Supporting Information

Encapsulation of Enzyme in Large Mesoporous Material with Small Mesoporous Windows

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(i) Materials

Tetraethylorthosilicate (TEOS), Cetyltrimethylammonium bromide (CTAB), 3-aminopropyltriethoxy-silane (APTES), 1, 3, 5-triisopropyl benzene (TIPB), Amino guanidine hydrochloride (AG.HCl), 4-Pentynoic acid, N-Hydroxy succinimide, Rhodamine B isothiocyanate (RBITC, mixed isomers), Trypsin and dialysis tubing cellulose membrane (cut off molecular weight 12kD) were obtained from Sigma Aldrich. Polyethelene glycol monomethyl ether (PEG, average molecular weight 1000) and 5 (6)-carboxy fluorescein-N-hydroxy succinimidyl ester were obtained from fluka. $CuSO_4$, Sodium ascorbate, triethyl amine, were obtained from Merck, India. All the chemicals used were of extra pure for biochemistry or of analytical grade and used as received. 3-azido-propyltriethoxysilane (AzPTES)[1] and tris(3-hydroxypropyltriazolylmethyl)amine (THPTA)[2] were prepared as reported earlier. For all the experiments de-ionized water was used.

(ii) Synthesis of *N*-(4-Pentynoyloxy)succinimide

N-(4-Pentynoyloxy)succinimide was synthesized by following the procedure reported by Galibert et al.[3] To a stirred solution of pent-4-ynoic acid (0.5 g, 5.1 mmol) and *N*-hydroxysuccinimide (0.6 g, 5.1 mmol) in ethyl acetate/dioxane (60 mL, 1:1) at 0 °C was added DCC (1.05 g, 5.1 mmol). The resulting mixture was stirred at room temperature for 6 h. The solid of DCU formed was filtered off and the filtrate concentrated under reduced pressure. The obtained residue was dissolved in ethyl acetate (200 mL), and the solution was washed with 5% aqueous NaHCO3 (2 x 50 mL), water (50 mL), and brine (50 mL). The organic layer was separated, dried over anhydrous Na_2SO_4, filtered and concentrated. Recrystallization from dichloromethane/n-pentane to give *N*-(4-Pentynoyloxy)succinimide as white solid which was used without further purification (yield 0.83 g). ^{1}H NMR (200.13 MHz, $CDCl_3$): δ = 2.04 (1H, t), 2.59 (2H, td), 2.83 (4H, s), 2.86 (2H, t); ^{13}C NMR (50.32 MHz, $CDCl_3$): δ = 14.04, 25.53, 30.27, 69.99, 80.82, 166.98, 168.87.

