Video Article

Nanosponge Tunability in Size and Crosslinking Density

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Abstract

We describe a protocol for the synthesis of linear polyesters containing pendant epoxide functionality and their incorporation into a nanosponge with controlled dimensions. This approach begins with synthesis of a functionalized lactone which is key to the pendant functionalization of the resulting polymer. Valerolactone (VL) and allyl-valerolactone (AVL) are then copolymerized using ring-opening polymerization. Post-polymerization modification is then used to install an epoxide moiety on some or all of the pendant allyl groups. Epoxy-amine chemistry is employed to form nanoparticles in a dilute solution of both polymer and small molecule diamine crosslinker based on the desired nanosponge size and crosslinking density. Nanosponge sizes can be characterized by transmission electron microscopy (TEM) imaging to determine the dimension and distribution. This method provides a pathway by which highly tunable polyesters can create tunable nanoparticles, which can be used for small molecule drug encapsulation. Due to the nature of the backbone, these particles are hydrolytically and enzymatically degradable for a controlled release of a wide range of hydrophobic small molecules.

Video Link

The video component of this article can be found at https://www.jove.com/video/56073/

Introduction

Precisely tuning the size and crosslinking density of nanoparticles based on intermolecular crosslinking is of great importance to influence and guide the drug release profile of these nanosystems[1]. Designing nanosponge tunability, *i.e.*, preparing particles of different network densities, is reliant upon the pendant functionality of the precursor polymer and the equivalents of the hydrophilic crosslinker incorporated. In this approach, the concentration of the precursor and crosslinker in the solvent is important to form nanoparticles of a discrete size rather than a bulk gel. Utilizing quantitative nuclear magnetic resonance spectroscopy (NMR) as a characterization technique allows for the precise determination of incorporated pendant functionality and polymer molecular weight. Once nanoparticles are formed, they can be concentrated and solubilized in organics without having the character of a nanogel.

Recent work in nanoparticle drug delivery has focused on the use of poly(lactic-*co*-glycolic acid) (PLGA) self-assembled nanoparticles[2,3,4,5,6]. PLGA has degradable ester linkages which make it suitable for drug delivery applications and is often combined with poly(ethylene glycol) (PEG) due to its stealth properties[7]. However, due to the self-assembled nature of PLGA particle formation, the particles cannot be solubilized in organics for further functionalization. In contrast to PLGA nanoparticles, the proposed method provides covalent crosslinking forming a nanoparticle with defined sizes and morphology, which are stable in organics and degrade in aqueous solutions[1]. Advantages of this approach are the ability to further chemically functionalize the surface of the nanosponge[8], and its stability in organic solvents can be used for the post-loading of the particles with pharmaceutical compounds[1,9]. With this method, encapsulation of hydrophobic small molecules can be achieved by precipitation into aqueous media. The hydrophobicity of the polyester backbone together with the hydrophilic short crosslinker gives these particles an amorphous character at body temperature. Furthermore, after drug loading, the particle can form fine suspensions in aqueous media to be readily injected *in vivo*. It is our goal in this work to evaluate the parameters for the synthesis of these polyester nanosponges and determine those which are vitally important to the design and control of size and morphology.

Protocol

1. Synthesis and Characterization of AVL

1. **Place a magnetic stir bar inside a 2 neck 500 mL round bottom flask (Flask 1) and seal with an appropriate sized rubber septum and steel wire. Flame dry the flask to remove moisture by purging with nitrogen gas connected through an inlet needle and open outlet needle in the septum, while using a butane flame torch to gently heat the outside of the flask by moving the flame along the surface.**

   1. Continue heating the entire flask by running the flame across the surface until moisture clouding the inside of the flask is not seen. Remove the flame and let the flask cool to room temperature while continuing the nitrogen flow throughout the reaction.

