Modelling the Internal Architecture of Fluorescent Silica Particles via a Template-Free Approach

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ABSTRACT

Particles with an open, porous structure can be used to deliver payloads. It is often of interest to detect such particles in tissue or materials, which is facilitated by addition of dye. A

straightforward approach leading to fluorescent, porous silica particles is described. The particles are etched with 3 mM aqueous sodium hydroxide, taking advantage of the etching rate difference between normal silica and an interior band of silica that contains covalently attached dye. No additional steps, such as dye labeling or thermal annealing, are required. Etching modeled the internal structure of the fluorescent silica particles by creating meso, macropores and voids, as reflected by nitrogen absorption measurements. In order to investigate whether a polymer shell influences etching, certain composite particles are top-coated with poly(L-lysine) representing neutral or positive charged surfaces under typical pH conditions in living systems. The polypeptide-coated fluorescent silica cores exhibit the same porous morphology as uncoated homologs. The polypeptide topcoat does little to alter the permeation by the etching agent. Preservation of size during etching, confirmed by dynamic light scattering, transmission electron microscopy and small-angle X-ray scattering, simplifies the use of these template-free porous fluorescent particles as platforms for drug encapsulation, drug carriers and in-vivo imaging.

Keywords: Stöber silica, fluorescent silica, mesoporous and macroporous silica, polypeptide composite particles, NaOH etching

INTRODUCTION

Colloidal silica features many desirable characteristics such as low cytotoxicity, facile preparation, control over interparticle interactions, and stability in the physiological pH range (6-8). The ease of surface modification qualifies colloidal silica as an excellent platform for the design of materials for biomedical applications including drug encapsulation, drug delivery, cell separation and cell imaging.[1-6] Typically, silica colloids are prepared from alkylorthosilanes in

1 a sol-gel process pioneered by Stöber, Finn and Bonn in 1968 and are known as Stöber silica.[7]
2 Efforts to modify the Stöber procedure to improve particle uniformity have been reported.[8, 9]
3 Narrow size distributions are particularly desirable for biomedical and biotechnology
4 applications where consistency and stability are valued.

5 Physically or covalently loaded with dyes, fluorescent silica particles are used as probes
6 for *in vivo* imaging purposes. While many fluorescent agents can be used, fluorescein
7 isothiocyanate, FITC, is attractive because its absorption maximum (~490 nm) matches the
8 strong 488-nm line of argon ion lasers. Another important feature is the high quantum efficiency
9 relative to self-quenching, as reported by Viger *et al.*[10] These researchers made fluorescent
10 silica-coated silver particles in two steps using 3-aminopropyltriethoxy silane, APS, as the
11 coupling agent. First, the FITC-dye was chemically bound to the APS. Next, the resulting adduct
12 was introduced into a reactor where the final product was prepared in a reverse micelle
13 procedure. The fluorescent silica-primed silver particles exhibited high detectability and low
14 self-quenching but the lack of a spacer layer to locate the dye at a distance from the silver core
15 induced a decrease in the fluorescence lifetime.

16 A similar approach was used by Santra *et al.* in the preparation of FITC-labeled silica
17 nanoparticles without metal cores for bio-imaging applications.[11] Lu *et al.* improved the
18 method and prepared fluorescent silica particles in a two-step process involving the sol-gel
19 technique.[12] Compared to the approach of Viger *et al.*,[10] these particles were treated with a
20 supplemental amount of TEOS to build a silica outer layer. In order to close any pores at the
21 silica surface, the suspension was treated with sodium silicate, Na_2SiO_3 , at a basic pH. Because
22 the absorption and fluorescence of FITC depends on pH, the last step was performed to prevent
23 the access of any ions that could influence the FITC fluorescence properties.

Mesoporous silica is an increasingly important subclass of colloidal particles. Particles with complex morphologies, composition and internal structures are attractive for many applications including catalysis, separations, diagnostics, coatings, optics and electronics.[13, 14] Several techniques have been used to develop porous structures, such as template swelling with supercritical carbon dioxide,[15, 16] microfluidic devices,[17, 18] spray drying[19-21] and etching with hydrofluoric acid.[22-26] A recent report presents a selective etching approach of complex inorganic particles comprised of Ru-B amorphous alloy, carbon, cetyltrimethylammonium bromide, CTAB surfactant as template and silica, Ru-B/m-Carbon-CTAB/SiO₂. [27] Reasonable control over silica porosity was achieved using some of these techniques, but all require a template (polymers or block copolymers), surfactants and additional steps to purify the final material from organic ingredients. In many cases the template removal necessitates high temperature that raises the cost of the final product. None of the techniques listed above are ideal; some necessitate advanced setup, others are prone to safety issues, and all seem to work better for micrometer-sized particles than for nanoparticles.[28]

A technique that is gaining traction uses a strong base such as NaOH as an etchant to tailor the internal morphology of the colloid.[29] Overall, the approach using base as the etchant is much milder than the alternative acidic route.[30, 31] Morphologies ranging from rattle-type or yolk-shell to sponge-like have been produced, and such configurations are very appealing, as demonstrated by Zhang *et al.*[32] Usually, particle sizes have been in the nanometer scale, suitable for biomedical applications. Whether the method involved was the so-called “surface-protected etching”[32] or just simple aging, the use of NaOH facilitated the creation of desirable silica morphologies.[32, 33] Until now, basic etchants have required a template, or scaffold, to either provide for surface protection or to design interior details (e.g., rattles). Building the

particles around a template necessitates subsequent steps to remove it and, if desired, to add a fluorescent label. Further, the nature of the template (usually organic molecules, e.g., polymers and surfactants) can constitute a drawback if biological applications are intended. For instance, template constituents may be toxic towards living cells, and therefore supplemental characterization steps must be taken to ensure that the final product is template-free.

Here, modified Stöber and seed regrowth procedures to make fluorescent particles with interior layer structures are presented (Scheme 1). The dye is covalently bound and blended with TEOS layers in the particle interior. NaOH etching is used to build fluorescent meso/macroporous structures in an approach that obviates the need for either a template or surface protection and does not require further steps to obtain fluorescent beads. Rather, the porous structures can be produced by taking advantage of etching rate differences between the more heavily crosslinked zones within the particle's silica network and more lightly crosslinked zones. The role of the TEOS-FITC adduct in altering the zonal density is considered. The template-free approach is economical, easy to scale up, and facilitates high yield of fluorescent meso/macroporous silica.

MATERIALS AND METHODS

Materials. *N*^ε-carbobenzyloxy-L-lysine, CBL (99%) and triphosgene (98%) were purchased by Acros Organics. MP Biomedicals was the vendor for o-benzyl-L-tyrosine, BTYR (99%). Tetraethylorthosilicate, TEOS (>90%), 3-aminopropyltriethoxy silane, APS, sodium bicarbonate, fluorescein isothiocyanate, FITC (99%), anhydrous tetrahydrofuran, anhydrous ethyl acetate and anhydrous hexanes were obtained from Aldrich and used as received. Absolute ethyl alcohol, EtOH ACS/USP grade was obtained from Pharmco-AAPER. Ammonium hydroxide 28-30%

ACS grade was purchased from BDH Aristar. Deionized water (18 mΩ) was withdrawn from a Barnstead Nanopure instrument.

