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Preparation of Poly(pentafluorophenyl acrylate) Functionalized SiO₂ Beads for Protein Purification

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Dr. Bing Wu
Review Editor
JoVE

Dear Dr. Wu,

Thank you very much for reviewing our manuscript entitled "Preparation of Poly(pentafluorophenyl acrylate) Functionalized SiO₂ Beads for Protein Purification" (JoVE58843). Point-by-point responses to both the editorial and reviewers' comments are attached in the next pages. We have revised our original manuscript to accommodate all the valuable comments raised, and the changes have been tracked in the revised version.

I sincerely hope that the revised manuscript would resolve all the comments. Thank you very much for your input and careful handling of our manuscript.

Sincerely,

A handwritten signature in black ink, appearing to read "Sheng Li", is written over a light blue horizontal line.

Sheng Li
Assistant Professor
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KAIST

TITLE:

Preparation of Poly(pentafluorophenyl acrylate) Functionalized SiO₂ Beads for Protein Purification

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KEYWORDS:

Poly(pentafluorophenyl acrylate), 3-aminopropyltriethoxysilane, reactive polymer brush, post-polymerization functionalization, antibody immobilization, immunoprecipitation

SHORT ABSTRACT:

A protocol for the preparation of poly(pentafluorophenyl acrylate) (poly(PFPA)) grafted silica beads is presented. The poly(PFPA) functionalized surface is then immobilized with antibodies and used successfully for the protein separation through immunoprecipitation.

LONG ABSTRACT:

We demonstrate a simple method to prepare poly(pentafluorophenyl acrylate) (poly(PFPA)) grafted silica beads for antibody immobilization and subsequent immunoprecipitation (IP) application. The poly(PFPA) grafted surface is prepared via a simple two-step process. In the first step, 3-aminopropyltriethoxysilane (APTES) is deposited as a linker molecule onto the silica surface. In the second step, poly(PFPA) homopolymer, synthesized via the reversible addition and fragmentation chain transfer (RAFT) polymerization, is grafted to the linker molecule through the exchange reaction between the pentafluorophenyl (PFP) units on the polymer and the amine groups on APTES. The deposition of APTES and poly(PFPA) on the silica particles are confirmed by X-ray photoelectron spectroscopy (XPS), as well as monitored by the particle size change measured via dynamic light scattering (DLS). To improve the surface hydrophilicity of the beads, partial substitution of poly(PFPA) with amine-functionalized poly(ethylene glycol) (amino-PEG) is

also performed. The PEG-substituted poly(PFPA) grafted silica beads are then immobilized with antibodies for IP application. For demonstration, an antibody against protein kinase RNA-activated (PKR) is employed, and IP efficiency is determined by Western blotting. The analysis results show that the antibody immobilized beads can indeed be used to enrich PKR while non-specific protein interactions are minimal.

INTRODUCTION:

Reactive polymer brushes have received much interest in recent years. They can be used to immobilize functional molecules on organic or inorganic materials to create activated surfaces with applications in areas such as detection and separation¹⁻⁵. Among the reactive polymers reported, those containing pentafluorophenyl ester units are particularly useful due to their high reactivity with amines and resistance toward hydrolysis⁶. One such polymer is poly(PFPA), and it can be readily functionalized post-polymerization with molecules containing primary or secondary amines⁷⁻¹⁰. In one example, poly(PFPA) brushes were reacted with amino-spiropyrans to create light-responsive surfaces⁷.

The preparation of poly(PFPA) and its applications have been described in a number of previous publications⁶⁻¹⁷. In particular, Theato and co-workers reported the synthesis of poly(PFPA) brushes via both “grafting to” and “grafting from” methods^{7,8,10-12}. In the “grafting to” approach, a poly(methylsilsequioxane)-poly(pentafluorophenyl acrylate) (poly(MSSQ-PFPA)) hybrid polymer was synthesized^{8,10-12}. The poly(MSSQ) component was able to form strong adhesion with a number of different organic and inorganic surfaces, thus allowing the poly(PFPA) component to form a brush layer on the coated material surface. In the “grafting from” approach, surface initiated reversible addition and fragmentation chain transfer (SI-RAFT) polymerization was employed to prepare poly(PFPA) brushes⁷. In this case, a surface immobilized chain transfer agent (SI-CTA) was first covalently attached to the substrate via silica-silane reaction. The immobilized SI-CTA then participated in the SI-RAFT polymerization of PFPA monomers, generating densely packed poly(PFPA) brushes with stable covalent linkage to the substrate.

By utilizing the poly(PFPA) brushes synthesized via SI-RAFT polymerization, we recently demonstrated the immobilization of antibodies on poly(PFPA) grafted silica particles and their subsequent application in protein purification¹⁸. The use of poly(PFPA) brushes for antibody immobilization was found to resolve a number of issues associated with current protein separation through IP. Conventional IP relies on the use of Protein A/G as a linker for antibody immobilization¹⁹⁻²¹. Since the use of Protein A/G allows the antibodies to be attached with a specific orientation, high target antigen recovery efficiency is achieved. However, the use of Protein A/G suffers from non-specific protein interaction as well as the loss of antibodies during protein recovery, both of which contribute to a high level of background noise. To resolve these shortcomings, direct crosslinking of the antibodies to a solid support has been explored²²⁻²⁴. The efficiency of such techniques is typically low due to the random orientation of the crosslinked antibodies. For the poly(PFPA) grafted substrate, the immobilization of antibodies is permanent, achieved through exchange reaction between PFP units and amine functionalities on antibodies. Although the antibody orientation is still random, the system benefits from having many reactive PFP sites, controllable by the degree of polymerization. Furthermore, we showed that by partial

substitution of PFP units with amino-PEG, surface hydrophilicity can be tuned, further improving the protein recovery efficiency of the system¹⁸. Overall, the poly(PFPA) grafted silica particles were shown to be an effective alternative to traditional IP with reasonable efficiency as well as much cleaner background.

In this contribution, we report an alternative method to prepare poly(PFPA) grafted surface for antibody immobilization and IP application. In a simple two-step process, as illustrated in **Figure 1**, an APTES linker molecule is first deposited onto the silica surface, then the poly(PFPA) polymer is covalently attached to the linker molecule through the reaction between the PFP units on the polymer and the amine functions on APTES. This preparation method allows for the permanent crosslinking of poly(PFPA) to a substrate surface, but avoids the many complications associated with SI-CTA synthesis and SI-RAFT polymerization of poly(PFPA) brushes. Partial substitution of the PFP units with amino-PEG can still be performed, allowing fine-tuning of the polymer brush surface properties. We show the poly(PFPA) grafted silica beads thus prepared can be immobilized with antibodies and used for protein enrichment via IP. The detailed bead preparation procedure, antibody immobilization, and IP testing are documented in this article, for readers interested in seeking an alternative to conventional Protein A/G based IP.

PROTOCOL:

1. Preparation of Poly(PFPA) Homopolymer

1.1. Recrystallization of AIBN

1.1.1. Combine 5 g of 2,2'-azobis(2-methylpropionitrile) (AIBN) with 25 mL of methanol in a 250 mL beaker. Immerse the beaker in a 60 °C oil bath, then vigorously stir the mixture with a stir bar until AIBN is fully dissolved.