(iii) Synthesis of fluorescein labeled trypsin

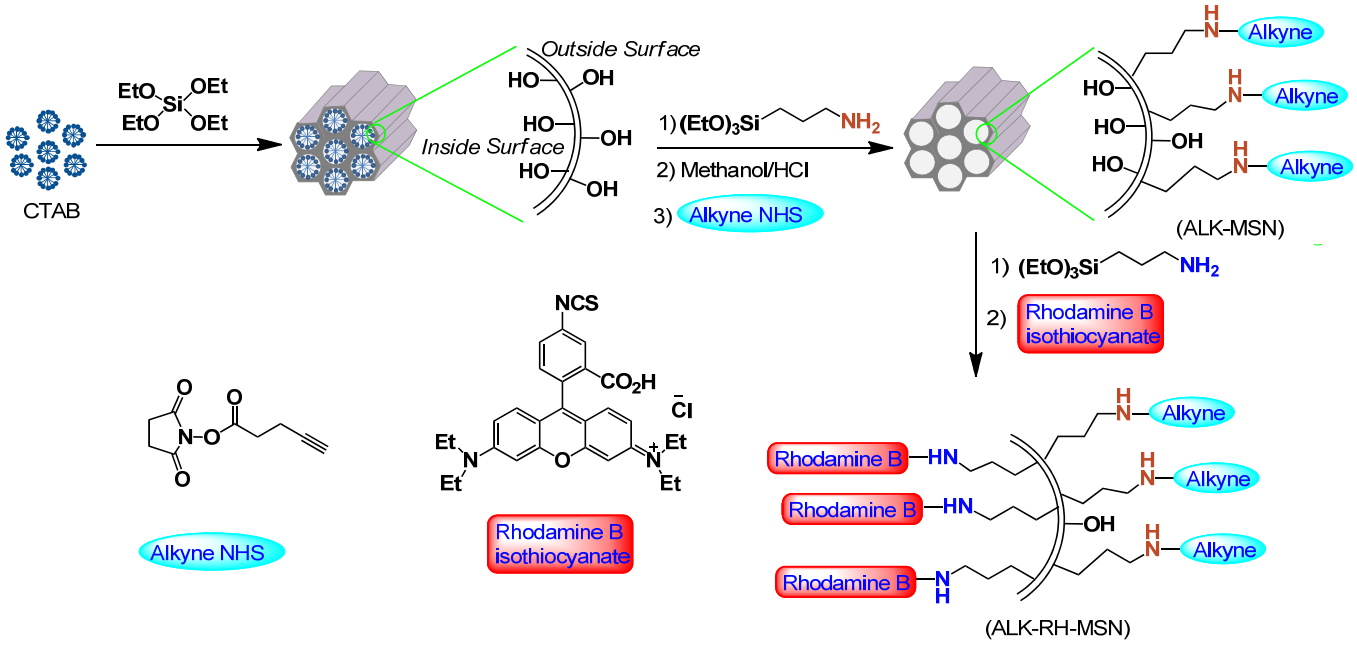
10 mg of trypsin was added to 10 mL of phosphate buffer (50 mM, pH 7.4) containing 0.4 mg of 5 (6)-carboxy fluorescein-N-hydroxy succinimidyle ester and stirred at 4 °C for 12 h. After 12 h the solution was dialyzed using cellulose membrane (cut off molecular weight 12kD) in 10 mM ammonium bicarbonate buffer (4 x 500 mL). Finally the solution was lypholized to obtain fluorescein labeled trypsin in solid form.

(iv) Synthesis of Azide functionalized Spherical SBA-15

Spherical SBA-15 mesoporous material was prepared by following the procedure reported by Katiyar et al. with slight modification using 1, 3, 5-triisopropyl benzene (TIPB) as swelling agent.[4] In a typical batch process triblock copolymer P123 (3 g) was dissolved in 1.5 M HCl (60 ml) using a mechanical stirrer. To this solution a mixture of CTAB (0.6 g) and TIPB (0.517g) in 25 mL of de-ionized water and 20 mL absolute ethanol were added. The resulting surfactant solution was stirred vigorously at 35 °C for 15 minutes and then TEOS (10 mL) was added drop by drop and stirred vigorously at 35 °C for 45

minutes. The resulting mixture was transferred into an air tight metallic reactor lined with teflon and subjected to hydrothermal synthesis at 75 °C. for 10 h and then aging at 125 °C for 48 h. After 48 h cooled to room temperature, the white precipitate was recovered by filtration and washed with copious amount of water until neutral to pH paper. The white solid was air dried and template was removed by calcination at 550 °C for 6 h at a slow heating rate of 1 °C per minute (Yield 2.45 g). This calcined SBA-15 was denoted as CAL-SBA.

To a suspension of 1 g of CAL-SBA in 100 mL of dry toluene, AzPTES (0.247 g, 1 mmol) and triethyl amine (0.02 g, 0.1 mmol) were added, and the mixture was stirred for 18 h at 80°C under nitrogen atmosphere. After the completion of reaction, the contents were cooled, filtered and washed with toluene until it became free from AzPTES. The sample was then dried at 80°C for 8h in a vacuum oven and preserved under argon atmosphere for further use. Yield: ~1.05 g. This material was referred as AZP-SBA. Elemental analysis : C, 2.23; H, 0.45; N, 2.3%



Scheme S1. Synthesis of outer surface alkyne functionalized and inner surface rhodamine B functionalized MSNs

