Selective External Surface Functionalization of Large-Pore Silica Materials Capable of Protein Loading

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Abstract

Differentiating the chemical properties of the external and pore surface of sol-gel derived mesoporous materials by selective functionalization is important to advancing their application as platforms for biological catalysis, sensing and drug delivery. Prior selective functionalization approaches to concentrating functional groups at the external surface of particles have been limited to small pores (≤ 5.5 nm diameter) incapable of loading large biological molecules. This work investigates the selective exterior surface functionalization by amines of larger-pored (>7 nm diameter) mesoporous silica particles, which are synthesized by dual surfactant templating and hydrothermal aging. Previously developed selective functionalization techniques rely on choice of functionalization precursor, functionalization reaction time, or pore blocking (by leaving pore templates in as-synthesized materials). The effectiveness of these strategies are compared for larger-pored materials using the precursors (3-aminopropyl)tris(methoxyethoxyethoxy)silane (APTMEES) and (3-aminopropyl)triethoxysilane (APTES). The extent of amine functionalization is determined as a function of precursor reaction time (10 or 20 minutes) in both as-synthesized and template-extracted materials by confocal laser

scanning microscopy of the ~10 micrometer diameter particles tagged with fluorescein isothiocyanate. Reaction time, regardless of pore template presence, is demonstrated to be the controlling variable for achieving selective exterior functionalization in these larger pored mesoporous materials. Under the conditions used, 10 min of functionalization with APTMEES localizes amine groups at the exterior of the particles, while 20 min functionalizes both the exterior and the interior pore surfaces. Protein accessibility within pores, before and after selective and full functionalization, is visually confirmed by confocal fluorescence imaging of Rhodamine B tagged lysozyme loaded particles.

Keywords: protein capture, selective functionalization, amine functionalization, mesoporous silica, interfacial engineering

1. Introduction

Complementary advances in the synthesis of mesoporous silica materials (MSMs) and the uses of proteins in biotechnology present new opportunities for enzyme catalysis, protein delivery, and protein-based sensing using silica platforms [1-7]. The synthesis of templated mesoporous silica materials with tunable ordered pores of size appropriately large for protein loading (5 nm – 12 nm) has become routine [8, 9]. These materials can be synthesized in a variety of morphologies such as thin films and particles [10, 11]. The uses of enzyme loaded mesoporous silica materials range from biosensors to biocatalysts for the detection of a variety of compounds such as carbohydrates, aromatics and aquatic toxins [12-17].

The benefits of using mesoporous silica materials for the entrapment of proteins stem from the tunable properties of the materials. SBA type materials, synthesized with the tri-block copolymers such as Pluronic P-123 ((ethylene oxide)₂₀(propylene oxide)₇₀(ethylene oxide)₂₀), have transformed the synthesis of ordered large-pored metal oxides with a variety of tunable morphologies such as thin films and particles with different ordered mesostructures [10, 11, 18]. The addition of the ionic surfactant cetyltrimethylammonium bromide (CTAB) permits the synthesis of SBA-15 particles with spherical morphology and diameters in the micron range [10]. Hydrothermal aging of particles has proven to be an effective means of tuning pore diameters from 3.5 nm to 11.6 nm using temperatures between 60°C and 130°C [19-21]. Tunable pore sizes within mesoporous silica particles can be used for protein protection, protein separations and to sustain protein activity [21, 22].

Protein loading within the pores of mesoporous silica is frequently established by bulk measurements of protein concentration or activity assays before and after exposure to particles

[15, 23]. However, activity measurements can be difficult to interpret clearly due to limited accessibility of the substrate to the enzyme within pores. In addition, neither method can directly determine the location of proteins within the materials. Confocal laser scanning microscopy (CLSM), on the other hand, has the benefit of using fluorescent imaging to visualize the location of proteins on the surface or within the particles [22, 24]. For example, the diffusional resistance of enhanced green fluorescent proteins has been visualized within particles with pore sizes between 2.9 nm and 5.5 nm in both rod shaped and spherical particles [25]. Size selective protein separations have been visually confirmed using fluorescently tagged lysozyme and bovine serum albumin within 12 nm diameter pores of SBA-15 silica [22]. Recently, our group has demonstrated the use of spherical MSMs for pore-size dependent protection of proteins within SBA-15 spherical particles in the presence of hydrolyzing proteases [21]. Hydrothermal aging was used to tune pore sizes between 5.4 nm and 11.6 nm in diameter where an optimum diameter of 7.3 nm permitted access of a smaller fluorescent protein, while protecting it from a larger protease. CLSM provided both confirmation of fluorescent activity and location of the protein within the particles.

The synthesis of separate organic functional domains, either on the exterior particle surface or in the pores, has the potential to further enhance the application of large-pored organic – inorganic porous structures for protein applications. Selective functionalization could regulate particle interactions with their environment, covalently anchor proteins either within pores or on the exterior surface of particles, screen molecules entering and exiting pores or be used in particle targeting with antibodies. Functionalization of mesoporous silica with amines, in particular, has been shown to stabilize enzymes in mesopores [26], to modulate interactions of peptides and proteins with silica [27-31] and to control particle uptake and cytotoxicity [32-35].

