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6. AUTHOR(S) Judy S. Riffle				
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Macromolecular Carriers for Nanomedicine and Nano-devices

AFOSR report

Introduction

Magnetic nano- and microspheres coated with polymers that have the right combination of characteristics have enormous potential for applications in drug delivery, biological sensor technologies, pathogen diagnostics, antibody-antigen and intracellular targeting, and more recently, for nano-motors and other nano-devices. For *in-vivo* use in all of these applications, the surface properties of the macromolecular nanospheres or microspheres must be tailored to 1) disperse them in physiological media, and 2) avoid immune response. Moreover, surfaces that can target specific cell populations or pathogens are also of great interest. This DARPA-AFOSR project has helped to address macromolecular concepts relative to tailoring the surface properties of biodegradable nanospheres and microspheres for *in-vivo* blood-contacting applications. The project goals have been to define relationships among chemical composition, processing parameters, nanosphere sizes and size distributions, and surface structure. Our accomplishments include 1) a facile method for achieving magnetite-poly(lactide) nanospheres that can be dispersed in aqueous media, 2) methods for functionalizing the termini of the hydrophilic brushes on the nanospheres in order to conjugate targeting moieties, 3) development of a nanosphere processing approach that yields nanospheres in the desired size range with a narrow distribution of sizes, and 4) maintenance of all of these characteristics with up to approximately 60 weight percent of magnetite incorporated into the nanospheres.

Approach

Our approach has been to prepare poly(D,L-lactide)s with molecular weights that are sufficiently high to afford good mechanical properties, functionalize one end of the poly(lactides) with carboxylic acids that adsorb well onto hydrophobic magnetite nanoparticles, and complex the polymer to the magnetite. This poly(lactide)-magnetite complex comprises the base nanosphere material. We also prepare a poly(ethylene oxide-*b*-D,L-lactide) (PEO-*b*-PDLA) block copolymer and functionalize the hydrophilic PEO terminus with a chemical group that can covalently bond targeting moieties onto the nanosphere surfaces after they are fabricated. We have constructed a precise confined impingement jet (CIJ) mixer for fabricating the nanospheres through controlled nucleation and growth of nanospheres. Low concentrations of the PEO-*b*-PDLA (i.e., 2 weight percent) are blended with the base poly(lactide)-magnetite nanosphere material in DMSO (a solvent for both) and this is placed in one jetstream of the mixer. The other stream comprises alcohol-water mixtures that are non-solvents for the nanospheres, but that extract the DMSO. The nanospheres are fabricated in the CIJ process and the block copolymer self-assembles at the nanosphere-nonsolvent interface. We have demonstrated this process with amine and maleimide functional groups at the surfaces of the nanospheres. The methods and results are discussed below. Some of the synthetic methods have been published and others have not yet been described in the literature. Thus, references are provided for the published procedures and examples of the (as yet) unpublished procedures are provided in this document.

Synthesis of diblock PEO-*b*-PDLA copolymers with functional groups on the PEO termini to provide tailored nanosphere surfaces through self-assembly

Synthesis of new vinylsilane initiators

Heterobifunctional PEO with a hydroxyl group on one chain end and one to three functional groups on the other were synthesized utilizing new vinylsilylpropanol initiators. These initiators containing one, two, or three vinyl groups were prepared from 3-chloropropylchlorodimethylsilane, 3-chloropropyldichloromethylsilane, and 3-

chloropropyltrichlorosilane, respectively, by reaction with vinylmagnesium chloride.^{1,2} The alkyl chlorides were then converted to the corresponding alcohols. The alkoxide initiators for PEO were prepared by reacting the appropriate vinylsilylpropanol with potassium naphthalide in THF (1 mole of vinylsilylpropanol:0.95 mole of potassium naphthalide). A slight deficiency of potassium naphthalide ensured that the vinyl groups were preserved during alkoxide formation and subsequent ethylene oxide polymerization. The anionic initiator was added to ethylene oxide and reacted at room temperature, then the polymerizations were terminated with acetic acid. The ratio of endgroup protons observed via ¹H NMR matched the theoretical values (3:2:2:2, 9:2:2:2, and 6:2:2:2 for the mono-, di-, and tri-vinylsilane initiators, respectively), confirming the structures of the heterobifunctional polymers. Molecular weights obtained by ¹H NMR and size exclusion chromatography (SEC) matched well with the targeted values based on the monomer to initiator ratios. Molecular weight distributions were narrow (≤ 1.13).

