Supporting Information for

Enhanced Uptake of Porous Silica Microparticles by Bifunctional Surface Modification with a Targeting Antibody and a Biocompatible Polymer

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Detailed Instrumental Methods

Fluorescence confocal laser scanning microscopy (CLSM). Cells treated with particles were stained 4, 8, or 24 h after treatment and were washed twice with PBS (100 mM, pH 7.4), fixed with paraformaldehyde (4% in PBS) for 5 min at room temperature, washed in PBS again, and permeabilized using Triton X-100 (0.1% in PBS) for 10 min at room temperature. After washing with PBS, 4',6-diamidino-2-phenylindole (DAPI) was used as a nuclear stain. The cells were then washed twice, and the coverslips removed from the wells and mounted onto slides using 90% glycerol in H_2O and analyzed by CSLM (Carl Zeiss LSM 510 META laser scanning microscope). Dyes were excited at 405, 488 nm, and/or 568 nm. Samples studied by CSLM were first slurried in Type FF immersion oil, and then the slurries were mounted using a 25 × 75 × 1 mm micro slide and a 22 × 22 cover glass (thickness 1). 63 X and 100 X oil-immersion lenses were used to image the samples. Graphic image analysis was performed using Adobe Photoshop. Three-dimensional reconstruction was accomplished using the Confocal Assistant or Zeiss LSM Image Browser.

Scanning electron microscopy (SEM). MM cells were grown on Thermanox plastic coverslips (Nalge Nunc International, Naperville, IL) in 12-well plates as described above, and treated with APMS particles (APMS-TEG, APMS-TEG(ME1), or APMS-ME1, 0.1 mg particles/mL of medium to give a final concentration of 1.5×10^7 particles/mL) for various amounts of time. The processing protocol for SEM was a fixation method for minimizing shrinkage and cracking of cells.[1] Coverslips were washed twice with PBS (100 mM, pH 7.4) for 5 min, and then fixed using glutaraldehyde (2% in 100 mM PBS) at 4 ˚C for 45 min. Samples were then washed twice for 5 min with PBS and once for 5 min with cacodylate buffer (50 mM $NaAsO_2(CH_3)_2 \cdot 3\ H_2O$ in H_2O, pH 7.2), and post-fixed using OsO_4 (1% in 50 mM cacodylate buffer) for 1 h at room temperature. After this time the samples were rinsed three times for 5 min with 50 mM cacodylate buffer, and the samples were then immersed in 50 mM cacodylate buffer containing tannic acid (1%) for 1 h at room temperature. After rinsing again three times for 5 min with 50 mM cacodylate buffer, samples were incubated with 0.5% uranyl acetate ($UO_2(CH_3COO)_2 \cdot 2\ H_2O$, 0.5%

in distilled H_2O) for 1 h at room temperature. After further washing with 50 mM cacodylate buffer, samples were then dehydrated in graded ethanols from 35% to 100%. Samples were subjected to critical point drying using liquid CO_2 as the transition fluid in a Samdri PVT-3B critical point dryer (Tousimis Research Corporation, Rockville, MD). Specimens were mounted on aluminum specimen stubs using conductive graphite paint and after drying were sputter-coated for 4 to 5 min with Au/Pd in a Polaron sputter coater (Model 5100). Specimens were then examined with a JSM 6060 scanning electron microscope (JEOL USA, Inc., Peabody, MA) with energy dispersive x-ray (EDAX) capability.

Transmission electron microscopy (TEM).[1] MM cells were grown on Thermanox plastic coverslips (Nalge Nunc International, Naperville, IL) in 12-well plates and treated with various APMS particles as described above. Coverslips were washed twice for 5 min with Millonig's phosphate buffer (100 mM Na_2HPO_4/NaH_2PO_4, 0.5% NaCl, pH 7.4), then fixed in a 1:1 H_2O dilution of Karnovsky's fixative (2.5% glutaraldehyde, 1% paraformaldehyde in Millonig's phosphate buffer, total volume = 2 mL per well) at 4 ˚C for 45 min. Samples were then rewashed with Millonig's phosphate buffer and post-fixed using OsO_4 (1% in Millonig's phosphate buffer) at 4 ˚C for 30 to 45 min. Samples were then dehydrated in graded ethanols from 35% to 100%, 3 times in propylene oxide (PO), and then infiltrated with Spurr's resin (Electron Microscopy Sciences) according to the following procedure: (100% PO:Spurr's resin) 3:1 for 30 min; 1:1 for 30 min; 1:3 for 30 min; and 100% Spurr's resin for 30 min. Flat embedding molds were filled with Spurr's resin and coverslips were placed onto the surface of the resin, cell side down. Resin was then polymerized overnight at 70 ˚C. Polymerized blocks were plunged into liquid nitrogen to facilitate removal of the coverslips from the resin block, and the resin blocks were cut into pieces and remounted onto blank blocks for sectioning. Semi-thin sections (1µm) were cut using glass knives on a Reichert Ultracut microtome, stained with methylene blue-azure II, and evaluated for areas of cells. Ultra-thin sections (60 ~ 80 nm) were cut with a diamond knife, retrieved onto 150 mesh copper grids, contrasted with uranyl acetate ($UO_2(CH_3COO)_2 \cdot 2\ H_2O$, 2% in 50% EtOH) and lead citrate ($Pb(C_6H_2O_7)_2 \cdot 2\ H_2O$, 1% in 50% EtOH), and examined with a JEM 1210 TEM (JEOL USA, Inc.) operating at 60kV.