Due to the versatile chemistry of primary amines on the surface of silica, amine functionalization allows for direct tagging with fluorophores or use as a building block for more advanced functionalization [24, 36, 37]. Precursors typically used to modify mesoporous silica are aminopropyl alkoxysilanes (e.g. 3-aminopropyltriethoxysilane (APTES) and 3-aminopropyltris(methoxyethoxyethoxy)silane (APTMEES)).

Selective interior or exterior functionalization of ordered mesoporous silica has been demonstrated by using pore blocking in mesoporous silica (where an organic functionality is grafted while the pore template still resides in the pores) [38-42], by functionalizing non-selectively followed by diffusion-limited deprotection [43], by adding combinations of precursors at different times to generate layers with different functionality [44, 45], by intentionally functionalizing with bulky functional groups, sterically hindered from pore accessibility [46] and by passivating the external surface with an inert organic group followed by adding a second reactive silane to functionalize the internal pore surface [38]. Specifically for amines, selective external functionalization of silica with small mesopores (less than 5.5 nm) has been demonstrated through the selection of an amino silane precursor of suitable size and reactivity, and via pore blocking by the templating surfactant [45, 47-49]. For example, Gartmann and Brühwiler demonstrated exterior functionalization using the larger and less reactive aminosilane APTMEES for particles with 2.9 nm pores [24]. By functionalizing mesoporous materials in the presence of the unextracted P123/CTAB template, exterior surface functionalization was demonstrated for pore sizes up to 5.5 nm using APTMEES as the precursor. By varying the solvent, Gartmann and Brühwiler concluded that slow diffusion of (perhaps partially hydrolyzed) APTMEES from a nonpolar medium was the most likely cause for external functionalization, although partitioning and diffusion effects were not considered

separately in their work [24]. Selective exterior aminosilane functionalization has been interpreted from FTIR and NMR data, [47, 48] and visual confirmation of functional groups location via fluorescent tagging has been demonstrated with CLSM [24, 37, 43]. However, the techniques used to functionalize small pored materials (pore blocking, choice of aminosilanes, and reaction time for functionalization) have not been translated to selective functionalization of large pore porous materials (pore diameter > 5.5 nm) [24, 45, 47]. Pores for protein loading and protection from environmental destabilization are required to be large in diameter (in excess of 5 nm) to accommodate protein dimensions, so a need exists to test and develop methods for selective functionalization to large pored materials [21, 50].

The goal of this work is to demonstrate strategies for the selective exterior functionalization of large-pore (>7 nm diameter) mesoporous silica particles with aminosilanes. SBA-15 sphere (SBAS) materials are employed in this study, which are micron-scale particles with tunable large mesopores obtained through dual-surfactant templating and hydrothermal aging. Synthesis conditions have been chosen (90°C hydrothermal aging temperature) to obtain 5 μm – 15 μm particles, appropriate for imaging by CLSM, with 7.4 nm diameter pores. The aminosilanes, APTES and APTMEES, are selected for their differing reactivity and size. The density of amine functional groups on the silica particles as a function of reaction time with the precursor (10 or 20 minutes) is examined for both as-synthesized (materials with the pore template left in the pores) and template extracted materials using FTIR and by a fluorescamine assay following dissolution of the particles. Functional group location and protein accessibility in the functionalized material is confirmed via imaging using confocal microscopy after fluorescent tagging.

2. Experimental

2.1 Materials

Hydrochloric acid (12.1 M, ACS certified) and hexanes were purchased from Fisher Scientific. Tetraethyl orthosilicate (TEOS, $\geq 98\%$) and Fluorescamine (Synthetic, 100% pure) were purchased from Acros Organics. Cetyltrimethylammonium bromide (CTAB, 98%) was purchased from Research Organics. Ethanol (200 proof) was purchased from Decon Labs. Rhodamine B isothiocyanate (TRITC), fluorescein isothiocyanate (FITC, 90%), 3-aminopropyltriethoxy silane (APTES, 99%) and Pluronic P123 triblock copolymer ((EO)₂₀(PO)₇₀(EO)₂₀ where EO is an ethylene oxide unit and PO is a propylene oxide unit, MW_{avg} = 5800) were purchased from Sigma Aldrich. 3-aminopropyltris(methoxyethoxyethoxy) silane (APTMEES, 95%) was purchased from Gelest Inc. All amino-silanes were stored under nitrogen after initial use. Handling was performed under nitrogen in a glove bag. Lyophilized lysozyme (LYS, $\geq 20,000$ units/mg) was purchased from MP Biomedicals. Proteins were purified after tagging with TRITC using Thermo Scientific Pierce polyacrylamide desalting spin columns with 6K MW cutoffs from Fisher Scientific. All dilutions were made using 18.2 MΩ DIUF water purchased from Fisher Scientific.