Experimental Procedures

1. *Synthesis of silica seeds*[7, 9, 34-36] (STEP I, Scheme 1). Silica seed particles were prepared according to a well-known protocol as follows: In a two-neck 2000 mL round-bottomed flask connected to an overhead mechanical stirrer, 1250 mL of EtOH was mixed with 100 mL of ammonium hydroxide (solution S1, Table 1) to ensure a basic pH (9-10, pH paper). Moderate stirring of the mixture (150-200 rpm) was performed over 15-20 min. to obtain a homogeneous solution. Then solution S2 was added quickly ~10-15s (using a glass funnel with a long

Table 1. Components of the Solution 1 and 2 used in Silica Seed Production.

Solution S1	Solution S2
1250 mL of EtOH and 100 mL of ammonium hydroxide	132.5 mL of EtOH and 33.25 mL of TEOS

and wide leg) under vigorous stirring (~400 rpm) at 22-25°C and reacted for 4h. The particles were allowed to rest overnight undisturbed and then stored in a well-capped bottle (the cap was wrapped in parafilm). A part of this batch ($R = 105$ nm by DLS) was functionalized with poly(o-benzyloxy-L-tyrosine). The rest was used as seed particles in the dye-labeling and protection steps.

Typical yield: 11.25 g

2. *Synthesis of the adduct fluorescein isothiocyanate- 3-aminopropyl triethoxysilane,*

FITC – APS. [37] Fluorescein isothiocyanate (50 mg, 0.12 mmol) and 10 mL of absolute ethanol were suspended in a 50 mL round-bottomed flask capped with a septum. The mixture was magnetically stirred for 30 min. and APS (0.5 mL, 1.2 mmol) was injected into the solution through the septum. The reaction proceeded for 18 h, in the dark, to prevent the dye from bleaching.

3. *Synthesis of covalently labeled fluorescent silica particles* [12, 38, 39] (STEP II, Scheme 1). After the formation of the silica seeds, a separatory funnel containing 75 mL of TEOS five times diluted with EtOH (375 mL) was attached to the two-neck flask. The flow of the dropping solution was adjusted for 1 drop per minute ($\sim 50 \mu\text{L}$). Using a syringe, a volume of 8 mL of FITC–APS adduct was injected into the silica seed suspension. The round-bottomed flask was wrapped in aluminum foil to prevent dye bleaching. The reaction proceeded for 36 h under continuous stirring (200 rpm) at room temperature. Fluorescent particles were washed copiously with alcohol by repeated centrifugation-dispersion cycles to a loose precipitate settled on the bottom of the PTFE tubes until the washing alcohol did not fluoresce visibly when illuminated with a 488 nm wavelength laser. In order to vary the thickness of the fluorescent band, the volume of the FITC-APS adduct can be adjusted (e.g. 2 mL, 4mL, 6ml, 10 mL, 12 mL). The procedure also permits the variation of the TEOS amount. Control over the TEOS volume can be achieved by using the below calculations.

Typical yield: 19 g

4. *Protective coating of fluorescent silica particles*. [9] Silica coating was performed on particles as described in the previous step in order to prevent dye bleaching. Control over the size of the final particle was designed using the calculations by Zhang et al. [9]:

$$d = d_0(V/V_0)^{1/3}$$

where d is the final target diameter of the fluorescent silica particles, 375 nm, d_0 is the initial size of the silica seeds, V_0 is the initial volume of the TEOS used in silica seeds preparation and V is the TEOS needed for the target particle size. Several sizes were prepared ranging from 200 nm to 1.3 μm . The following paragraph describes the detailed procedure for a target size of 350 nm.

From the original fluorescent suspension (3), 100 mL (4.74 g) was taken and diluted to 200 mL total volume in a beaker. This mixture was first sonicated with a probe ultrasonicator operating at 40 amps for 5 min to ensure a good dispersion of particles within the solution. Then the suspension was quickly transferred into a 500 mL two-neck round bottom flask connected to an overhead mechanical stirrer and wrapped in aluminum foil. Ammonia, 43 mL was added to create a basic environment (pH=9) validated by pH paper. This mixture was stirred for 15–20 min for homogenization. Separately 6 mL TEOS was diluted four times with absolute EtOH (24 mL) and the solution was swirled for homogenization. This mixture was transferred into a small separatory funnel (125 mL) attached to the two-neck flask containing the fluorescent particles. The flow rate was 50 μL per minute under continuous vigorous stirring (~ 400 rpm) at room temperature. The reaction continued over 24 h. Particles were washed 3-4 times with ethanol by centrifugation-dispersion to a loose precipitate and stored in brown bottles as an alcoholic suspension. These silica beads were used for growing poly(N^ϵ -carbobenzyloxy-L-lysine) polypeptide hairs on their surface.

Typical yield: 5.92 g

5. *Preparation of polypeptide composite particles.* Polypeptide composite particles having either poly(L-lysine) or poly(L-tyrosine) shells were obtained by ring-opening polymerization of the corresponding N-carboxyanhydrides, N^ϵ -carbobenzyloxy-L-lysine and o-benzyl-L-tyrosine

1 N-carboxyanhydride respectively, initiated by the amino-functionalized fluorescent silica
2 particles. This method is known by the colloid community as the *grafting from* method.

3 *5.1 Functionalization of silica surface with amino groups.*[40-42] The amino-
4 functionalization procedure was applied to silica seeds from STEP I (770 mg). The
5 functionalization with amino groups used 3-aminopropyltriethoxysilane, APS. In a typical
6 preparation, 10 mL of the silica-protected fluorescent particles (770 mg, 7.7% solids) was diluted
7 to 100 mL with absolute ethanol. The suspension was probe-sonicated for five minutes prior use
8 and quickly transferred into a 250 mL two-neck round bottom flask attached to an overhead
9 mechanical stirrer and protected with aluminum foil. Afterwards 15 μ L of APS was injected into
10 the mixture with a syringe. The reaction proceeded for 18 h under stirring (200 rpm) at room
11 temperature. The particles were washed by centrifugation-dispersion to a loose precipitate with
12 ethanol until the ninhydrin test was negative indicating the absence of free amino groups in the
13 suspension. For testing, small aliquots (1 mL) of supernatant from the PTFE centrifuge tubes
14 were loaded in glass test tubes placed in a water bath set at 45°C. Two drops ($\sim$ 100 μ L) of a 10%
15 alcoholic solution of ninhydrin was added. The presence of free amino group was indicated by a
16 purple to blue coloration. Afterwards, the particles were washed by centrifugation-dispersion
17 twice with anhydrous THF and stored in a dark capped vial and suspended in anhydrous THF.
18 Typical yield per batch: 650 mg.

