Chapter 6

Brain-Penetrating Nanoparticles for Analysis of the Brain Microenvironment

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Abstract

The past decade has witnessed explosive growth in the development of nanoparticle-based therapies for the treatment of neurological disorders and diseases. The systemic delivery of therapeutic carriers to the central nervous system (CNS) is hindered by both the blood–brain barrier (BBB) and the porous and electrostatically charged brain extracellular matrix (ECM), which acts as a steric and adhesive barrier. Therapeutic delivery to the brain is influenced by changes in the brain microenvironment, which can occur as a function of physiology, biology, pathology, and developmental age. Brain-penetrating nanoparticles (BPNs) are an optimal platform not only for therapeutic delivery to the brain, but also for evaluating changes in the brain microenvironment. BPNs possess both the capability to readily move within their local environment to survey their surroundings and the ability to reach the diffuse disease cells often associated with CNS disorders. To achieve effective delivery of BPNs to specific locations within the brain requires careful control over the nanoparticle's transport properties. Here, we describe the process of conjugating a dense layer of poly(ethylene glycol) (PEG) to the surface of nonbiodegradable nanoparticles to achieve brain-penetrating capabilities.

Key words Brain-penetrating nanoparticle, PEGylation, Polystyrene, Carboxyl-amine reaction, PEG density

1 Introduction

Nanoparticle delivery to specific locations within the brain requires the ability to control the transport properties of the nanoparticle platform. As neurological injury or disease is not generally limited to one location or one cell type, a therapeutic or diagnostic platform must have the ability to move from the point of access in the brain to diffuse disease-related cells. This is especially important in diseases like cancer or mediated by neuroinflammation, where access across an impaired blood–brain barrier (BBB) is heterogeneous and variable. Importantly, delivery platforms that can readily move within the brain, regardless of how they access the brain microenvironment, can reach these diffuse disease cells to maximize therapeutic effect [1]. Nanoparticles with no electrostatic,

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hydrophobic or hydrogen bonding interactions within the brain are classified as brain-penetrating [2]. These brain-penetrating nanoparticles (BPNs) can also probe the steric limitations of nanoparticle transport within the brain microenvironment, and be used to characterize microrheological properties of the brain extracellular space. BPNs are therefore an optimal platform for evaluating changes in the brain microenvironment as a function of normal development, developmental age, disease etiology, or disease progression, and can then be a more effective platform for therapeutic intervention.

For a nanoparticle to efficiently penetrate within the brain microenvironment, it must avoid any steric limitation it would experience as it moves between cells, vessels, and extracellular matrix (ECM) components. The particle must also avoid any adhesive interactions, via hydrogen bonding, hydrophobic interactions, or electrostatic interactions between the particle surface and a cell membrane, protein, or ECM component. Steric limitations are primarily influenced by the brain's volume fraction and tortuosity. The volume fraction of the brain is a ratio of the volume of the extracellular space to the total brain volume. This volume fraction changes with sleep, position (supine or upright), stress, and chronic inflammation. Tortuosity in the brain microenvironment is defined by a molecule's hindrance to diffusion within the brain. To maximize diffusion within the brain and accurately assess the steric limitations that occur due to changes in volume fraction and tortuosity, it is important to have a bio-inert particle that will avoid adhesive interactions. Here, we describe the process of conjugating a dense layer of PEG to the surface of nonbiodegradable polymer nanoparticles to create a bio-inert particle that can achieve brain-penetrating capabilities to survey the brain microenvironment in normal and diseased states (Fig. 1). PEG is a hydrophilic, FDA approved polymer that, when coated on the surface of nanoparticles, can provide a bio-inert nanoparticle platform that minimizes interactions with the environment. Although we only focus on polystyrene particles, this protocol can also apply to quantum dots with similar starting surface chemistry and surface functionality density [2].