2.2 Materials Synthesis

Spherical SBA-15 particles were prepared using synthesis procedures adapted from Gartmann and Brühwiler, as modified from the work of Katiyar and Pinto [22, 24]. Initially, 3.10 g of P123 were heated in a 250 mL round bottom flask in a 50°C oven until melted. After this, 0.465 g of CTAB dissolved in 20 mL of deionized water was added to the P123. This solution was placed in a water bath at 30°C and stirred vigorously while 7.8 mL of 200 proof

ethanol and 45.9 mL of 1.5 M HCl were added. After the P123 completely dissolved, 10 mL of TEOS was slowly added drop wise. This solution was mixed for 2 hours. After 2 hours, the solution was poured into a Parr 4748 Teflon lined reactor, sealed, and heated at 90°C for 3 days. The sample was then homogenized in a high speed mixer and filtered over Whatman #5 filter paper in a 55 mm Büchner funnel. The synthesized material was split into two portions prior to amine functionalization (described below). In one portion (labeled ‘extracted’), the template was removed via Soxhlet extraction with 200 mL refluxing ethanol over 24 hours. The extracted materials were then filtered and dried in the oven at 80°C. The ‘as-synthesized’ sample was placed in an oven at 80°C overnight, leaving the template present in the materials.

2.3 Amine Functionalization of Mesoporous Silica

Extracted or as-synthesized material (200 mg) was dispersed in 10 mL of hexane in a 50 mL nitrogen-purged two-necked flask. To this solution, 190 µL of either APTES or APTMEES was added under vigorous mixing. This solution was stirred for either 10 or 20 minutes under a nitrogen atmosphere. The materials were labeled as AsSyn- XM or Extracted- XM for as-synthesized or extracted, functionalized for X minutes (0M (non-functionalized), 10M or 20M). After the functionalization period, the particles were filtered from solution over Whatman #5 filter paper in a 55 mm Buchner funnel and rinsed with 25 mL hexane. These materials were then cured at 80°C for 24 hours. After curing, the materials were washed for 24 hours in 100 mL ethanol to remove any remaining surfactant (in both as-synthesized and extracted materials), filtered, and dried in an oven at 80°C overnight.

2.4 Fluorescent Tagging of Functionalized Materials

Washed and dried functionalized APTMEES materials were re-suspended in 10 mL hexane in a nitrogen-purged 50 mL two-necked flask. To this solution, 15 mg of FITC was added and mixed for 10 minutes. After mixing, particles were filtered over Whatman #5 filter paper, rinsed with 25 mL hexane and immediately placed in 100 mL ethanol under vigorous mixing to remove excess FITC. After 24 hours, materials were filtered from solution again and rinsed with copious quantities of ethanol over filter paper until the supernatant became colorless, indicating that the non-covalently bound FITC had been removed. After rinsing with ethanol, the materials were dried in an 80°C oven for 24 hours. APTES functionalized materials were tagged immediately following functionalization with 15 mg of FITC for 10 minutes followed by filtration over Whatman #5 paper and curing at 80°C overnight. Samples were washed in 100 mL 200 proof ethanol for 24 hours, centrifuged out of solution and dried.

2.5 Particle Characterization

Pore diameter and surface area were measured from nitrogen adsorption measurements (Micromeritics Tristar 3000) conducted at 77 K. Samples were degassed at 120°C for a minimum of 4 hours under flowing nitrogen gas before analysis. Specific surface area was estimated using the Brunauer, Emmett and Teller (BET) isotherm and the pore diameter was estimated as the peak in the pore size distribution calculated by the method of Barrett, Joyner and Halenda (BJH) using the adsorption branch of the nitrogen adsorption-desorption isotherm [51-53]. Micropore volume, mesopore volume, and external surface area were estimated using as comparative adsorption plots (a.k.a. α_s plots) as described by Sayari et al. [54] using reference adsorption data from macroporous silica [55]. The particles were imaged using a Hitachi S-4300 Scanning Electron Microscope (SEM). SEM samples were prepared by sprinkling the particles onto double sided carbon tape and adhering the tape to 15 mm aluminum mounts. Excess silica

materials were blown off of the sample with nitrogen. Samples were prepared 24 hours in advance and left in a desiccator prior to being sputter coated using an Emscope SC400 with a gold-palladium alloy before analysis. Particle diameters were measured from the captured SEM images using ImageJ software. X-Ray Diffraction analysis was performed using a Bruker-AXS D8 Discover diffractometer. Samples were prepared in a powder sample holder and tapped flat with a spatula, followed by scanning at 0.5 °/min for 2 θ values from 1-4°. Data analysis was performed with the Bruker Diffrac-Suite software.

2.6 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR was used to observe the removal of template during the functionalization procedure. The removal of the surfactant pore template was examined by exposing as-synthesized particles to hexane (the solvent used during functionalization) for 10 and 20 minutes. The FTIR spectra of the original as-synthesized particles and the hexane-exposed particles were analyzed for the presence of the pore template. FTIR also provided a complementary method to particle dissolution (below) for the determination of the extent of aminosilane functionalization of the materials. All FTIR samples were ground at a concentration of 0.5 – 1.0 % by weight in anhydrous KBr. Pellets were pressed and spectra taken using a desiccated and sealed ThermoNicolet Nexus 470 with a DTGS detector at room temperature. Within the FTIR spectra, the areas beneath the CH₂ stretching (2800 cm⁻¹ to 3000 cm⁻¹), Si-OH stretching (900 cm⁻¹ to 984 cm⁻¹), and N-H bending (1570 cm⁻¹ to 1740 cm⁻¹) bands were calculated using Thermo OMNIC software, and were normalized using the area of the Si-O-Si stretching (980 cm⁻¹ to 1330 cm⁻¹) band to account for small changes in the mass of the bulk silica sample being prepared.