Flow Cytometry. MM and A549 cells were grown in 60 mm dishes to 60 to 70% confluence and then were serum starved in media containing 0.5% serum for 24 h. APMS-TEG(ME1) or APMS-TEG(BSA) particles that had been labeled with Alexa-488 (0.1 mg of particles/mL of medium, to give a final concentration of 1.5×10^7 particles/mL) were then added and cell growth was continued for several fixed periods of time (see manuscript). Hoechst 33342 nucleic acid dye (16.2 µM in 100 mM PBS, pH 7.4) (Molecular Probes) was added 15 min prior to harvesting cells. (1:1000, at 37°C). Cells were washed once in PBS (100 mM, pH 7.4) and removed from dishes using Accutase cell detachment solution (5

min), pelleted by centrifugation (100 rgm for 4 to 5 min), resuspended in PBS (0.5 mL, 100 mM, pH 7.4) and kept on ice until analyzed. Samples were transferred to flow cytometry tubes (12 × 75 mm), placed on ice, and analyzed using a BD LSRII flow cytometer (BD Bioscineces, San Jose, CA) equipped with a Sapphire 488 (Coherent, Santa Clara, CA) laser which emitted at 488 nm to excite the Alexa-488 dye, and a solid state Xcite (Lightware) which emits at 355 nm to excite the Hoechst 333342 nucleic acid dye. Data analysis was performed at the time of acquisition using Flow Jo. This software package is an experiment-based flow cytometry data analysis package designed for multi-color research. Populations of cells and of particles were selected based on their locations on histograms and were sorted, collected and mounted on glass slides using cytocentrifugation. Cell sorting was accomplished using a BD FACSARIA (BD Biosciences) equipped with the following lasers: a Sapphire 488 (Coherent) which emits at 488 nm to excite the Alexa-488 dye, and an IFlex 2000-P1-405 which emits at 407 nm to excite the Hoechst 33342 nucleic acid dye. The fluorescent signals from Alexa 488 were detected using 530/30 BP and 505 LP filters. Hoechst staining was detected using 440/40 BP filter. Identification and quantification of populations on cytospins were performed manually with phase contrast and fluorescence microscopy using an Olympus BX50 microscope.

Detailed Synthetic Procedures

Synthesis of APMS Particles.[2] Briefly, cetyltrimethylammonium bromide (1.8 g, 4.94 mmol) was dissolved in an aqueous acidic solution composed of deionized H_2O (39.6 g), EtOH (11.1 g, 241 mmol), and concentrated HCl (36.5 wt%, 4.4 g, 44 mmol). TEOS (4.0 g, 19.2 mmol) was added to this solution and stirring was continued at 800 rpm for 5 min, and then NaF (0.5 M aqueous solution, 4.76 g, 2.33 mmol) was added and the mixture was stirred until a white precipitate was observed (~90 s), at which point it was immediately transferred to a 125 ml Teflon bottle and placed into a 100 ºC oven for 40 min. The resulting mixture was then cooled to room temperature, the white precipitate was collected by vacuum filtration and rinsed with 2 x 10 mL deionized H_2O, and dried under heat and vacuum for 48 hours to yield as-synthesized APMS. The surfactant could be removed by calcination at 550 ºC for 6 h under air to produce calcined APMS.

Synthesis of Stöber silica nanoparticles (1). Monodisperse silica spherical nanoparticles were prepared according to the Stöber method,[3] which involved the hydrolysis and condensation of tetraethoxysilane in the presence of ethanol, water and ammonia. Briefly, TEOS (7.1 g, 34 mmol) was added to a flask containing concentrated NH_4OH (10.26 g, 0.162 mol) and EtOH (228 mL) with vigorous stirring. The mixture was stirred at 400 rpm for 12 h at room temperature. The resulting nanoparticles were collected

by centrifugation at 16000×g for 15 min, and then were washed thoroughly by repeated dispersion and centrifugation in absolute EtOH at least 5 times.

Synthesis of thiol-modified Stöber silica nanoparticles (Stöber-SH). The silica nanoparticles prepared above were stirred in MeCN to eliminate residual NH_3. The solid content was determined from the amount of solids remaining after centrifugation and solvent evaporation in a vacuum oven at room temperature. After this step, the nanoparticles (200 mg) were suspended in dry MeCN (20 mL), the mixture was heated to 50 ˚C, and 3-mecaptopropyl trimethoxysilane (MPTMS, 0.539 g, 513.5 µl, 2.63 mmol) was quickly added with vigorous stirring. The mixture was allowed to stir for 24 h at this temperature. The amount of MPTMS used in this reaction was approximately 100 times greater than that necessary for reaching a complete coating of nanoparticles, based on an assumption that the coverage of the trimethylsilyl group was 40 $Å^2$. The resulting nanoparticles were collected by centrifugation at 16000×g for 15 min and were washed thoroughly by repeated dispersion and centrifugation in MeCN at least five times. The number of thiol groups grafted onto the solid was determined using Ellman's reagent.[4] For this experiment, modified Stöber particles were suspended in MeCN (1.0 mL) and sonicated for 10 min to produce an even dispersion. An aliquot of this disperson (8 µL) was added to deoxygenated reaction buffer (342 µL of 10 mM phosphate buffered saline (PBS) containing 1 mM EDTA, pH 7.2, bubbled with Ar) and 50 µL of Ellman's reagent (5,5'-dithio-bis-(2-nitrobenzoic acid), DTNB) in MeCN, 2 mg/mL). The reaction was allowed to proceed for 4 h at room temperature with vigorous shaking, and the extent of the disulfide exchange reaction was easily monitored after this time by the intense yellow color of the reaction mixture due to the release of 2-nitro-5-thiobenzoic acid (TNB). The color was monitored by isolating the supernatant by centrifugation and analyzing it using UV-Visible spectroscopy at 412 nm. The number of thiol groups on the particles were then calculated from a calibration curve, which was obtained following the manufacturer's protocol (Pierce Biotechnology) except that 3-mecaptopropionic acid was used instead of L-cysteine as the titrant.

