or coalescence. B and C) Observed distributions of PEG_8-VS/PEG_8-amine microspheres formed from pre-reacted solutions of PEG_8-VS and PEG_8-amine ($d_{PCS} \cong 100$ nm) diluted to 2% (w/v) in PBS + 0.6 M sodium sulfate and incubated at 37°C for 45 min at: (B) 7.4 (C) 8.5. Histograms were constructed using 100 bins for 500 randomly selected microspheres for each pH.

3.4 Power Law Plots

The slopes of the power law plots revealed identical scaling exponent values. Log-log plots of mean microsphere diameter both versus fractional time remaining to the gel point (Figure 3.11) and versus the pH-based reaction rate (Figure 3.12) yielded slopes of 0.24. Thus, the growth of the mean PEG-rich domain diameters during coarsening for all observed conditions approximately scaled as $\bar{d} \propto time^{1/4}$. Note that for the pH-based plot, microspheres formed at pH 6 and 6.5 were not included in the analysis because their swollen sizes were far from asymptotic at 45 min as judged by the results in Figure 3.6. As a result, these microspheres thus should swell to much greater degrees than microspheres produced at the higher pHs.

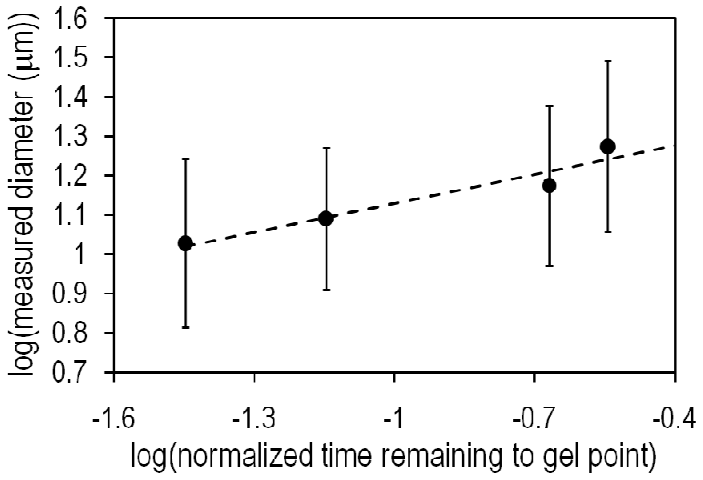

Figure 3.11 – Power Law Plot of Fraction Time Remaining to Gelation versus Microsphere Size. The log-log plot of mean microsphere diameters from Figure 3.6 against the fractional (normalized) time remaining to the gel point at corresponding extents of pre-reaction. Linear regression yielded a slope of 0.24, suggesting a $R \propto time^{1/4}$ growth law.

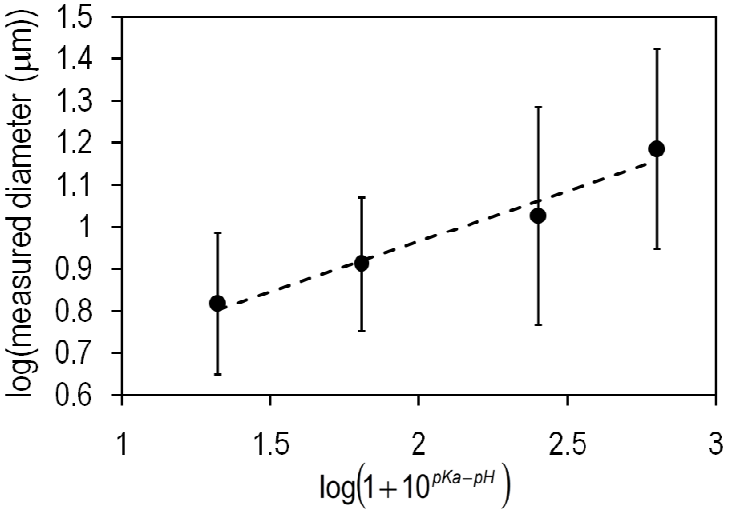

Figure 3.12 – Power Law Plot of pH-based Reaction Rate versus Microsphere Size. The log-log plot of mean microsphere diameters from Figure 3.8 against the derived pH-based reaction rate at corresponding pHs. Linear regression yielded a slope of 0.24, suggesting a $R \propto time^{1/4}$ growth law.

4. Discussion

Several factors influenced the production of 100% PEG microspheres via thermally induced phase separation in the absence of surfactants or organic solvents. Whether or not the PEG derivatives were pre-reacted was the most prominent influence on fabrication, as without pre-reaction stable microspheres were not observed to form under mild conditions (pH 7.4, 37°C). Under these conditions, PEG_8-VS but not PEG_8-amine was observed to phase separate by cloud point measurements. Compared to amine groups, vinylsulfone groups are relatively hydrophobic due to their aliphatic vinyl group. Additionally, the pK_a of amine groups is 9.8, leading to a predominance of protonated, positively charged amine groups at pH 7.4 (Brown, Devadas et al. 1997). Hence, the difference in cloud points was likely due to the increased hydrophobic hydration cost and diminished hydrogen bonding capability of PEG_8-VS relative to PEG_8-amine. This same logic also explains the even lower cloud point of PEG_8-acrylate.