2.7 Quantification of Amines in Dissolved Particles

The amount of amine incorporated on the particles was quantified from the presence of amines in solution following particle dissolution at basic conditions. Fluorescamine, which covalently binds to primary amines to form a fluorescent pyrrolinone, was used to quantify the amines in solution [56]. Functionalized particles were dissolved over a 4 hour period in 30 mL of 0.02 M NaOH at room temperature under vigorous stirring. A 100 μ L aliquot of the dissolved particle solution and 1.0 mL of 1.0 mM solution of fluorescamine in acetone was added to 2.0 mL of phosphate buffered saline (PBS) at pH 7.4. The maximum fluorescence of this solution at 480 nm was monitored after excitation at 366 nm using a Varian Cary Eclipse fluorescence spectrophotometer. Both excitation and emission slits were held at 5 nm. A calibration curve with known amounts of aminosilanes was prepared by dissolving 30 mg of extracted, non-functionalized material in 30 mL of 80, 160 and 320 μ M APTMEES solutions in 0.02 M NaOH. These calibration solutions (100 μ L) were added to the PBS solution with fluorescamine and analyzed as described above.

2.8 TRITC-labeled Lysozyme to Probe Pore Accessibility

Lysozyme, at a concentration of 10 mg/mL in 1 mL PBS, was labeled at a ratio of 1:1 molar with TRITC for 24 hours at 4°C. After 24 hours, samples were purified in 100 μ L aliquots according to the instructions provided with the Thermo Scientific spin columns (#89849) [57]. New columns were unsealed and centrifuged for 1 minute at 1,500 x g to remove excess gel liquid. Columns were equilibrated in PBS by applying 100 μ L PBS to columns and centrifuging at 1,500 x g for 1 minute. Proteins were purified by pipetting 100 μ L of TRITC-Lys solution on the columns followed by centrifugation at 1,500 x g for 2 minutes. The accessibility of the

particles to TRITC-labeled lysozyme before and after amine functionalization was examined by diluting the protein solution to 200 $\mu\text{g/mL}$ in ethanol and mixing 1 mL of the protein solution with 10 mg of particles for 1.5 hours. After 1.5 hours, samples were imaged using confocal microscopy.

2.9 Confocal Laser Scanning Microscopy (CLSM)

Particles were prepared for CLSM (with a LEICA TSP SP5 confocal Microscope) by dispersing 10 mg of fluorescently tagged silica particles in 1 mL ethanol via vortex shaking for 24 hours. After 24 hours, one or two drops of the solution was placed on a glass slide, covered with a glass cover slip and sealed to prevent ethanol evaporation. An argon laser was used to excite the fluorophores over a 63X objective. The gain voltage on the laser was between 400 and 700 V. The scan speed was 400 frames per second. FITC emission was detected via a standard photomultiplier tube detector. Equatorial slices through the core of particles were taken using the z-scanning feature of the confocal microscope. The resolution of the z-scan was within 0.1 μm . Particles exposed to TRITC-labeled lysozyme were used as prepared and dropped onto glass slides for imaging, as described above. TRITC emission was detected with a photon counting high definition detector.

3. Results and Discussion

3.1 Characterization of Mesoporous Particles

Selective external amine functionalization of mesoporous silica has previously been demonstrated for pore diameters ranging from 2.9 nm to 5.5 nm [24, 38, 45, 47]. Selective functionalization for the largest pore diameter (5.5 nm) was achieved with APTMEES using as-

synthesized (pore blocked) materials and verified visually using CLSM [24]. For the purpose of extending selective functionalization techniques to larger-pore materials appropriate for protein loading, spherical silica particles with tunable pore sizes are synthesized by combining mixed cationic and non-ionic surfactant templating methods with hydrothermal aging [10, 22]. In our previous work, 5 μm to 15 μm spherical particles were synthesized with a pore size range of 5.4 nm to 11.6 nm (corresponding to hydrothermal aging temperatures between 60°C and 120°C) [21]. These spherical particles are appropriate for the visualization of protein loading by CLSM based on their large pore sizes and easily recognizable spherical morphology. Adsorption of proteins into pores with diameters that are greater than the largest diameter of the protein by 1 nm to 2 nm was demonstrated. Here, a synthesis temperature of 90°C was chosen to produce pore diameters of 7.4 nm, with a goal of producing accessible mesoporous silica materials for the model protein, lysozyme (3.0 nm x 3.0 nm x 4.5 nm).