(v) Synthesis of Alkyne functionalized MSN

Selectively outer surface alkyne functionalized MSN was synthesized in two steps by following procedures reported in literature with slight modifications (scheme S1).[5, 6, 7] In a typical batch synthesis, CTAB (1 g, 2.744 mmol) was dissolved in 480 mL of water and 2M aqueous NaOH (3.5 mL, 7 mmol). The mixture was stirred thoroughly at 600 rpm for 30 min at 80 °C to dissolve the surfactant completely. To this clear solution, TEOS (4.75g, 22.83 mmol) was injected rapidly. A white precipitate was observed within 1-2 min after the addition was completed. The resultant reaction mixture was allowed to stir at 600 rpm for 2 h at 80 °C . The hot contents were then filtered and the white residue was washed with copious amounts of water and methanol and dried under vacuum at 100 °C over night (yield ~1.7 g). This as-synthesized MSNs was denoted as AS-MSN

1 g of AS-MSN was suspended in 200 mL of dry toluene by sonication for 10 minutes. To this APTES (0.179 g, 1 mmol) was added, and the mixture was stirred for 18 h at 80°C under nitrogen atmosphere. After the completion of reaction, the contents were cooled, filtered and washed with toluene until it became free from APTES. The sample was then dried at 100°C for 8h in a vacuum oven The template was extracted by stirring the as-synthesized sample (1 g) in 200 mL methanol and 2 ml concentrated hydrochloric acid at 60 °C for 6 hr. The resulting template removed solid product, was filtered and washed with methanol (100 mL) and 1% triethyl amine in methanol (50 mL). Finally, again washed with methanol (50) then dried under vacuum at 100 °C over night (yield ~0.64 g). This material will be referred as NH_2-MSN. Elemental analysis : C, 3.1; H, 0.88; N, 1.1%

0.5 g of NH_2-MSN was suspended in 100 mL of dry DCM by sonication for 10 minutes. To this *N*-(4-Pentynoyloxy)succinimide (0.225 g, 2.3 mmol) was added, and the mixture was stirred for 12 h at room temperature. After completion of reaction, the contents were filtered and washed with DCM. The sample was then dried at 80°C for 3 h in a vacuum oven (yield ~0.51 g). This material was referred as ALK-MSN. Formation of alkyne fuctionalized MSN (ALK-MSN) was confirmed by simple ninhydrin and $KMnO_4$ tests and ^{13}C CPMAS NMR spectroscopy (Fig. S6 (b)). In ninhydrin test, ALK-MSN gave no blue color to the solution, while NH_2-MSN gave intense blue color, which shows absence of primary

amines in ALK-MSN, due to formation of amide linkage. While in $KMnO_4$ test, ALK-MSN showed quick de-colorization of dilute alkaline $KMnO_4$ solution in comparison to slow de-colorization by NH_2-MSN. Quick de-colorization by ALK-MSN was due to presence of easily oxidisable C-C triple bond.

(vi) Synthesis of outer surface alkyne functionalized and inner surface rhodamine functionalized MSN

0.1 g of ALK-MSN was suspended in 20 mL of dry toluene by sonication for 10 minutes. To this APTES (0.018 g, 0.1 mmol) was added, and the mixture was stirred for 18 h at 80°C under nitrogen atmosphere. After the completion of reaction, the contents were cooled, filtered and washed with toluene until it became free from APTES. The sample was then dried at 100°C for 8h in a vacuum oven (yield ~0.1 g). This material was referred as ALK-NH_2-MSN.

0.02 g of ALK-NH_2-MSN was suspended in 5 mL of dry DCM by sonication for 10 minutes. To this rhodamine B isothiocyanate (RBITC, 2 mg, 3.7 μmol) was added, and the mixture was stirred for 12 h at room temperature. After completion of reaction, the contents were filtered and washed vigorously with DCM, methanol and phosphate buffer (100 mM, pH 7) to remove unreacted RBITC. The sample was then dried at 80°C for 3 h in a vacuum oven. This material was referred as ALK-RH-MSN.