1.1.2. Filter the warm solution through filter paper (5-8 µm particle retention) and store the filtrate at 4 °C to allow the crystals to form slowly.

1.1.3. Collect the recrystallized AIBN by filtration. Combine the collected product with 25 mL of fresh methanol and repeat the recrystallization process.

1.1.4. Dry the 2x recrystallized AIBN in a vacuum oven at room temperature (RT) overnight. Store the product in the dark at < -10 °C.

1.2. Synthesis of benzyl dithiobenzoate²⁵

1.2.1. Prepare a 500 mL three-neck round-bottom flask equipped with a magnetic stir bar, a refluxing condenser, a dropping funnel, and a rubber septum. Connect the flask to the nitrogen gas line through the refluxing condenser and flush out the inside air with nitrogen. Insert a thermometer through the septum. Add 41 mL (0.041 mol) of 1 M solution of phenylmagnesium bromide in tetrahydrofuran (THF) via a syringe through the same septum.

1.2.2. Warm the phenylmagnesium bromide solution to 40 °C in an oil bath. Then add 3.1 g (0.041 mol) of carbon disulfide through the dropping funnel slowly, maintaining the solution temperature at 40 °C.

1.2.3. Add 7.1 g (0.042 mol) of benzyl bromide to the resultant mixture through the dropping funnel over 15 min. Increase the reaction temperature to 50 °C. Continue stirring at this temperature for 45 min.

1.2.4. Transfer the reaction mixture into a separatory funnel and dilute with 15 mL of ice cold water. Extract the product by adding 15 mL of diethyl ether and remove the lower water layer. Repeat the extraction with diethyl ether two more times.

1.2.5. Wash the combined organic phases with copious amount of water, then brine (solution of 50% (w/v) NaCl in water) and dry the product over anhydrous magnesium sulfate.

1.2.6. Remove the solvent in vacuum at 4 °C using a rotary evaporator.

1.2.7. Purify the product by column chromatography using 400 mL of silica gel (pore size 60 Å, 63-200 mesh particle size) and petroleum ether as the eluent, yielding 5 g of benzyl dithiobenzoate (BDB) as red oil. Confirm the product purity by ¹H NMR (400 MHz, CDCl₃): δ 8.02-7.99 (2H, m), 7.55-7.50 (1H, m), 7.41-7.29 (7H, m), 4.60 (2H, s).

1.3. Synthesis of poly(PFPA) via RAFT polymerization^{9,26}

1.3.1. Commercially available PFPA monomer contains small amount of inhibitors. Before polymerization, remove the inhibitors by passing the monomer through a disposable syringe packed with basic alumina.

1.3.2. Add 0.4 mg (0.0024 mmol) of recrystallized AIBN, 4.3 mg (0.018 mmol) of BDB, 1012 mg (4.25 mmol) of inhibitor-free PFPA, and 0.7 mL of anhydrous anisole to a 20 mL Schlenk flask.

1.3.3. Connect the flask to Schlenk line and degas the mixture with at least three freeze-pump-thaw cycles. Briefly, freeze the reaction mixture in a liquid nitrogen bath. Apply vacuum to remove the gas in headspace. Seal the flask then remove away from liquid nitrogen to allow the content to thaw at RT.

1.3.4. Place the flask in a 70 °C oil bath, and react for 4 h under N₂ purge.

1.3.5. To terminate the reaction, remove the flask from the oil bath and expose the reaction content to air.

1.3.6. Precipitate the polymer in cold methanol, then dry the recovered polymer in a vacuum oven at 40 °C overnight.

1.3.7. To measure the polymer molecular weight, use gel permeation chromatography (GPC). Use THF as the mobile phase at 35 °C with a 1 mL/min flow rate and construct the calibration curve using monodisperse polystyrene standards. To acquire GPC measurement, dissolve the polymer in THF (1-2 mg/mL) and filter through 0.2 µm disposable polytetrafluoroethylene (PTFE) filter. Inject 100 µL of the sample into the GPC instrument. Convert the measured sample retention time to molecular weight using the polystyrene calibration curve.

2. Preparation of Poly(PFPA) Functionalized SiO₂ Beads

2.1. Treatment of SiO₂ beads with APTES

2.1.1. SiO₂ particles are available in the form of a 5% (w/v) aqueous suspension. Combine 0.8 mL of SiO₂ suspension with 40 mg of APTES and 8 mL of methanol in a 20 mL scintillation vial equipped with a stir bar.

2.1.2. Allow the reaction to proceed at RT for 5 h with vigorous stirring.

2.1.3. Transfer the solution to a conical tube. To isolate the APTES functionalized SiO₂ beads, centrifuge the solution at 10000 x g for 5 min, then remove the supernatant. Wash the beads by re-dispersing them in 3 mL of fresh methanol. Shake the tube by hand for mixing, but if necessary, improve the dispersion by sonication in a water bath for a few seconds. Centrifuge the beads at 10000 x g for 5 min. Remove the supernatant and repeat the wash step one more time.

2.1.4. Combine the methanol washed SiO₂ beads with 3 mL of dimethyl sulfoxide (DMSO). Shake the mixture by hand until the beads are fully dispersed in DMSO. Centrifuge the beads at 10000 x g for 5 min, then remove the supernatant. Repeat the step to ensure complete solvent exchange from methanol to DMSO.

Note: The final suspension contains the APTES functionalized SiO₂ beads dispersed in 4 mL of DMSO.

2.1.5. To check the particle size distribution, perform DLS analysis. Take one drop of the suspension prepared in Step 2.1.4 and place into a disposable UV cuvette. Dilute the sample by filling the cuvette with fresh DMSO until it is 2/3 full. Insert the sample into the cell holder to begin data acquisition. For particle size measurement, use the following setup parameters: Temperature: 25 °C; Equilibration Time: 120 s; Measurement Duration: Automatic.

2.1.6. To check the surface composition, perform XPS analysis. Dry a small sample from the suspension prepared in Step 2.1.4 in vacuum oven at 40 °C overnight. Take the dried polymer and pack evenly onto a 0.5 cm x 0.5 cm sample holder. Load the sample into the high vacuum chamber (10⁻⁸ torr) and begin data acquisition. For the particular XPS instrument used, generate the photoelectrons using a monochromatic Al Kα X-ray operated at 15 kV and 6.7 mA, and collect using hybrid mode magnification with the analyzer at a 50 eV pass energy for high resolution

spectra, and a 100 eV pass energy for elemental surveys.

2.2. Grafting poly(PFPA) to APTES functionalized SiO₂ beads

2.2.1. Prepare the poly(PFPA) solution by dissolving 20 mg of poly(PFPA) in 2 mL of DMSO in a 20 mL scintillation vial.

Note: In this study, a relatively low molecular weight poly(PFPA) (20 kg/mol) is used. Thus, despite the high polymer concentration (10 mg/mL), no evidence of polymer crosslinking is observed. If a higher molecular weight polymer is used, then polymer solution concentration may need to be adjusted to avoid possible crosslinking.

2.2.2. Add 1 mL of APTES functionalized SiO₂ beads suspended in DMSO (from Step 2.1.4) to the poly(PFPA) solution. React at RT for 1 h with vigorous stirring.