Synthesis of succinimide linker-modified Stöber silica (Stöber-SS-Su, 2). Thiol-modified Stöber silica (50 mg, 1.0~2.0 $\times 10^{-4}$ mol thiol/g) were treated with *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP, 3.1 mg, 0.010 mmol) in MeCN (0.5 mL) under nitrogen, and the reaction was stirred for 12 h. The extent of reaction was determined by removing an aliquot, centrifuging to remove particles, and determining the absorption of pyridine-2-thione (TP) at 365 nm (ε = 7000 M^{-1} cm^{-1}). After the reaction was complete, the resulting solid was collected by centrifugation (16000 ×g for 15 min), washed by repeated dispersion and centrifugation in MeCN at least 5 times, and then dried under vacuum.

Synthesis of amine-modified APMS (APMS-NH_2, 3). A calcined sample of APMS (0.30 g) was suspended in dry toluene (10 mL) under nitrogen. 3-triethoxysilyl-propyl)-carbamic acid 9H-fluorenylmethyl ester (Fmoc-APTES, 0.444 g, 1 mmol) was then added, and the mixture was refluxed at 110 ˚C for 24 h. After the mixture was cooled to room temperature, the white powder was recovered by filtration under a flow of N_2, washed with toluene and methanol, and then vacuum dried for 24 h prior to further use. Selective deprotection of Fmoc, giving free amines on the external surface of APMS but leaving the amines inside the pores protected, was then performed by a previously reported method.[5] The amount of deprotected amine per gram of solid varied from sample to sample, and was accurately determined from a calibration curve created from a series of known Fmoc-APTES solutions that had been treated under identical conditions.

Attachment of dye to free amines of functionalized APMS. APMS-NH_2 (5.0 mg) was suspended in a solution of $NaHCO_3$ (200 µL, 100 mM). The suspensions were then transferred to vials containing either Alexa Fluor 488 carboxylic acid, succinimidyl ester (10 µg, 0.0155 µmol), or Alexa Fluor 568 carboxylic acid, succinimidyl ester (10 µg, 0.0126 µmol). After incubation for 4 h at room temperature with continuous shaking, the samples were recovered by centrifugation and washed successively with a 100 mM $NaHCO_3$ solution, distilled water, and MeOH. They were then air dried and stored at 4 ˚C in the dark.

Synthesis of Stöber-APMS conjugates (4). In a typical preparation, Stöber-ss-Su **(2)** was suspended in CH_2Cl_2 (0.96 mL) and sonicated for 10 min. Several concentrations of **2** were used (1, 5, 10, 20, 30 and 40 mg/mL). In a separate vial, APMS-NH_2 (**3**, 12 mg) was suspended in CH_2Cl_2 (0.24 mL) and sonicated for 1 min. The second suspension was slowly added to the first suspension with vigorous stirring. The mixture was then shaken for 6 h at room temperature and the resulting APMS-Stöber conjugates were collected by mild centrifugation (2000 ×g, 2 min, 5 times), followed by filtration/centrifuge (0.45 µm spin filter, Life Sciences, NANOSEP MF) and washing with CH_2Cl_2. The resulting particles were dried under vacuum at room temperature.

Synthesis of bifunctional APMS (APMS-NH_2(SH), 5). Any remaining active succinimide groups on the Stöber silica were deactivated by stirring the Stöber-APMS conjugate **(4)** in a glycine buffer (20 mL glycine in 10 mM PBS) for 30 min. The conjugates were collected by filtration, washed, and then dispersed in EtOH containing dithiothreitol (DTT, 50 mM) for 4 h under an inert atmosphere. Repeated filtration/washing/sonication cycles were performed to remove the released Stöber silica from the suspension. The resulting particles were dried under vacuum at room temperature.