(vii) Encapsulation of trypsin in Spherical SBA-15 using MSN

For the encapsulation of trypsin in spherical SBA-15 using MSN via Cu(I) catalyzed azide-alkyne cycloaddition reaction (CuAAC), first trypsin was adsorbed on the azide functionalized SBA-15 (AZP-SBA) in phosphate buffer (100 mM, pH 7) at 4 °C, and then incubated with the alkyne functionalized MSN, THPTA (2.5 equivalent), $CuSO_4$ (0.5 equivalent), AG.HCl (4 equivalent) and sodium ascorbate (4 equivalent). In a typical trypsin encapsulation click reaction, AZP-SBA (12 mg, 7.2 μmol of azide) in 1.1 mL, 100 mM phosphate buffer was freeze pump thawed thrice and then to this 0.6 mg of trypsin was added under inert atmosphere and stirred at 4 °C for 12 h to adsorb trypsin in the pores of AZP-SBA. After 12 h, centrifuged and washed once with 1mL, 100 mM freeze pump thawed phosphate buffer.

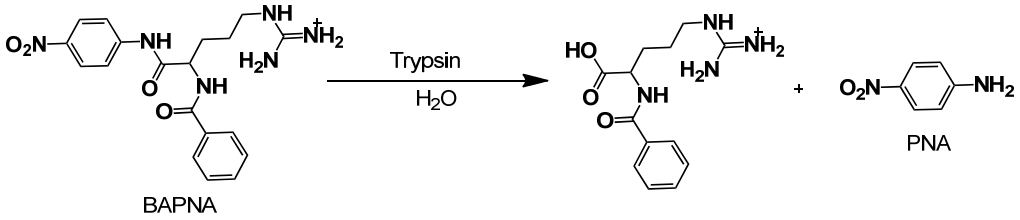
Centrifugate and washing were collected together and absorbance at 280 nm noted to determine amount to trypsin present in solution. Residue was redispersed in the 1 mL phosphate buffer. To this a mixture of ALK-MSN (6 mg) in 1mL, 100 mM phosphate buffer containing THPTA (7.8 mg, 18 μmol), $CuSO_4$ (0.9 mg, 3.6 μmol), AG.HCl (3.2 mg, 28.8 μmol) freeze pump thawed thrice was added. Then sodium ascorbate (5.7 mg, 28.8 μmol) was added under inert atmosphere and the mixture was stirred for 24 h. After completion of reaction, the reaction mixture was centrifuged and the residue was washed with 10% PEG solution in 50 mM, pH 8 tris buffer in order to leach out physically adsorbed trypsin on the outer surface and then twice with 50 mM, pH 8 tris buffer.[8] This material will be referred as Trypsin-SBA-MSN. The amount of trypsin encapsulated in Trypsin-SBA-MSN was determined by TGA (Fig. S7). The residue was preserved at 4 °C and further used for activity determination of trypsin using BAPNA in 50 mM, pH 8 tris buffer.

For the comparison of activity of trypsin, two control experiments were carried out by following the above procedure. In one of the control experiment, after adsorption of trypsin on AZP-SBA only ALK-MSN was added and all other click reaction reagents were excluded, as a result, trypsin was only physically adsorbed on surface. Finally this material was centrifuged and quickly once washed with 1 mL phosphate buffer. Centrifugate and washing were collected together and absorbance at 280 nm noted to determine of amount of trypsin remained unadsorbed. This material was referred as PHY-SBA-MSN. In second control experiment, PHY-SBA-MSN was treated with 1.5 mL of 10% PEG solution in tris buffer (50 mM, pH 8) for 1 h on rotaspin in order to study the leaching of the adsorbed trypsin. Finally this material was centrifuged and the amount of trypsin leached out with PEG solution was estimated by reading the absorbance at 280 nm in UV-Vis spectrophotometer. This material was referred as PEG-SBA-MSN.

Similarly other click reactions were carried out as described above for the purpose of characterization of hierarchical material, one click reaction was carried out with AZP-SBA and ALK-MSN without adsorption of trypsin. After completion of reaction, the reaction mixture was centrifuged and the residue was first washed twice with phosphate buffer and then sequentially washed with 10 mM

N,N-diethyldithiocarbamate sodium solution in 100 mM phospate buffer and acetone respectively.[1, 7] The last two washings were repeated thrice. Finally, the residue obtained was dried at 80°C in vacuum oven for 8 h. This material will be referred as CLICK-SBA-MSN. Another click reaction carried out using AZP-SBA and rhodamine B labeled MSNs (ALK-RH-MSN).