2.2.3. Isolate the poly(PFPA) grafted SiO₂ beads by centrifugation at 10000 x g for 5 min, followed by the removal of the supernatant. Wash the beads by adding 3 mL of DMSO and mix by either shaking with hand or few seconds of sonication. Centrifuge the beads at 10000 x g for 5 min, then remove the supernatant. Repeat washing of the poly(PFPA) grafted SiO₂ beads with DMSO twice.

2.2.4. Wash the beads two times more with triple distilled water (TDW). In this step, combine the beads with 3 mL of TDW, then mix by shaking with hand or few seconds of sonication. Centrifuge the beads at 10000 x g for 5 min, then remove the supernatant.

2.2.5. To check the particle size distribution, perform DLS following the procedure described in Step 2.1.5. To check the surface chemistry, perform XPS following the procedure described in Step 2.1.6.

3. Preparation of SiO₂ Beads Grafted with PEG-Substituted Poly(PFPA)

3.1. To prepare the poly(PFPA) solution, dissolve 20 mg of poly(PFPA) in 2 mL of DMSO in a 20 mL scintillation vial.

3.2. To prepare PEG solution, dissolve amine-functionalized PEG in 1 mL of DMSO. The exact amount of PEG used is determined by the desired degree of PFP substitution, determined by the equation shown below:

$$\text{Amount of amino-PEG (g/g-poly(PFPA))} = (N_{\text{poly(PFPA)}} \times \% \text{ PEG-Sub}) \times (\text{MW}_{\text{PEG}} / \text{MW}_{\text{poly(PFPA)}})$$

where $N_{\text{poly(PFPA)}}$ = poly(PFPA) degree of polymerization

% PEG-Sub = percent PEG substitution

MW_{PEG} = molecular weight of amino-PEG

MW_{_} poly(PFPA) = molecular weight of poly(PFPA)

3.3. Transfer the PEG solution to the poly(PFPA) solution. React at RT for 1 h with vigorous stirring.

3.4. To prepare APTES functionalized SiO₂ beads suspended in DMSO, follow the same steps shown in Step 2.1. Transfer 1 mL of the bead suspension into the PEG-substituted poly(PFPA) solution prepared in Step 3.3. Allow the grafting between poly(PFPA) and APTES functionalized SiO₂ beads to proceed at RT for 1 h with vigorous stirring.

3.5. Isolate the beads by centrifugation at 10000 x g for 5 min, followed by the removal of the supernatant. Wash the beads by adding 3 mL of DMSO and mix by either shaking with hand or few seconds of sonication. Centrifuge the beads at 10000 x g for 5 min, then remove the supernatant. Repeat the DMSO wash twice.

3.6. Wash the beads two times more with TDW. In this step, combine the beads with 3 mL TDW, then mix by shaking with hand or few seconds of sonication. Centrifuge the beads at 10000 x g for 5 min, then remove the supernatant.

3.7. Dry the beads at 40 °C in a vacuum oven overnight.

4. Antibody Immobilization on Poly(PFPA) Grafted SiO₂ Beads

Note: The same procedure is used regardless of percent PEG substitution on poly(PFPA). Prepare phosphate buffered saline (PBS) by dissolving PBS tablet in TDW. Prepare 0.1% (v/v) phosphate buffered saline with Tween-20 (PBST) by adding 1/1000 of Tween-20 to PBS.

4.1. Add 5 mg of poly(PFPA) grafted SiO₂ beads to a 1.5 mL microcentrifuge tube.

4.2. Wash the beads by adding 800 µL of PBS and mix well by vortexing. Centrifuge the beads at 10000 x g at RT for 1 min. Remove the supernatant and repeat the wash step three times.

4.3. Add 350 µL of fresh PBS, 50 µL 0.1% (v/v) PBST, and 6.67 µg of the antibody. Incubate ~20 h on a rotator at 4 °C.

4.4. Wash the beads to remove unbound antibodies. Centrifuge the beads at 400 x g and 4 °C for 1 min. Remove the supernatant and add 400 µL of lysis buffer carefully. Gently re-suspend the beads by pipetting up and down for five times.

Note: Lysis buffer used to wash the beads should be the same one used during cell lysis and IP (see Step 5).

4.5. Repeat this wash step three times. After the final wash, remove the supernatant as much as possible.

5. Cell Lysis and Immunoprecipitation

5.1. Preparation of lysis buffer and wash buffer

5.1.1. Prepare the lysis buffer (50 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.5% (v/v) NP-40, 10% (v/v) glycerol, 1 mM dithiothreitol (DTT), and protease inhibitor cocktail).

5.1.2. Prepare the wash buffer (50 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1 % (v/v) NP-40, and 10% (v/v) glycerol).

5.1.3. Store the buffer solutions at 4 °C.

5.2. Preparation of the cells

5.2.1. Seed the cells (HeLa cells) one or two days before IP experiment, and grow the cells at 37 °C and 5% CO₂.

5.2.2. Collect about 1.4×10^7 cells with a cell scraper and transfer into a 15 mL conical tube. Centrifuge the cells at 380 x g at RT for 3 min. Remove the supernatant and re-suspend with 1 mL of cold PBS and transfer into a 1.5 mL microcentrifuge tube.

5.2.3. Centrifuge the cells at 10000 x g at 4 °C for 30 s. Remove the supernatant cleanly. Cell pellets can be stored at -80 °C after removing the supernatant.

5.3. Preparation of cell lysates

5.3.1. Re-suspend the cell pellet with 400 µL of the lysis buffer. Sonicate the cells using an ultrasonicator.

5.3.2. After the sonication, vortex briefly and centrifuge the lysate at 20000 x g at 4 °C for 10 min.

5.3.3. Transfer the supernatant to a new 1.5 mL centrifuge tube.

5.4. Immunoprecipitation

5.4.1. Transfer 300 µL of cell lysate to previously prepared antibody incubated poly(PFPA) grafted SiO₂ beads. Retain 30 µL of the cell lysate as the input sample in a new microcentrifuge tube. Store the input sample at 4 °C.

Note: The total amount of protein in cell lysate should be approximately 4 mg.

5.4.2. Incubate the lysate/beads mixture for 3 h on a rotator at 4 °C.

5.4.3. Centrifuge the mixture at 400 x g at 4 °C for 1 min. Remove the supernatant and add 400 µL of wash buffer carefully. Gently re-suspend the beads by pipetting up and down about five times.

5.4.4. Repeat this wash step three times. After the final wash, remove the supernatant as much as possible.

5.4.5. Prepare 2x sodium dodecyl sulfate (SDS) loading dye (25% (v/v) glycerol, 0.1% (w/v) bromo phenol blue (BPB), 60 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, and 2.75 mM 2-mercaptoethanol). Store 2x SDS loading dye at -20 °C. Add 30 µL of 2x SDS loading dye to the beads and the stored input sample, and heat them for 10 min at 95 °C.