This discrepancy in cloud points likely resulted in PEG solutions that were not pre-reacted containing only spherical PEG_8-VS-rich domains above the cloud point. In this scenario, PEG_8-amine still in solution would be sequestered from phase-separated PEG_8-VS with which it has to react to form microspheres. Alternatively, phase-separated PEG-rich domains in pre-reacted solutions should contain oligomers and microgels such that both derivatives are sequestered together at very high local

concentrations. Both of these factors should result in faster reaction rates within the PEG-rich domains for pre-reacted solutions. The combination of pre-reacted solutions being much closer to the gel point and their faster crosslinking reactions should have substantially reduced the time to reach the gel point relative to non-pre-reacted solutions, leading to the stable formation of microspheres within 45 min.

Sizes of microspheres formed from pre-reacted PEG solutions were affected by the extent of pre-reaction as well as the pH, temperature and duration of the crosslinking reaction above the cloud point. These results demonstrated that the final, swollen microsphere sizes were governed primarily by two factors: (1) the degree of PEG-rich droplet coarsening prior to reaching the gel point, and (2) the extent of crosslinking within microspheres beyond the gel point. These dependencies provide insight into the mechanisms underlying the formation and swelling of cloud-point microspheres.

4.1 Coarsening Prior to Reaching the Gel Point

Coarsening of the phase-separated PEG-rich domains occurred by a combination of Ostwald ripening and coalescence. Although the two mechanisms follow the same growth law of $R \propto time^{1/3}$, they can be distinguished by the resulting microsphere size distributions (Crist and Nesarikar 1995). During coarsening, the sizes

of phase-separated domains are polydisperse and the observed microsphere size distributions likely reflected the polydispersity of the phase-separated domains at the time of gelation (Lifshitz and Slyozov 1961; Wagner 1961; Friedlander and Wang 1966; Crist and Nesarikar 1995; Vemury and Pratsinis 1995). The leftward-shifted means of the distributions are indicative of coarsening dominated by coalescence rather than Ostwald ripening (Figure 3.10) (Crist and Nesarikar 1995). Our lab has also frequently observed coalescence of fluorescently labeled PEG-rich domains by confocal microscopy under typical formation conditions, further suggesting the dominance of this process[**].

Coarsening was halted by gelation and thus the length of time required to reach the gel point was a major determinant of microsphere size. One method used to alter the time to gelation was changing the rate of the crosslinking reaction by varying the temperature. The rate of reaction increases with increasing temperature according to the Arrhenius equation, and thus the gel point should be reached earlier (Jenkins, Kratochvil et al. 2009). With less time for coarsening, PEG-rich domains are smaller when they reach the gel point. Consequently, smaller hydrogel microspheres would be expected, which was in agreement with our observation (Figure 3.9). Coarsening should also proceed more quickly at high temperatures, which should lead to the formation of larger microspheres. However, the rate of reaction increases exponentially with

[**] Evan A. Scott designed and carried out these experiments.

temperature while the rate of coarsening only increases linearly, overwhelming any apparent qualitative increase in coarsening rate (Friedlander 1977; Marqusee and Ross 1984).

Reaction rate was also modulated by varying the pH, with more acidic conditions resulting in larger microspheres. The rate of the second-order reaction between vinylsulfone and amine groups is decreased at lower pH due to a greater proportion of less nucleophilic, protonated amines. Analysis of the effect of pH (detailed in Section 2.9.1) was performed and predicted that mean microsphere diameters should scale with pH as $\bar{d} \propto (1+10^{pKa-pH})^{\alpha}$, where α is a scaling factor. As classical coarsening possesses a $R \propto time^{1/3}$ power law, an α value of 1/3 was expected. Instead, linearization and linear regression revealed a value of 0.24, which suggested a $R \propto time^{1/4}$ power law (Figure 3.12).

Alternatively to changing the reaction rate, the time required to reach the gel point was also changed by the proximity to the gel point at the beginning of the reaction. Pre-reaction of the PEG derivatives should simply bring the solution closer to the gel point. With less time required for gelation upon phase separation, the gel point would occur earlier in coarsening, resulting in smaller microspheres. As detailed in Section 2.9.2, this effect was expressed as a fractional time remaining to the gel point via the power law $\bar{d} \propto t_{frac}{}^{\alpha}$ that predicts that mean diameters of microspheres fabricated

from different pre-reacted PEG solutions should again scale with some factor α. Surprisingly, linearization of the data again revealed an α value of 0.24 for a $R \propto time^{1/4}$ growth law instead of the relationship expected for classical coarsening (Figure 3.11).