The pore diameters and surface areas of pore template extracted and amine functionalized mesoporous silica particles synthesized by mixed surfactant templating were measured using nitrogen adsorption. All materials had Type IV nitrogen adsorption isotherms (**Figure S1**), which is consistent with the well-defined mesoporous nature of these materials. Surface areas were calculated using the BET method (**Table 1**) and modes of the pore diameter distribution were determined from the BJH pore size distribution plots (**Figure 1**). Comparative α_s plot analysis (**Figure S2**) was performed on template extracted and functionalized materials to determine contributions to nitrogen adsorption from microporosity and external surfaces of the particles. The BET surface area of template extracted particle is 524 m^2/g , 17% of which can be associated with the external surface of the particle by α_s plot analysis (**Table 1**). Micropores represent only 3-5% of pore volume while mesoporosity represents between 91% and 95% of

pore volume in extracted and functionalized materials (**Table 1**). The remaining porosity is associated with textural porosity between particles. XRD analysis of materials after template removal indicates long range order (**Figure S3**). The peak at 1.5 degrees corresponds to a d-spacing of 5.9 nm. Since that is smaller than the 7.4 nm pores, it represents the second order reflection associated with a primary d-spacing of 11.8 nm. Particles with spherical morphology and diameter of $7.3 \pm 1.8 \mu\text{m}$ ($n = 21$ particles for this estimate) are obtained from this synthesis procedure, as seen in the representative SEM micrograph in **Figure 2**. This morphology makes the particles easily identifiable under a confocal microscope. Additionally, locating fluorescent protein probes and functional groups on the surface or interior of the particle is possible with spherical particles by performing z-scans in the third dimension to define particle boundaries during imaging.

3.2 Comparison of Functionalization Approaches

A time-dependent functionalization method was chosen to selectively modify both as-synthesized and template extracted materials. The location of the grafted aminopropyl silane groups on the particles was determined by tagging the amine groups with the fluorescent probe (FITC) and imaging the particles using CLSM. The effects of functionalization time (10 and 20 minutes) on aminopropyl functional group (APTMEES) location on as-synthesized and extracted materials are compared in **Figure 3**. The removal of FITC from non-functionalized materials with ethanol washing is effective, resulting in no fluorescence of non-functionalized particles after washing. Short exposure time (10 minutes) of APTMEES to both as-synthesized and extracted materials results in selective functionalization near the exterior of the particles (**Figure 3**). In contrast, after 20 minutes of exposure to APTMEES in hexane, incorporation of the amine group both on the exterior of the particles and in the pores throughout the particles is observed.

This is an indication of a time dependent, diffusion based functionalization of pores as reactive species (APTMEES) diffuse into pore spaces and functionalize pore walls.

For comparison, another common aminosilane, APTES, was examined in extracted particles after 10 minutes of functionalization. Relative to APTMEES, APTES has significantly higher hydrolysis reactivity due to the reduced size of its alkoxy groups [58]. APTES would be expected to diffuse and adsorb more quickly within the pores; for example in hydrated silica gel with 7.6 nm diameter pores, adsorption of APTES was reported to reach equilibrium within 1 minute [59]. In spite of higher reactivity of APTES, selective surface functionalization is also observed for APTES grafted material after 10 minutes of exposure to particles in hexane (**Figure S4**), suggesting a similar time-dependence for selective functionalization as in APTMEES. This contrasts with the results of Gartmann and Brühwiler, who reported functionalization throughout the particles after 10 minutes of exposure of 20 mg/mL particles to 2 mM APTES in hexane. While APTES was in greater excess in our study (45 mM), the solvent and environmental reaction conditions and particle preparation (e.g., drying) were also different. Therefore, in addition to pore size (2.9 nm versus 6.1 nm in this study), differences in hydration of the particle may contribute to differences in time dependent functionalization. Hydrated silica gels exhibit a greater degree of APTES functionalization than those heat treated to remove water [60].

Characterization of the effects of the reaction time for amine functionalization (0 (no functionalization), 10 or 20 min) on the surface area and the overall extent of functionalization was performed using nitrogen adsorption, FTIR, and an amine assay on the dissolved particles. Only template-extracted materials were characterized due to potential effect of remaining templating agents on the FTIR and nitrogen adsorption results. The effect of APTMEES functionalization on the pore diameters for extracted particles is similar for 10 and 20 minutes of

functionalization (**Table 1**). Pore diameters of non-functionalized and APTMEES functionalized samples are 7.4 nm and 6.1 nm, respectively. Amino propyl silanes are approximately 0.9 nm in length [61] and after hydrolysis and condensation onto silica pore wall surfaces have been documented to extend approximately 0.5 nm into the pore space [62]. The reduction in pore size can be attributed either to the presence of amine functional groups on pore walls or shifting of the mode of the pore size distribution to a smaller diameter due to blocking of a fraction of the larger pores. The accumulation of aminosilane functional groups at the pore opening of smaller (3.1 to 3.9 nm) pores is well documented as a known artifact of post-synthesis functionalization [61, 62]. Along with the reduction of pore diameter, the pore volume and BET surface area of the particles decrease in response to amine functionalization.