5.4.6. After heating, analyze the sample using Western blotting²⁷, or store the sample at -20 °C.

REPRESENTATIVE RESULTS:

A schematic for the preparation of poly(PFPA) grafted SiO₂ beads, with or without PEG substitution is shown in **Figure 1**. To monitor the APTES and poly(PFPA) grafting process, bare SiO₂ beads, APTES functionalized SiO₂ beads, and poly(PFPA) grafted SiO₂ beads are characterized by both DLS (**Figure 2**) and XPS (**Figure 3**). IP efficiencies of the beads are determined by Western blotting. **Figure 4** shows the Western blotting results for IP using 1% PEG-substituted poly(PFPA) grafted beads, where the beads are incubated with no antibody, a non-specific antibody, or anti-PKR antibody. **Figure 5** shows the Western blotting results for IP using 0% PEG-substituted poly(PFPA) grafted beads and 1% PEG-substituted poly(PFPA) grafted beads, both incubated with anti-PKR antibodies.

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic for the preparation of poly(PFPA) grafted SiO₂ beads using APTES as a linker molecule. (a) Poly(PFPA) grafted beads. (b) Partially PEG-substituted poly(PFPA) grafted beads.

Figure 2. DLS measurements for (a) bare SiO₂ beads (SiO₂), (b) APTES functionalized SiO₂ beads (APTES-SiO₂), and (c) poly(PFPA) grafted SiO₂ beads (poly(PFPA)-SiO₂), dispersed in DMSO. The Z-average diameter (d) and polydispersity index (PDI) of each sample are reported.

Figure 3. XPS spectra for bare SiO₂ beads (SiO₂), APTES functionalized SiO₂ beads (APTES-SiO₂), and poly(PFPA) grafted SiO₂ beads (poly(PFPA)-SiO₂). The peaks examined correspond to (a) Si 2p, (b) O 1s, (c) N 1s, and (d) F 1s.

Figure 4. Western blotting results for IP using 1 % PEG-substituted poly(PFPA) grafted beads, treated with no antibody (lane 2), a non-specific antibody mixture, normal rabbit IgG (lane 3), or anti-PKR antibody (lane 4). Lane 1 shows the input protein mixture before IP.

Figure 5. Western blotting results for IP using 0 % PEG-substituted poly(PFPA) grafted beads (lane 2) and 1% PEG-substituted poly(PFPA) grafted beads (lane 3), both are treated with anti-

PKR antibodies. Lane 1 shows the input protein mixture before IP.

DISCUSSION:

The synthesis of poly(PFPA) grafted SiO₂ beads is illustrated in **Figure 1**. By employing APTES as a linker molecule, poly(PFPA) brushes covalently grafted to SiO₂ substrate can be prepared via a simple two-step process. Although some of the PFP units are sacrificed for the reaction with APTES, a large number of the PFP units are expected to remain available for later reaction with either amino-PEG or antibodies. The PFP groups are known to form low energy surfaces so poly(PFPA) brushes do not solvate well in water²⁸. For IP application, the antibodies need to be immobilized on the poly(PFPA) brushes, and this exchange reaction is done in aqueous buffer solution in order to preserve the activity of the antibodies. As reported in our previous publication, partial substitution of the PFP units with hydrophilic molecules such as amine-functionalized PEG can improve surface hydrophilicity, leading to increased antibody immobilization efficiency¹⁸. In this study, partially PEG substituted poly(PFPA) is also prepared, then grafted to the SiO₂ surface using the same APTES linker molecule. Overall, the preparation methods illustrated in **Figure 1** allow the preparation of poly(PFPA) grafted surfaces with different degrees of PEG substitution. These polymer brushes with tunable surface properties provide an ideal platform for antibody immobilization and subsequent IP application.

The bead preparation process is monitored by both DLS and XPS. The DLS results for various functionalized SiO₂ beads in DMSO are summarized in **Figure 2**. The bare SiO₂ beads exhibit hydrodynamic diameter of 666 nm, in agreement with the manufacturer reported bead size (0.676 μ m; SD = 0.03 μ m). After APTES treatment, the bead diameter increases to 740 nm; and with poly(PFPA) treatment, the bead diameter further increases to 1889 nm. It is important to point out that the polydispersity index (PDI) for the poly(PFPA) grafted beads is rather large (PDI = 0.76), which is indicative of poor quality sample containing large aggregates. Although the DLS curve only shows one nano-sized peak, small amount of aggregates may be present in the suspension. The functionalized SiO₂ beads are also examined by XPS to determine surface composition (**Figure 3**). Following APTES treatment, N 1s peak associated with the amine groups on APTES is detected. And, following poly(PFPA) treatment, F 1s peak associated with the PFP units on the polymer is detected. Together these data show the successful functionalization of the SiO₂ surface, first with APTES, then with poly(PFPA).

To show that the poly(PFPA) grafted beads can be used for protein enrichment through IP, we used 1% PEG-substituted poly(PFPA) grafted beads, and incubated them with no antibody, a non-specific rabbit IgG antibody mixture, or anti-PKR antibody. Cell lysate containing the target PKR was extracted from cell, and PKR enrichment was then performed through IP using the three type of beads. To determine IP efficiency, the eluted protein samples were analyzed against two different antibodies via Western blotting. Anti-PKR antibody was used to visualize the amount of PKR recovered. And, anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody was used as a negative control as GAPDH is an abundant protein that does not interact with PKR. As shown in **Figure 4**, beads immobilized with no antibody or non-specific antibody mixture result in no PKR recovery. In contrast, the beads incubated with anti-PKR antibody can successfully enrich PKR, as indicated by the presence of a strong PKR band and the absence of GAPDH band.

These results suggest PEG-substituted poly(PFPA) brushes can indeed be functionalized with antibodies and used for selective enrichment of target protein. Note when protein recovery efficiencies of different bead systems are compared, the IP experiments as well as the subsequent western blotting analyses should be done simultaneously. Due to the inherent variations in performing these experiments, data obtained on separate trials should not be directly compared.

As reported before, the surface hydrophilicity of poly(PFPA) brushes plays a key role in IP efficiency¹⁸. **Figure 5** shows the Western blotting data for IP recovered protein samples using 0% PEG-substituted poly(PFPA) grafted beads and 1% PEG-substituted poly(PFPA) grafted beads. In both cases, the beads were immobilized with anti-PKR antibodies. While the use of 0% PEG-substituted poly(PFPA) results in low PKR recovery efficiency, the 1% PEG-substituted poly(PFPA) shows significant improvement, indicated by the selective enrichment of the target PKR over non-target GAPDH. In agreement with our previous publication¹⁸, the PEG treatment increased the surface hydrophilicity of the poly(PFPA) brush, allowing more PFP units to be accessible for antibody immobilization, leading to the observed improvement in IP efficiency. Note, the percent PEG substitution reported in this study cannot be directly compared to that reported in our previous study which used SI-RAFT synthesized poly(PFPA) brushes. The two cases employ very different polymer brush preparation methods, so the amount of PFP units available with equal PEG loading is expected to be very different. However, the observations from the two studies do agree qualitatively, both pointing to surface hydrophilicity as a key control parameter for achieving high IP efficiency.