Both power law plots supported a $R \propto time^{1/4}$ growth law instead of a $R \propto time^{1/3}$ growth law, which is plausible based on previous results in the literature for off-critical polymer compositions. Immediately following phase separation, the PEG-rich domains may be connected in a percolated, network-like structure (Lauger, Lay et al. 1994). Surface tension drives the flow from the thin, connected web of phase separated polymer into larger clusters (McMaster 1975; Siggia 1979). During this initial flow-driven period, a relatively rapid $R \propto time$ growth law is followed (McMaster 1975; Lauger, Lay et al. 1994; Crist 1996; Termonia 1997). Upon reaching the percolation-to-cluster transition (PCT), the growth law slows dramatically to $R \propto time^{1/4}$, eventually evolving to the classical $R \propto time^{1/3}$ growth law (Crist 1996; Termonia 1997).

Additional evidence for the occurrence of a PCT during coarsening was found by DLS. Monitoring the d_{PCS} of PEG domains just after phase separation, we have

observed growth to be linear during the first few minutes of coarsening††. This indicated coarsening proceeded according to a $R \propto time$ power law initially following phase separation. Soon after, growth apparently plateaued. These results of a growth regime shift have been previously reported in studies following polymer coarsening via light scattering (Lauger, Lay et al. 1994). Additionally, molecular dynamics simulations suggest a transition from the $R \propto time$ growth law to a $R \propto time^{1/4}$ growth law (Termonia 1997). The $R \propto time^{1/4}$ growth law observed here may reflect that the gel point is reached during the intermediate period after the PCT but before the $R \propto time^{1/3}$ regime of classical coarsening is achieved. Together these results imply that the pre-reacted PEG is at an off-critical concentration and phase separation occurs at least in part by SD to initially form a percolated structure that subsequently breaks down into the gelling spherical PEG-rich domains.

Despite the above conclusion being logically sound, the potential exclusion of some smaller microspheres from distributions sized could have skewed the observed α value and thus the power law. Some smaller microspheres may have been lost during buffer exchange, and those surviving the wash step with diameters < 1.5 μm were too small to accurately size from phase-contrast photomicrographs at 20X. These experimental shortcomings would have been more pronounced for microspheres with smaller average diameters, because a larger proportion of the population would be

†† Evan A. Scott designed and carried out these experiments.

prone to these effects. In such cases the apparent mean size would be lower than the actual size, and this discrepancy would have been more marked for smaller means. Thus for faster gelling conditions, the slope of the power law plots could be skewed downward due to overestimation of the average microsphere size, leading to smaller values of α. Nonetheless, the remarkably identical exponents obtained in two separate sets of experiments suggest that the $R \propto time^{1/4}$ growth law governs the sizes of microspheres formed under these conditions.

4.2 Additional Crosslinking Beyond the Gel Point

Final microsphere sizes were established from the swelling of unswollen microspheres upon buffer exchange into PBS. Hydrogel swelling in this case likely resulted from externally higher osmotic pressure driving water into microspheres below their cloud point, a phenomenon observed for similarly thermoresponsive pNIPAm hydrogels (Wu, Hoffman et al. 1992; Dusek 1993; Yoshida, Uchida et al. 1995). Above the LCST, the aqueous phase effectively becomes a poor solvent and is extruded from the gel, driving polymeric collapse and causing phase separation analogous to that of hydrophilic copolymers in organic solvent (Holtz and Asher 1997; Toyotama, Sawada et al. 2006). Buffer exchange into PBS should bring the microspheres below the cloud point, restoring the aqueous phase as a good solvent and causing swelling.

While pH affected the sizes of both swollen and unswollen microspheres, the length of incubation beyond the gel point only affected the sizes of the swollen microspheres (Figure 3.7). Coarsening should be halted after the PEG-rich domains reached the gel point and further reaction should not alter the sizes of unswollen microspheres. However, further reaction beyond the gel point should increase the mean density of crosslinks within the microspheres and consequently reduce the degree of swelling Q upon buffer exchange.

The Flory-Rehner equation predicts the relationship between the mean crosslink density of a polymeric network and its degree of swelling given a number of physical and thermodynamic parameters. For gels (1) exhibiting a high degree of swelling ($Q >$ 10) and (2) possessing an average molecular weight between crosslinks $\overline{M}_c$ much smaller than the MW of the polymer, the Flory-Rehner equation can be simplified to $Q \propto \overline{M}_c^{3/5}$ (Flory 1950; Flory 1953; Anseth, Metters et al. 2002). As the crosslinking proceeds, $\overline{M}_c$ should decrease based on the reaction rate and the combinatorics of the endgroups. Expected for gels of the same composition, the asymptotic values of Q were the ultimately the same ($Q_{asymp} \cong 7$) for microspheres formed at both pHs. The faster rate of crosslinking at the higher pH decreased the value of $\overline{M}_c$ this value more quickly, leading to Q_{asymp} being reached sooner. Though Q_{asymp} was less than the value of 10 assumed by the simplified form of the Flory-Rehner equation, the observed

asymptotic decrease in microsphere swelling was qualitatively in agreement with the relationship and thus its usage seemed appropriate. While quantitative analysis of the data in the context of the full Flory-Rehner equation would be illuminating, detailed knowledge of network defects and kinetics of the reaction would be required. Such analysis is beyond the scope of this investigation.