19 *5.2 Synthesis of N^ε-carbobenzyloxy-L-lysine N-carboxyanhydride, CBL – NCA and o-benzyl*
20 *L-tyrosine N-carboxyanhydride, BTY-NCA.*[43, 44] The amounts of the chemicals involved in
21 the preparation are listed in Table 2.

Table 2. NCA Name and Ingredients Used in the Polypeptide Precursor Synthesis.

NCA Name	Solvent	NCA precursor, CBL/oBTYR			Triphosgene		
	Anh. EtOAc/ mL	Mass/ g	Molarity/ mmol	M_w / $\text{g}\cdot\text{mol}^{-1}$	Mass/ g	Molarity/ mmol	M_w / $\text{g}\cdot\text{mol}^{-1}$
CBL-NCA	150	5.021	18.0	280.32	1.8	6.0	296.75
BTY-NCA	150	4.66	18.4	271.31	1.82	6.1	296.75

In a N₂-filled glove bag, anhydrous (Sigma) ethyl acetate, EtOAc, 150 mL was mixed with the amount of the NCA precursor in a flame-dried three-neck round bottom flask. The flask was immersed in an oil bath and connected to dry N₂ (passed through Drierite with indicator). A condenser attached to a cooling bath was also connected to the flask and capped with a glass stopper. Under N₂ bubbling and magnetic stirring the slurry was brought to reflux. In a separate vial the desired amount of the triphosgene was weighed inside the hood in a glove bag filled with dry N₂. The content of the vial was quickly added to the refluxing mixture and the reaction continued until the solution became clear (4-5 h). In the situation where turbidity persisted beyond 90-120 min, supplemental amounts (0.2 g) of solid triphosgene were fed into the mixture. After the solution turned clear, the reflux was maintained for another hour. The clear solution was allowed to cool to room temperature while stirring and bubbling nitrogen continued. Overnight, the solution was kept in the freezer at -5°C. Removal of unreacted amino acid and HCl was performed by washing oily NCA in a separatory funnel with icy water (75 mL) and icy solution (0.5%) of NaHCO₃ (75 mL). Two layers formed each time and the upper was the organic phase of interest. Afterwards, the NCA was dried over anhydrous MgSO₄ until the drying powder did not show clumping. The clear solution of NCA was then filtered by suction through Whatman ($D=90$ mm) filter paper. The drying steps and the filtration were performed in a glove bag. The volume of the mixture was reduced to 1/3 by evaporation inside the glove bag placed in the hood. Finally dry, cold (Aldrich) hexane (100 mL) was poured slowly in the NCA

1 solution. White crystals of NCA started to form. The crystallization took place overnight in the
2 freezer at -5°C. The recrystallization was repeated twice in a glove bag filled with dry nitrogen
3 as follows: NCA was dissolved in a minimum amount (15-20 mL) of dry THF and then cold, dry
4 hexane was poured slowly and the flask gently swirled until white crystals began to form. The
5 flask was capped with a septum and placed inside the freezer overnight for a complete
6 crystallization. The NCA crystals were recovered by suction filtration inside the glove bag.
7 Yield: CBL-NCA- 4.43g, 86%; BTY-NCA-4.18g, 82%.

8 **NOTE A:** *The dissolution of the NCA in THF during recrystallization steps might require*
9 *warming the mixture. The flask containing NCA solution should be attached to a bubbler to*
10 *release the pressure caused by the THF vapors. Preheated water (T~ 30-35°C) or a sparkless*
11 *heat gun can be used to warm the mixture with continuous swirling.*

12 **NOTE B.** *Heating NCA mixture with a heat gun near a volatile solvent should be done with*
13 *ventilation.*

14 **NOTE C.** *Triphosgene is a toxic chemical. The reaction is, therefore, conducted in a well-*
15 *ventilated fume hood and the HCl/phosgene gas formed during reaction should be passed*
16 *through an ammonia hydroxide solution for neutralization. This is realized by connecting the*
17 *bubbler to the flask containing the basic solution. Triphosgene should be weighed inside the*
18 *hood.*

19 5.3 *Synthesis of poly(N^ε-carbobenzyloxy-L-lysine)-based fluorescent silica particles, PCBL*
20 *silica.*[40, 42, 45] Into a flame-dried 250 mL round-bottom flask was loaded 50 mL of anhydrous
21 (Aldrich) THF. To this amount of solvent, 2.5 g of CBL-NCA was added and a stir bar. The flask
22 was capped with a rubber septum and stirred until the CBL-NCA completely dissolved to a clear
23 solution. To this mixture, 20 mL (400 mg) of amino-functionalized particles were added under

the inert atmosphere (N_2) in a glove bag. The flask, immersed in a water bath set to 35°C , was rapidly connected to a bubbler to release the CO_2 formed during the reaction. After three days the production of the gas stopped; an indication for reaction completion. The particles were subjected to centrifugation and dispersion five-six times with THF and finally suspended in THF. They were stored in dark vials.

Yield: 600 mg

5.4 Synthesis of poly(o-benzyl-L-tyrosine)-based silica particles, PBTY. Anhydrous tetrahydrofuran, THF, (Aldrich, 50 mL) was loaded into a flamed dried 150 mL round-bottom flask. Polypeptide precursor, *o*-benzyl-L-tyrosine N-carboxyanhydride, BTY-NCA, (2g, 6.7 mmol) was added and the flask capped with a septum. The mixture was swirled until it became clear. Then, 0.196 g of amino-functionalized silica ($R = 105\text{ nm}$) particles (non-fluorescent) were added along with a stir bar. The reaction was performed in a glove bag under inert atmosphere of nitrogen. The flask was connected to a bubbler to release the CO_2 formed during reaction. The reaction continued for 72 h at 35°C . After the gas release stopped the particles were washed $6\times$ with THF by centrifugation/dispersion and finally stored in anhydrous THF.

Yield: 366 mg

6. Deprotection of the side chains.[46, 47]A stock suspension of polypeptide-coated particles in anhydrous THF was placed in a Teflon tube and solvent reduced until the mixture became a slurry. Then a volume of $\text{HBr}/\text{CH}_3\text{COOH}$ (33%) (Aldrich) was added inside the hood. The tube was quickly capped and the mixture stirred magnetically for 45 min. The light brown mixture was washed with toluene, acetone, neutralized with 3 mM NaOH and washed with water twice. Finally particles were dispersed in water. The amounts of the chemicals used in the synthesis are listed in Table 3.

Table 3. Sample, Amounts of Particles Subjected to Deprotection, Volume of Debenzylation Agent and Yields of Deprotected Particles.