FTIR was used to qualitatively evaluate the functionalization of the particle surface with aminopropyl silane functional groups. Specific vibrational modes analyzed were the CH_2 stretching associated with the aminopropyl silane, Si-OH stretching of surface silanols and the Si-O-Si stretching of the bulk silica sample. All spectra are normalized by the area of their Si-O-Si stretching peaks (980 cm^{-1} - 1330 cm^{-1}), which is representative of the amount of bulk silica prepared in each KBr crystal sample. The FTIR spectra in the region 800 cm^{-1} to 1500 cm^{-1} (**Figure 4A**) highlights the Si-O-Si stretching (1085 cm^{-1}) and the Si-OH stretching (970 cm^{-1}). The greatest absorbance at 970 cm^{-1} occurs prior to functionalization and is attributed to hydroxyl groups on the surface of the particles. This peak decreases as the surface silanols react with silanes to form siloxane bonds bound to aminopropyl silane groups during functionalization. After surface functionalization of materials for 10 and 20 minutes, a significant reduction in Si-OH stretching is seen on the surface of particles. This net reduction in silanol groups is consistent with one or two siloxane bonds to the surface along with condensation

among silane units at the surface (type I surface modification as defined by Vrancken et al. [60]. As can be seen in **Figure 4B**, minimal CH₂ stretching (2800 cm⁻¹ – 3000 cm⁻¹) occurs in non-functionalized, extracted samples and can be associated with small amounts of remaining reaction by-products and pore template. CH₂ stretching associated with the propyl portion of the aminopropyl silanes increases substantially after 10 and 20 minutes of functionalization, as compared to non-functionalized samples (as seen in **Figure 4B**). Coupled with the reduction in surface silanol content, the increase in CH₂ stretching indicates surface functionalization with the aminopropyl silane after 10 and 20 minutes of functionalization. The intensity of the CH₂ stretching band appears to be similar in both functionalized samples; this may be because of a multilayer of partially hydrolyzed APTMEES precursor near the exterior of the 10 min particles after recovery and drying, and also due to the nonquantitative nature of infrared spectroscopy. Thus, although FTIR qualitatively describes particle functionalization, a more quantitative method of particle dissolution was employed to measure the amount of aminosilane grafted to the particles.

Quantification of grafted APTMEES was performed via a fluorescence based amine assay following the dissolution of the silica particles in dilute sodium hydroxide [62]. After particle dissolution, fluorescamine, which covalently binds to the free primary amines to create a fluorescent pyrrolinone product, was added to the solution. The quantity of APTMEES grafted to the particles was determined from the fluorescence intensity of the solution and quantified using a calibration curve (**Figure S5**) developed from known quantities of APTMEES. After 10 minutes of APTMEES functionalization, grafting quantities reached 623 mg APTMEES per gram of silica sample. If this quantity of amine grafted only to the external surface of the particles, it must have grafted in multilayers. Monolayer coverage of 623 mg/g APTMEES

corresponds to 423 m²/g grafted area assuming 50 Å²/ molecule.[63] α_s analysis of nitrogen adsorption isotherms indicates only 87 m²/g of external surface area (Table 1), thus ATPMEES is grafted in 4.9 fold excess of a monolayer. Note that there may be some amine grafting near the openings of the pores as well, but multilayer coverage is still likely. After an additional 10 minutes of grafting, the amount of amine increased by 45% to 906 mg/g. At this point, the mesopore surface area, which is significantly larger than the external surface area (**Table 1**) has been grafted, making the amount of grafted aminosilane approach monolayer coverage (1.2× monolayer coverage).

While the total amount of amine grafted increased upon going from 10 to 20 minutes of treatment with APTMEES, it is somewhat surprising that a drastic change in the distribution of amines is observed in the CLSM images (**Figure 3**). However, because these are *ex situ* measurements of the amines after the particles have been collected and dried, the difference most likely represents changes due to the diffusion of (partially hydrolyzed) APTMEES into the pores of the material. This is consistent with the reaction conditions during particle functionalization. Although anhydrous solvents were used in nitrogen purged reaction flasks, water physisorbed to the particle surface or present at small concentration in the reaction flask could induce hydrolysis and condensation between aminosilanes in solution and on the surface of particles, leading to greater than monolayer coverage. Vrancken et al. showed that treatment of silica at significantly higher temperatures (200 °C or higher) is necessary to remove adsorbed water and prevent multilayer formation.[60] After 10 minutes of exposure to APTMEES, hydrolysis of the precursor at the surface of the particles would have begun, leading to adsorption of a significant amount of silane near the exterior of the particles (and possibly near the pore openings). This (partially) hydrolyzed precursor most likely became covalently bound upon collection and drying

of the particles, leading to over a multilayer of aminosilane coating near the exterior of the particles as observed by CLSM. Exposure of the particles to APTMEES for 20 minutes allowed time for diffusion of the hydrolyzed precursor into the pores as well as addition of more silanes. This process may be autoaccelerating in nature, as hydrolysis of the precursors would enhance the driving force for interaction with the silica pore space, leading to rapid establishment of uniformly functionalized pores, as shown in **Figure 3**. Consistent with this picture, the adsorbed multilayer present at 10 min of exposure leads to a significant amount of amine attachment and pore volume decrease (possibly due to partial blocking of pores). This silane is able to diffuse further into the particles after 20 min of exposure, increasing the amount of aminosilane attached without significantly reducing the pore volume. While spatially detailed kinetic measurements beyond the scope of this work would be required to verify this mechanism, it is consistent with the characterization of the particles.