4.3 Inherent Characteristics of the Method

Microspheres produced by this method were both polydisperse and observed to cluster during and after fabrication. These clusters were usually easily dispersed by tituration, but if incubated for longer durations microsphere aggregates formed. Residual vinylsulfone and amine functional groups present on the surfaces of microspheres likely reacted with complimentary groups on other microspheres to form inter-microsphere crosslinks. As more of these bonds were formed, larger groups of microspheres that were more difficult to disperse were produced. Also, though most observed microspheres were spherical, the geometry of some appeared as though gelation occurred during coalescence of two or more particles (Figure 3.2). The characterstics of polydispersity and clustering intrinsic to this technique must be suitable for the ultimate application of the microspheres.

The presented method of microsphere fabrication differs from alternative techniques. Compared to methods producing microparticles in a serial fashion, this solution-based method offers the easy production of large quantities of microspheres but lacks control over particle shape (Rivest, Morrison et al. 2007). By thermally inducing phase separation of PEG using sodium sulfate, organic solvents and surfactants that could be time consuming to remove and negatively affect biocompatibility are avoided. Though most solution-based methods require these additives, they have also been avoided in variations of emulsion polymerization, precipitation polymerization and gelatin coacervation (Arshady 1990; Franssen and Hennink 1998; Nolan, Reyes et al. 2005). Instead of an organic solvent, dextran has been used to promote phase separation of PEG and make free-radical polymerized microspheres (Franssen and Hennink 1998). However, dextran is more expensive and may be more difficult to remove than sodium sulfate. Additionally, most of these solution-based strategies rely on stirring/agitation to reduce the size of polymer-rich domains prior to crosslinking or on surfactants to stabilize the particles and control growth. Our method works in the absence of mixing because gelation is timed to occur early in coarsening, generating microspheres of controllable size without the use of surfactants. The mild nature of the method offers the possibility of conjugating chemically reactive biomolecules to the microspheres during and/or after their formation. Together, the simplicity, effectiveness, and flexibility of this technique provides a promising route to engineer highly biocompatible microspheres for a host of applications.

4.4 Conclusions

In summary, microsphere sizes were established primarily by the extent of coarsening that occurred prior to gelation and the degree of crosslinking within the microspheres beyond the gel point. Analysis of microsphere sizes in conjunction with quantitative coarsening data suggested that phase separation occurred by off-critical spinodal decomposition, initially forming a web-like structure that quickly broke down into spherical PEG-rich domains by a percolation-to-cluster transition. Size distributions of microsphere sizes indicated that coarsening of the PEG-rich domains was likely dominated by coalescence. Using the principles outlined here, the properties of 100% PEG microspheres can be engineered for specific applications such as affinity-based purification systems or as surface coatings (Singh, Bridges et al. 2007; Scott, Nichols et al. 2008). However, they are perhaps best-suited as components of modular tissue engineering scaffolds due to their high biocompatibility, residual reactivity and ability to be formed under mild conditions that biomolecules could survive (Scott, Nichols et al. In preparation).

References

Anseth, K. S., A. T. Metters, et al. (2002). In situ forming degradable networks and their application in tissue engineering and drug delivery.

Arshady, R. (1990). "MICROSPHERES AND MICROCAPSULES, A SURVEY OF MANUFACTURING TECHNIQUES .2. COACERVATION." Polymer Engineering and Science **30**(15): 905-914.

Bae, Y. C., S. M. Lambert, et al. (1991). "CLOUD-POINT CURVES OF POLYMER-SOLUTIONS FROM THERMOOPTIC MEASUREMENTS." Macromolecules **24**(15): 4403-4407.

Bailey, F. E. and R. W. Callard (1959). "Some properties of poly(ethylene oxide) in aqueous solution." J Appl Polym Sci **1**: 56-62.

Bailey, F. E. and J. V. Kaleske (1976). Poly(Ethylene Oxide). New York, Academic Press.

Banerjee, P., D. J. Irvine, et al. (2000). "Polymer latexes for cell-resistant and cell-interactive surfaces." Journal of Biomedical Materials Research **50**(3): 331-339.

Beebe, D. J., J. S. Moore, et al. (2000). "Functional hydrogel structures for autonomous flow control inside microfluidic channels." Nature **404**(6778): 588-+.

Bhavsar, M. D. and M. M. Amiji (2007). "Polymeric nano- and microparticle technologies for oral gene delivery." Expert Opinion on Drug Delivery **4**(3): 197-213.

Bhavsar, M. D. and M. M. Amiji (2008). "Development of novel biodegradable polymeric nanoparticles-in-microsphere formulation for local plasmid DNA delivery in the gastrointestinal tract." Aaps Pharmscitech **9**(1): 288-294.

Borowiec, J. W., P. Venge, et al. (1997). "Biomaterial-dependent blood activation during simulated extracorporeal circulation: a study of heparin-coated and uncoated circuits." Thoracic and Cardiovascular Surgeon **45**(6): 295-301.

Bostrom, M., D. R. M. Williams, et al. (2001). "Specific ion effects: Why DLVO theory fails for biology and colloid systems." Physical Review Letters **87**(16).

Boutris, C., E. G. Chatzi, et al. (1997). "Characterization of the LCST behaviour of aqueous poly(N-isopropylacrylamide) solutions by thermal and cloud point techniques." Polymer **38**(10): 2567-2570.