This chapter will only cover nonbiodegradable nanoparticle platforms. Biodegradable polymeric nanoparticles and DNA nanoparticles with high PEG density have also been reported in the literature, demonstrating that improvement in diffusive capability in the brain can improve therapeutic outcome [3–5]. DNA nanoparticles with high PEG density show increased penetration and a larger area of transfection compared to nonpenetrating DNA platforms [6, 7]. The PEG density on the surface of a biodegradable polymeric particle is equally important for diffusion within the brain; yet the surfactant used for emulsifying the particles will also play a role and should be further evaluated for brain-penetrating capabilities. There is evidence that commonly used surfactants,

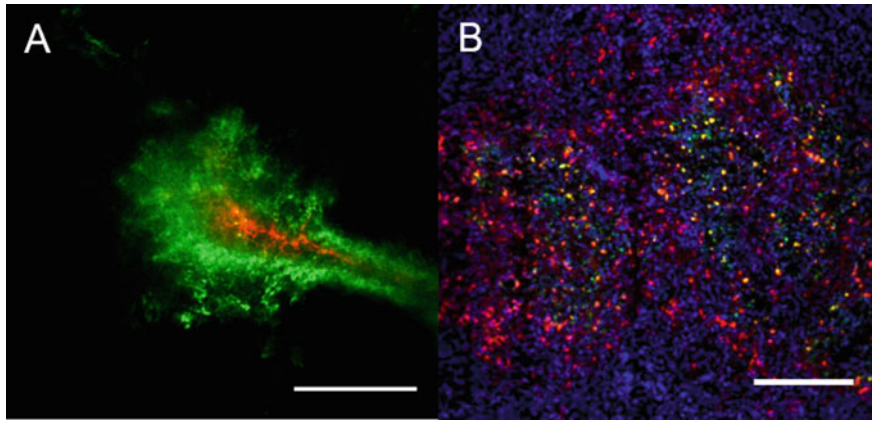

Fig. 1 BPN distribution in the rodent brain. (**a**) PS-PEG (BPN, *green*) penetrate in vivo in living mice away from the site of injection more readily compared to nonpenetrating PS-COOH (*red*). Scale bar: 50 μm. From Sci Transl Med. 2012 Aug 29;4(149): 149ra119. Reprinted with permission from AAAS. Copyright 2012 AAAS. (**b**) PS-PEG (BPN, *red*) distribute more evenly through a 9 L gliosarcoma following direct injection intracranially into the tumor, compared to PS-COOH (*green*). Cell nuclei are stained with DAPI (*blue*). *Yellow* in both panels represent overlay of the two particle platforms. Scale bar: 100 μm. Reprinted in part with permission from ACS Nano. 2014 Oct 28; 8(10): 10655–10664. Copyright 2014 American Chemical Society

including polysorbate 80 [8, 9], poly(vinyl alcohol) [4, 10], and pluronics [11, 12], do not provide similar diffusive capability in biological environments. The effect of these surfactants in combination with PEG on the diffusion of biodegradable nanoparticles in the brain should be further characterized and understood before classifying a particle as brain-penetrating. PEG provides steric stabilization, shielding of hydrophobic domains on the particle surface, and a near-neutral surface charge. However, not all nanoparticle platforms will require PEG to enable brain penetration. Lipophilic and hydrophilic particles, like dendrimers, with near-neutral surface chemistry, can also readily move within the brain to reach disease associated cells [13, 14].

The end goal of this protocol is to obtain nanoparticles coated with a high density of PEG. PEGylation has been used to create stealth nanoparticles [15], increase circulation time of nanoparticles, and aid in penetration of nanoparticles across mucosal barriers [16]. Unlike many protocols that focus on specific size ranges (40–100 nm [17] or larger than 200 nm [18]), or require bottom-up fabrication and layer-by-layer techniques like PRINT [19], this protocol focuses on utilizing a single step, stable carboxyl-amine chemistry to achieve dense PEG coatings for particles ranging from 20 nm up to micron-sized particles. In addition, materials other than PEG, including zwitterionic compounds [20], surfactants [10], and polymers [21], can provide stealth-like qualities, increased circulation time, and improved penetration of nanoparticles within a tissue environment. Many of these materials

have emerged to address the limitation that repeat administration of PEG conjugated nanoparticles has shown to induce a PEG-specific immune response [22]. The concept of nanoparticle penetration within the brain microenvironment is relatively new, where most of the focus on drug delivery to the brain has been on developing nanoparticles that can bypass or overcome the BBB. However, it cannot be assumed that coatings that provide stealth properties also provide brain-penetrating properties, and thus, many of these coatings that could create bio-inert nanoparticles, like zwitterions or surfactants, have not thoroughly been tested to determine if they maintain brain-penetrating properties. This is an area for future exploration that may greatly expand the nanoparticle platforms that can be utilized as BPNs.