3.3 Pore Template Removal during Functionalization

Amine functionalization is observable in the pores deep within the particles after 20 minutes of functionalization for both the as synthesized and template-extracted materials (**Figure 3**). This finding is in contrast to an accepted approach to exterior-only functionalization of mesoporous silica - functionalizing as-synthesized materials while leaving the template in the pores to limit the accessibility of the reactive organic silanes to the interior pore walls. For small-pore silica materials (less than 5.5 nm), selective exterior functionalization using pore blocking with silanes has been confirmed with combinations of NMR, nitrogen adsorption, CLSM and FTIR [24, 38, 47]. The effectiveness of this pore-blocking approach for the larger-pore silica materials was examined by quantifying the presence of the pore template in the materials after exposure to hexane, the solvent used for the functionalization with APTMEES.

FTIR spectra were measured for as-synthesized, 10 minute hexane-exposed, 20 minute hexane-exposed and Soxhlet extracted samples to compare the CH₂ stretching band associated with the pore template. A comparison between each sample can be made by normalizing the CH₂ stretching (between 2800 cm⁻¹ and 3000 cm⁻¹) to the bulk silica samples by dividing the area under the CH₂ stretching band by the area under the Si-O-Si stretching band (980 cm⁻¹ to 1330 cm⁻¹). The raw FTIR spectra with marked CH₂ and Si-O-Si stretching ranges are presented in **Figure S6** of the supporting information. The normalized CH₂ stretching associated with the pore template can be seen in **Figure 5**. There is a minor reduction in the quantity of template present after exposure to hexane; after 10 and 20 minutes of exposure to hexane, 92% and 83%, respectively, of the template present in the as-synthesized sample remains. In contrast, the Soxhlet ethanol extraction process removes over 90 percent of the original pore template. These results suggest that while some template may be extracted by hexane during functionalization, its removal is largely incomplete during the amine functionalization procedure used for the as-synthesized silica particles. These results suggest that interior pore functionalization occurs independently of pore template presence in these hydrothermally processed, large-pored materials.

The demonstrated functionalization of the silica pores in the presence of pore template suggest that the pore blocking techniques used to achieve selective exterior functionalization of small pore mesoporous silica may not be effective in materials prepared by hydrothermal expansion of P123 micelles. Prior researchers have used mesoporous materials with smaller pore diameters, 2 nm – 4 nm, with CTAB as a pore blocking agent, and found evidence for selective external functionalization even with relatively long (on the order of hours) exposure periods to silanes in solution [38, 47]. Extended functional group exposure periods on the hour time scale

are incompatible with the selective functionalization of the large-pored materials used here, which can be seen with full particle functionalization after 20 minutes. Aminosilanes have previously been shown to remove pore template by displacing surfactants within 4.9 nm pores and to rapidly hydrogen bond to surface silanols within pores [59, 64]. APTES, as well as the similarly structured 3-aminopropyltrimethoxysilane (APTMS), have been shown to displace the positively charged head group of CTAB (the templating surfactant) during a 10-hour period of refluxing, thus simultaneously displacing the template and functionalizing the pore walls [64]. This method of template removal and surface functionalization would not be evident in hexane exposure studies (**Figure 4**) due to the absence of aminosilane functional groups in the solution. Functionalization of both the external surface and the interior pore surface at longer functionalization times is consistent with the greater diffusion of aminosilanes into the pores, and perhaps increased template replacement with aminosilanes. In addition to displacement of the pore template, more rapid diffusion of (potentially hydrolyzed) APTMEES in the hydrothermally expanded CTAB and P123-based particles may be possible due to greater separation of the PEO blocks of the template from the silica walls due to increased hydrophobicity at elevated temperature [20]. At increased temperatures, water becomes a poorer solvent for the ethyleneoxide blocks of P123. Therefore at higher temperatures they retract into the hydrophobic core of the micelle, reducing their pore wall interactions. This reduced pore template – pore wall interaction, caused by high temperature synthesis, may increase the accessibility of aminosilanes to pore wall surfaces in the presence of P123 based templates.

3.4 Protein Accessibility within Functionalized Materials

This work establishes through visualization a selective, surface functionalization strategy for mesoporous particles with pore sizes appropriate for the protein loading. The accessibility of

the pores of these functionalized particles is verified using a model protein, fluorescently tagged lysozyme, which has previously been used to demonstrate pore accessibility in non-functionalized mesoporous silica materials as well as size selective protein separations in these materials [15, 22, 65]. Lysozyme, a 14.6 kDa protein with dimensions of 4.5 nm x 3.0 nm x 3.0 nm [66], has been shown in literature to adsorb into pore diameters as narrow as 6.5 nm [15, 22]. In this study, the favorable properties of the silica particles for CLSM imaging are used to visualize the accessibility of TRITC-tagged lysozyme for functionalized particles. The difference in the excitation and emission wavelengths of the TRITC tagged lysozyme and FITC tagged amine groups on the surface of the particles allow their location to be imaged simultaneously.