Synthesis of glycol-modified, amine-reactive APMS (APMS-TEG(Su), 10). A series of steps were performed to prepare this material. First, APMS-NH_2(SH) (**5**, 10 mg) was reacted with 2,2'-dithiobis(5-nitropyridine) (0.5 mL, 20 mM in 10 mM PBS/MeCN, v/v 2:1) for 3 h at room temperature. The resulting particles, APMS-NH_2(Npys) **(6)**, were collected by repeated filtration/washing/sonication cycles and dried under vacuum at room temperature for 24 h. They were then suspended in anhydrous MeCN (1.0 mL) and succinic anhydride (5 mg, 0.05 mmol) and NEt_3 (12.5 µL, 9.1 mg, 0.09 mmol) were added followed by stirring for 2 h at 60 ˚C. These particles were then recovered from solution by filtration, washed with copious amounts of MeCN and trifluoroacetic acid solution (0.1%, aqueous solution), and dried overnight under vacuum at 25 ˚C to yield APMS-COOH(Npys) **(7)**. A negative result from the Kaiser test and a positive result from the Malachite Green test confirmed that most of the amine groups had reacted with succinic anhydride. **7** was then resuspended in 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC, 6.3 mg, 0.025 mmol, 5 to 10-fold excess relative to the COOH groups) and N-hydroxysuccinimide (NHS, 3 mg, 0.025 mmol) in MeCN (1 mL). The mixture was then stirred for 2 h at room temperature and then the product was collected by filtration and washed with MeCN. The resulting particles were suspended in a solution of 2-(2-(2-hydroxyethoxy)ethoxy)ethoxy) ethylamine (TEG-NH_2, 5 mg, 0.025 mmol) in MeCN (1 mL). (TEG-NH_2 was prepared by modifying a reaction described by Xie et al.)[6] The suspension was then stirred for 6 h at room temperature and these particles (APMS-TEG(Npys), **8**) were captured by filtration, washed with MeCN, and dried under vacuum to remove the remaining solvent. In the next step, the thiol protecting group was removed by cleavage with DTT as described in the synthesis of **5**, forming APMS-TEG(SH) **(9)**. The number of free thiol groups on the particles, which was equimolar to the number of molecules of 5-nitro-2-pyridinethiol (TNP) released into solution, was quantitatively determined using UV-Visible spectroscopy at 395 nm. The molar extinction coefficient of TNP was 13560 $M^{-1}cm^{-1}$ at 395 nm and pH = 6.0. Finally, **9** was reacted with a heterobifunctional crosslinker, N-(β-maleimidopropyloxy)succinimide ester (BMPS). Typically, **9** (10 mg) was suspended in deoxygenated reaction buffer (0.5 mL, MeCN/10 mM PBS, 1:1 (v/v), pH 6.8, bubbled with Ar) and BMPS (20 mM in MeCN, 2 to 5-fold molar excess based on the number of free thiols on APMS-TEG(SH)) was added. Conjugation of BMPS to APMS-TEG(SH) was continued for 3 h at room temperature by covering the reaction vial with foil and shaking vigorously. The final product, APMS-TEG(Su) **(10)** was recovered by centrifugation, washed with MeCN/H_2O (1:1, v/v), and stored at 4 ˚C in the dark.

Protein immobilization on APMS-TEG(Su). Amine-reactive APMS-TEG(Su) (5 mg) was suspended in PBS (0.2 mL, 10 mM, pH 7.2) containing either bovine serum albumin (BSA, as a generic control for

binding studies) or human mesothelin antibody (mAb ME1). Initial protein concentrations of BSA and ME1 were initially determined by UV-Visible absorption at 280 nm. After sonicating briefly, the coupling was allowed to proceed for 3 h at room temperature Conjugation efficiency was determined by UV-Visible spectroscopy of the supernatant. For these determinations, supernatant was removed at predetermined time intervals followed by centrifugation and washing with PBS. Since the NHS-ester leaving group can absorb strongly at 280 nm, the concentration of protein remaining in the solutions were determined by absorbance at 595 nm using the Bradford method,[7,8] which used 150 μL of dye reagent (Coomassie Blue G250) and 150 μL of protein samples followed by vortexing and incubation at room temperature for at least 5 min. The linear ranges of the assays were between 1 and 20 μg/mL. Triplicate aliquots were assayed for protein concentrations. The particles were subsequently washed and sonicated to remove any physically adsorbed protein, and were either used immediately or stored in PBS at 4 ˚C for later use. Bifunctionally modified materials are designated APMS-TEG(BSA) or APMS-TEG(ME1), depending on the type of immobilized protein.

Synthesis of APMS-ME1 (Scheme 2). Direct coupling of proteins onto APMS-NH_2 produced a material with a similar functionalization to APMS-TEG(ME1) but without the TEG chains. A series of steps were performed to prepare this material. APMS-NH_2 (200 mg) was suspended in anhydrous MeCN (5 mL) and succinic anhydride (100 mg, 1 mmol) and NEt_3 (0.25 mL, 182 mg, 1.8 mmol) were added. The mixture was then allowed to stir for 12 h at 60 ˚C. The resulting solid was recovered by filtration and washed with copious amounts of MeCN and trifluoroacetic acid solution (0.1%, aqueous solution), and dried overnight under vacuum to yield carboxylated APMS (APMS-COOH). These particles were then resuspended in EDAC (126 mg, 0.5 mmol, 5-10 fold excess relative to the carboxylic acid groups) and NHS (58 mg, 0.5 mmol) in MeCN (10 mL). The mixture was then stirred for 4 h at room temperature. The resulting product was collected by filtration and washed with MeCN. Finally, these particles (150 mg) were suspended in a solution of anti-mesothelin, ME1 (10 μg protein per 20 mg APMS) in PBS buffer (6 mL). The suspension was then stirred for 3 h at room temperature. The resulting APMS-ME1 were collected by filtration, washed with PBS, and used immediately or stored in PBS at 4 ˚C for later use.

Synthesis of APMS-TEG (Scheme 2). Direct coupling of tetraethylene glycol onto APMS-NHMe produced a material with a similar functionalization to APMS-TEG(ME1) but without the protein.[2] APMS-NHMe was prepared by suspending a calcined sample of APMS (300 mg) in dry toluene (10 mL) under nitrogen. 3-(*N*-methylamino)propyltrimethoxysilane (387 mg, 394 μL) was then added, and the mixture was refluxed at 110 ˚C for 24 h. After the mixture was cooled to room temperature, the white powder was recovered by filtration under a flow of argon, washed with toluene and methanol, and then

vacuum dried for 24 h prior to further use. To attach the TEG chains, 300 mg of APMS-NHMe was suspended in 10 mL of anhydrous acetonitrile that contained triethylamine (100 µL, 0.7 mmol) and tosyl-TEG (174 mg, 0.5 mmol. Tosyl-TEG was prepared by modifying a reaction described by Xie et al.)[6] After the mixture was refluxed for 6 hours under an inert atmosphere, the resulting APMS-TEG was captured by filtration, washed with ethanol, and dried under vacuum.