Sample	Amount of the Particles/g	Volume of HBr/CH ₃ COOH/mL	Yield of deprotected particles/g
PCP PCBL	0.2	2	0.14
PCP PBTY	0.21	5	0.15

7. *Preparation of meso/macroporous particles.* Bare silica particles subjected to aging (~100 mg) were brought to slurry by removal of almost all storage solvent (anhydrous ethanol), using centrifugation. The 3 mM NaOH solution (8 mL) was poured over the thick suspension of particles. The aging took place in a glass vial under gentle stirring. Each day small aliquots (~1 mL) were collected for analysis. Each time, particles were washed six times with deionized water by low-speed centrifugation (3,000 rpm)-dispersion and stored in water. The low-speed centrifugation performed at all steps is necessary to avoid solid pellets that will yield aggregated particles when re-dispersed.

Yield/day: 20 mg

Polypeptide composite particles were each immersed overnight in a 3 mM NaOH solution (5 mL) inside glass vials under magnetic stirring. Then particles were washed six times with water by centrifugation-redispersion.

Yield: PLYS-silica meso/macroporous: 100 mg, PTYR-silica sponge-like: 110 mg

8. *Plant uptake.* Three leaves from an *Arabidopsis thaliana* plant grown in a laboratory greenhouse (25°C) were detached from the plant branch by performing a cut with a small scissors. The freshly cut tail of each leaf was immersed in small beakers containing aqueous suspensions of uncoated meso/macroporous particles (~1wt%). They were let to rest for three

days (25°C) and then visualized with a epi-fluorescence microscope using an FITC filter set. The leaves were placed on a clean microscope slide without glass cover.

Methods

Transmission electron microscopy, TEM. Transmission electron microscopy images of magnetic particles at different stages were obtained using a JEOL 100-CX electron microscope with an accelerating voltage of 80kV. A dilute sample solution was prepared by dispersion of 20µL of particles in 1mL of appropriate solvent (water). The sol was sonicated 1-2 min to ensure a good dispersion of particles in the solution. From this solution a drop was placed on top of a 400 mesh carbon-coated grid (Electron Microscopy Sciences) and dried either in the air overnight or 2-3 hours under vacuum. Particles were visualized without staining. At 100,000× magnification and higher some images were intentionally under focused to capture the Fresnel fringes and to guard against possible artifacts. Particle size visualized in the transmission electron microscope was analyzed using NIH Image J software.

Thermogravimetric Analysis, TGA. Thermogravimetric analysis, of samples was obtained with a TGA Q50 apparatus from TA Instruments under nitrogen flow. The heating rate was 10°C min⁻¹.

Dynamic Light Scattering, DLS. A Malvern Zeta Pals instrument operating at 173° external scattering angle was used to evaluate the size of the silica particles. Clear polystyrene squared cells free of dust were used to prepare the aqueous dispersion of silica. A volume of 1.5 mL of deionized water filtered through a 0.1 µm Millipore filter in the cell and then approximately 20-40 µL of silica sol was added. The size was calculated as an average over three measurements each consisting of ten individual 60s long runs. The temperature was kept at 25°C.

Fluorescence Microscopy. A Leica fluorescence microscope was used to record the images of the particles and the leaf. PCPs were placed inside rectangular capillaries from VitroCom.

1 *Small-angle X-ray scattering, SAXS.* Small angle X-ray scattering experiments were carried out
2 at Stanford Synchrotron Radiation Light source (SSRL), beam line 1-5. The beam is highly
3 collimated with a spot size of 300 μm x 300 μm and an approximate flux of 10^{11} photons/second.
4 The sample to detector distance was 3 meters and a Rayonix 165 CCD camera was used as the
5 detector. Samples were placed in 1 mm round glass capillaries and exposed for 1-5 seconds for
6 15 runs. For transmission correction and background subtraction an empty capillary and one
7 filled with DI water were exposed in the same manner as the sample. Data reduction was
8 performed using the Nika package for Igor Pro (version 6.36) and fitting was done using
9 SasView.

10 *Porosimetry.* The particle surface area (BET method) and pore size distributions (BJH) method
11 were determined by N_2 adsorption-desorption in a Micromeritics ASAP-2020P. The samples
12 tested were initially dried under vacuum at 200°C.

14 **RESULTS AND DISCUSSION**

15 Silica cores were prepared following modified Stöber (STEP I) and seed regrowth (STEP
16 II and STEP III) approaches,[7, 9, 34-36] as depicted in Scheme 1. Silica seeds were prepared in
17 STEP I by a sol-gel procedure using a four-fold dilution of tetraethylorthosilicate, TEOS with
18 absolute ethanol, EtOH. A layer of TEOS along with the fluorescent FITC-APS adduct was
19 added to the existing seeds in STEP II by hydrolysis/condensation. In the third and final step,
20 STEP III, TEOS diluted four times with EtOH was added in a dropwise manner to build a silica
21 layer on top of the dye and also to obtain a thicker external layer to protect the dye against
22 bleaching. Details on the preparatory steps appear in Materials and Methods. Transmission
23 electron microscopy, TEM images of particles prepared at each step are available in
24 Supplementary Content. The fluorescent silica particles were further used as supports to

construct meso/macroporous silica architectures. They will be referred to as uncoated fluorescent silica.

Scheme 1. Schematic illustration for transforming simple silica into covalently labeled fluorescent silica. Not drawn to scale.

An aqueous NaOH solution, 3mM, was chosen as the etching agent. This base concentration was selected to ensure a slow etching rate to create openings on the silica particles. Fig. 1 shows the structure of uncoated fluorescent silica subjected to etching, as seen by TEM. In these images, the particles are not stained.

Fig. 1. TEM micrographs recorded at different stages of etching the uncoated fluorescent silica cores with 3 mM NaOH: (A) day0, (B) day1, (C) day2, (D) day3 and (E) day4.

More TEM images are available in the Supplementary Content.

Before exposure to NaOH, the silica particles exhibited good size uniformity, as seen in Fig. 1A ($D = 176.5 \pm 9$ nm). They showed a tendency to pack into 2D hexagonal arrays on the TEM support. After one day of base incubation, Fig. 1B, no significant size difference was

1 observed ($D = 164 \pm 7.5$ nm). A possible indication of pore formation and/or surface roughening
2 was discernible by day2, Fig. 1C, and the particle diameter was 171 ± 15 nm. Although TEM
3 under these contrast conditions produces a projection image, the fuzzy edges of the particles at
4 this stage compared to the images at the time zero (Fig. 1A) suggests most of the etching
5 occurred at the surface. After day3, interior voids or surface craters (TEM is silent on this
6 difference) began to form, as shown in Fig. 1D. Further base action enabled the voids/craters to
7 merge into larger formations by day4, Fig. 1E. The size of the particles as determined from TEM
8 analysis is summarized in Fig. 2. Overall, the particles did not change dramatically in size after 3
9 days of base treatment when compared with the original spheres (Fig. 1A, silica day0).
10 Furthermore, the images do not suggest any change in shape. Before moving on to the effects of
11 continued base exposure, a word about TEM sizing is in order. All particles in Fig. 1A-E were
12 common objects on the grids, but only a subset of the imaged particles were counted to get an
13 idea of their size from TEM because the experimental design also relied on DLS and SAXS tools
14 which sample many times more particles than TEM under typical conditions. Additional TEM
15 images and size distribution histograms appear in Supplementary Content.