There are multiple methods for calculating PEG density [19]. PEG chains have a Flory radius defined as $R_f \sim \alpha N^{3/5}$, where N is the degree of polymerization and α is the effective monomer length. For example, an unconstrained 5000 Da PEG chain has a diameter of 5.4 nm and occupies a surface area of 22.7 nm^2 (assuming an unconstrained random walk). Although not covered in detail in this chapter, PEG surface densities, provided by the ratio of number of PEG molecule chains to 100 nm^2 of nanoparticle surface are, for these nonbiodegradable polystyrene (PS) nanoparticles can be calculated using NMR analysis, using an adapted method published previously [23]. The average PEG surface density (chains/100 nm^2) on the surface of the NPs is then determined by taking into account the total quantity of PEG detected by NMR and the total NP surface area. The surface area of PS NPs is calculated by assuming that the NPs are made of individual particles of diameter equal to that measured by laser Doppler anemometry, with smooth surfaces, and a density of 1.055 g/mL, as provided by the manufacturer. In general, this protocol will produce nonbiodegradable nanoparticles with PEG densities greater than nine PEG per 100 nm^2, which allows penetration in the brain [2]. The packing of PEG can be determined by calculating the ratio of PEG chains per 100 nm^2 to the number of unconstrained PEG molecules that occupy a surface area of 100 nm^2 on a nanoparticle. The required PEG density necessary for other nanomaterials to achieve rapid brain penetration is dependent on the PEG molecular weight (MW), the PEG structure (linear vs branched), and the material properties of the particle core, including its composition, porosity, size, and MW.

2 Materials

Prepare all solutions using deionized water or ultrapure water and analytical-grade reagents. Prepare and store all reagents at room temperature or as otherwise noted below. Follow all local and

university waste-disposal regulations when disposing of waste materials from this protocol.

2.1 Reaction Solutions

1. Reaction buffer: 200 mM borate buffer, pH 8.2. Add 200 mL of ultrapure water to a 500 mL glass pyrex bottle. Weigh 3.71 g of boric acid and transfer to the bottle. Add ultrapure water to a total volume of 300 mL. Mix well and adjust pH to 8.2 using 1 N NaOH. Store at room temperature (*see* **Note 1**).
2. Working suspension of fluorescent PS-COOH nanoparticles: 4× dilution (*see* **Note 2**). Fluorescent PS-COOH nanoparticles may be purchased from a variety of vendors (*see* **Note 3**). In a water bath sonicator, place the stock bottle in a floating tube holder and sonicate the stock polystyrene nanoparticle solution for 10 min. In a 1.5 mL siliconized centrifuge tube, add 50 μL of fluorescent polystyrene nanoparticles from the stock bottle to 150 μL of ultrapure water (*see* **Note 4**). Particles should be sonicated in a water bath sonicator for 5 min after dilution. Stock particle solutions sometimes contain trace amounts of sodium azide (*see* **Note 5**).

2.2 Reagents

Allow all reagents to thaw for 20 min prior to starting the reaction.

1. Methoxy-PEG-NH_2: Remove methoxy-PEG-NH_2 (mPEG-NH_2, PEG MW: 5000 Da) from the −20 °C freezer. Do not open until ready to weigh out desired quantities. Keep mPEG-NH_2 stored in a jar containing indicating desiccant.
2. *N*-Hydroxysulfosuccinimide (Sulfo-NHS): Remove Sulfo-NHS sodium salt from the 4 °C refrigerator. Do not open until ready to weigh out desired quantities. Keep NHS in a jar containing indicating desiccant.
3. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC): Remove EDC from the −20 °C freezer. Do not open until ready to weigh out desired quantities. Keep EDC stored in a jar containing indicating desiccant.

2.3 Characterization Solutions

1. Size and zeta-potential measuring solutions: 10 mM NaCl, pH 7.4. Add 200 mL of ultrapure water to a 500 mL glass pyrex bottle. Weight 175.32 mg of NaCl and transfer to the bottle. Add ultrapure water to a total volume of 300 mL. Mix well and use 1 N HCl to adjust pH to 7.4. Store at room temperature.
2. In situ measuring solution: artificial cerebrospinal fluid (ACSF), pH 7.4. Add 200 mL of deionized water to a 500 mL glass pyrex bottle. Add 2.07 g NaCl (119 mM), 660 mg $NaHCO_3$ (26.2 mM), 56 mg KCl (2.5 mM), 36 mg NaH_2PO_4 (1 mM), 37 mg $MgCl_2$ (1.3 mM), and 540 mg glucose (10 mM). Mix the solution thoroughly then bring the final volume to 300 mL with deionized water. Gas the

solution with 5% CO_2/95% O_2 for 10–15 min. Then add 83 mg $CaCl_2$ (2.5 mM). Filter using a 0.22 μm filter apparatus. Store at 4 °C (*see* **Notes 6** and **7**).