Synthesis of the diblock PEO-b-PDLA copolymer and functionalization of the PEO terminus with an amine by reaction of the vinylsilane with cysteamine

PEO is typically polymerized via anionic ring-opening polymerization techniques. These reactions proceed by nucleophilic attack of the anionic initiator on the ethylene oxide methylenes. Initiators include hydroxides, alkoxides, oxides, and metal alkyls/aryls such as potassium naphthalide. These reactions are living polymerizations, which are characterized by excellent control over the molecular weight and narrow molecular weight distributions. Such reactions can produce heterobifunctional polymers by utilizing one functional group on the initiator and another on the terminator. In this particular case, the PEO was heterobifunctional with one vinylsilane terminus and one hydroxyl terminus.

The ethylene oxide reactions are conducted under 40 psi of pressure at room temperature. As the ethylene oxide polymerization proceeds, the pressure drops to approximately 30 psi indicating that nearly all of the ethylene oxide has been consumed. The PEO is quenched with acetic acid and washed twice with water to neutralize the resulting potassium acetate. In addition, these polymerizations were conducted utilizing a slight deficiency of base in preparing the initiator solution. This ratio served to preserve the vinylsilane groups during the initiator alkoxide formation and during the polymerization. It was observed that 1 mol initiator:0.95 mol base functioned well in preserving the vinyl moieties. A representative procedure is provided below.

Synthesis of poly(ethylene oxide) with a vinyl dimethylsilylpropoxy group at one end and a hydroxyl group at the other end. A 300-mL Parr pressure reactor equipped with a mechanical stirrer, thermocouple, and valve-controlled gas inlets and outlets was cooled to -50 °C utilizing an isopropanol-dry ice bath. Ethylene oxide (EO, 0.227 mol, 10 g) was distilled from a lecture bottle into the cold pressure reactor that had been placed under vacuum. The EO lecture bottle was weighed before and after addition to determine the amount of EO that was charged. 3-Hydroxypropylvinyl dimethylsilane initiator (0.002 mol, 0.29 g) was added to a flame-dried, septum-sealed roundbottom flask via syringe. THF (10 mL) was added to the vessel containing the initiator. Potassium naphthalide (1 mol initiator:0.95 mol base, 1.95 mL of a 0.98 M solution of potassium naphthalide in THF) was added to the flask containing the initiator to form the alkoxide. The color changed from colorless to yellow. The initiator solution was added to the Parr pressure reactor (while the reactor was stirring) via syringe. THF (10 mL) was deoxygenated with a nitrogen purge through the solution for 1 h and added to the Parr reactor via syringe. The reactor was allowed to warm to room temperature and the reaction was stirred for 24 h. The reaction progress was monitored by noting a drop in

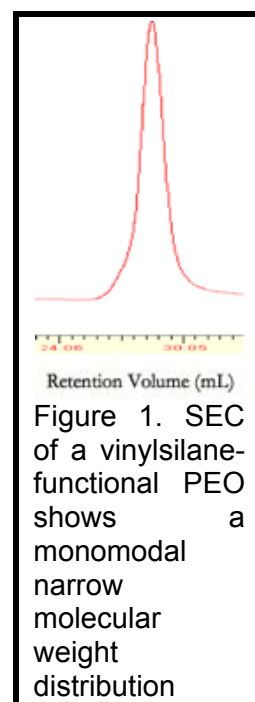


Figure 1. SEC of a vinylsilane-functional PEO shows a monomodal narrow molecular weight distribution