16 Continued treatment with the basic etchant appeared to cause the silica particles to swell
17 to 264 ± 11 nm on the 4th day as suggested by TEM analysis. This sudden increase may be an
18 artifact of the drying down of low-density particles on the copper grid during sampling because
19 the sizes from DLS and SAXS do not increase (see below). But the TEM images contain a
20 surprise: as illustrated in Fig. 1E, after 4 days base etching of uncoated fluorescent silica allows
21 access to a range of morphologies. Some particles have a dark-light-dark contrast profile,
22 observing from the center to the outside, while other particles are just the opposite. Most of them
23 appeared dark-light-dark on the TEM grid. Additional images of these “banded” particles appear

1 in the Supplementary Content. The appearance of ring patterns is a common artifact in many
2 forms of microscopy, including TEM. To guard against being misled, we deliberately attempted
3 to produce ring images by over-focusing and under-focusing samples of non-etched silica.
4 Images similar to Fig. 1E could not be produced in these controls. The images in Fig. 1E are
5 consistent with the notion that the base penetrates the interior, and the dye-containing zone acts
6 as a sacrificial layer which may decrease the etching rate of the more crosslinked zones. This
7 possibility is discussed later in the paper. Whatever the mechanism, etching may cause particles
8 to shrink in size or to swell (for example, if material excavated from the interior is re-deposited
9 on the exterior). Because significant size deviations were measured by TEM on day4, and
10 because TEM is always subject to sample size limitations, other techniques capable of analyzing
11 a large number of particles without sample preparation artifact were applied.

12 Dynamic light scattering was used to evaluate base-treated particle size to measure more
13 accurately the particle size over the etching period. In addition to sampling thousands of
14 particles, DLS does not present issues with particle drying-down artifacts as does TEM. Fig. 2
15 compares particle diameter variation over time from TEM and DLS (and SAXS; see below). The
16 trend observed from the DLS measurements generally agrees with the TEM results presented
17 above, and particle size does not vary significantly during etching. The large increase in diameter
18 seen from TEM results on day4 was most likely an artifact due to low and high mass density
19 distribution within the silica. In addition, the polydispersity index as determined by DLS
20 remained approximately the same, confirming that the spherical shape and uniformity of the
21 silica particles are unaffected. Based on these results it can be concluded that the slight size
22 deviations recorded by both TEM and DLS are within experimental error. The colloidal silica
23 particles preserved their shape and size over the etching period.

Fig. 2. Radius (nm) versus time (days) from dynamic light scattering, DLS (red, empty star) small-angle X-ray scattering, SAXS (blue, empty circle), transmission electron microscopy, TEM (black, empty square) and polydispersity index versus time (days), PDI from DLS (magenta, empty diamonds).

The radius from DLS is a hydrodynamic value, which could be affected by solvent draining through porous particles. The SAXS radius does not share this problem, and was obtained by fitting the scattering profiles in Fig. 3.

Fig. 3. SAXS data representing the intensity *versus* the magnitude of scattering vector, q , measured at room temperature: comparison of the scattering traces and fit as a function of etching time: (A) day0, (B) day1, (C) day2, (D) day3 and (E) day4. The experimental data are displayed as open symbols. The solid lines show the fits performed with the scattering function for monodisperse hard spheres.

1 Because the SAXS settings were selected to highlight internal structural features, the scattered
2 intensity of the fluorescent silica particles did not satisfy the Guinier approximation, $q_{min}R < 1$,
3 regardless of the etching time. Therefore evaluation of the radius of gyration radius, R_g , was not
4 possible by performing linear regression on Guinier plots [$\ln I(q)$ vs q^2]. Even though the
5 particles contain internal structural features, at least according to TEM, the next step in the
6 analysis was to fit the data to the uniform sphere form factor (see details in Supplementary
7 Content).[48] The apparent radius values were approximately the same as those from DLS and
8 (with the exception of the day4 sample) close to those from TEM, as shown in Fig. 2.

9 Turning now to discuss the fidelity of the data to the scattering function for monodisperse
10 spheres with a uniform internal structure, the measured scattering signals from all five silica
11 dispersions (day0 to day4) deviate near the nodes. The complete destructive interference
12 expected from the hard sphere model is not observed experimentally due to some combination of
13 size polydispersity, shape polydispersity, small baseline offsets and even smearing of the
14 scattering angles. Even so, some conclusions can be made. For the non-etched silica (day0), the
15 theoretical uniform sphere model fits coincides with all of the peaks and supports the appearance
16 of spherical particles from TEM images. Beginning with day1, the scattering profile deviates
17 from the model at high q . A small shoulder appears at $q \cong 0.04 \text{ \AA}^{-1}$; perhaps this is the effect of
18 surface roughening (see Fig. 1B) or the appearance of some correlated structure with a spacing at
19 $\sim 160 \text{ \AA}$ (i.e., $2\pi/0.04 \text{ \AA}^{-1}$). The same trend was observed for day2, though the shoulder is even
20 more weakly evident (perhaps coincidentally, the day2 TEM images reveal smooth surfaces
21 again). Starting with day3, the scattering profile begins to deviate overall from the monodisperse
22 uniform sphere model; the peaks simply do not align at the q -range extremities. The
23 misalignment of the fitted (manually) peaks with the experimental data for day3 and day4

particles means the uniform, smooth sphere model is no longer valid. Unfortunately, although SAXS supports the TEM observations that etching leads to internal structures, it is difficult to extract details without absolutely uniform particles and/or more sophisticated modeling. The effects of size polydispersity were explored, as described in the Supplementary Content.

Thermogravimetric analysis provided quantitative information regarding the percentage of mass loss in the temperature region where dye degradation occurs, as seen in Fig. 4.

Fig. 4. (A) TGA profiles of silica-etched particles and FITC (inset) and (B) Percentage of mass loss measured in the 300-600°C temperature range. Scan rate was 10°C·min⁻¹.