3 Methods

Each carboxyl-modified fluorescent PS bead batch has a charge (milliequivalents, mEq), percent solid, and density (particles/mL). These numbers need to be obtained from the Certificate of Analysis (CoA) provided by the manufacturer prior to starting the protocol. There are four stages of the protocol: (1) calculation to determine the amount of reagents needed, (2) the PEGylation reaction, (3) collection of the nanoparticles, and (4) characterization of the nanoparticles' physicochemical properties.

3.1 Calculation of Number of COOH Per Bead

The reaction protocol is based on calculations that associate the mole ratio of number of COOH per bead to the number of moles of each reagent, which are often added in excess. Therefore, the number of COOH per bead needs to be calculated first. These calculations will be different for each size polystyrene particle and for each lot number of a specific particle type. A summary of sample calculations is shown in Table 1.

1. Number of beads per gram: Calculate density provided in CoA divided by the percent solid provided in CoA.
2. Number of milliequivalents per bead: Divide the charge provided in CoA by the number of beads per gram calculated in **step 1**.
3. Number of COOH groups per bead: Multiply the number of mEq per bead calculated in **step 2** by Avogadro's Number (6.022×10^{23}) and divide by 1000 (*see* **Note 8**).

Table 1
Sample calculation to determine number of COOH per bead

Starting particles	100 nm PS-COOH red	Notes
% solid	0.02	In CoA, based on nanoparticle size
Density (particles/mL)	2.73×10^{13}	In CoA, based on lot number
Charge (mEq)	0.309	In CoA, based on lot number
	Value	**Calculation**
Number of beads per gram	1.37×10^{15}	$2.73 \times 10^{13}/0.02$
Number of millieq per bead	2.26×10^{-16}	$1.37 \times 10^{15}/0.309$
Number of COOH per bead	1,36,322	$(2.26 \times 10^{-16}) \times 6.022 \times 10^{23}$

3.2 Calculation of Number of Moles of PEG Needed

The following calculations are based on a starting volume of 50 μL of 100 nm PS-COOH nanoparticles. A summary of sample calculations is shown in Table 2.

1. The number of beads in the working nanoparticle solution is calculated by multiplying the volume of stock beads (50 μL) by the density of beads provided in the CoA, divided by 1000.
2. The total number of COOH in the working solution is the number of beads added in **step 1** of Subheading 3.2 multiplied by the number of COOH groups per bead calculated in **step 3** of Subheading 3.1.
3. The number of moles of PEG needed is calculated by dividing the total number of COOH in the working solution by Avogadro's Number (*see* **Notes 9** and **10**).

3.3 PEGylation Reaction

The following reaction protocol is based on a starting volume of 50 μL of 100 nm PS-COOH nanoparticles. A sample calculation is provided in Table 3. The reaction schematic for PEGylation of PS nanoparticles is shown in Fig. 2.

Allow all reagents to thaw for 20 min prior to use.

Table 2
Sample calculation for number of moles of PEG needed for the conjugated of mPEG-NH_2 to PS-COOH

Starting particles	100 nm PS-COOH	Notes
% solid	0.02	In CoA, based on nanoparticle size
Density (particles/mL)	2.73×10^{13}	In CoA, based on lot number
Charge (mEq)	0.309	In CoA, based on lot number
	Value	**Calculations**
Number of beads per gram	1.37×10^{15}	$2.73 \times 10^{13}/0.02$
Number of millieq per bead	2.26×10^{-16}	$1.37 \times 10^{15}/0.309$
Number of COOH per bead	1,36,322	$(2.26 \times 10^{-16}) \times 6.023 \times 10^{23}$
Volume of beads added (μL)	50	
Dilution factor	4	*See* **Note 2**
Number of beads added	1.37×10^{12}	$50 \times 2.73 \times 10^{13}/1000$
Total number of COOH	1.86×10^{17}	$(1.37 \times 10^{12}) \times 136322$
Number of mole of PEG needed	3.09×10^{-7}	$1.86 \times 10^{17}/6.023 \times 10^{23}$
Molar mass of PEG (Da)	5000	*See* **Note 9**
Excess of PEG	4	*See* **Note 10**
Mass of PEG needed (mg)	6.18	$(3.09 \times 10^{-7}) \times 5000 \times 4 \times 1000$