2. Add 156.25 mL anhydrous tetrahydrofuran (THF) *via* a 30 mL syringe to Flask 1. Cool Flask 1 to -78 °C with a dry ice/acetone bath. Monitor the temperature with a thermometer throughout the reaction and add dry ice as needed to maintain -78 °C. Begin stirring the magnetic stir bar using a magnetic stir plate to create a gentle vortex.
3. Add *N,N*-diisopropylethylamine (DIPA) (3.30 mL, 2.3 x 10^{-2} mol) via a 5 mL syringe, followed by the dropwise addition of 2.5 M *n*-Butyllithium (9.34 mL, 2.3 x 10^{-2} mol) *via* a 10 mL glass syringe equipped with a needle locking mechanism and metal needle to Flask 1. Continue to stir using a magnetic stir plate for 15 min at -78 °C.
4. Flame dry (as described in step 1.1) and purge with argon gas (as described in step 1.1 with nitrogen gas) a 100 mL round bottom flask (Flask 2). Remove the argon gas inlet and outlet at the same time once the flask is cool enough to touch. Add 56 mL anhydrous THF *via* a 30 mL syringe and δ-valerolactone (VL) (1.97 mL, 2.1 x 10^{-2} mol) *via* a 5 mL syringe.
5. Add the solution in Flask 2 to Flask 1 *via* a small cannula dropwise over 45 min while continuously stirring *via* a magnetic stir bar. Once Flask 2 is empty, remove the cannula and allow Flask 1 to continue stirring for 15 min at -78 °C.
6. Flame dry (as described in step 1.1) and purge with argon gas (as described in step 1.1 with nitrogen gas) a 25 mL round bottom flask (Flask 3). Remove the argon gas inlet and outlet at the same time once the flask is cool enough to touch, to maintain positive pressure inside the flask. Add ally bromide (2.02 mL, 2.3 x 10^{-2} mol) and hexamethyphosphoramide (HMPA) (4.43 mL, 2.5 x 10^{-2} mol) each *via* a 5 mL syringe.
7. **Add Flask 3 to Flask 1 *via* a small cannula dropwise. Once Flask 3 is empty, remove the cannula.**
   1. Remove as much dry ice and cold acetone as possible from the dry ice/acetone bath in which Flask 1 is submerged using a scoop and plastic cup. Replace with room temperature acetone to raise the bath to -40 °C and allow Flask 1 to continue stirring for 2 h while monitoring the temperature. If the temperature increases above -40 °C, add a few pieces of dry ice to cool the acetone.
   2. Warm to -10 °C by removing the cold acetone and dry ice and replace with room temperature acetone.

8. Remove the septum from Flask 1 and quench the reaction with a 150 mL saturated ammonium chloride solution while stirring *via* a magnetic stir bar. Place Flask 1 in a freezer at -20 °C overnight for storage.
9. Remove Flask 1 from the freezer and allow to warm to room temperature. Pour the solution from Flask 1 into a 500 mL separatory funnel.
10. Add 50 mL dichloromethane (DCM), cap the separatory funnel, and rock gently to mix the DCM and aqueous/THF mixture to extract the product into the organic layer. Collect the organic layer into a 500 mL beaker containing an appropriately sized magnetic stir bar by opening the separatory funnel stopcock and set aside. Repeat two times.
11. Collect all the organics into the same beaker and stir over magnesium sulfate (to remove any residual water) using a magnetic stir plate until the magnesium sulfate no longer clumps when added to solution. Discard the aqueous waste.
12. Remove the magnesium sulfate solid from the organic product solution by using a filter paper inside a glass funnel and collect the organics in a 250 mL round bottom flask. Rinse the filter paper and solid magnesium sulfate with excess DCM to ensure that all the product is collected.
13. Remove the solvent from the product by rotary evaporation while heating at 30 °C and using a water aspirator as the vacuum source.
14. Purify the crude product by column chromatography[10] using a silica gel as the stationary phase and eluting beginning with 3% ethyl acetate/hexanes for 1 column volume (CV), increasing the gradient to 29% ethyl acetate/hexanes over 5 CVs. Collect the fractions in 16 x 150 mm^2 borosilicate glass tubes.
15. Perform thin layer chromatography using 20% ethyl acetate/hexanes as eluent to determine the product fractions.
16. Combine all product fractions into an appropriately sized round bottom flask and remove the solvent using rotary evaporation at 40 °C. Transfer to a pre-weighed product vial and place under a high vacuum at 0.05 torr to obtain pure AVL. Characterize the product by ^{1}H NMR[11] ($CDCl_3$, 400 MHz): δ 5.75 (m, 1H), 5.06 (m, 2H), 4.26 (m, 2H), 2.54 (m, 3H), 2.28 (m, 1H), 2.03 (m, 1H), 1.87 (m, 1H), 1.54 (m, 1H). Yield: 2.07 g (70.56%).