While surface hydrophilicity influences the amount of antibody attachment to the poly(PFPA) brushes, it also has a significant effect on IP background due to non-specific enrichment. In a typical IP experiment, many washing steps are performed to remove unbound proteins. When the beads are very hydrophobic, such as the ones with 0% PEG substitution, they tend to form large aggregates that are difficult to break apart. In this case, non-specific proteins can be trapped inside the aggregate structures, and washing cannot sufficiently remove them, leading to an increase in background. Therefore, when performing IP, it is important to optimize the bead surface property, and attention should be paid to ensure the beads are reasonably dispersed.

Overall, we demonstrated a simple two-step process to prepare poly(PFPA) grafted SiO₂ beads, and showed that the surface hydrophilicity of the beads can be fine-tuned with partial substitution of the PFP units with amino-PEG. These polymer brushes were successfully used for target protein enrichment through IP, presenting itself as an alternative to traditional Protein A/G based IP technique. We expect poly(PFPA) brushes to find application in many other areas requiring biomolecule immobilization.

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DISCLOSURES:

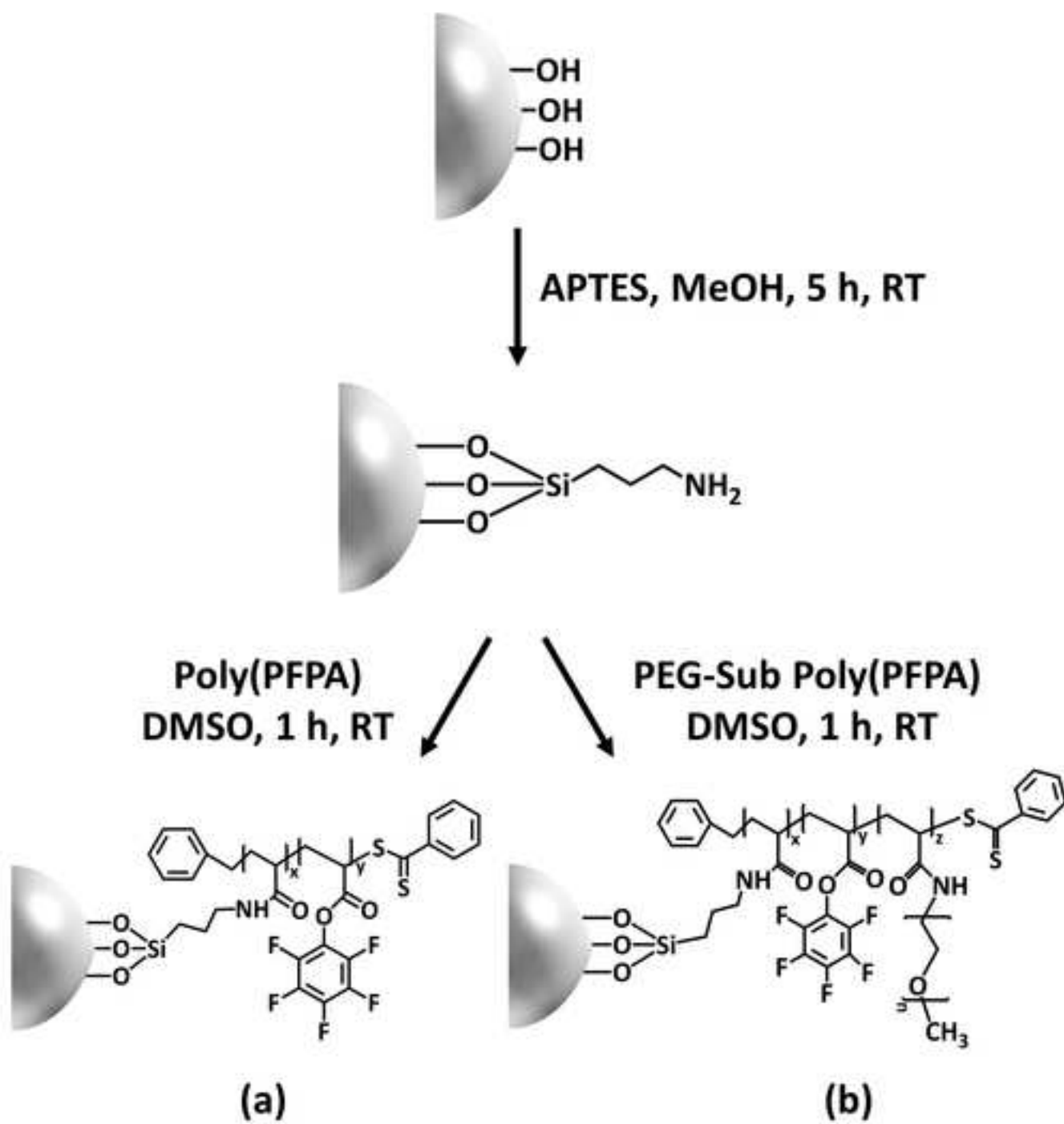
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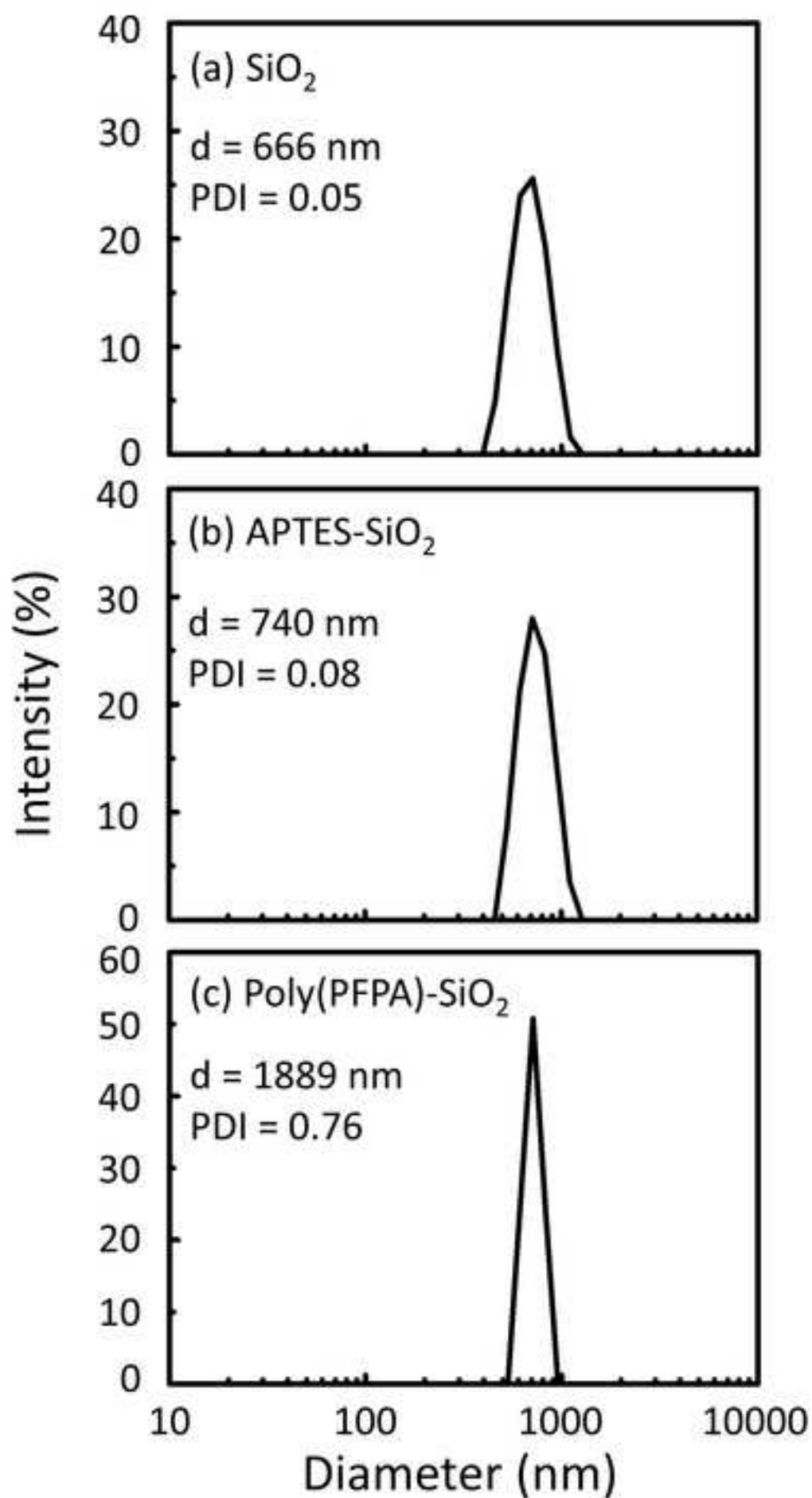
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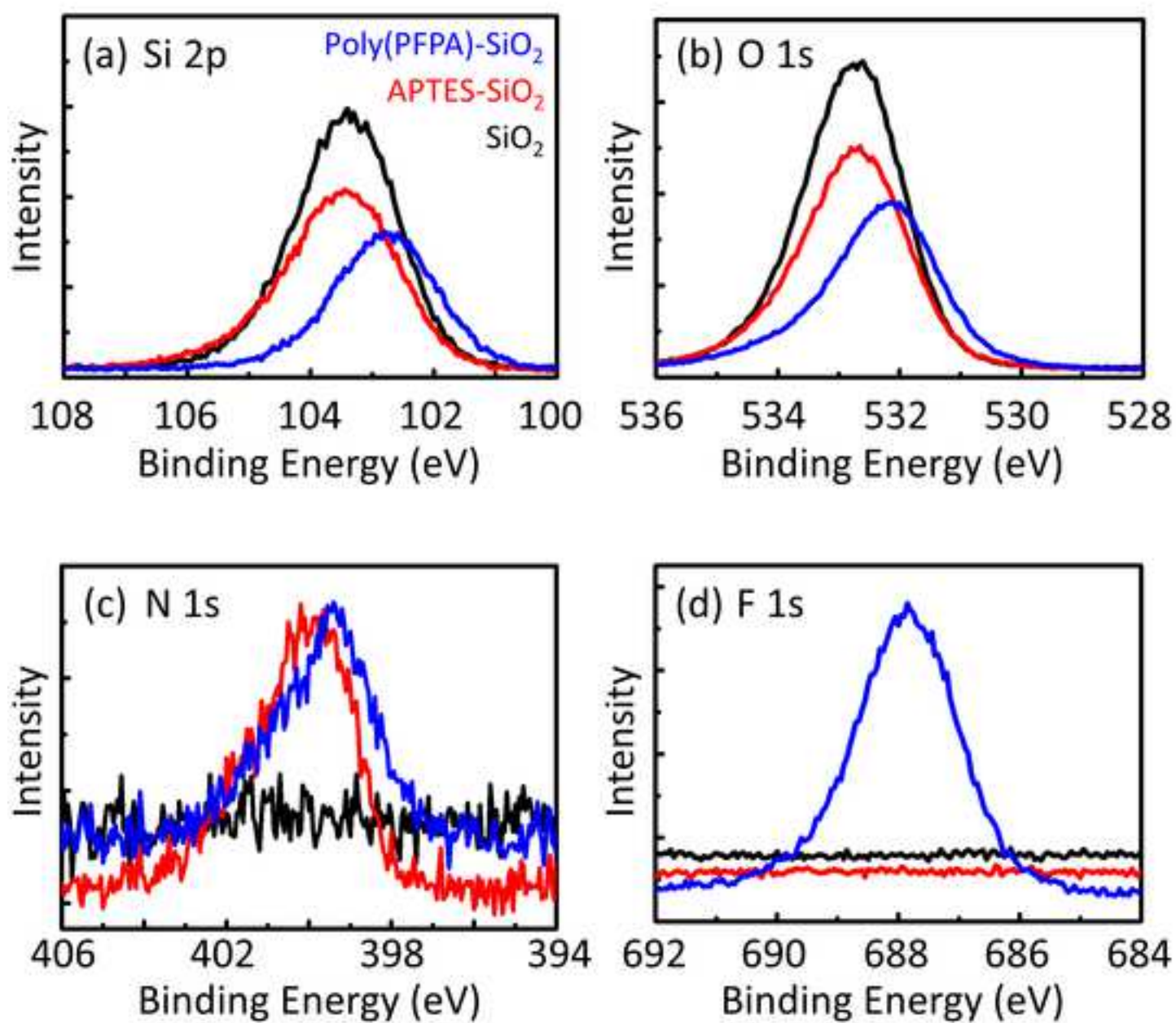
486 **REFERENCES:**