(viii) Determination of activity of trypsin



Scheme S2. Hydrolysis of BAPNA by trypsin

Preparation of 2 mM BAPNA solution: 22 mg of BAPNA dissolved in 0.4 mL of DMSO and diluted to 25 mL.

Activity Determination: 1.5 mL of BAPNA solution (2 mM) was added to the residue containing encapsulated enzyme in a 2 mL eppendorf tube and rotated on a rotator at 30 rpm for 20 minutes. At 20th minute solution was centrifuged and quickly washed with tris buffer (50 mM, pH 8) three times (1 x 1.5 mL and then 2 x 1 mL). Centrifugate and washings were collected together (total volume ~5 mL) and absorbance was noted at 405 nm. Trypsin encapsulated solid was used for the next cycle. The activity of the trypsin was calculated as micromoles of paranitroaniline (PNA) formed per gram of trypsin per minute.

(ix) Characterization techniques

Powder X-ray diffraction of all the samples was carried out in a PANalytical X'pert Pro dual goniometer diffractometer. A proportional counter detector was used for low angle experiments and an X'celerator solid state detector was employed in the low angle experiments. The radiation used was Cu Kα (1.5418 Å) with a Ni filter and the data collection was carried out using a flat holder in Bragg–Brentano geometry (0.5 to 10°; 0.2° min^{-1}). Care was taken to avoid sample displacement effects. SEM images were obtained on Leica Stereoscan 440 microscope. HR-TEM images were taken on a FEI Technai F30 operating at 300 kV with FEG. The samples were prepared by dispersing a large number of solid particles in isopropanol by sonication, and dropping the resulting suspension on a copper grid of 400 mesh and allowed to dry in air. Nitrogen adsorption and desorption studies were carried out using Quantachrome instrument. Samples were preheated at 100°C for 18 hours in the vacuum line. Multi point BET surface area was obtained from adsorption isotherm from P/P_0 0.1-0.3. Pore size distributions were calculated from adsorption isotherm using the BJH method. Semi-quantitative FT-IR spectra were recorded on Perkin Elmer FT-IR spectrum GX instrument by making KBr pellets. Pellets were prepared by mixing 3 mg of sample with 97 mg of KBr. Yields for CuAAC reactions were calculated from corrected area under the curve characteristic for the azide stretch at ~2100 cm^{-1}. UV-Vis experiments were carried out on Perkin Elmer PL Lambda 950 spectrophotometer using 1 mL cuvettes with 10 mm path length. ^{13}C Cross Polarization Magic Angle Spinning (CPMAS) NMR experiments were carried out on a Bruker AVANCE 300 wide bore spectrometer equipped with a superconducting magnet with a field of 7.1Tesla operating at 75.4MHz. The samples were packed into a 4mm zirconia rotor and loaded into a 4mm BL MAS probe and spun about the magic angle (54.74) at 10KHz using a standard ramp-CP pulse sequence was used for the experiment. The RF-powers was 60kHz ^{13}C CPMAS experiments. The contact times was 3ms for ^{13}C CPMAS experiments. All the chemical shifts were referenced to TMS. Typically 10,000 to 25,000 scans with a recycle delay of 3s were collected depending on the sensitivity of the sample. Confocal laser scanning microscopy (CLSM) images were taken with Carl Zeiss confocal system equipped with a 20x objective. Optical slices in the center of the particle were selected.