2. Synthesis and Characterization of VL- *co-* AVL

1. **Flame dry and purge with nitrogen gas a 25 mL round bottom flask equipped with a magnetic stir bar and septum.**
   1. Flame dry the flask to remove moisture by purging with nitrogen gas connected through an inlet needle and open outlet needle in the septum, while using a butane flame torch to gently heat the outside of the flask by moving the flame along the surface.
   2. Continue heating the entire flask by running the flame across the surface until moisture clouding the inside of the flask is not seen. Remove the flame and let the flask cool to room temperature while continuing the nitrogen flow throughout the reaction.

2. Quickly remove the septum from the round bottom and add tin(II) triflate (2.5 mg, 5.9 x 10^{-6} mol) to the very bottom of the flask using a spatula. Replace the septum.
3. Add 3-methyl-1-butanol (72.6 µL, 6.6 x 10^{-3} mol) *via* a 100 µL microsyringe and 1.33 mL anhydrous DCM *via* a 2 mL syringe sequentially. Stir via a magnetic stir plate for 10 min.
4. Add AVL (0.48 mL, 3.7 x 10^{-3} mol) and VL (1.37 mL, 1.4 x 10^{-2} mol) sequentially *via* a syringe. Allow to continue stirring for 18 - 20 h.
NOTE: The AVL and VL percentage in the final copolymer can be altered by stoichiometry in this step.
5. Quench the reaction by adding ~5 mL methanol (MeOH). Add ~100 mg solid-support tin scavenger and stir for 2 h. Filter by gravity filtration to remove the solid.
6. Remove the solvent under rotary evaporation with a water aspirator as the vacuum source while heating at 30 °C until the solution is viscous. Precipitate the solution dropwise into 500 mL cold MeOH (chilled with dry ice for at least 1 h) to produce flakes of white solid. Filter the solution by vacuum filtration into a funnel containing a fritted glass disc with filter paper to collect the solid.
NOTE: If precipitation produces a cloudy supernatant or fine powder which goes through the filter paper, then the product solution is too dilute. All supernatant solvent should be removed by rotary evaporation with a water aspirator as the vacuum source while heating at 30 °C until the solution is viscous and then precipitation should be performed again.
7. Transfer the solid product to a pre-weighed vial *via* a spatula and dry overnight via high vacuum pressure of 0.05 torr to collect the white, flaky solid.
8. Characterize the polymer by ^{1}H NMR[11] ($CDCl_3$, 400 MHz): δ 5.71 (m, 1H), 5.03 (t, 2H), 4.08 (m, 4H), 3.65 (t, 2H), 2.15 - 2.50 (m, 5H), 1.47-1.78 (m, 9H), 0.91 (d, 6H). 81.42% VL, 18.58% AVL, 2,940 g/mol, 578 g/mol repeat unit of AVL. Yield: 1.43 g, (73.43%).

3. Post-polymerization Epoxidation to Produce Epoxy-valerolactone (EVL) Copolymer Units

1. Add VL-*co*-AVL copolymer (500 mg, 1.7 x 10^{-4} mol, 2,940 g/mol) to a 6-dram vial with a magnetic stir bar. Add 6.15 mL anhydrous DCM to the vial and vortex to solubilize the polymer.
2. Add meta-chloroperoxybenzoic acid (mCPBA) (74.53 mg, 4.3 x 10^{-4} mol) to a second 6-dram vial. Add 6.15 mL anhydrous DCM and vortex until the mCPBA is solubilized completely.
   NOTE: The conversion ratio of allyls to epoxides can be varied by altering the stoichiometry in this step. The final allyl concentration in the solvent should be 0.065 M.
3. Transfer the mCPBA solution to the VL-AVL solution. Cap the reaction and cover with a plastic paraffin film. Allow to stir for 48 h.
4. **Transfer the reaction mixture to a 50 mL separatory funnel. Add 15 mL saturated sodium bicarbonate, cap the separatory funnel, and rock gently to mix. Collect the organic layer containing the product into a 50 mL Erlenmeyer flask with an appropriate sized magnetic stir bar (product flask).**
    1. Add 5 mL DCM to the aqueous layer that is still in the separatory funnel, cap, and then rock gently. Collect the organics into the product flask. Discard the aqueous waste. Transfer the organic layer back into a separatory funnel. Repeat this aqueous wash two times.