Particle Characterization Data

Solid-State ^{29}Si MAS NMR. ^{29}Si MAS-NMR spectra provide direct evidence for covalent binding between organic functional groups and the silica framework. The spectrum of calcined APMS, shown in Figure X, exhibits three signals at δ = -111.7, -103.2 and -93.6 ppm, which are assigned to siloxane (Q^4, $Si(OSi)_4$), single silanol (Q^3, $Si(OSi)_3OH$)) and geminal silanediol (Q^2, $Si(OSi)_2(OH)_2$) sites, respectively.[9-11] The relative populations of the silicon resonances were obtained by deconvolution of the spectra (**Table 1**). A spectrum of amine-functionalized APMS (**Figure 1**) shows two additional overlapping resonances at approximately δ = -68, -58 ppm, which represents T^3 ($RSi(OSi)_3$) and T^2 ($RSi(OSi)_2(OMe)$) sites, respectively.[12] The increases in the population of silicon sites in Q^3 and Q^2 environments and a decrease in Q^4 are due to the reaction of silanol groups with the organosilanes, and the presence of silicon sites in T^3 and T^2 environments in the functionalized APMS samples further indicates the existence of the covalent linkage between the organic moieties and silica walls.

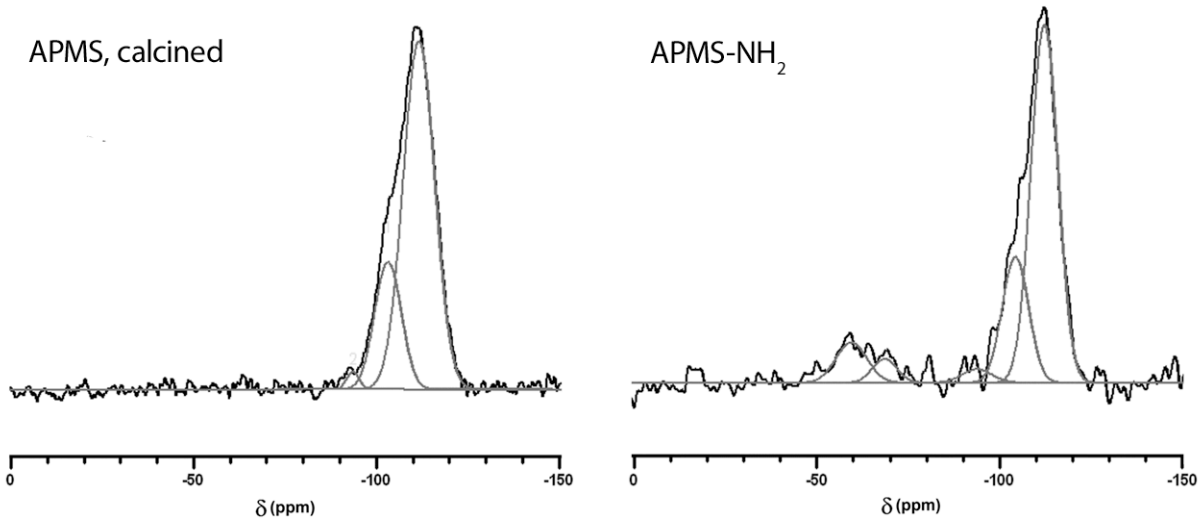

Figure 1. ^{29}Si MAS-NMR spectra of calcined APMS and APMS-NH_2.

Table 1. Tabulated ^{29}Si MAS NMR data of unmodified APMS and modified APMS. Chemical shifts from deconvolution of the spectra are listed, and relative peak areas are given in parentheses.

Samples	Q^4 (%)	Q^3 (%)	Q^2 (%)	T^3 (%)	T^2 (%)

calcined APMS	-111.7 (76.4)	-103.2 (21.8)	-93.6 (1.8)	-	-
APMS-NH_2	-112.5 (64.1)	-104.6 (22.6)	-94.7 (3.5)	-67.9 (2.8)	-58.8 (6.9)

Modification of Stöber Nanoparticles. The Stöber spheres were first converted into thiol-terminated nanoparticles by reaction with 3-mercaptopropyl trimethoxy silane (MPTMS). The amount of grafted mercaptopropyl groups on the various Stöber particle surfaces was assessed by thermogravimetry (TGA) and spectrophotometric analyses (Ellman's method)[4], as summarized in **Table 2**. The TGA-based loadings are quite close to theoretical maximum loadings based on simple geometric arguments. Spectrophotometric measurements using Ellman's reagent ((5,5'-dithiobis-(2-nitrobenzoic acid)) corroborated the TGA results. The thiolated Stöber spheres were further functionalized with a heterobifunctional crosslinker (SPDP) to introduce amine-reactive succinimidyl ester groups. The progress of the immobilization of SPDP to the Stöber spheres was followed quantitatively by measuring the appearance of the pyridine-2 thione group (ε_{365} = 7000 M^{-1} cm^{-1}). With 65 nm Stöber particles, we achieved a loading of 0.135 mmol g^{-1} of activated esters, or roughly 77 % yield based on the thiol loading (**Table 2**). Experiments with Stöber particles of larger sizes followed the expected trend of decreasing loading with increasing size due to the decrease in exterior surface area.

Table 2. Characterization data of the immobilization of thiol groups onto Stöber particles.