Brodbeck, W. G., E. Colton, et al. (2003). Effects of adsorbed heat labile serum proteins and fibrinogen on adhesion and apoptosis of monocytes/macrophages on biomaterials.

Brown, D. L., B. Devadas, et al. (1997). "Replacements for lysine in L-seryl-L-lysyl dipeptide amide inhibitors of Candida albicans myristoyl-CoA: Protein N-myristoyltransferase." Bioorganic & Medicinal Chemistry Letters **7**(3): 379-382.

Cavanagh, H. M. A., D. Dingwall, et al. (2001). "Cell contact dependent extended release of adenovirus by microparticles in vitro." Journal of Virological Methods **95**(1-2): 57-64.

Cho, Y., Y. Zhang, et al. (2008). "Effects of Hofmeister anions on the phase transition temperature of elastin-like polypeptides." J Phys Chem B **112**(44): 13765-71.

Collins, K. D., G. W. Neilson, et al. (2007). "Ions in water: Characterizing the forces that control chemical processes and biological structure." Biophysical Chemistry **128**(2-3): 95-104.

Collins, K. D. and M. W. Washabaugh (1985). "THE HOFMEISTER EFFECT AND THE BEHAVIOR OF WATER AT INTERFACES." Quarterly Reviews of Biophysics **18**(4): 323-422.

Cortesi, R., E. Esposito, et al. (1994). "GELATIN MICROSPHERES AS A NEW APPROACH FOR THE CONTROLLED DELIVERY OF SYNTHETIC OLIGONUCLEOTIDES AND PCR-GENERATED DNA FRAGMENTS." International Journal of Pharmaceutics **105**(2): 181-186.

Crist, B. (1996). "On "pinning" domain growth in two-phase polymer liquids." Macromolecules **29**(22): 7276-7279.

Crist, B. and A. R. Nesarikar (1995). "COARSENING IN POLYETHYLENE COPOLYMER BLENDS." Macromolecules **28**(4): 890-896.

Del Valle, E. M. M., E. P. Herrero, et al. (2006). "Development of a new technology for the production of microcapsules based in atomization processes." Chemical Engineering Journal **117**(2): 137-42.

Di Serio, F., C. Gechtman, et al. (2006). "Analytical evaluation of a new microparticle enzyme immunoassay (MEIA) for the determination of Sirolimus and

comparison with High-Performance Liquid Chromatography Tandem Mass Spectrometry (HPLC-MS/MS)." Clinical Chemistry **52**(6): B72.

Dusek, K., Ed. (1993). Responsive Gels: Volume Transitions II. Berlin, Springer.

Edman, P., B. Ekman, et al. (1980). "Immobilization of proteins in microspheres of biodegradable polyacryldextran." J Pharm Sci **69**(7): 838-42.

Elbert, D. L. and J. A. Hubbell (1996). "Surface treatments of polymers for biocompatibility." Annual Review of Materials Science **26**: 365-394.

Elbert, D. L. and J. A. Hubbell (2001). "Conjugate addition reactions combined with free-radical cross-linking for the design of materials for tissue engineering." Biomacromolecules **2**(2): 430-41.

Flodin, P. (1965). Process for preparing hydrophilic copolymerization and product obtained thereby. US.

Flory, P. J. (1950). "Statistical mechanics of swelling of network structures." J Chem Phys **18**: 108-11.

Flory, P. J. (1953). Principles of Polymer Chemistry. Ithaca, Cornell University Press.

Franks, F., Ed. (1973). Water: A Comprehensive Treatise. New York, Plenum.

Franssen, O. and W. E. Hennink (1998). "A novel preparation method for polymeric microparticles without the use of organic solvents." International Journal of Pharmaceutics **168**(1): 1-7.

Friedlander, S. K. (1977). Smoke, Dust and Haze. New York, Wiley.

Friedlander, S. K. and C. S. Wang (1966). "The self-preserving particle size distribution for coagulation by brownian motion." Journal of Colloid and Interface Science **22**: 126-32.

Groll, J., W. Haubensak, et al. (2005). "Ultrathin coatings from isocyanate terminated star PEG prepolymers: patterning of proteins on the layers." Langmuir **21**(7): 3076-83.

Groth, T., K. Derdau, et al. (1992). "THE HEMOCOMPATIBILITY OF BIOMATERIALS INVITRO - INVESTIGATIONS ON THE MECHANISM OF THE WHOLE-BLOOD CLOT FORMATION TEST." Atla-Alternatives to Laboratory Animals **20**(3): 390-395.

Gunton, J. D., M. S. Miguel, et al. (1983). Phase Transitions and Critical Phenomena. London, Academic Press.

Gupta, V., S. Nath, et al. (2002). "Role of water structure on phase separation in polyelectrolyte-polyethyleneglycol based aqueous two-phase systems." Polymer **43**(11): 3387-3390.

Gurau, M. C., S. M. Lim, et al. (2004). "On the mechanism of the Hofmeister effect." Journal of the American Chemical Society **126**(34): 10522-10523.