5. Add magnesium sulfate (to remove any residual water) to the product flask while stirring over a magnetic stir plate. Continue adding small scoops of magnesium sulfate until it no longer clumps when added. Use a glass funnel fitted with a filter paper to remove the solid magnesium sulfate while transferring the mixture to a 50 mL round bottom flask.
6. Remove the solvent by rotary evaporation with a water aspirator as the vacuum source and heating at 25 °C. Transfer the contents of the round bottom flask to a pre-weighed product vial and remove the solvent by rotary evaporation. Place on high vacuum at 0.05 torr overnight to produce a white, waxy solid.
7. Characterize by ^{1}H NMR[11] ($CDCl_3$, 400 MHz): δ 5.71 (m, 1H), 5.03 (t, 2H), 4.08 (m, 4H), 3.65 (t, 2H), 2.94 (d, 1H), 2.90 (s, 1H), 2.74 (s, 1H), 2.15-2.50 (m, 5H), 1.47-1.78 (m, 9H), 0.92 (d, 6H). 84.53% VL, 9.65% AVL, 5.82% EVL, 2,855 g/mol, 1,841 g/mol repeat unit of EVL. Yield: 417.3 mg, (83.46%).

4. Nanosponge Synthesis and Characterization

1. Dissolve the VL-*co*-AVL-*co*-EVL polymer (200 mg, 7.0 x 10^{-5} mol, 2,855 g/mol) in 20.01 mL anhydrous DCM for an epoxide concentration of 0.0054 M. Transfer to a 100 mL round bottom flask with a 14/20 neck.
   NOTE: To catalyze the reaction, 100 µL aqueous saturated sodium bicarbonate can be added in this step.
2. Place the reaction flask in an oil bath at 50 °C. Stir the solution with a fast vortex and add 2,2'-(ethylenedioxy)bis(ethylamine) (21.45 µL, 2.9 10^{-4} mol) *via* a microsyringe dropwise. Fit the neck of the flask with a water-jacketed condenser with cool water flowing through it, fitted with a 14/20 neck adapter, and reflux the solution for 12 h. Ensure a very tight seal between the neck of the flask and the condenser to avoid solvent evaporation.
3. Remove excess solvent from the reaction flask by rotary evaporation at 25 °C until a viscous solution is obtained.
4. **Place a large magnetic stir bar into a 2 L beaker. Cut an approximately six-inch section of 10 K molecular weight cut-off (MWCO) dialysis tubing and fold one end then close with a dialysis clip.**
    1. Transfer the product to 10 K MWCO dialysis tubing. Rinse the flask with excess DCM and transfer it to the tubing.Fold the top of the tubing and close with a dialysis clip that has a wire for hanging. Hang the dialysis tubing on the side of the beaker using the wire and fill the beaker with DCM until the dialysis tubing is completely submerged.
    2. Place on a magnetic stir plate and stir gently. Cover the beaker with aluminum foil to prevent solvent evaporation. Remove the solvent by pouring into a waste container and replace with fresh DCM three times daily for 48 h to remove the unreacted polymer and crosslinker.

5. Remove all solvent from the beaker and transfer the contents of the dialysis tubing to a 10 mL syringe fitted with a 0.45 µm polytetrafluoroethylene (PTFE) syringe filter. Push the solution through the filter directly into a pre-weighed product vial to remove any remaining impurities. Remove the solvent by rotary evaporation at 25 °C, then place on high vacuum at 0.05 torr overnight to collect the light yellow, waxy solid.
6. Characterize by ^{1}H NMR[11] ($CDCl_3$, 400 MHz): δ 5.71 (m, 1H), 5.03 (t, 2H), 4.08 (m, 4H), 3.60-3.67 (t, 14H), 2.94 (d, 1H), 2.90 (s, 1H), 2.74 (s, 1H), 2.15-2.50 (m, 5H), 1.47-1.78 (m, 9H), 0.92 (d, 6H). Yield: 176.9 mg (88.45%).