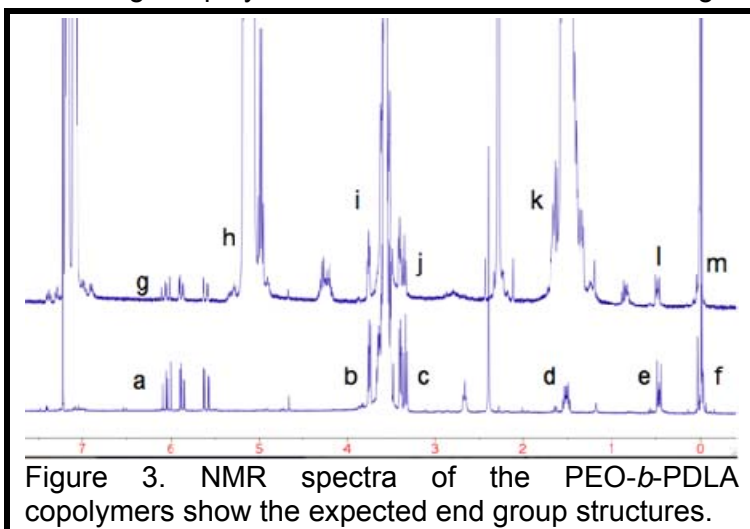
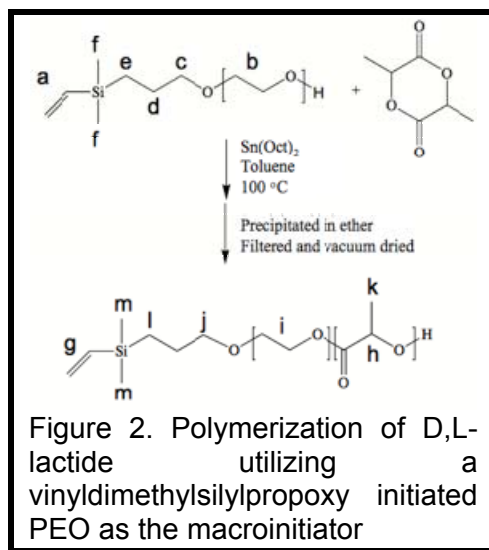
pressure from 40 to 30 psi. The polymerization was quenched with acetic acid (0.002 mol, 0.9 mL of a 2.5 M solution of acetic acid in THF) under nitrogen before opening the reactor (care should be taken to neutralize the polymer prior to exposure to oxygen). The poly(ethylene oxide) was dissolved in 200 mL of dichloromethane and filtered. The PEO/dichloromethane solution was washed twice with water and ~90% of the dichloromethane was removed via rotary evaporation. The PEO was precipitated into cold diethyl ether and dried under vacuum at 40 °C overnight. The reaction yielded 92 % by weight and SEC showed that the polymer had a M_n of 4800 g mol⁻¹ with a PDI of 1.04 (fig. 1). Proton NMR shows a molecular weight of 6049 based on the ratio of the vinyl protons to the methylene protons in the backbone.

*Synthesis of poly(ethylene oxide-*b*-D,L-lactide) with a vinyl dimethylsilylpropoxy group at the PEO terminus and a hydroxyl group at the PDLA terminus (fig. 2).* D,L-lactide (27.88 g, 0.193 mol), and toluene (70 mL) were charged to a flame-dried, nitrogen-purged, roundbottom flask equipped with a magnetic stir bar. The flask was placed in an oil bath at 65 °C to dissolve the lactide monomer. The PEO having the vinyl dimethylsilylpropoxy group at the initiator end and a hydroxyl at the other (4.21 g, 6.96 x 10⁻⁴ mol) was added to the reaction and dissolved. Stannous octoate (16.6 mg) was charged to the flask and the temperature of the oil bath was increased to 100 °C and reacted for 18 h. The polymer was isolated by precipitation into cold diethyl ether followed by washing several times with ether. The copolymer was vacuum dried at 40 °C for 18 h.

End group analysis was performed via ¹H NMR to ensure that the end groups remained intact during the polymerization and that molecular weight

could be targeted and controlled. Figure 3 depicts the ¹H NMR spectra obtained from the vinylsilane initiated PEO and the subsequent vinylsilane-functional PEO-*b*-PDLA. The ratio of the end group protons matched closely with the theoretical values. SEC showed a molecular weight distribution of 1.45 and indicated a M_n of 49,000 (fig. 4). Proton NMR yielded a M_n value of 51,000 by comparing the integrals of the resonances due to the vinyl groups to the backbone. The targeted M_n was 46,000, and the difference between this value and the molecular weights derived from SEC and NMR are within experimental error of that value.

*Functionalization and neutralization of the vinyl dimethylsilylpropoxy-functional PEO-*b*-PDLA with cysteamine hydrochloride.* A vinyl dimethylsilylpropoxy-functional PEO-*b*-PDLA (described above) (5.188 g, ~1 x 10⁻⁴ mol) was charged into a roundbottom flask equipped with a magnetic stir bar. DMF (30 mL) was added via syringe. AIBN (0.1501 g, 9.14 x 10⁻⁴ mol) was added and the reaction was purged with nitrogen. Cysteamine hydrochloride (2.77 g, 0.024 mol) was added and the reaction was allowed to stir at 70 °C in an oil bath for 18 h. The solution was cooled to room temperature and the polymer was isolated by precipitation into cold water and