Harris, J. M. (1997). "Introduction to biomedical and biotechnical applications of polyethylene glycol." Abstracts of Papers of the American Chemical Society **213**: 21-POLY.

Hofmeister, F. (1888). "Zur Lehre von der Wirkung der Salze." Arch Exp Pathol Pharmakol **24**: 247-260.

Holtz, J. H. and S. A. Asher (1997). "Polymerized colloidal crystal hydrogel films as intelligent chemical sensing materials." Nature **389**(6653): 829-32.

Janatova, J. (2000). "Activation and control of complement, inflammation, and infection associated with the use of biomedical polymers." Asaio Journal **46**(6): S53-S62.

Jenkins, A., P. Kratochvil, et al. (2009). Compendium of Polymer Terminology and Nomenclature: IUPAC Recommendations 2008. Cambridge, UK, The Royal Society of Chemistry.

Jenney, C. R. and J. M. Anderson (1999). "Effects of surface-coupled polyethylene oxide on human macrophage adhesion and foreign body giant cell formation in vitro." Journal of Biomedical Materials Research **44**(2): 206-216.

Jeon, S. I., J. H. Lee, et al. (1991). "PROTEIN SURFACE INTERACTIONS IN THE PRESENCE OF POLYETHYLENE OXIDE .1. SIMPLIFIED THEORY." Journal of Colloid and Interface Science **142**(1): 149-158.

Kao, W. J., S. Sapatnekar, et al. (1996). "Complement-mediated leukocyte adhesion on poly(etherurethane ureas) under shear stress in vitro." Journal of Biomedical Materials Research **32**(1): 99-109.

Karlsson, C., H. Nygren, et al. (1996). "Exposure of blood to biomaterial surfaces liberates substances that activate polymorphonuclear granulocytes." Journal of Laboratory and Clinical Medicine **128**(5): 496-505.

Kawaguchi, H., K. Fujimoto, et al. (1992). "HYDROGEL MICROSPHERES .3. TEMPERATURE-DEPENDENT ADSORPTION OF PROTEINS ON POLY-N-ISOPROPYLACRYLAMIDE HYDROGEL MICROSPHERES." Colloid and Polymer Science **270**(1): 53-57.

Kemp, R. B., R. W. Meredith, et al. (1983). "A rapid cell culture technique for assessing the toxicity of detergent-based products in vitro as a possible screen for eye irritancy in vivo." Cytobios **36**(143-144): 153-9.

Khademhosseini, A., G. Eng, et al. (2006). "Micromolding of photocrosslinkable hyaluronic acid for cell encapsulation and entrapment." Journal of Biomedical Materials Research Part A **79A**(3): 522-532.

Khademhosseini, A., R. Langer, et al. (2006). "Microscale technologies for tissue engineering and biology." Proceedings of the National Academy of Sciences of the United States of America **103**(8): 2480-2487.

Koga, Y., P. Westh, et al. (2004). "Toward understanding the Hofmeister series. 1. Effects of sodium salts of some anions on the molecular organization of H2O." Journal of Physical Chemistry A **108**(40): 8533-8541.

Kondo, A. and H. Fukuda (1997). "Preparation of thermo-sensitive magnetic hydrogel microspheres and application to enzyme immobilization." Journal of Fermentation and Bioengineering **84**(4): 337-341.

Kunz, W., P. Lo Nostro, et al. (2004). "Hofmeister phenomena." Current Opinion in Colloid & Interface Science **9**(1-2): VII-VII.

Kunz, W., P. Lo Nostro, et al. (2004). "The present state of affairs with Hofffieister effects." Current Opinion in Colloid & Interface Science **9**(1-2): 1-18.

Kwok, K. K., M. J. Groves, et al. (1991). "Production of 5-15 microns diameter alginate-polylysine microcapsules by an air-atomization technique." Pharm Res **8**(3): 341-4.

Lauger, J., R. Lay, et al. (1994). "THE PERCOLATION-TO-CLUSTER TRANSITION DURING SPINODAL DECOMPOSITION OF AN OFF-CRITICAL POLYMER MIXTURE - OBSERVATION BY LIGHT-SCATTERING AND OPTICAL MICROSCOPY." Journal of Chemical Physics **101**(8): 7181-7184.

Lifshitz, I. M. and V. V. Slyozov (1961). "The Kinetics of Precipitation from Supersaturated Solid Solutions." J Phys Chem Solids **19**: 35-50.

Liu, V. A. and S. N. Bhatia (2002). "Three-dimensional photopatterning of hydrogels containing living cells." Biomedical Microdevices **4**(4): 257-266.

Liu, W. G., M. Griffith, et al. (2008). "Alginate microsphere-collagen composite hydrogel for ocular drug delivery and implantation." Journal of Materials Science-Materials in Medicine **19**(11): 3365-3371.

Lopez, J. E. and N. A. Peppas (2004). "Effect of poly (ethylene glycol) molecular weight and microparticle size on oral insulin delivery from P(MAA-g-EG) microparticles." Drug Development and Industrial Pharmacy **30**(5): 497-504.