5. TEM Imaging of Nanosponge Morphology and Size

1. Filter 5 mL cell culture water through a 0.2 µm PTFE syringe filter.
2. Place 0.5 mg nanosponges into a 1.5 mL centrifuge tube. Add 1 mL filtered cell culture water. Use a probe sonicator (20 kHz, 40 a) to sonicate the solution with 2 s bursts 4 - 5 times at room temperature until the particles have developed a fine suspension. Do not use prolonged sonication or let the solution heat up as this will cause aggregation.
3. Add 30 mg phosphotungstic acid hydrate (PTA) to 1 mL filtered cell culture water in a 1.5 mL centrifuge tube. Vortex on the highest setting for 10 s or until the PTA is completely solubilized to produce 3% PTA solution.
4. Use a 1 mL syringe with 22 G needle to draw up 0.5 mL 3% PTA solution. Add 4 drops of the 3% PTA solution to the particles and vortex on the highest setting for 10 s. Use a pair of self-closing tweezers to pick up a TEM grid then dip it into the particles solution quickly, three times. Let the grid dry for 5 h under a cover to reduce dust collection on the grid.
5. Perform TEM imaging[12] of the sample using high contrast and 40 µm objective.

Representative Results

To evaluate the relationship between the synthesis parameters of the nanosponge and its resultant size, the concentration and pendant functionality of each polymer precursor is important. In **Figure 1**, a successfulsynthetic scheme of nanosponges is carried out under reflux conditions after incorporating both precursor polymer and diamine crosslinker in DCM for 12 h. The concentration of epoxides in the solution is also critical to forming discrete particles. Once nanosponges were synthesized, TEM imaging was used to determine the precise dimensions of a set of particles. In **Figure 2**, a collection of various nanosponge experiments was analyzed based on their polymer precursor molecular weight and pendant functionality incorporation to determine if a relationship between the two could have an effect on the nanosponge size. In **Figure 2**, a trend of increasing particle size is seen as molecular weight is increased for both a 6% and 8% EVL incorporation with one diamine crosslinker per epoxide (2 amines per epoxide).

Figure 3 shows that increasing both the epoxide percentage and crosslinker equivalents would have a similar effect while maintaining a similar molecular weight between nanosponge sets. Again, a trend in increasing nanosponge size while changing these parameters is seen. It is important to understand how the synthesis of polymer precursors can play a vital role in the resultant nanoparticle size to precisely tune nanosponges for various applications. It is also important to maintain a reproducible and reliable method for nanosponge synthesis which has small deviations among individual particle size, as shown by **Figure 4**. By utilizing these parameters, a range of sizes and a formula for reliably reproducing a nanosponge of a particular size can be developed for a given application or desired goal, proving this to be a versatile and practical nanosponge chemistry.

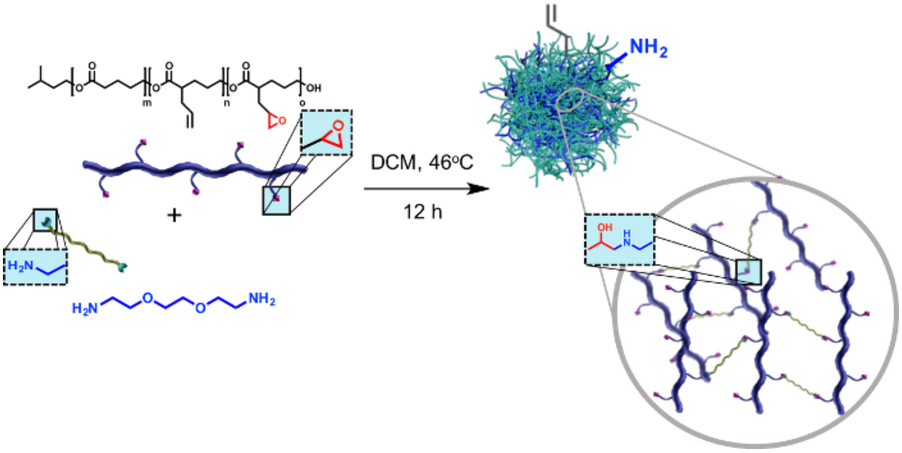

Figure 1: Reaction Scheme for Nanosponge Synthesis. A linear polyester copolymer containing pendant allyl and epoxide functional groups is reacted with a diamine crosslinker to form discrete nanoparticles with size dimensions of approximately 100 nm. Please click here to view a larger version of this figure.