Thermogravimetric analysis (TGA) of the silica nanoparticles were carried out using a TA Instrument SDT Q600 analyzer between 100 and 800°C in air (flow 25 ml min^{-1}) at a heating rate of 5° min^{-1}. All samples were stirred in water overnight, centrifuged and dried under vacuum at 80°C overnight prior to TGA runs. The amount of trypsin encapsulated on the silica surface was determined by TGA using the following equation[7]:

$$\text{Trypsin encapsulated } (\mu\text{mol/g}) = \frac{\left(\frac{W_{\text{Trypsin-SBA-MSN(150-750)}}}{100 - W_{\text{Trypsin-SBA-MSN(150-750)}}} \times 100\right) - \left(\frac{W_{\text{CLICK-SBA-MSN(150-750)}}}{100 - W_{\text{CLICK-SBA-MSN(150-750)}}} \times 100\right)}{M \times 100} \times 10^6$$

where $W_{\text{Trypsin-SBA-MSN(150-750)}}$ is the weight loss between 150^0C and 750^0C corresponding to the decomposition of the organic substance from Trypsin-SBA-MSN corrected from the thermal degradation**,** while $W_{\text{CLICK-SBA-MSN(150-750)}}$ represents the weight loss between 150^0C and 750^0C from CLICK-SBA-MSN. M is the molecular weight of the decomposed organic substance.

(x) Figures S1-S12

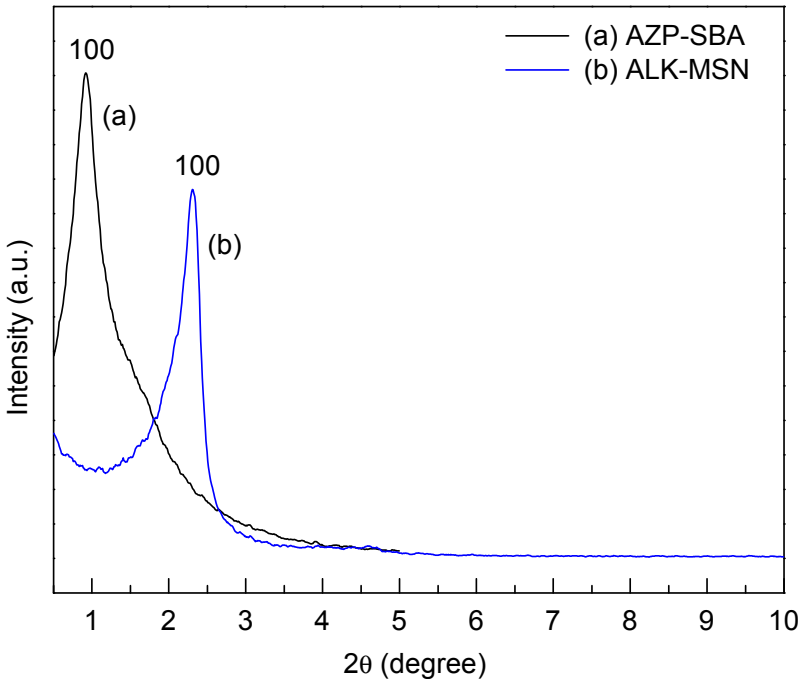


Figure S1. Powder XRD patterns of various silica materials: (a) Powder XRD pattern of AZP-SBA shows characteristic high intensity 100 peak at 2θ ~0.91° indicating formation of mesoporous structure. Absence of higher order 110 and 200 peaks indicates lack of long range order in mesopores, as observed and described by Ma et al.[9] (b) Powder XRD pattern of ALK-MSN shows characteristic high intensity 100 peak at 2θ ~2.3° indicating that well-ordered two-dimensional hexagonal mesoporous channels were formed.