Table 3
Sample calculation table for PEGylation reaction

	100 nm PS-COOH	Calculations/notes
Volume of beads added (μL)	50	
Dilution factor	4	*See* **Note 2**
Number of beads added	1.37×10^{12}	$50 \times 2.73 \times 10^{13}/1000$
Total number of COOH	1.86×10^{17}	$(1.37 \times 10^{12}) \times 136322$
Number of mole of PEG needed	3.09×10^{-7}	$1.86 \times 10^{17}/6.023 \times 10^{23}$
Molar mass of PEG (Da)	5000	*See* **Note 9**
Excess of PEG	4	*See* **Note 10**
Mass of PEG needed (mg)	1.55	$(3.09 \times 10^{-7}) \times 5000 \times 1000$
Mass of PEG to add (mg)	6.18	1.55×4
Mass of NHS to add (mg)	2.68	$(3.09 \times 10^{-7}) \times 4 \times 217.13 \times 10 \times 1000$
Amount of Borate buffer to add (μL)	800	*See* **Note 11**
Mass of EDC to add (mg)	2.37	$(6.18/5000) \times 191.7 \times 10$

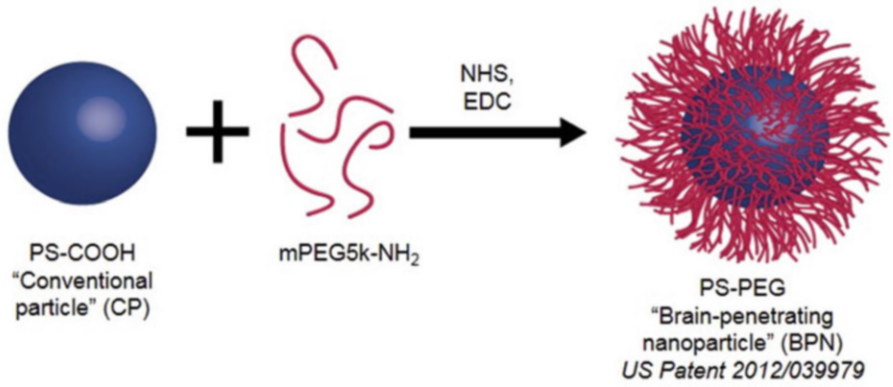

Fig. 2 Conjugation of methoxy PEG (5000 Da)-NH_2 to carboxy-modified polystyrene nanoparticles, using NHS- and EDC-assisted chemistry

1. In a water bath sonicator, sonicate the diluted working nanoparticle solution prepared as described in Subheading 2.1, **item 2** for 5 min.
2. Add 1.55 mg mPEG-NH_2 to the 1.5 mL centrifuge tube containing the diluted particle solution. Vortex ~30 s to 1 min to mix (*see* **Notes 9** and **10**).
3. The next steps should be done quickly. Add 2.68 mg Sulfo-NHS to the 1.5 mL centrifuge tube containing the particle solution. Vortex briefly (~1–5 s) to mix solution.

4. Add 800 μL 200 mM borate buffer, pH 8.2, to the 1.5 mL centrifuge tube containing the particle solution. Vortex briefly (~5 s) to mix. The amount of borate is based on a 4× dilution from the starting dilute PS volume (200 μL in this example) (*see* **Note 11**).
5. Add 2.37 mg 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) to the centrifuge tube containing the particle solution. Vortex ~30 s to 1 min to mix thoroughly.
6. Wrap 1.5 mL tube in aluminum foil and place on a rotary incubator for 4 h at 25 °C (*see* **Note 12**).

3.4 Nanoparticle Collection

Particle collection methods are based on the size of the particle and are therefore provided below based on the size of the particles used.