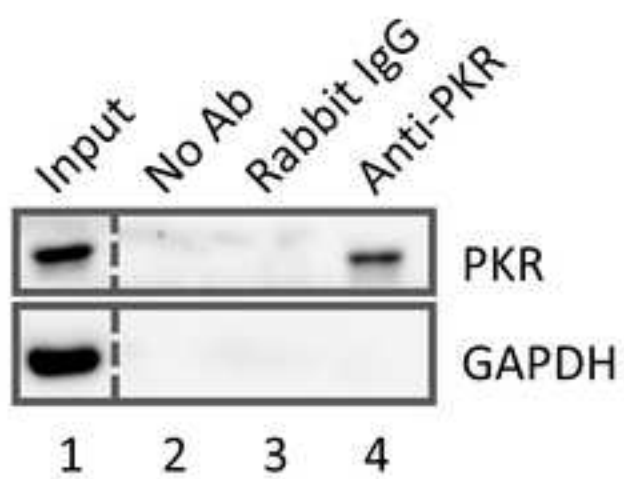
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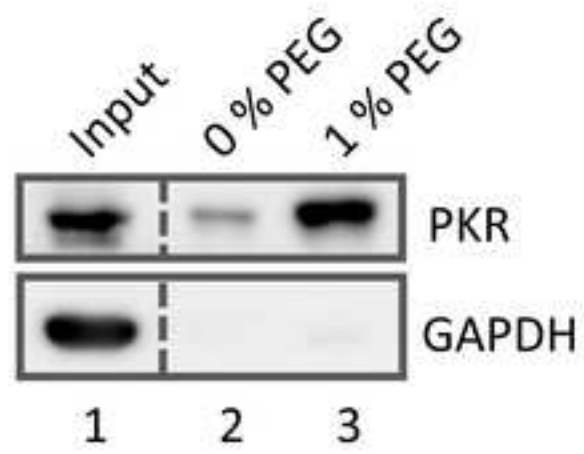
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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
2,2-Azobisisobutyronitrile, 99%	Daejung Chemicals	1102-4405	
Methyl alcohol for HPLC, 99.9%	Duksan Pure Chemicals	d62	
Phenylmagnesium bromide solution 1.0 M in THF	Sigma-Aldrich	331376	
Carbon disulfide anhydrous, ≥99%	Sigma-Aldrich	335266	
Benzyl bromide, 98%	Sigma-Aldrich	B17905	
Petroleum ether, 90%	Samchun Chemicals	P0220	
Ethyl ether, 99%	Daejung Chemicals	4025-4404	
Magnesium sulfate anhydrous, powder, 99%	Daejung Chemicals	5514-4405	
Pentafluorophenyl acrylate	Santa Cruz Biotechnology	sc-264001	contains inhibitor
Aluminium oxide, activated, basic, Brockmann I	Sigma-Aldrich	199443	
Sodium Chloride (NaCl)	Daejung Chemicals	7548-4400	
Anisole anhydrous, 99.7%	Sigma-Aldrich	296295	
Silica nanoparticle	Microparticles GmbH	SiO2-R-0.7	5% w/v aqueous suspension
3-Aminopropyltrimethoxysilane, >96.0%	Tokyo Chemical Industry	T1255	
Dimethyl sulfoxide for HPLC, ≥99.7%	Sigma-Aldrich	34869	
Amino-terminated poly(ethylene glycol) methyl ether	Polymer Source	P16082-EGOCH3NH2	
Phosphate buffered saline tablet	Takara	T9181	
Tween-20	Calbiochem	9480	
Tris-HCl (pH 8.0)	Invitrogen	AM9855G	
KCl	Invitrogen	AM9640G	
NP-40	VWR	E109-50ML	
Glycerol	Invitrogen	15514-011	
Dithiothreitol	Biosesang	D1037	
Protease inhibitor	Merck	535140-1MLCN	
Bromo phenol blue	Sigma-Aldrich	B5525-5G	
Tris-HCl (pH 6.8)	Biosolution	BT033	
Sodium dodecyl sulfate	Biosolution	BS003	
2-Mercaptoethanol	Gibco	21985-023	
PKR Antibody	Cell Signaling Technology	12297S	
GAPDH Antibody	Santa Cruz Biotechnology	sc-32233	
Normal Rabbit IgG	Cell Signaling Technology	2729S	
HeLa	Korea Cell Line Bank	10002	