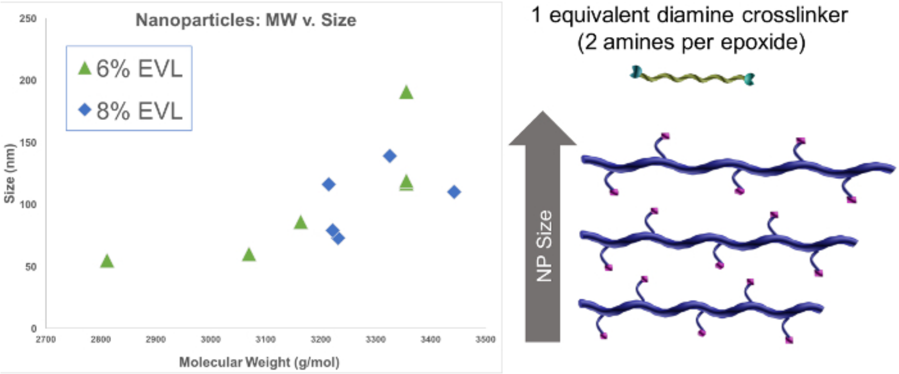

Figure 2: Analysis of Nanosponge Tunability Based on Molecular Weight and Pendant Functionality. By evaluating the change in the nanosponge size based on the molecular weight of the precursor polymer while keeping the relative pendant functionality the same, an increase in the particle size as the molecular weight increases can be shown for both 6% and 8% EVL polymers. Please click here to view a larger version of this figure.

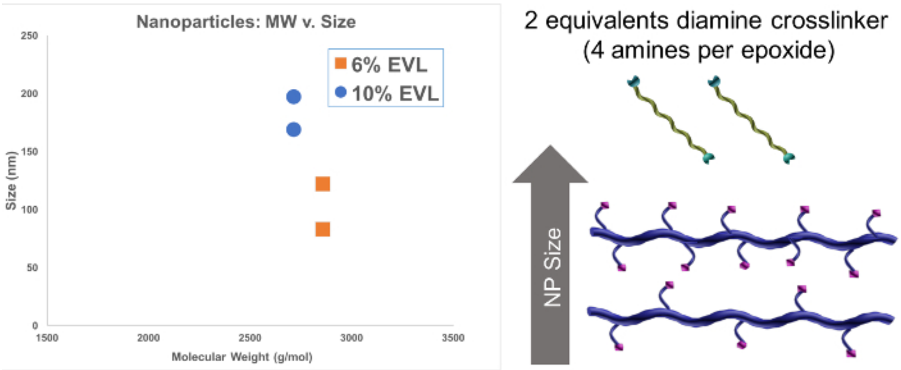

Figure 3: Analysis of Nanosponge Tunability by Crosslinker and Pendant Functionality Equivalencies. By holding the crosslinker equivalent steady, a higher pendant functionality will result in higher crosslinker incorporation. In this figure, four amines per epoxide (two diamine crosslinker equivalents per epoxide) were added to both a 6% and 10 % EVL polymer. As more crosslinker is incorporated into the nanosponge due to more epoxides per polymer and higher crosslinker equivalents, the size increases. Please click here to view a larger version of this figure.