Lussi, J. W., D. Falconnet, et al. (2006). "Pattern stability under cell culture conditions - A comparative study of patterning methods based on PLL-g-PEG background passivation." Biomaterials **27**(12): 2534-2541.

Margel, S. (1983). "AGAROSE-POLYALDEHYDE MICROSPHERE BEADS - SYNTHESIS AND BIOMEDICAL APPLICATIONS - CELL LABELING, CELL-SEPARATION, AFFINITY-CHROMATOGRAPHY, AND HEMOPERFUSION." Applied Biochemistry and Biotechnology **8**(6): 523-539.

Marqusee, J. A. and J. Ross (1984). "THEORY OF OSTWALD RIPENING - COMPETITIVE GROWTH AND ITS DEPENDENCE ON VOLUME FRACTION." Journal of Chemical Physics **80**(1): 536-543.

Masri, M. S. and M. Friedman (1988). "PROTEIN REACTIONS WITH METHYL AND ETHYL VINYL SULFONES." Journal of Protein Chemistry **7**(1): 49-54.

McGuigan, A. P. and M. V. Sefton (2006). "Vascularized organoid engineered by modular assembly enables blood perfusion." Proc Natl Acad Sci U S A **103**(31): 11461-6.

McMaster, L. P. (1975). Copolymers, Polyblends, and Composites. Washington DC, American Chemical Society.

Nisisako, T., T. Torii, et al. (2002). "Droplet formation in a microchannel network." Lab on a Chip **2**(1): 24-26.

Nolan, C. M., C. D. Reyes, et al. (2005). "Phase transition behavior, protein adsorption, and cell adhesion resistance of poly(ethylene glycol) cross-linked microgel particles." Biomacromolecules **6**(4): 2032-9.

Odian, G. (2004). Principles of polymerization. Hoboken, NJ, John Wiley & Sons, Inc.

Omta, A. W., M. F. Kropman, et al. (2003). "Negligible effect of ions on the hydrogen-bond structure in liquid water." Science **301**(5631): 347-349.

Osikwoicz, G., P. Fries, et al. (1988). "IMX FERRITIN - A FULLY AUTOMATED MICROPARTICLE ENZYME-IMMUNOASSAY (MEIA) FOR THE DETERMINATION OF FERRITIN IN HUMAN-SERUM." Clinical Chemistry **34**(6): 1155-1155.

Pegram, L. M. and M. T. Record (2006). "Partitioning of atmospherically relevant ions between bulk water and the water/vapor interface." Proceedings of the National Academy of Sciences of the United States of America **103**(39): 14278-14281.

Peppas, N. A., J. Z. Hilt, et al. (2006). "Hydrogels in biology and medicine: From molecular principles to bionanotechnology." Advanced Materials **18**(11): 1345-1360.

Pini, L. A., D. Gallesi, et al. (2006). "Switching from HPLC/UV to MEIA for whole blood sirolimus quantitation: Comparison of methods." Journal of Clinical Laboratory Analysis **20**(6): 239-244.

Rabel, F. M. (1980). "USE AND MAINTENANCE OF MICROPARTICLE HIGH-PERFORMANCE LIQUID-CHROMATOGRAPHY COLUMNS." Journal of Chromatographic Science **18**(9): 394-408.

Ratke, L. and P. W. Voorhees (2002). Growth and Coarsening: Ostwald Ripening in Material Processing. Berlin, Springer-Verlag.

Rivest, C., D. W. G. Morrison, et al. (2007). "Microscale hydrogels for medicine and biology: Synthesis, characteristics and applications." Journal of Mechanics of Materials and Structures **2**(6): 1103-1119.

Robeson, L. M. (2007). Polymer Blends: A Comprehensive Review. Cincinnati, OH, Hanser Gardner Publications, Inc.

Saeki, S., N. Kuwahara, et al. (1976). "Upper and lower critical solution temperatures in poly(ethylene glycol) solutions." Polymer **17**: 685-9.

Schellman, J. A. (2003). "Protein stability in mixed solvents: A balance of contact interaction and excluded volume." Biophysical Journal **85**(1): 108-125.

Schuster, P., G. Zundel, et al. (1976). The Hydrogen Bond. Amsterdam, Elsevier.

Scott, E. A., M. D. Nichols, et al. (2008). "Protein adsorption and cell adhesion on nanoscale bioactive coatings formed from poly(ethylene glycol) and albumin microgels." Biomaterials **29**(34): 4481-93.

Scott, E. A., M. D. Nichols, et al. (In preparation). "Modular bioactive scaffolds assembled from poly(ethylene glycol) cloud point microspheres with macroporation using a non-cytotoxic porogen."

Siggia, E. D. (1979). "Late stages of spinodal decomposition in binary mixtures." Physical Review A **20**: 595.

Singh, N., A. W. Bridges, et al. (2007). "Covalent tethering of functional microgel films onto poly(ethylene terephthalate) surfaces." Biomacromolecules **8**(10): 3271-5.

Smith, J. D., R. J. Saykally, et al. (2007). "The effects of dissolved halide anions on hydrogen bonding in liquid water." Journal of the American Chemical Society **129**(45): 13847-13856.