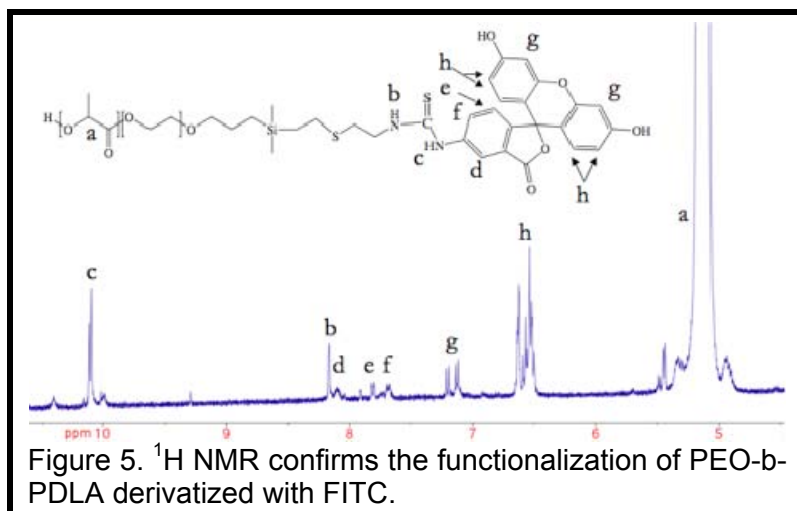
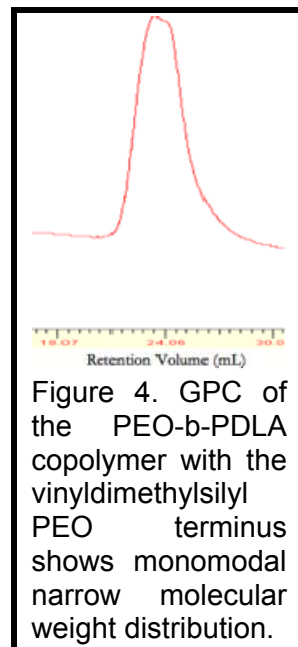
filtered. Additional water was used to wash the polymer, and then the polymer was freeze-dried at $-45\text{ }^{\circ}\text{C}$ for 18 h. The ammonium-functionalized block copolymer (1.3349 g, $\sim 2.6 \times 10^{-5}$ mol) was charged into a roundbottom flask and DMF (8 mL) was added via syringe. NaHCO_3 (2.8 mg, 3.33×10^{-5} mol) was added and the reaction flask was shaken for 10 min. The neutralized aminofunctional polymer was isolated by precipitation into cold water and filtered. Additional water was used to wash the filtered polymer, which was then freeze-dried at $-45\text{ }^{\circ}\text{C}$ for 18 h.

Functionalization of the amino-functional PEO-b-PDLA block copolymer with fluorescein isothiocyanate (FITC) to use as a standard for calibrating fluorescence.

The aminofunctional copolymer was utilized to modify the surfaces of the PDLA nanospheres, and the amount of PEO that self-assembled onto the nanosphere surfaces was quantified with fluorescence studies. Thus, the aminofunctional copolymer was derivatized with fluorescein isothiocyanate (FITC) so that it could be utilized as a standard for calibrating the fluorescence measurements. A $51,000\text{ g mol}^{-1}$ aminofunctional PEO-*b*-PDLA (0.1354 g, $\sim 2.6 \times 10^{-6}$ mol) was charged into a roundbottom flask equipped with a magnetic stir bar and wrapped in aluminum foil. DMSO (3 mL) was added via syringe. FITC (3.0 mg, 7.69×10^{-6} mol) was added and the reaction was stirred at room temperature for 18 h. The reaction mixture was diluted with H_2O (15 mL) and transferred to a dialysis bag and dialyzed for 6 days to remove remaining FITC, exchanging the water daily. The polymer was isolated by freeze drying the solution at $-45\text{ }^{\circ}\text{C}$ for 18 h. End group analysis was performed via ^1H NMR to confirm that FITC had reacted with the amino terminus of the PEO-*b*-PDLA copolymer (fig. 5). As will be described later in this report, we have developed a fabrication procedure for the magnetite-PDLA nanospheres that utilizes blends of the block copolymer surface modifiers, and the PEO that assembles onto the nanosphere surfaces forms a brush layer that allows the nanospheres to disperse in aqueous physiological media. We were able to quantify the self-assembly process utilizing the aminofunctional copolymers and reacting the surfaces with FITC after the nanospheres were formed.