Sonicator	DAIHAN Scientific	WUC-D10H
Ultrasonicator	BMBio	BR2006A
Centrifuge I	Eppendorf	5424 R
Centrifuge II	LABOGENE	1736R
Rotator	FINEPCR	ROTATOR/AG
Vacuum oven	DAIHAN Scientific	ThermoStable OV-30
Gel permeation chromatography (THF)	Agilent Technologies	1260 Infinity II
X-ray photoelectron spectrometer	Thermo VG Scientific	Sigma Probe
Dynamic light scattering	Malvern Instruments	ZEN 3690



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Author(s): Sura Kim, Jayoung Ku, Jaemin Park, Raisa Kharbash, Sheng Li

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Rebuttal Letter

We thank the editors and reviewers for the detailed comments. Point-by-point responses to the comments are listed below. We have also revised the manuscript to reflect these changes.

Editorial Comments

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: We have thoroughly proofread the manuscript.

2. Please spell out each abbreviation the first time it is used.

Response: We have checked the manuscript to spell out all abbreviations the first time they appeared.

3. Please remove commercial language: Bioruptor.

Response: We removed Bioruptor (line 336).

4. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Some examples:

1.1.2: What is the pore size of the filter?

Response: Please see line 119 “Filter the warm solution through filter paper (5-8 μm particle retention)”.

1.2.3: Please how to confirm completion of the reaction with TLC.

Response: We decided to remove this line because in this step checking reaction progress via TLC is unnecessary, and it is also not used in the original publication (Ref 25, Chong et al., *Macromolecules*, 36 (7), 2256-2272, (2003)).

1.2.7: Please mention how to perform column chromatography and NMR. For instance, mention sample preparation, NMR solvent, parameters used, etc.

Response: Please see line 153 “1.2.7 Purify the product by column chromatography using 400 mL of silica gel (pore size 60 \AA , 63-200 mesh particle size) and petroleum ether as eluent, yielding 5 g of benzyl dithiobenzoate (BDB) as red oil. Confirm product purity by ^1H NMR (400 MHz, CDCl_3) δ 8.02-7.99 (2H, m), 7.55-7.50 (1H, m), 7.41-7.29 (7H, m), 4.60 (2H, s).”

1.3.6: Please mention how to determine polymer molecular weight using GPC. Alternatively, references may be provided.

Response: Please see line 180 “1.3.7 To measure polymer molecular weight, gel permeation chromatography (GPC) is used. The GPC system uses THF as the mobile phase at 35 $^\circ\text{C}$ with a 1 mL/min flow rate, and the calibration curve is constructed using monodisperse polystyrene standards. To acquire GPC measurement, dissolve polymer in THF (1-2 mg/mL) and filter through 0.2 μm disposable polytetrafluoroethylene (PTFE) filter. Inject 100 μL of sample into the GPC instrument. Convert the measured sample retention time to molecular weight using the polystyrene calibration curve.”

2.1.4, 2.1.5, 2.2.4: What volume of DMSO is used?

Response: The exact volume of DMSO used for each step has been added.

2.1.5: Please describe how DLS and XPS analyses are done.

Response: Please see line 210. The following paragraphs have been added.

“2.1.5 To check particle size distribution, DLS analysis is performed. Take one drop of the suspension prepared in step 2.1.4 and place into a disposable UV cuvette. Dilute the sample by fill the cuvette with fresh DMSO until it is 2/3 full. Insert the sample into the cell holder to begin data acquisition. For particle size measurement, the following setup parameters are used: Temperature: 25 °C; Equilibration Time: 120 s; Measurement Duration: Automatic.

2.1.6 To check surface composition, XPS analysis is performed. Dry a small sample from the suspension prepared in step 2.1.4 in vacuum oven at 40 °C overnight. Take the dried polymer and pack evenly onto a 0.5 cm x 0.5 cm sample holder. Load the sample into the high vacuum chamber (10^{-8} torr) and begin data acquisition. For the particular XPS instrument used, photoelectrons are generated using a monochromatic Al K α X-ray operated at 15 kV and 6.7 mA, and they are collected using hybrid mode magnification with the analyzer at a 50 eV pass energy for high resolution spectra, and a 100 eV pass energy for elemental surveys.”

5. 1.3.1, 1.3.3, 1.3.5, 1.3.6, 2.1.5, 2.2.5, 2.2.6, etc.: Please write the text in the imperative tense.

Response: The text has been changed to imperative tense.

6. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

7. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

8. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Response to comments 6-7: Sections for filming have been indicated with highlight.

9. Figure 1: Please label structures a and b. Please include a space between the number and the time unit (i.e., 5 h, 1 h, etc.).

Response: Figure 1 has been updated to reflect the recommended changes.