1 The inset in Fig. 4A shows that FITC dye degradation occurs mainly in 300-600°C temperature
2 range. The decomposition seen up to 200°C can be assigned to water either physically adsorbed
3 or H-bonded to the particle surface. The non-etched fluorescent silica exhibited slightly more
4 mass loss, probably due to the presence of a higher amount of dye when compared to the etched
5 particles, and also a higher level of silanol, Si—OH, groups at the surface which also decompose
6 up to 600°C.[49] For samples etched over the course of 4 days, the mass loss between 300-
7 600°C was essentially constant, as shown in Fig. 4B but lower, as percentage, than day0
8 samples. Two samples (silica day0 and silica day3) were run at higher temperature, 1000°C, to
9 test whether such constancy was recorded only at lower temperatures. Indeed Fig. 4A shows a
10 flat decomposition profile between 600-1000°C after the third day. The difference in mass loss
11 seen on day1 versus day0 may be due to the outer silica network hydrolysis to silanol groups.
12 Basic conditions can enable surface exfoliation (Fig. 1B), a process that also facilitates silica
13 pore opening. The mechanism appears in a recent review.[50] The exfoliation is possible because
14 the silica network at the surface of particles from STEP III is less compact, as explained in the
15 preceding paragraphs. Once the pores widen, the base etchant can penetrate easily into deeper
16 layers. The constant mass loss for samples etched over a period of four days suggests the silica
17 particle loses some amount of the dye adduct but, overall, preserves enough dye to remain
18 visibly fluorescent. Covalently reacting the dye in a layer of silica might also protect against its
19 rapid thermal degradation. Consequently, the TGA traces suggest that enough dye was not lost
20 and survived the accelerated etching process. The particles kept their yellowish coloration over
21 the duration of the experiment (See Supplementary Content) and fluoresced when visualized
22 with an epifluorescence microscope.

The porosimetry results for BET surface specific area, S_{BET} , [51] and pore size distribution [52] are summarized in Table 4 and Fig. 5. The data obtained for day0, Fig. 5A, day2, Fig. 5B, and day4, Fig. 5C, represent the initial, intermediate and final steps of silica etching.

Table 4. Sample Code, BET Surface Specific Area, Pore Diameter, Volume Average Pore, Range of Pore Size, Total Nitrogen Adsorbed and Total Pore Volume for Fluorescent Silica Etching

Sample Code	S_{BET} $\text{m}^2 \text{g}^{-1}$	Volume-Averaged D_{pore}^* nm	D_{pore} Range nm	Total Pore Volume $\text{cm}^3 \text{g}^{-1}$
Day0	20.5 ± 0.5	70 ± 7.0	20-200	0.19
Day2	92 ± 5.0	39 ± 3.0	30-55	0.78
Day4	24 ± 2.0	85 ± 13.0	30-300	0.27

*From integration of the distribution with respect to volume

The surface specific area increased over the first two days of base etching from 20.5 to $92 \text{ m}^2 \text{g}^{-1}$ but decreased in the last day to $24 \text{ m}^2 \text{g}^{-1}$. The volume-averaged pore diameter decreased from 70 nm (day0) to 39 nm (day2) and then increased again with a broader pore-size distribution observed on day4. The etching not only decreased the pore size on day2 but it also made the pores more uniform as reflected in the observed D_{pore} size range. The overall effect was to carve out more pore volume, which increased significantly to $0.78 \text{ cm}^3 \text{g}^{-1}$ by day2. Base-catalyzed closure of smaller pores, accompanied by further etching, ultimately reduced the pore volume to $0.27 \text{ cm}^3 \text{g}^{-1}$ by day4.

Fig. 5. Nitrogen adsorption isotherms for meso/macroporous silica particles after day0 (A), day2 (B) and day4 (C) of base etching.

The pore size distribution narrowing observed on day2 silica particles can be associated with a “focusing” effect. The base etchant hydrolyzes the silica network creating pores and, at the same time, deposits some silica oligomers on the outer parts of the sphere. This process, resembling that of ants building their mound, continues until the energy penalty associated with the newly established outer silica frameworks becomes too high. Then the hydrolyzed silica starts to fill the existing large pores, narrowing their size. The base carves the silica particle interior and redistributes the excavated silica oligomers, leading to overall particle size conservation. TEM, DLS and SAXS results confirm this interpretation. After four days of etching an increase in specific surface area and pore volume would be expected for pure silica networks,[53, 54] but, in the case of the fluorescent particles they decreased. This result underlines the importance of the dye-embedded layer to the particle’s constitution. Once the base penetrates deep into the particle interior, it hydrolyzes the less crosslinked FITC-APS network (see below). While the hydrolyzed pure silica is re-attached, the fluorescent adduct apparently solubilizes to small oligomers that depart to solution leaving behind large voids. The large empty zones created as a result of FITC-APS network removal have essentially null contribution to S_{BET}

1 and a relatively small (but observable) contribution to the pore size distribution, which becomes
2 broader (Table 4). Indeed on day4, there still exist some small pores, but S_{BET} , total amount
3 adsorbed, and pore volume decreased significantly when compared to day2. This observation is
4 consistent with pore closure and large void formation, and it is supported by the TEM analysis.

5
6 **Fig. 6.** TEM images of (A) PLYS-coated meso/macroporous fluorescent silica,
7 with no staining, show light-dark-light (blue arrow) alongside with dark-light-
8 dark particles, as well as the “struts” separating denser layers (red arrows) and (B)
9 meso/macroporous PLYS-coated fluorescent silica at lower magnification
10 (sample not stained). The green arrow points to a particle probably made from a
11 secondary nucleation event during STEP III of Scheme1.

12
13 To expand the pool of potential applications and also to learn whether a polymer shell will affect
14 how the base etches the particles, the surface of the parent fluorescent silica particles was grafted
15 with a polypeptide, poly(L-lysine), PLYS, obtained from poly(N^{ϵ} -carbobenzyloxy-L-lysine
16 through side chain deprotection (details on the preparation in Materials and Methods). Their
17 morphologies, after base treatment, are shown in Fig. 6. The etched PLYS-silica morphologies
18 closely resemble those seen for uncoated etched particles. The presence of such architectures
19 indicates first, that the etchant molecules were able to penetrate through the polypeptide shell

1 and the protective TEOS layer to create openings. Second, the presence of the voids for coated
2 fluorescent particles suggests that they formed in the same manner as for non-coated silica,
3 possibly as a consequence of faster etching of the FITC-APS (see below). The appearance of the
4 overall meso/macroporous cores demonstrates the reliability of the NaOH method to obtain
5 fluorescent meso/macroporous silica in a reproducible manner, regardless of the outer functional
6 moieties. Fig. 6 also indicates that the spherical shape of the polypeptide-coated fluorescent
7 particles core was conserved. The level of polypeptide coverage did not appear to impact the
8 structure formation. In contrast with the uncoated and fluorescent silica batch, the PLYS-coated
9 fluorescent cores tended to change slightly in size ($D = 275 \pm 18$ nm before etching versus $D =$
10 234 ± 16 nm after etching by TEM) but this is almost within experimental error. The negligible
11 effect of polypeptide shell on the formation of the meso/macroporous architecture points to the
12 APS-FITC sacrificial etching as the key to these structures.