Synthesis of a maleimide initiator and of maleimide-terminated PEO

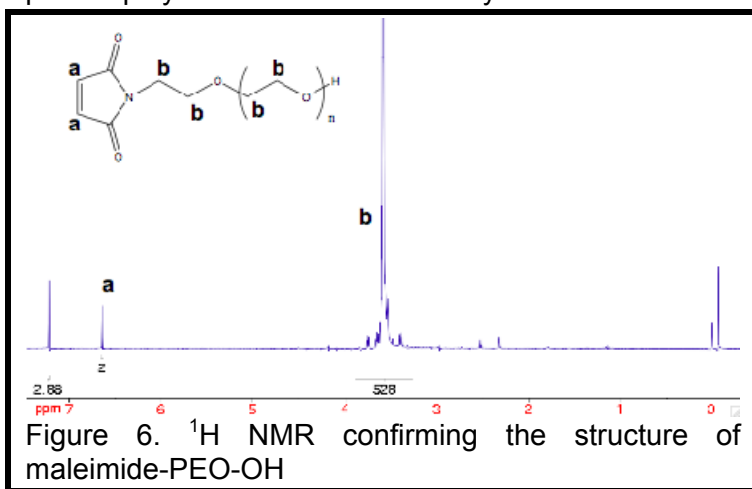
It was desirable to derivatize the surface-modifying copolymers with a versatile functional group such as a maleimide that could subsequently be reacted with targeting reagents. The maleimide reacts at room temperature with sulfhydryl reagents such as cysteine-functional antibodies, and these reactions proceed well even with the nanospheres in the solid state. Thus, maleimide-PEO-*b*-PDLA can be blended with the nanosphere base material to form dispersible nanospheres that can be targeted to specific pathogens or cell populations.



A coordination catalyst was utilized to polymerize EO from a heterobifunctional initiator, N-(2-hydroxyethyl)maleimide, to yield a heterobifunctional PEO with a maleimide group on one end and a hydroxyl group at the other.¹ Although polymerizations of EO are usually carried out with a basic initiator, the sensitivity of N-(2-hydroxyethyl)maleimide toward base prohibited this approach.³⁻⁵ The zinc hexacyanocobaltate catalyst allowed for polymerizing EO utilizing N-(2-hydroxyethyl)maleimide as the initiator with retention of the maleimide functionality.

The heterobifunctional initiator was prepared in three steps.⁶ Maleic anhydride was first protected by a Diels-Alder reaction with furan, and this was followed by reacting ethanolamine with the anhydride under anhydrous conditions. Thermal deprotection of the double bond produced N-(2-hydroxyethyl)maleimide. The N-(2-hydroxyethyl)maleimide initiator was utilized in batch polymerizations of EO in the presence of Bayer Impact 3 zinc hexacyanocobaltate. It has been previously shown that batch epoxide polymerizations activated by such coordination

catalysts produce polymers with broader molecular weight distributions as compared to the base-catalyzed polymers, but the maleimide groups withstand these conditions quantitatively. As expected from previous studies, the molecular weight distributions obtained from these polymerizations were broad (~3.3). As determined from ¹H NMR, the molecular weights of the polymers were consistent with the targeted values based on the monomer to initiator ratios, not the monomer to catalyst ratio. Analysis via ¹H NMR



confirmed that the maleimide endgroup was retained during polymerization (fig. 6).

*Synthesis of the PEO-*b*-PDLA utilizing the maleimide-terminated PEO*

A 4,300 M_n maleimide-PEO-OH homopolymer was prepared with N-(2-hydroxyethyl)maleimide as the initiator. EO (14.7 g) was charged into a 300-mL pressure reactor under a 20 psi head of N_2 . An initiator solution consisting of N-(2-hydroxyethyl)maleimide (0.64 g, 4.5 mmol), THF (10 mL), and the zinc hexacyanocobaltate (Impact 3) catalyst (1.1 mL of a 1.0 mg mL^{-1} dispersion) was prepared in a separate flame-dried, 100-mL, roundbottom flask. The initiator solution was added to the reaction mixture via syringe followed by 10 mL of THF. The pressure reactor was heated to 90 °C, and the polymerization was conducted until a decrease in pressure was no longer observed (~4 h). The reactor was then allowed to cool to room temperature and purged with nitrogen for one hour to remove any residual EO. The reactor was opened and its contents were transferred to a 250-mL, roundbottom flask. The solvent was removed under vacuum at room temperature and the product was redissolved in a minimal amount of dichloromethane (~20 mL). The polymer was precipitated into cold diethyl ether to yield 13.7 g of maleimide-PEO-OH.

A 4,300-43,000 M_n maleimide-PEO-*b*-PDLA-OH diblock copolymer was prepared with the 4,300 M_n maleimide-PEO-OH as a macroinitiator. D,L-lactide (14.0 g) was charged into a flame-dried, 100-mL, roundbottom flask equipped with a magnetic stir bar. Dry toluene (30 mL) was added to the reaction via syringe and the mixture was heated to 110 °C to dissolve the monomer. An initiator solution consisting of the 4,300 M_n maleimide-PEO-OH (1.4 g) and toluene (10 mL) was prepared in a separate flame-dried, 50-mL roundbottom flask. A catalyst solution consisting of stannous octoate (1.25 g, 3.1 mmol) and 20 mL toluene was prepared in a separate flame-dried, 50-mL, roundbottom flask. The macroinitiator solution was added to the monomer solution via syringe, and then 0.31 mL of the catalyst solution (18 mg mL^{-1}) was

added. The polymerization was conducted at 110 °C for 48 h while stirring. The final product was precipitated into cold methanol to yield 13.4 g of maleimide-PEO-*b*-PDLA-OH.