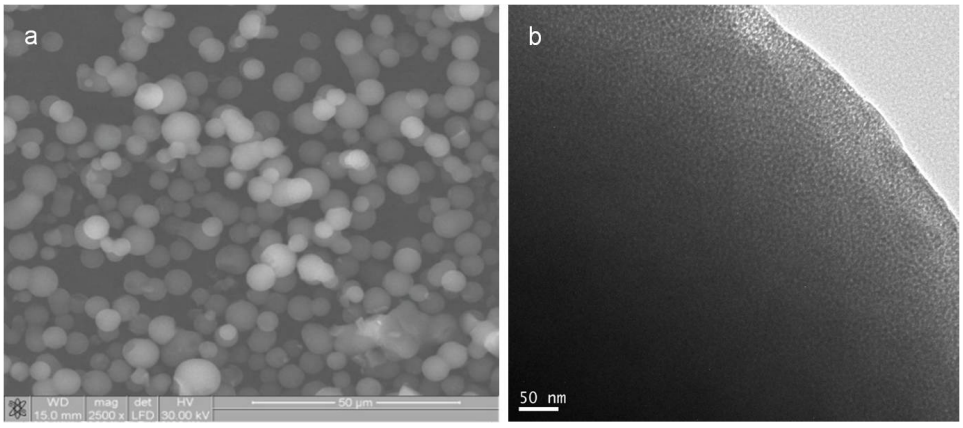


Figure S2. (a) SEM and (b) TEM images of AZP-SBA showed formation of spherical morphology particles with particle size of 5-8 μm and disordered mesoporous structure.[9]

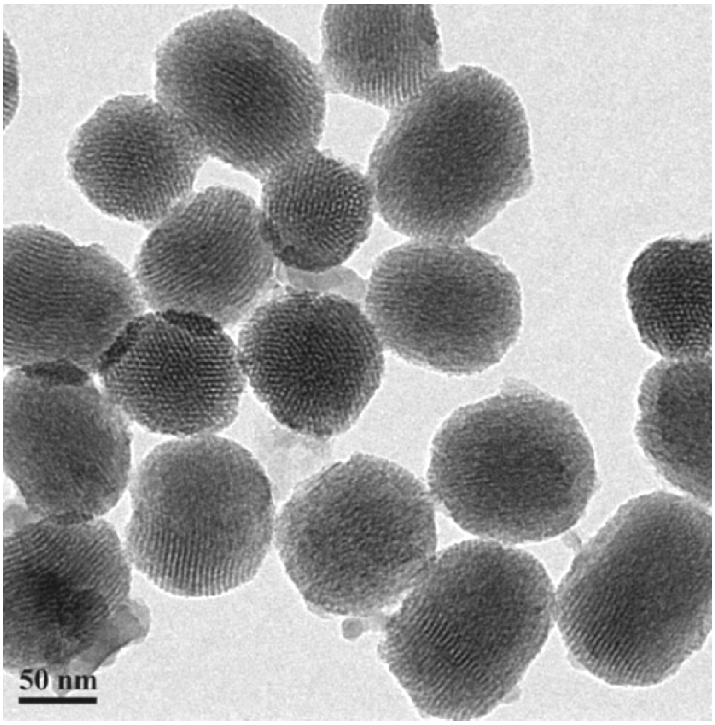


Figure S3. TEM of ALK-MSN showed formation of well-ordered two-dimensional hexagonal mesoporous particles with a spherical morphology having particle size of 90±10 nm