Sample	Weight Loss (%) 650-100 °C	Weight Change (%)	Linker Loading ($\times 10^{-3}$mol/g) by TGA	SH Loading ($\times 10^{-3}$mol/g) (Ellman's method)	Calculated Maximum SH Loading ($\times 10^{-3}$mol/g)[a]
Stöber, 65 nm	8.81	-	-	-	-
Stöber-SH	10.21	1.40	0.186	0.176	0.202

[a] The calculations were based on an assumed 40Å^2 area for a trimethylsilyl group.[13] The corresponding equation was:

$$\Gamma_{SH} = \frac{4\pi r^2}{S_{SH}} \times \frac{1}{D_{Si}} \times \frac{1}{4/3\pi r^3} \times \frac{1}{N_A} = \frac{3}{S_{SH} D_{Si} N_A} \cdot \frac{1}{r} (mol/g) \quad (1)$$

Alternately,

$$S_{SH} = \frac{3}{\Gamma_{SH} D_{Si} N_A} \cdot \frac{1}{r} \ (\text{Å}^2) \quad (2)$$

where S_{SH} is the area for a trimethylsilyl group on the silica wall (~40 Å^2), D_{Si} is the density of the silica wall (1.9 g/ml), r is the radius of Stöber silica, and N_A is the Avogadro's number.

Determination of the amount of incorporated ligands on APMS. When the Stöber spheres were attached to the APMS surface, only a certain portion of their surface areas was in contact with each other (**Figure 2**). After removal of the Stöber particles by reduction, the regions of contact left behind free thiol groups on the external surface of APMS. Thus, the amount of incorporated functional ligands on the APMS surface was based on the total contact areas, which could be influenced by various factors, such as the density of bifunctional linkers on the Stöber spheres, the diameter of the Stöber spheres, the

distribution and array of the Stöber spheres on the APMS surface, the loading of amine groups, and the length of bifunctional linkers.

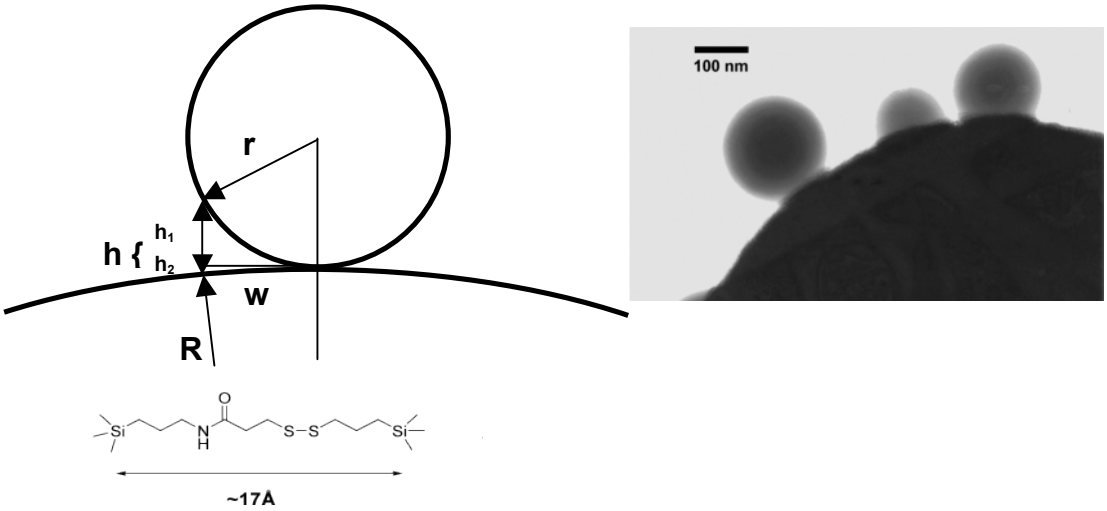

Figure 2. Left Panel: Schematic representation of APMS-Stöber conjugation. where r is the radius of Stöber, R is the radius of APMS, h is the maximum length of linker ($h = h_1 + h_2$), and w is the apparent contact-radius. Right Panel: TEM image of APMS conjugated to Stöber particles. Bottom: the lengths of the linkers between Stöber and APMS were estimated by MM2 calculation (ChemOffice).

As represented in **Figure 2**, we assume that the maximum distance between Stöber and APMS particles was not longer than the length of the organic linkers, estimated to be 17 Å. The maximum distance *h* is defined as the length of the linker, which consists of two parts and can be calculated by the following equations. The radius *w* and the contact area (S_{CA}) between Stöber sphere and APMS particle is obtained from equations 3-5:

$$h_1 = \frac{2Rh - h^2}{2(R + r - h)} \quad (3)$$

$$w = \sqrt{r^2 - (r - h_1)^2} = \sqrt{r^2 - (r - \frac{2Rh - h^2}{2(R + r - h)})^2} \quad (4)$$

$$S_{CA} = \pi w^2 \quad (5)$$

where R and r are the radii of the APMS and Stöber sphere, respectively. The percentage of surface coverage (*SC*%) of contact area relative to the whole surface area can be calculated by the following equation:

$$SC\% = \frac{\pi}{\sqrt{12}} \cdot \frac{4\pi(R + r)^2}{\pi r^2} \cdot \frac{\pi w^2}{4\pi R^2} = \frac{\sqrt{3}\pi}{6} \cdot \frac{(R + r)^2 w^2}{R^2 r^2} \quad (6)$$

and finally, Γ_{max} (the maximum amount of incorporated thiol) is given by:

$$\Gamma_{max} = SC\% \cdot \Gamma_{NH_2} \ , \quad (7)$$

where Γ_{NH2} is the amount of deprotected amine groups on the surface of APMS. These calculations and measurements of the thiols with Ellman's reagent yielded the loading data in **Table 3**.

Table 3. Determination of the amount of incorporated thiol groups on APMS-NH_2 reacted with Stöber particles, d = 65 nm.

w (Å)	SC (%)	Maximum incorporated thiol groups ($\times 10^{-6}$ mol/g) calculated by Eq.9	Incorporated thiol groups ($\times 10^{-6}$ mol/g) measured by Ellman's method	Yield (%)[a]
102.22	9.56	10.43	1.18	11.3

[a]the ratio of the amount of thiol groups measured by Ellman's method to the calculated value (Eqn. 7).