10. Table 1: Please define PDI.

Response: Per reviewer #3's comment, Table 1 has been deleted, and its contents have been replaced by Figure 2. The phase PDI has been defined in the legend of Figure 2.

11. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

Response: Per editor's suggestion, following paragraphs have been added to the Discussion section.

Line 442: “Note when protein recovery efficiencies of different bead systems are compared,

the IP experiments as well as the subsequent western blotting analyses should be done simultaneously. Due to the inherent variations in performing these experiments, data obtained on separate trials should not be directly compared.”

Line 464: “While surface hydrophilicity influences the amount of antibody attachment to the poly(PFPA) brushes, it also has a significant effect on IP background due to non-specific enrichment. In a typical IP experiment, many washing steps are performed to remove unbound proteins. When the beads are very hydrophobic, such as the ones with 0 % PEG substitution, they tend to form large aggregates that are difficult to break apart. In this case, non-specific proteins can be trapped inside the aggregate structures, and washing cannot sufficiently remove them, leading to an increase in background. Therefore, when performing IP, it is important to optimize the bead surface property, and attention should be paid to ensure the beads are reasonably dispersed.”

12. References: Please do not abbreviate journal titles.

Response: Reference section has been updated to show full journal titles.

Reviewer #1 Comments:

Major Concerns: none.

Minor Concerns:

1. The polymer concentration of poly(PFPA) for the SiO₂ attachment appear rather high and I wonder if the authors observed partial cross-linking of the solution. A comment on the choice of suitable concentrations would be helpful to the reader.

Response: We thank the reviewer for the comment. The following paragraph has been added

Line 229: “Note: In this study, a relatively low molecular weight poly(PFPA) (20 kg/mol) is used. Thus despite the high polymer concentration (10 mg/mL), no evidence of polymer crosslinking is observed. If a higher molecular weight polymer is used, then polymer solution concentration may need to be adjusted to avoid possible crosslinking.”

2. Citation of PFPA literature is rather selective. Considering the enormous utilization in polymer science, the seminal work of the group of Theato in this area could be better stated.

Response: We added six new references (Ref 10, 12, 13, 14, 15, 16) to better reflect the works done by the Theato group in the area of poly(PFPA) synthesis and utilization.

Reviewer #2 Comments:

Major Concerns:

Protocol section 1.2 is vague in its description of the reactor setup. What is the reactor? A multi-neck round bottom with septa seems likely but should be described. How are things added; by syringe through septa, poured, etc?

Response: We thank the reviewer for the valuable comment. Section 1.2 has been rewritten to better describe the reaction setup.

“1.2.1 Prepare a 500 mL three-neck round-bottom flask equipped with magnetic stir bar, refluxing condenser, dropping funnel, and rubber septum. Connect the flask to nitrogen gas line through the refluxing condenser and flush out the inside air with nitrogen. Insert a

thermometer through the septum. Add 41 mL (0.041 mol) of 1 M solution of phenylmagnesium bromide in tetrahydrofuran (THF) via a syringe through the same septum.

1.2.2 Warm the phenylmagnesium bromide solution to 40 °C in an oil bath. Then add 3.1 g (0.041 mol) of carbon disulfide through the dropping funnel slowly, maintaining the solution temperature at 40 °C.

1.2.3 Add 7.1 g (0.042 mol) of benzyl bromide to the resultant mixture through the dropping funnel over 15 min. Increase the reaction temperature to 50 °C. Continue stirring at this temperature for 45 min.

1.2.4 Transfer the reaction mixture into a separatory funnel, and dilute with 15 mL of ice cold water. Extract the product by adding 15 mL of diethyl ether, and remove the lower water layer. Repeat the extraction with diethyl ether two more times.

1.2.5 Wash the combined organic phases with copious amount of water, then brine (solution of 50 % (w/v) NaCl in water), and dry the product over anhydrous magnesium sulfate.

1.2.6 Remove the solvent in vacuum at 40 °C using a rotary evaporator.

1.2.7 Purify the product by column chromatography using 400 mL of silica gel (pore size 60 Å, 63-200 mesh particle size) and petroleum ether as eluent, yielding 5 g of benzyl dithiobenzoate (BDB) as red oil. Confirm product purity by ¹H NMR (400 MHz, CDCl₃) δ 8.02-7.99 (2H, m), 7.55-7.50 (1H, m), 7.41-7.29 (7H, m), 4.60 (2H, s)."

Minor Concerns:

While mostly well written there are numerous issues with plurals, tense and article placement within the text. Many instances of 'poly(PFPA) brush' should read 'brushes' and 'antibody' 'antibodies'. The symbol for degrees changes in protocol #4. Protocol 1.2.7 (line 149) should list the ¹H NMR spectroscopy chemical shifts used to confirm the product purity. Given values in % have inconsistent spacing (i.e X% vs X %) and do not always specify volume or mass or mole. For instance line 267 gives '0.1% (v/v)' while lines 492, 495, and 302 do not specify. These should be made more consistent. The XPS peak labeling in Figure 2 caption (line 370) should contain spaces to be consistent with the text (line 404); i.e. 'O 1s'. Line 268 reads 'Tween (PBST)' and should specify the Tween used; i.e. 'Tween-20 (PBST)'

Response: We have carefully proofread the manuscript and corrected all the mistakes and inconsistencies pointed out by the reviewer.

Reviewer #3 Comments:

Major Concerns: I do not have major concerns about this manuscript.

Minor Concerns:

1. Line 71-72 mentions "specially synthesized SI-RAFT chain transfer agent." The SI-CTA in the citation (reference 7) was actually previously synthesized, and was not made 'specially' for that particular work.

Response: We thank the reviewer for providing this information. The words "specially synthesized" have been removed.

2. Line 72: "silica-silane reaction" seems to more accurately describe the reaction than "silane reaction"

Response: Again, we thank the reviewer for the valuable comment. We changed "silane reaction" to "silica-silane reaction".

3. Line 399: "diameter" here seems to be referring to "hydrodynamic diameter" as measured through DLS.

Response: The word "diameter" has been changed to "hydrodynamic diameter".

4. Table 1: There seems to be a large size change from APTES-SiO₂ to poly(PFPA)-SiO₂. I wonder if this is from the formation of covalent aggregates induced by poly(PFPA) treatment (a single strand of poly(PFPA) may link multiple APTES-SiO₂ beads). It would be nice to have the actual DLS profile of each of the three samples mentioned in the table. I do recognize that the formation of covalently-bound aggregates will not negatively influence the procedure described in the manuscript. However, having actual DLS curves will help users by providing a more visual comparison.

Response: Per reviewer's suggestion, we replaced Table 1 with Figure 2, and the DLS curves for the differently functionalized beads are shown. For the poly(PFPA) functionalized beads, although the DLS curve only shows one nano-sized peak, the sample PDI is large (PDI=0.76). We suspect that there are indeed large aggregates in the suspension, but the amount may be small. We added the following explanation in the Discussion section.

Line 420 "It is important to point out that the polydispersity index (PDI) for the poly(PFPA) grafted beads is rather large (PDI = 0.76), which is indicative of poor quality sample containing large aggregates. Although the DLS curve only shows one nano-sized peak, small amount of aggregates may be present in the suspension."