3.4.1 For Sub-100 nm Particles

1. At the end of the incubation time, add 500 μL of the PEGylated particle solution to an Amicon Ultra 0.5 mL 100 kDa molecular weight cut-off (MWCO) spin filter tube.
2. Place in a microcentrifuge at 4 °C.
3. Centrifuge particles for 12 min at 14,000 × *g* (*see* **Note 13**).
4. Pour off supernatant and add 500 μL ultrapure water to the top of the filter. Pipet up and down to resuspend particles. Particles should resuspend easily if well-PEGylated (*see* **Note 14**).
5. Repeat **steps 3** and **4**. This wash step should be repeated twice. More washes can risk loss of particles or cause the filter membrane to break.
6. After the last wash step, invert the filter membrane upside down in a new tube. Spin at 1000 × *g* for 2 min to collect the particles from the membrane. The volume of particles will be 20–50 μL.
7. If multiple tubes were used, combine into one 1.5 mL centrifuge tube. Bring the total volume to 100 μL in ultrapure water and store at 4 °C in the dark until use.

3.4.2 For 100 nm and Larger Particles

The centrifugation speed and time is dependent on the particle diameter and is outlined for different size particles that are greater than 100 nm in Table 4. All other steps in the collection process are the same.

1. At the end of the incubation time, remove the aluminum foil and place the 1.5 mL tube in a microcentrifuge at 4 °C. These particles, and particles larger than 100 nm, do not use filter membranes.
2. Centrifuge the particles for 25 min at 21,000 × *g*.
3. Pipet off the supernatant and add 200 μL of ultrapure water to the 1.5 mL tube. Pipet up and down to resuspend the particles. Particles should resuspend easily if well PEGylated (*see* **Note 14**).

Table 4
Suggested collection times and speeds for nonfiltration centrifugation for 100 nm PS particles or larger

Particle diameter (nm)	Centrifugation speed (×*g*)	Centrifugation time (min)	Number of wash steps
100	21,000	25	2
200	18,000	15	2
500	15,000	15	2
1000 or larger	10,000	10	2

4. Bring final volume in tube to 1 mL using ultrapure water. Repeat **step 2**. This wash step (**steps 2** and **3**) should be repeated twice. More washes can risk loss of particles.
5. After the last wash step, pipet off the supernatant. Bring the total volume to 100 μL in ultrapure water and store at 4 °C in the dark until use (*see* **Note 15**).

3.5 Brain-Penetrating Nanoparticle Characterization

The net surface charge (ξ-potential), polydispersity index (PDI), and hydrodynamic diameter should be measured for COOH- and PEG-coated fluorescent nanoparticles (NPs) of all sizes. COOH- and PEG-coated fluorescent NPs of all sizes can be measured by laser Doppler anemometry for net surface charge (ξ-potential) and dynamic light scattering (DLS) for PDI, and hydrodynamic diameter. The Malvern Zetasizer NanoZS is the most commonly employed instrument for these measurements.

1. In the instrument settings, polystyrene should be chosen for the material (for other materials, *see* **Note 16**).
2. In the instrument settings, ACSF needs to be added as a dispersant based on the concentrations provided in Subheading 2.3, **item 2**.
3. Dilute ACSF from 119 mM NaCl to 10 mM NaCl to obtain accurate ζ-potential measurements.
4. Dilute nanoparticle samples 1000-fold in ACSF at pH 7.0 to run the size and zeta potential measurements.
5. Perform size measurements and PDI at 25 °C at a scattering angle of 90°. A minimum of three separate measurements should be taken with a minimum of ten runs per measurement. Typical size, zeta potential, and PDI for particles referenced in this protocol are provided in Table 5.

Table 5
Representative physicochemical properties of PS nanoparticles after PEGylation. Mean diameter in ACSF at pH 7.0 was measured with dynamic light scattering. ζ-potential and PDI were measured in ACSF at pH 7.0. Adapted From Sci Transl Med. 2012 Aug 29;4(149): 149ra119. Reprinted with permission from AAAS. Copyright 2012 AAAS

Starting mean diameter of PS-COOH ± SEM (nm)	Mean diameter with PEG ± SEM (nm)	Mean Zeta potential with ± SEM (mV)	PDI
57 ± 2	69 ± 2	−2.8 ± 0.4	0.05
94 ± 3	106 ± 4	−4.4 ± 0.2	0.03
185 ± 1	198 ± 6	−7.8 ± 0.6	0.03