The amount of reactive thiol groups incorporated by the Stöber spheres on the APMS was determined by spectrophotometric analysis (Ellman's method). The measured results, listed in **Table 3**, are less than the calculated results because the Stöber spheres are not close-packed on the APMS particles, resulting in reduced surface coverage. Also, even in the contact areas, not all of the accessible and reactive linkers on the Stöber spheres reacted with amine groups on the APMS. Moreover, the density of organic ligands on the exterior surface of APMS was probably overestimated because a small portion of deprotected amine groups was located in the entrance of the pore channel. Finally, the density of bifunctional linkers on the Stöber spheres was not considered in the calculation. We note that in preliminary experiments, we used Stöber particles of two larger sizes (100 and 230 nm in diameter) and the loading achieved followed the trends predicted by the equations above.

Immobilization of Tetraethylene Glycol. TGA provided the best evidence for the immobilization of TEG to the exterior surfaces of the particles. The data in **Figure 3** shows that in the two-step reaction to immobilize TEG the particles gained 11.3 weight % of combustible organics (MW = 277 g mol^{-1}), yielding a loading of 0.40 mmol g^{-1}.

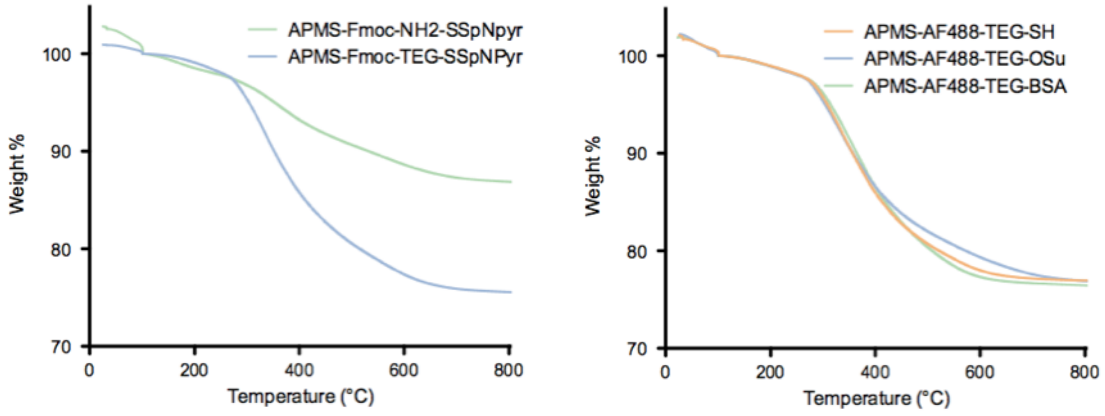

Figure 3. Left: TGA data showing the weight changes vs temperature for APMS before attaching TEG (NH_2, green) and after (TEG, blue). Right: TGA data for APMS before (SH, orange) and after (OSu, blue) reaction with BMPS, and after the immobilization of a protein (BSA, green).

Immobilization of BMPS and a Protein. After the deprotection of the surface thiols (see **Scheme 1** in the main text), TGA again provided evidence of the addition of the remaining functional groups to the multifunctional particles (**Figure 3)**. The addition of the heterobifunctional crosslinker BMPS resulted in an additional 0.139 weight %, equivalent to a loading of 5.2 µmol g^{-1} (MW = 266 g mol^{-1}). This result by TGA agrees well with the theoretical loadings calculated with equations 3-7 and those measured using Ellman's reagent in **Table 3**. Finally, reaction with the non-specific protein control, bovine serum albumen, resulted in an additional 0.41 weight %.

Infrared Spectroscopy. ATR-FTIR was performed on the particles at several stages of the synthesis, but with the sensitivity available with our instrument (Shimadzu IRAfiinity-1 with Pike Technologies MIRacle™ ATR attachment), changes to the functional groups on the surface yielded few insights. Nonetheless, we have included spectra of fully Fmoc-protected APMS, used to differentially modify the particle exteriors from the pores, and a completed APMS-AF647-TEG-OSu, with the dye in the pores and the TEG and succinimidyl ester on the surface (**Figure 4**). Major points of differentiation include the disappearance of the carbamate carbonyl at 1705 cm^{-1} due to the deprotection of the Fmoc-amines and the appearance of the amide I band at 1651 cm^{-1} from the attachment of TEG.