Song, J. D., R. Ryoo, et al. (1991). "ANION BINDING-PROPERTIES OF POLY(VINYLPYRROLIDONE) IN AQUEOUS-SOLUTION STUDIED BY HALIDE NMR-SPECTROSCOPY." Macromolecules **24**(8): 1727-1730.

Sun, T. and H. E. King (1998). "Discovery and modeling of upper critical solution behavior in the poly(ethylene oxide) water system at elevated pressure." Macromolecules **31**(18): 6383-6386.

Termonia, Y. (1997). "Molecular modeling of structure development upon quenching of a polymer solution." Macromolecules **30**(18): 5367-5371.

Toyotama, A., T. Sawada, et al. (2006). "Optical detection of nonequilibrium swelling behavior of a polymer gel upon solvent substitution." Langmuir **22**(5): 1952-1954.

Um, E., D. S. Lee, et al. (2008). "Continuous generation of hydrogel beads and encapsulation of biological materials using a microfluidic droplet-merging channel." Microfluidics and Nanofluidics **5**(4): 541-549.

Van Thienen, T. G., J. Demeester, et al. (2008). "Screening poly(ethyleneglycol) micro- and nanogels for drug delivery purposes." Int J Pharm **351**(1-2): 174-85.

Van Tomme, S. R., A. Mens, et al. (2008). "Macroscopic hydrogels by self-assembly of oligolactate-grafted dextran microspheres." Biomacromolecules **9**(1): 158-65.

Vemury, S. and S. E. Pratsinis (1995). "SELF-PRESERVING SIZE DISTRIBUTIONS OF AGGLOMERATES (VOL 26, 175, 1995)." Journal of Aerosol Science **26**(4): 701-701.

Videm, V. (2004). "Endpoint-attached heparin blocks neutrophil sticking and spreading." Biomaterials **25**(1): 43-51.

Wacker, B. K., E. A. Scott, et al. (2006). "Delivery of sphingosine 1-phosphate from poly(ethylene glycol) hydrogels." Biomacromolecules **7**(4): 1335-43.

Wagner, C. (1961). "Theorie der Alterung von Niederschlagen durch Umlosen." Zeitschrift fuer Elektrochemie **65**.

Weinstock, E., B. Khoobehi, et al. (1996). "A new biodegradable copolymer microsphere as an intravitreal drug delivery system." Investigative Ophthalmology & Visual Science **37**(3): 3569-3569.

Wu, X. S., A. S. Hoffman, et al. (1992). "SYNTHESIS AND CHARACTERIZATION OF THERMALLY REVERSIBLE MACROPOROUS POLY(N-ISOPROPYLACRYLAMIDE) HYDROGELS." Journal of Polymer Science Part a-Polymer Chemistry **30**(10): 2121-2129.

Xie, J. W., J. C. M. Marijnissen, et al. (2006). "Microparticles developed by electrohyd ro dynamic atomization for the local delivery of anticancer drug to treat C6 glioma in vitro." Biomaterials **27**(17): 3321-3332.

Xu, S., Z. Nie, et al. (2005). "Generation of monodisperse particles by using microfluidics: Control over size, shape, and composition (vol 44, pg 724, 2005)." Angewandte Chemie-International Edition **44**(25): 3799-3799.

Yeh, J., Y. Ling, et al. (2006). "Micromolding of shape-controlled, harvestable cell-laden hydrogels." Biomaterials **27**(31): 5391-8.

Yen, D. R., S. Raghavan, et al. (1996). "Fractional precipitation of star poly(ethylene oxide)." Macromolecules **29**(27): 8977-8978.

Yoshida, R., K. Uchida, et al. (1995). "COMB-TYPE GRAFTED HYDROGELS WITH RAPID DE-SWELLING RESPONSE TO TEMPERATURE-CHANGES." Nature **374**(6519): 240-242.

Zaslavsky, B. Y., T. O. Bagirov, et al. (1989). "STRUCTURE OF WATER AS A KEY FACTOR OF PHASE-SEPARATION IN AQUEOUS MIXTURES OF 2 NONIONIC POLYMERS." Polymer **30**(11): 2104-2111.

Zaslavsky, B. Y., N. M. Mestechkina, et al. (1983). "CHARACTERISTICS OF PROTEIN AQUEOUS-MEDIUM INTERACTIONS MEASURED BY PARTITION IN AQUEOUS FICOLL DEXTRAN BIPHASIC SYSTEM." Journal of Chromatography **260**(2): 329-336.

Zhang, Y. and P. S. Cremer (2006). "Interactions between macromolecules and ions: The Hofmeister series." Curr Opin Chem Biol **10**(6): 658-63.

Zhang, Y. J., S. Furyk, et al. (2005). "Specific ion effects on the water solubility of macromolecules: PNIPAM and the Hofmeister series." Journal of the American Chemical Society **127**(41): 14505-14510.

Vita

Michael D. Nichols

Date of Birth	August 7, 1984
Place of Birth	Atlanta, Georgia
Degrees	B.S. Biomedical Engineering, Washington University in St. Louis, May 2007
	M.S. Biomedical Engineering, Washington University in St. Louis, August 2009