13 Another polypeptide composite particle was tried, but without the fluorescent FITC-silica
14 layer. Poly(L-tyrosine), PTYR-coated particles with a simple silica core, when etched in the
15 same base conditions as the PLYS-coated fluorescent silica analogs, were not observed to form
16 banded structures, as seen in Fig. 7.

Fig. 7. TEM micrograph showing the core morphology of the non-fluorescent PTYR-coated silica particles (A) before and (B) after etching. Particles were not stained for shell visualization. Additional images appear in Supplementary Content.

Fig. 7A presents the appearance of the PTYR-coated silica non-fluorescent cores prior to etching ($D = 151 \pm 17$ nm by TEM). Before base treatment, the core particles had a clear and sharp surface corona, and the electron contrast suggested a uniform mass distribution. After etching, Fig. 6B, they had a significantly different morphology, similar to a sponge ($D = 136 \pm 18$ nm by TEM). Still their spherical shape was conserved. The insight obtained from etching non-fluorescent particles emphasizes the importance of the dye layer within the fluorescent silica particles.

Now finally it is appropriate to present a mechanism to explain the observed structures. The literature contains contradictory findings regarding possible pathways associated with base-induced etching of colloidal silica. Park, Kim and Park found that etching took place predominantly inside the particle,[55] while El-Toni *et al.* showed mesoporous structures formed by shell-to-core etching.[56] Liu *et al.* reported particle shrinkage in alkaline suspension,[57] yet

1 Chen *et al.*[58] and Zhao, Zhao and Han[59] showed particles that were slightly swollen after
2 alkaline treatment. The present results demonstrate that all sides of the controversy may be
3 observed. There is little evidence for overall change in size, but banded particles exhibiting light-
4 dark-light and dark-light-dark TEM contrast profiles were observed in the same batch.

5 Before acquiring their banded appearance in TEM (e.g., Fig. 1E), the particles take on a
6 “splotchy” look (e.g., Fig. 1D). In TEM as practiced here, it is not possible to tell whether the
7 structures related to the splotches are inside the particles (voids) or outside the particles (craters).
8 The following development assumes internal structures and meso/macroporous particles because
9 the FITC-silica adduct layer was placed inside by the synthetic design. When that layer is
10 present, the particles proceed to a banded structure even when coated with poly(L-lysine). When
11 the fluorescent layer is absent in poly(L-tyrosine)-coated particles, no such progression occurs.
12 Structural changes in silica without a FITC-silica layer or polypeptide coating is yet to be
13 investigated in detail. Meanwhile, a mechanism consistent with the observations is offered in
14 Scheme 2.

Scheme 2 Schematic illustration of meso/macroporous fluorescent silica formation.

Before etching, the fluorescent particles have an inner layer of condensed FITC-APS adduct sandwiched in-between an inner core and outer protective shell made of condensed TEOS. As a trifunctional silane, the FITC-APS adduct is a less potent crosslinker than the tetrafunctional TEOS. Additionally, it bears a large and slightly hydrophobic dye that can disrupt network formation during the base-catalyzed condensation that creates the particle. It is proposed that exposure to the aqueous alkaline etchant solution causes the Si-O-Si bonds from the FITC-APS network to hydrolyze to produce silanol, Si-OH, faster than the Si-O-Si bonds from the denser TEOS network. The etchant easily accesses the Si—O—Si bridges promoting the formation of

1 sparse, small voids within the fluorescent layer. Prolonged exposure to the basic environment
2 may cause the voids formed on day3 to evolve into a cave-like structure on day4 with thin struts,
3 reminiscent of stalactites and stalagmites. This etching mechanism was also used by Roca and
4 Haes to describe the behavior of gold-void-silica particles.[60] In the present interpretation, the
5 Si—O—Si bonds associated with the FITC-APS adduct are imagined to hydrolyze to form Si—
6 OH bonds at a faster rate due to a lower crosslink density when compared to the TEOS siloxanes
7 because the FITC-APS molecule has fewer arms available for crosslinking.[60]

8 It could be suggested that non-uniformity within the original, solid, spherical particles is
9 also responsible. It is known that pH influences the compactness within silica.[53, 61, 62] As
10 indicated by Scheme 1, the initial stages of synthesis starts at pH 9, but this value decreases by as
11 much as one pH unit during reaction as a result of byproducts, absorption of CO₂ and loss of
12 NH₃. It is possible then to form alternate, more compact structures when the pH is close to 9 and
13 less compact structures later in the diluted NH₄OH. During etching, at higher pH, the base can
14 selectively hydrolyze the FITC-APS layer and less compact zones within the silica network
15 while the compact areas are more resistant. If this kind of mechanism, essentially an
16 amplification of natural density fluctuations by the etching process, was dominant, we might
17 have expected to observe banded structures in the particles lacking any FITC-silica layer.

18 One of the most important qualities of the covalently labeled particles is that they remain
19 fluorescent after treatments required by their solution processing. It is additionally convenient if
20 the fluorescent signal is not greatly altered by the environment. Fig. 8A illustrates the parent
21 uncoated silica cores in the solid state. Fig. 8B shows that PCBL-coated silica (the precursor
22 material to PLYS-silica) fluoresces strongly in DMF solvent. Further, enough fluorophores
23 survived etching in basic conditions to generate meso/macroporous PLYS-silica (Fig. 6) and

1 their fluorescence signal is displayed in Fig. 8C. The particles survive processing conditions to
2 display strong fluorescence, proving the presence of the FITC dye, in widely varying
3 environments.

4
5 **Fig. 8.** Fluorescence images of (A) parent uncoated silica (day0) in solid
6 state, (B) PCBL-coated fluorescent silica dispersed in DMF, (C) PLYS-
7 coated fluorescent silica dispersed in water after etching and (D) uncoated
8 fluorescent silica particles that were taken into the vasculatory system of
9 an *Arabidopsis thaliana* leaf. The inset is a close-up of the area marked by

the white frame showing particles reaching to the plant's interior; see text for details.

Previously reported procedures for the fabrication of fluorescent mesoporous particles require supplemental steps including template presence:[63-65] after void formation, the colloid is labeled with FITC or an alternate fluorescent dye, usually by physical adsorption.[66] Additional coatings with silica are not feasible because the voids can be covered/blocked and the material loses its viability as a potential encapsulation candidate. Consequently, the dye might leak or be easily bleached. Applications such as *in vivo* imaging might thus be negatively affected because the free dye may be bleached and flushed quickly from the cell. As a result, cell visualization would be difficult due to low fluorescence contrast.[67, 68]

In comparison, the present colloid has an internal, covalently bonded layer that preserved enough fluorescence for convenient imaging, thus ensuring a longer lifetime for applications such as imaging. Fig. 8D shows the parent (day0) silica particles visualized using fluorescence imaging inside an *Arabidopsis thaliana* leaf, the model organism for a variety of biological studies.[69] The experiment exploits the natural low contrast in fluorescence imaging of *Arabidopsis thaliana*, which is explained by Littlejohn and Love.[70] The fluorescent light should arise predominantly from the excited (by ~490 nm wavelength illumination) FITC molecules covalently attached inside the silica particles. Despite their size (176 nm diameter from TEM), the fluorescent silica particles were able to flow into the mainstream of the leaf's capillary system (see Materials and Methods on sample preparation). The inset image of Fig. 7D indicates the presence of fluorescent silica in the leaf interior. The covalent labeling, combined with the ability to flow through the capillary system of a living body and penetrate to its deep

interior, are valuable properties for *in vivo* imaging. Further, the meso/macroporous particles can carry a polypeptide shell that can be loaded with a target drug.[71] Together with the meso/macroporous core, the resulting hybrid particles offer a platform to increase the drug loading and delivery.