Molecular weights and polydispersity indices of the 4,300 M_n maleimide-PEO-OH macroinitiator and the 4,300-43,000 M_n maleimide-PEO-*b*-PDLA-OH diblock copolymer were characterized via ¹H NMR and SEC. Figure 7 shows the ¹H NMR spectrum of the maleimide-PEO-OH confirming the structure and molecular weight of the homopolymer. The polydispersity index of the maleimide-PEO-OH homopolymer was 2.2. The polydispersity index of the maleimide-PEO-PDLA-OH diblock copolymer was 1.6.

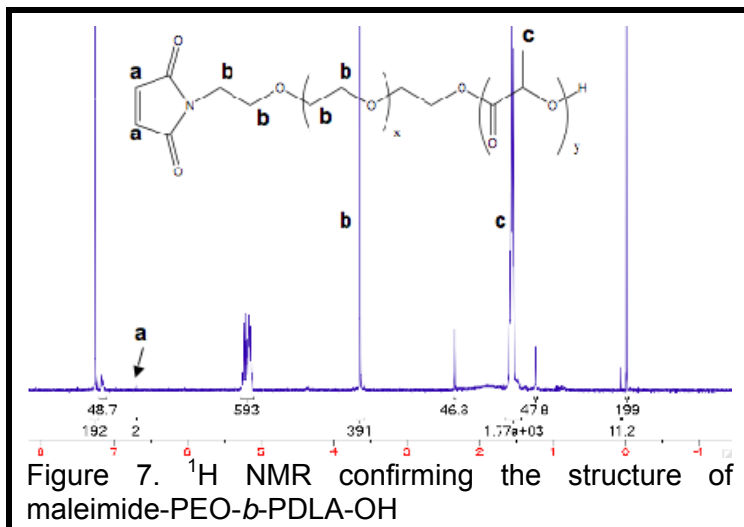


Figure 7. ¹H NMR confirming the structure of maleimide-PEO-*b*-PDLA-OH

Fabrication of biodegradable nanospheres

Confined impingement jet mixer

We continue to investigate a novel continuous particle precipitation process within a confined impingement jet (CIJ) mixer that can yield controlled particle sizes with low polydispersity (fig. 8).⁷⁻¹² We constructed this instrument based on collaborations with Prof. Prud'homme at Princeton University. In the CIJ mixer, liquid jets collide under highly-turbulent conditions in a confined mixing chamber where particle nucleation and precipitation occur. The particle size decreases as the jets' flow rates increase, i.e. as the characteristic mixing time τ_{mix} decreases, until τ_{mix} becomes smaller than the nuclei aggregation time, at which point the size stops changing. Under these conditions, particles form via nucleation and diffusion-limited growth that is quenched with a repulsive polymer brush barrier. In our case, the hydrophilic PEO component of the diblock copolymer surface-segregates on the nanospheres and provides the required steric repulsive barrier. We have used the CIJ process to make polylactide particles with an intensity diameter (measured by dynamic light scattering) of 74 nm and a polydispersity index of 0.057. By comparison, a polystyrene latex calibration standard has a PDI of $\sim < 0.03$. Thus, the polylactide nanospheres