4 Notes

1. Borate buffer is stable for 3–6 weeks when stored at room temperature. The solution should be clear and not contain any precipitates. If the solution becomes cloudy or has precipitates, a new solution should be made.
2. Dilution of the stock PS-COOH particles is based on the nanoparticle size. Each size has a different starting concentration, with smaller particles having a higher concentration than larger particles. Therefore, smaller particles should be diluted more. Particles with starting concentrations greater than 10^{14} particles/mL (i.e., 20 and 40 nm) should be diluted six-fold. Particles with starting concentrations of 10^{12}–10^{13} particles/mL (i.e., 100 and 200 nm) should be diluted four-fold. Particles with starting concentrations less than 10^{11} particles/mL should be diluted two-fold.
3. Fluorescent particles are necessary for visualization using confocal imaging. COOH surface functionalization is useful for creating a stable, nondegradable amine bond, using carboxyl-amine chemistry. However, other surface functionalities on the nanoparticle surface can be utilized depending on the desired degradation or removal of PEG coating on the surface of the particle.
4. The dilution of the stock particles based on the particle size and concentration, as described in **Note 2**, can be scaled to any starting volume of stock particle solution.
5. Stock solutions of PS particles may contain trace amounts of sodium azide. If this is a concern, the particles can be purified by adding up to 1 mL of ultrapure water to the solution, centrifuging the particles based on their size to form a pellet, then removing the supernatant as described in Subheading 3.4.

This process should be repeated at least three times to ensure adequate removal of the sodium azide.

6. Nanoparticles in the brain microenvironment are exposed to approximately a 300 mM ion concentration in both in vivo and ex vivo CSF; however, at this high ion concentration, the accuracy of ξ-potential measurements can be negatively impacted. Therefore dilution of ACSF down to a 10 mM NaCl concentration can provide more accurate and stable zeta potential measurements.
7. ACSF is stable for 3–4 weeks when stored at 4 °C. The solution should be clear and not contain any precipitates. If the solution becomes cloudy or has precipitates, a new solution should be made.
8. The number of COOH per bead can be replaced with any surface functionality, provided the surface functionality can be directly associated with the Charge (mEq) provided in the CoA.
9. The PEG MW used in this protocol to achieve brain penetration is 5000 Da. Linear PEG chains with MWs from 1000 to 20,000 Da have shown the ability to penetrate through biological mediums like mucus and tissue [24]; however, thus far, 5000 Da PEG has led to the highest effective diffusivities for nanoparticles in the brain microenvironment. Branched PEGs or PEG MWs above 10 kDa have not been extensively explored and are an avenue for future investigation, in addition to alternative surface coatings discussed in subheading 1.
10. PEG excess is dependent on the starting nanoparticle size. Generally speaking, given the high density of COOH groups on sub-100 nm particles, and the surface curvature, a one-to-one mole ratio of PEG to COOH groups is used. For particles 100 to sub-500 nm, a four-fold excess of PEG to COOH groups can be used. For larger particles, greater than 500 nm, a two- to four-fold or higher excess of PEG to COOH groups can be used. If too much PEG is used, it will be immediately obvious once the borate buffer is added. The solution will become gel-like or very viscous. This indicates potential cross-linking or entanglement of the PEG, which will lead to poor reaction outcomes.
11. Borate should be added at four times the diluted PS-COOH particle volume. However, when determining the starting volume, it is important to remember the max volume of microcentrifuge tubes is either 1.5 or 2.0 mL. This protocol can scale to volumes large enough to justify 15 mL conical tubes; however, preparing multiple small batches in 1.5–2.0 mL tubes is more reproducible, and the batches can be combined after the final wash step.

12. Particles can be incubated for 4–24 h at room temperature; however, there is no benefit to incubating longer than 4 h.
13. If all the particle solution has not filtered through the membrane, add five more minutes of centrifugation time. Continue to add 5 min until all particle solution has filtered through the membrane. It is better to add time instead of increasing the speed.
14. PS-PEG particles that do not readily resuspend after centrifugation suggest that the PEG conjugation was not efficient. After the first collection, add 200 μL of ultrapure water to the pellet and pipet up and down over the pellet. Vortex the solution. After the pellet is resuspended in this volume, bring the total volume up to 800 μL. If the pellet is not dissolving, then vortex the solution longer. Do not sonicate the solution, as the sonication could disrupt the PEG layer.
15. Particles can be resuspended to the starting volume of stock PS particles used, or can be resuspended to a two-fold dilution.
16. Depending on the instrument being used for the size and zeta potential measurements, quantum dot and other materials can be listed in the material setting. If the material is not listed, it should be added. The dielectric constant and refractive index of the material must be known to add it to the list.

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