The location of TRITC tagged lysozyme within non-functionalized particles shows full penetration through the particles after 1.5 hours of passive protein loading (**Figure 3**). Although there is a pore diameter reduction after 10 and 20 minute functionalization, this reduction does not have an impact on the diffusion of lysozyme into the pores. Both selectively functionalized and fully functionalized materials are capable of loading lysozyme within pores throughout the particle. Previous reports using CLSM have visually confirmed accessibility of lysozyme within non-functionalized particles with 7.4 nm diameter pores, but this is the first documentation of simultaneous protein localization with selective external or full particle functionalization [22]. Similar amino-silane grafting attempts on smaller pored silica materials (4.0 nm pore diameter) block pores and significantly reduced pore accessibility to proteins – to the extent they have been used to permanently trap proteins within pores [67]. Similar to selectively functionalized materials, full functionalization of pore walls after 20 minutes of functionalization does not result in the clogging of pores, which would prohibit lysozyme diffusion into the pores. This

observation is significant not only because it suggests that protein-accessible particles with engineered external surfaces can be prepared by controlling silane exposure times, but also because simultaneous imaging of functional groups attached to pore walls within close proximity to proteins inside of pores is an enabling technology for future studies of functional group / protein interactions in confined nanopore spaces.

4. Conclusion

The selective placement of functional groups on mesoporous particles allows for two distinct local interactions of a particle with a solute or with its environment. Selective functionalization of large-pored, protein accessible materials has been shown to be primarily dependent on the reaction time of the functionalization, where shorter exposure time (10 minutes vs. 20 minutes) to aminosilane precursors led to localization of functional groups only at the exterior of the particles. As indicated by CLSM, the location of grafted amine groups (on the exterior surface or in the pores) is identical for as-synthesized (non-extracted) materials and extracted materials, indicating the ineffectiveness of the pore blocking method in selectively functionalizing large-pored materials prepared in this case by hydrothermal expansion of a P123 template. Although extended functionalization periods (in excess of an hour) can provide selective exterior functionalization in small pore blocked materials, aminosilanes in larger pored materials effectively replace template along pore walls, leading to pore wall functionalization.

The accessibility of pores after functionalization is critical in maintaining the applicability of large pored-mesoporous particles. Pore diameters were found to be somewhat reduced after functionalization, from 7.5 nm to 6.1 nm, in both selectively and fully functionalized materials. This reduction is consistent with the length of an aminopropyl

functional groups extending into a pore opening. Although pore diameters were reduced, pores were still accessible to the model protein (fluorescently tagged lysozyme (14.6 kDa 3.0 nm x 3.0 nm x 4.5 nm)). Using both red and green fluorescent dyes, respectively, to tag proteins and particles, CLSM was used to simultaneously confirm the presence and full accessibility of lysozyme throughout non-functionalized, selectively external functionalized and fully functionalized particles. Mesoporous silica materials have proven to be versatile platforms for biological interactions, although the ability to selectively locate functional groups on distinct particle surfaces can be difficult to verify. As demonstrated here, the selective functionalization of mesoporous silica with pore sizes appropriate for protein loading permits tailored interactions on the particle surface while providing the opportunity for protein based biological function within pore environments.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:xx.yyy/zzzz.

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Tables

Table 1. Pore structure results from nitrogen adsorption of materials after template extraction and as a function of functionalization time using APTMEES

Functionalization Time	Pore Diameter (nm) ^a	Micropore Volume (cm ³ /g) ^b	Mesopore Volume (cm ³ /g) ^b	Total Pore Volume (cm ³ /g)	External Surface Area (m ² /g) ^b	BET Surface Area (m ² /g)
Extracted (0 min)	7.4 ± 1.0	0.04	1.15	1.26	87.1	524
10 min	6.1 ± 1.3	0.04	0.72	0.78	40.8	376
20 min	6.1 ± 1.4	0.04	0.78	0.82	29.1	389

^a The ranges are determined from the full width at half maximum (FWHM) of the BJH pore size distribution.

^b From α_s plot analysis.

Figure Captions

Figure 1. Pore size distributions for materials synthesized after varying synthesis steps – as-synthesized, after extraction of the templates, and after 10 or 20 minutes of functionalization by APTMEES in hexane.

Figure 2. SEM image of non-functionalized, extracted mesoporous silica (denoted Extracted-0M).

Figure 3. CLSM images of FITC tagged amine functional groups (green) before and after template extraction (left and center columns) for varying functionalization time. The materials after template extraction are simultaneously probed with TRITC tagged lysozyme (red). All scale bars are 5.0 μm wide.

Figure 4. FTIR spectra of extracted (non-functionalized) silica particles and silica particles after 10 or 20 min of functionalization using APTMEES. The regions are selected to highlight (A) the Si-OH stretching band (970 cm^{-1}) and (B) the CH_2 stretching band (2985 cm^{-1}). The FTIR spectra are normalized to the Si-O-Si stretching (1085 cm^{-1}) indicated in part in A.

Figure 5. Normalized CH_2 stretching of the pore template in as-synthesized material as a function of duration of exposure to hexane. Materials washed by Soxhlet extraction are presented as a reference, and denoted “extracted.”

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5