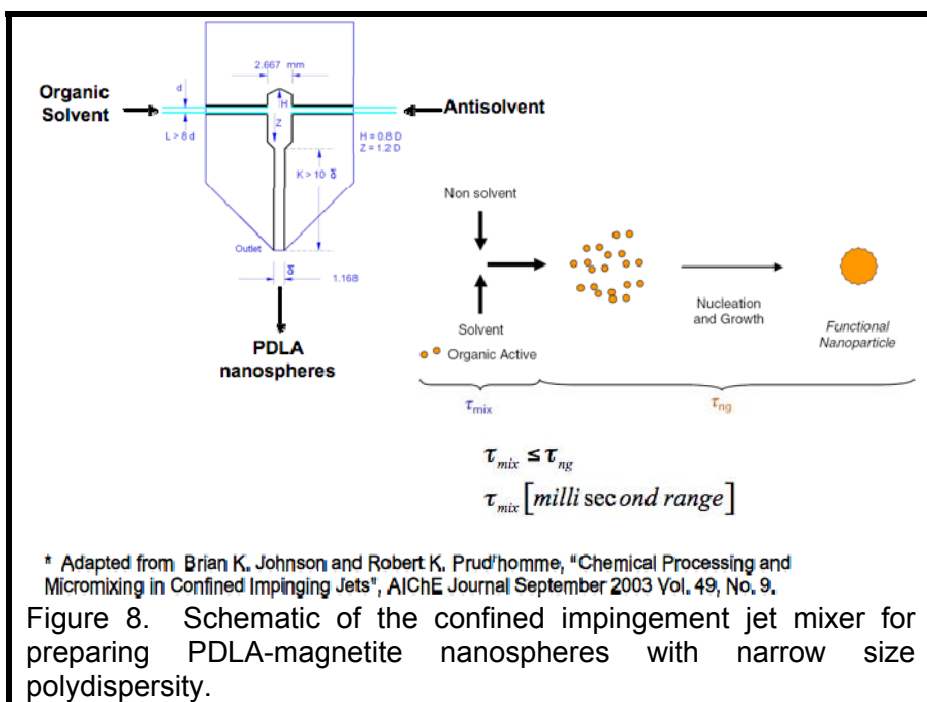


Figure 8. Schematic of the confined impingement jet mixer for preparing PDLA-magnetite nanospheres with narrow size polydispersity.

are formed under highly-turbulent conditions in a confined mixing chamber where particle nucleation and precipitation occur. The particle size decreases as the jets' flow rates increase, i.e. as the characteristic mixing time τ_{mix} decreases, until τ_{mix} becomes smaller than the nuclei aggregation time, at which point the size stops changing. Under these conditions, particles form via nucleation and diffusion-limited growth that is quenched with a repulsive polymer brush barrier. In our case, the hydrophilic PEO component of the diblock copolymer surface-segregates on the nanospheres and provides the required steric repulsive barrier. We have used the CIJ process to make polylactide particles with an intensity diameter (measured by dynamic light scattering) of 74 nm and a polydispersity index of 0.057. By comparison, a polystyrene latex calibration standard has a PDI of $\sim < 0.03$. Thus, the polylactide nanospheres

prepared by the CIJ process are only slightly more polydisperse than a latex calibration standard.

The confined impingement jet mixer consists of two separate liquid streams, one powered by a Harvard Apparatus PHD 4400 programmable syringe pump and the other by a KD Scientific 200 syringe pump. Each stream is fed at room temperature into the mixing chamber through 1/8 inch PTFE tubing that is connected to a blunt-tipped 20-gauge stainless steel needle that is inserted into the mixing chamber. A pressure relief valve from Swagelok is set at a relief pressure of 50 psi and pressure measurements can be taken with an in-line Omegadyne pressure transducer, whose signal is fed into a computer and recorded.

Figure 8 shows an illustration of the CIJ mixing head, whose dimensions can be used to calculate critical parameters, such as mixing or residence times. The product stream exits out the bottom of the mixing head and is collected in 50 mL volumetric flasks.