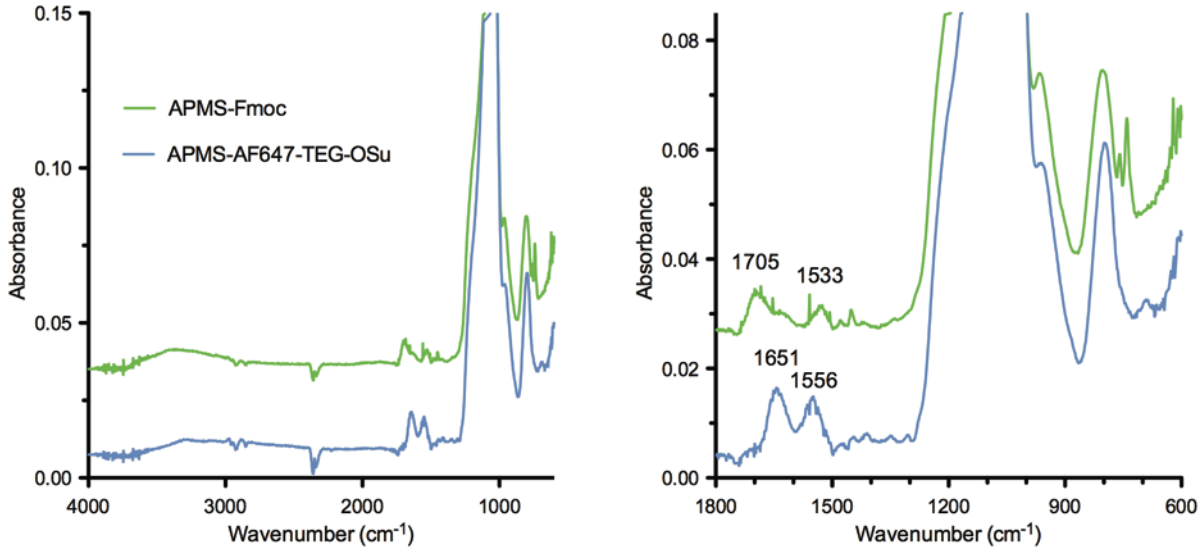

Figure 4. ATR-FTIR spectra (zoom on the right) showing minimal changes after the completion of the trifunctional sequence to produce APMS-AF647-TEG-OSu (blue) from fully Fmoc protected APMS (green).

Nitrogen Physisorption. Analysis of calcined APMS and the final multifunctional particles shows that the surface area and pore volume were reduced by roughly half after all of the functional groups and synthetic manipulations were performed. This is probably due to incomplete deprotection of the pores and subsequent attachment of the bulky dye molecules. The peak in the pore size distribution has remained the same. (**Figure 5** and **Table 4)**.

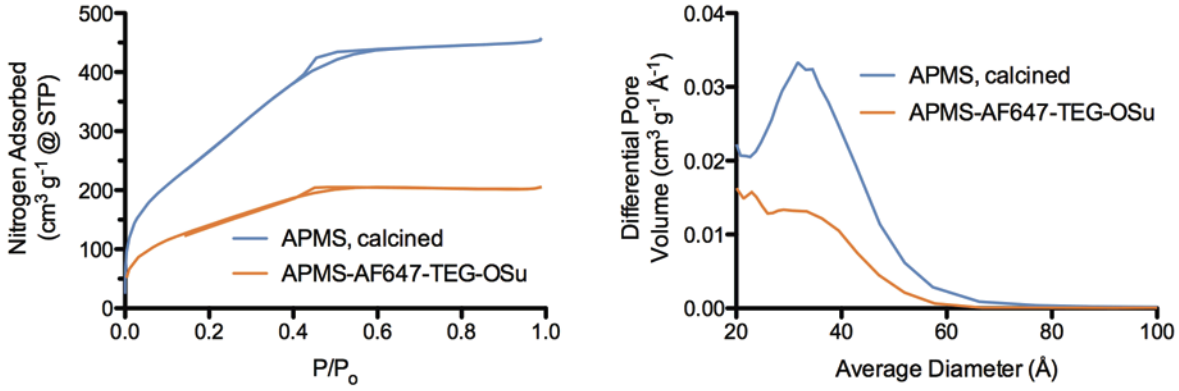

Figure 5. Nitrogen physisorption data showing the original, calcined APMS (blue) and the final particles with a bifunctional surface and dye-labeled pores (orange).

Table 4. Textural characterization by nitrogen physisorption. Surface area was calculated by the BET method and differential pore size distribution, by a modified BJH algorithm.

Sample	Surface Area (m^2 g^{-1})	Pore Volume (cm^3 g^{-1})	Mean Pore Size (Å)
APMS-	1000	0.99	33
APMS-AF647-TEG-OSu	527	0.40	33

References

1. Robards, A. W.; Wilson, A. J., *Procedures in electron microscopy*. Wiley: Chichester, **1993**.
2. Blumen, S. R.; Cheng, K.; Ramos-Nino, M. E.; Taatjes, D. J.; Weiss, D. J.; Landry, C. C.; Mossman, B. T. *Am. J. Respir. Cell Mol. Biol.* **2007,** *36*, 333.
3. Stöber, W.; Fink, A.; Bohn, E. *J. Coll. Interf. Sci.* **1968,** *26*, 62.
4. Ellman, G. L. *Arch. Biochem. Biophys.* **1959,** *82*, 70.
5. Cheng, K.; Landry, C. C. *J. Am. Chem. Soc.* **2007,** *129*, 9674.
6. Xie, H. Z.; Braha, O.; Gu, L. Q.; Cheley, S.; Bayley, H. *Chem. Biol.* **2005,** *12*, 109.
7. Bradford, M. M. *Anal. Biochem.* **1976,** *72*, 248.
8. Compton, S. J.; Jones, C. G. *Anal. Biochem.* **1985,** *151*, 369.
9. Zhao, X. S.; Lu, G. Q.; Whittaker, A. K.; Millar, G. J.; Zhu, H. Y. *J. Phys. Chem. B* **1997,** *101*, 6525.
10. Chong, A. S. M.; Zhao, X. S. *J. Phys. Chem. B* **2003,** *107*, 12650.
11. Zhao, X. S.; Lu, G. Q. *J. Phys. Chem. B* **1998,** *102*, 1556.
12. Feng, X.; Fryxell, G. E.; Wang, L. Q.; Kim, A. Y.; Liu, J.; Kemner, K. M. *Science* **1997,** *276*, 923.
13. Herzberg, W. J.; Marian, J. E.; Vermeulen, T. *J. Colloid Interf. Sci.* **1970,** *33*, 164.