CONCLUSIONS

A template-free approach combined with base-induced etching was used to tailor the internal architecture of simple fluorescent Stöber silica into fluorescent meso and macroporous particles. The formation of the porous particles can be explained by the accelerated hydrolysis of FITC-APS siloxane zones of the particle's network structure in the basic environment ensured by a 3mM NaOH solution. The evidence suggests this less-crosslinked FITC-APS zone evolves into local voids which finally merge to create a meso-macroporous structure. While template methods require supplemental steps, such as thermal degradation, to remove the sacrificial organic molecules, the method presented here enables the fabrication of porous particles in high yield without any additional steps. No high-temperature treatment is required, which is appealing from energy and cost perspectives. Particle etching was monitored by DLS, SAXS and TEM with good agreement. A quantitative assessment of the dye content was possible from TGA. The etched particles retain enough dye content to permit fluorescent visualization.

The method presented here is expected to be useful in a range of biological applications. The uncoated fluorescent silica are of a size that can permeate living cells for bio-imaging. The voids have the potential to carry molecules such as drugs, and the fluorescent motif can facilitate location and visualization of drug action. Further efforts are required to establish whether porous silica of smaller and larger sizes can be prepared by the approach used here, and these methods

1 supplement rather than supplant HF-based procedures.[72] While the effect of polypeptide
2 grafting density and polypeptide loading on the formation of the surface openings and the
3 interior voids remains to be studied in more detail, it was demonstrated that porous silica
4 morphology is related to the presence of the FITC-APS layer embedded within the solid silica.
5 Finally, the approach presented here is versatile; although discovered while adding fluorescent
6 dyes, other chemistries that insert a partly hydrophobic moiety by attachment of a silica
7 derivative deserve attention.

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Figure Captions

Scheme 1. Schematic illustration for transforming simple silica into covalently labeled fluorescent silica. Not drawn to scale.

Fig. 1. TEM micrographs recorded at different stages of etching the uncoated fluorescent silica cores with 3 mM NaOH: (A) day0, (B) day1, (C) day2, (D) day3 and (E) day4. More TEM images are available in the Supplementary Content.

Fig. 2. Radius (nm) versus time (days) from dynamic light scattering, DLS (red, empty star) small-angle X-ray scattering, SAXS (blue, empty circle), transmission electron microscopy, TEM (black, empty square) and polydispersity index versus time (days), PDI from DLS (magenta, empty diamonds).

Fig. 3. SAXS data representing the intensity *versus* the magnitude of scattering vector, q , measured at room temperature: comparison of the scattering traces and fit as a function of etching time: (A) day0, (B) day1, (C) day2, (D) day3 and (E) day4. The experimental data are displayed as open symbols. The solid lines show the fits performed with the scattering function for monodisperse hard spheres.

Fig. 4. (A) TGA profiles of silica-etched particles and FITC (inset) and (B) Percentage of mass loss measured in the 300-600°C temperature range. Scan rate was 10°C·min⁻¹.

Fig. 5. Nitrogen adsorption isotherms for meso- macroporous silica particles after day0 (A), day2 (B) and day4 (C) of base etching.

Fig. 6. TEM images of (A) PLYS-coated mesoporous fluorescent silica, with no staining, show light-dark-light (blue arrow) alongside with dark-light-dark particles, as well as the “struts” separating denser layers (red arrows) and (B) mesoporous PLYS-coated fluorescent silica at lower magnification (sample not stained). The green arrow points to a particle probably made from a secondary nucleation event during STEP III of Scheme1.

Fig. 7. TEM micrograph showing the core morphology of the non-fluorescent PTYR-coated silica particles (A) before and (B) after etching. Particles were not stained for shell visualization. Additional images appear in Supplementary Content.

Scheme 2 Schematic illustration of mesoporous fluorescent silica formation.

Fig. 8. Fluorescence images of (A) parent uncoated silica (day0) in solid state, (B) PCBL-coated fluorescent silica dispersed in DMF, (C) PLYS-coated fluorescent silica dispersed in water after etching and (D) uncoated fluorescent silica particles that were taken into the vasculatory system of an *Arabidopsis thaliana* leaf. The inset is a close-up

of the area marked by the white frame showing particles reaching to the plant's interior;
see text for details.

Tables

Table 1. Components of the Solution 1 and 2 used in Silica Seed Production.

Solution S1	Solution S2
1250 mL of EtOH and 100 mL of ammonium hydroxide	132.5 mL of EtOH and 33.25 mL of TEOS

Table 2. NCA Name and Ingredients Used in the Polypeptide Precursor Synthesis.

NCA Name	Solvent	NCA precursor, CBL/oBTYR			Triphosgene		
	Anh. EtOAc/ mL	Mass/ g	Molarity/ mmol	M_w / g·mol ⁻¹	Mass/ g	Molarity/ mmol	M_w / g·mol ⁻¹
CBL-NCA	150	5.021	18.0	280.32	1.8	6.0	296.75
BTY-NCA	150	4.66	18.4	271.31	1.82	6.1	296.75

Table 3. Sample, Amounts of Particles Subjected to Deprotection, Volume of Debenzylation Agent and Yields of Deprotected Particles.

Sample	Amount of the Particles/g	Volume of HBr/CH ₃ COOH/mL	Yield of deprotected particles/g
PCP PCBL	0.2	2	0.14
PCP PBTY	0.21	5	0.15

Table 4. Sample Code, BET Surface Specific Area, Pore Diameter, Volume Average Pore, Range of Pore Size, Total Nitrogen Adsorbed and Total Pore Volume for Fluorescent Silica Etching

Sample Code	S_{BET} $\text{m}^2 \text{g}^{-1}$	Volume-Averaged D_{pore^*} nm	D_{pore} Range nm	Total Pore Volume $\text{cm}^3 \text{g}^{-1}$
Day0	20.5±0.5	70±7.0	20-200	0.19
Day2	92±5.0	39±3.0	30-55	0.78
Day4	24±2.0	85±13.0	30-300	0.27

1 *From integration of the distribution with respect to volume

Graphical Abstract

Modelling the Internal Architecture of Fluorescent Silica Particles via a Template-Free Approach

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