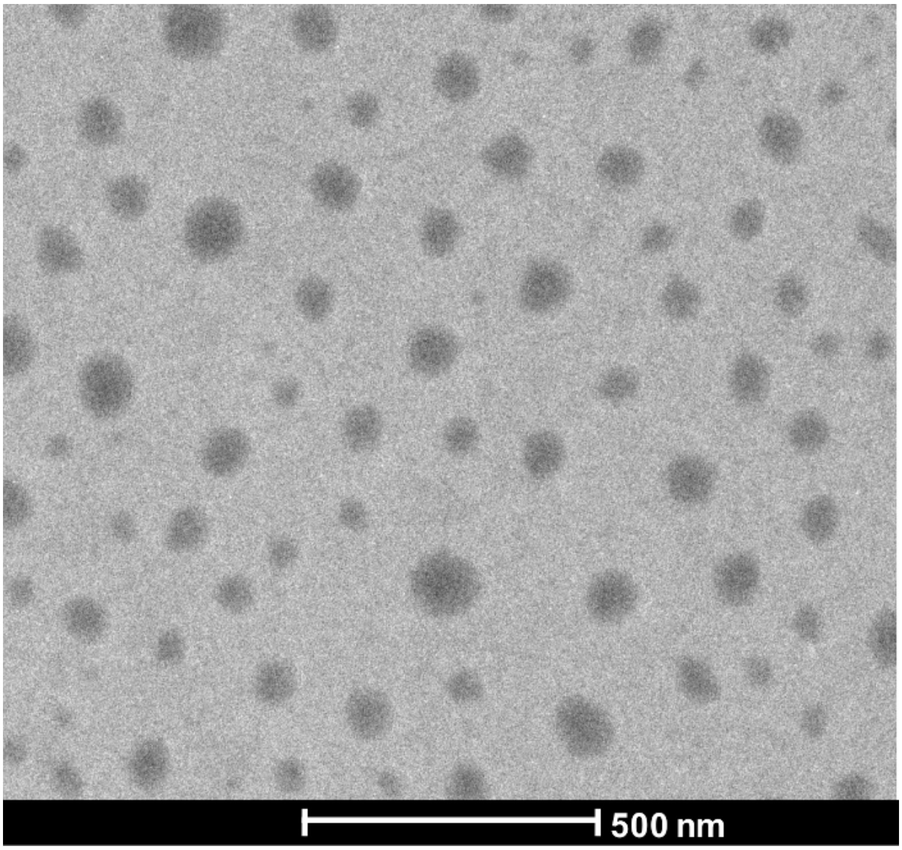

Figure 4: TEM Image of Nanosponges. A TEM image of covalently linked nanoparticles formed during synthesis. Indicated size of 79 ± 12 nm. Please click here to view a larger version of this figure.

Discussion

Obtaining reproducible nanosponge sizes is vital in drug delivery applications. Multiple parameters in polymerization and nanosponge synthesis affect the size and crosslink density of the resulting particle. Three important parameters were identified in our analysis: polymer molecular weight, epoxide pendant functionality, and crosslinker equivalents. In order to produce a range of molecular weights and epoxide functionalities for nanosponge synthesis, the stoichiometry of the VL-*co*-AVL copolymer must be altered. The concentration of the allyl functional group during epoxidation of the copolymer can be used to epoxidize either a desired percentage of allyls or all of them. If an excess of oxidizing agent is used, degradation of the polymer chain can occur; however, this can be remedied by reducing the amount of oxidizing agent. When all allyls are epoxidized, there are no pendant allyls on the surface of the nanosponge for further functionalization. It is also important for nanosponge synthesis that the concentration of epoxide in solution nanosponge synthesis is 0.0054 M.

The nanosponge reaction has been previously evaluated to determine an optimal concentration for desired nanosponge size ranges[13]. This concentration is calculated based on the repeat unit value for the epoxide functionality in the polymer. The repeat unit is the weight of polymer per one reactive unit, which is used to calculate the moles of reactive units in one polymer. For example, as shown below, if a polymer with a molecular weight of 2,000 g/mol contains 10 reactive monomer units (RMU) bearing pendant functionality, determined by quantitative NMR, the reactive unit of the polymer is 200 g/mol RMU. Using this value, the moles of reactive units can be calculated from the polymer weight in order to determine crosslinker equivalencies for nanosponge synthesis.

$$\frac{2000\ \frac{g}{mol}}{10\ RMU} = 200\ \frac{g}{mol\ RMU}$$

$$\frac{2.0\ g\ polymer}{200\ \frac{g}{mol\ RMU}} = 1.0 \times 10^{-2}\ mol\ RMU$$

As a general trend, increasing both the polymer molecular weight and epoxide functionality contributed to an increased nanosponge size independently. A narrow polydispersity achieves a narrow nanosponge size distribution (~ 10% standard deviation) and improves reproducibility of nanosponge synthesis.

The presented approach achieves a narrow polymer dispersity by use of a tin triflate catalyst[14]. The crosslinking equivalencies are calculated based on the amine per epoxide equivalents, and an increase in crosslinker equivalents is shown to increase the nanosponge size. However, using an excess of crosslinker is important due to the goal of consuming all available epoxides. Remaining amine functionality on the nanosponge surface can be used for further functionalization of the particle surface.

Compared to conventional methods for nanoparticle preparation, the advantages in this approach are the multiple parameters by which precise size and density control can be achieved, the ability to further functionalize the surface of the nanosponge, and the solubility in the organics for hydrophobic drug encapsulation.

Disclosures

The authors have nothing to disclose.

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