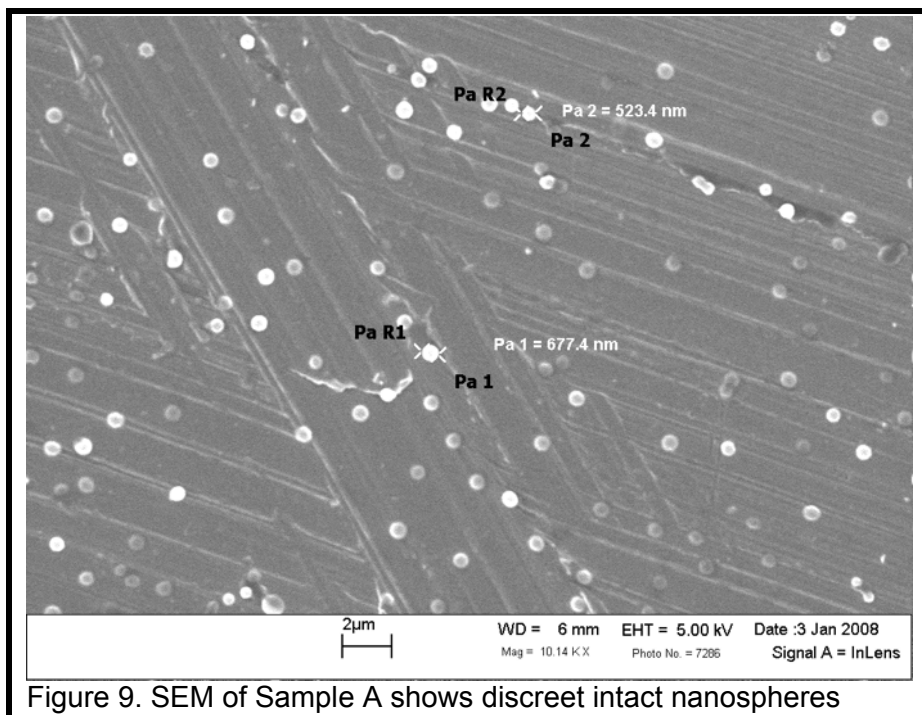


Figure 9. SEM of Sample A shows discrete intact nanospheres

Each experimental run is 30 seconds long to minimize any variation due to start-up or shut-down conditions.

Sample A (fig. 9) used a $27,000 \text{ g mol}^{-1}$ PDLA homopolymer blended with a $51,000 \text{ g mol}^{-1}$ NH_2 -PEO-*b*-PDLA copolymer dispersed in DMSO as a solvent at a concentration of 50 mg mL^{-1} . The flow rate for the solvent was 3.25 mL min^{-1} for 30 sec yielding a total of 1.625 mL. The nonsolvent was methanol and the flow rate was 60 mL min^{-1} for 30 sec yielding a total of 30 mL.

Aminofunctional nanospheres were precipitated from a separate process into a roundbottom flask wrapped in aluminum foil. A 5 % mol excess of FITC was syringed into the roundbottom flask and allowed stir at room temperature for 18 h. The mixture was dialyzed in water for 5 days, exchanging the solvent daily. The nanospheres were freeze-dried at $-45 \text{ }^\circ\text{C}$ for 18 h. Approximately 72% of the PEO self-assembled at the nanosphere surfaces, based on post-derivatization with FITC, followed by fluorescence analysis of the nanospheres.

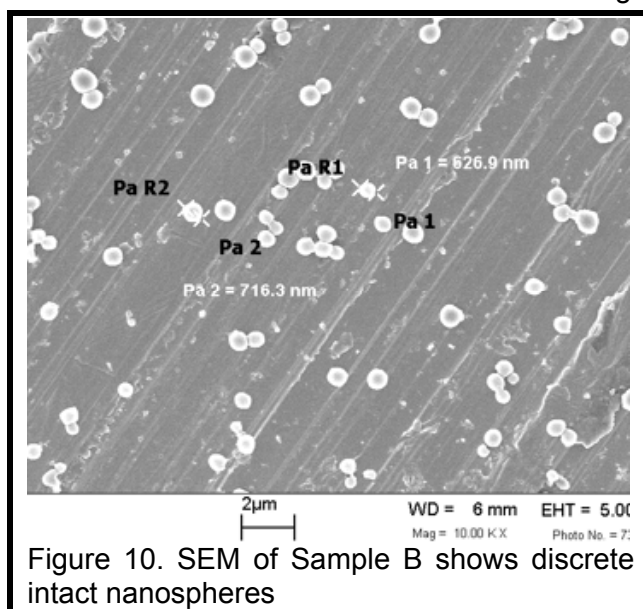


Figure 10. SEM of Sample B shows discrete intact nanospheres

Sample B (fig. 10) used a 70 wt % magnetite- 27,000 M_n PDLA complex blended with an $\sim 51,000$ g/mol NH_2 -PEO-PDLA copolymer dispersed in DMSO at a concentration of 50 mg mL^{-1} . The flow rate for the solvent was 3.25 mL min^{-1} for 30 sec yielding a total of 1.625 mL. The nonsolvent was methanol and the flow rate of 60 mL min^{-1} for 30 sec yielding a total of 30 mL.

Summary and recommendations

We have demonstrated that biodegradable polylactide nanospheres carrying up to 70 weight percent of superparamagnetic magnetite can be dispersed in water by tailoring their surfaces. The approach of simply blending two weight percent of an amphiphilic multiphase diblock copolymer with the magnetite-polylactide base material, and precipitating into a hydrophilic medium leads to rapid self-assembly of the PEO component at the surface. The effect is sufficient to render these nanospheres dispersible in water, even though they contain significant magnetite with a density of 5.17 g mL^{-1} . Moreover, when the termini of the PEO surface chains are amino groups, the amino groups have been shown to react under mild physiological conditions with a fluorescent dye (fluorescein isothiocyanate). This demonstrates that the blending technique leads not only to dispersibility, but also to nanospheres with reactive surfaces. The effect was also demonstrated with maleimide groups on the PEO termini. These were designed to react with targeting moieties such as antibodies bearing sulfhydryl groups, again under mild physiological conditions. These conjugations should be pursued in future studies.

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