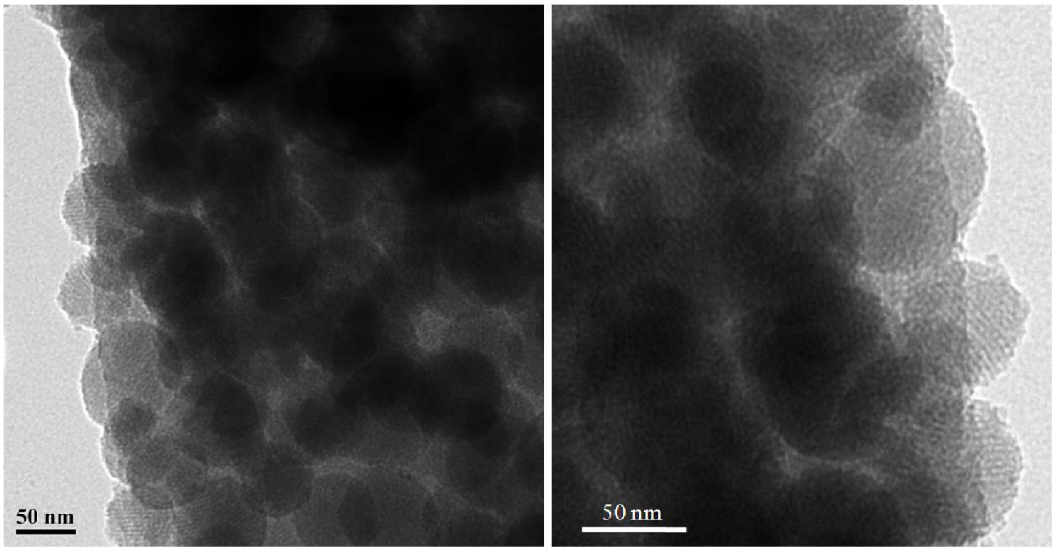


Figure S4. TEM images of Trypsin-SBA-MSN showing formation of hierarchical mesoporous structure

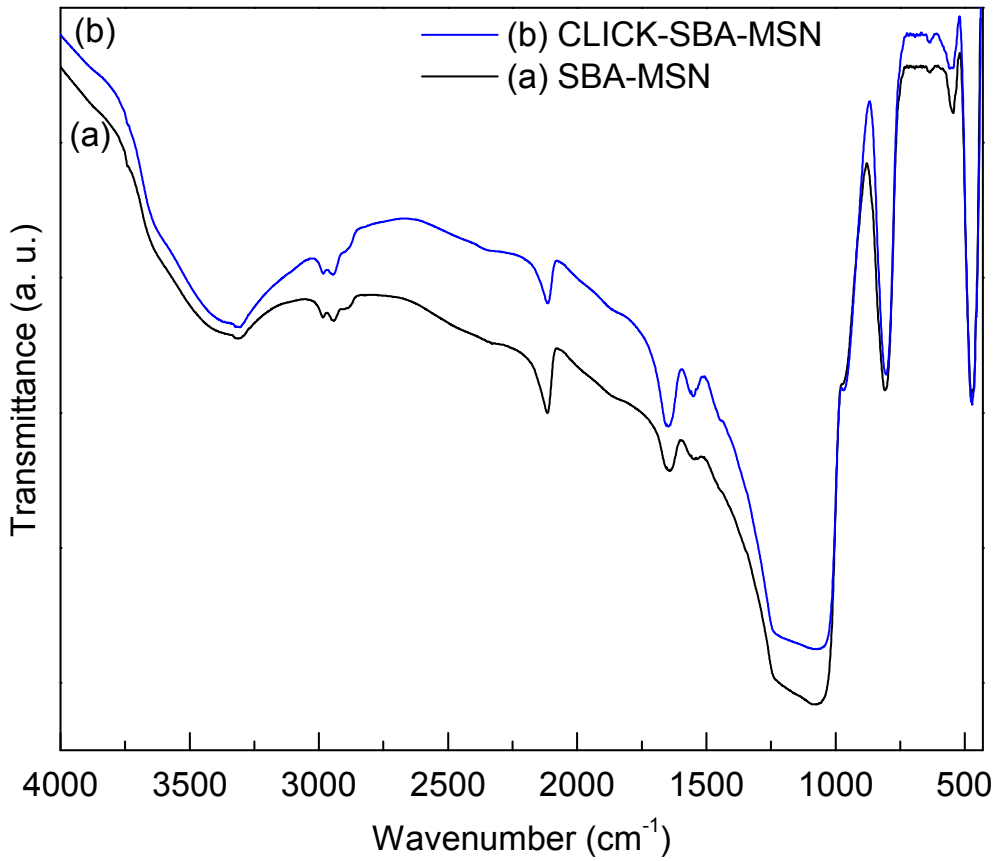


Figure S5. FT-IR spectra of (a) SBA-MSN display an absorbance at ~2100 cm^{-1} which is characteristic stretching vibration of any organic azide (N_3). SBA-MSN is a physical mixture of AZP-SBA and ALK-MSN in the same ratio used for CuAAC reaction, this sample is used as a control to compare the intensity with CLICK-SBA-MSN for semi-quantitative estimation of the conversion of the azide to triazole upon CuAAC (b) CLICK-SBA-MSN: FT-IR spectroscopy shows about 25% decrease in the integrated intensity of $\nu_{as}(N_3)$ at 2100 cm^{-1} in comparison to the sample SBA-MSN

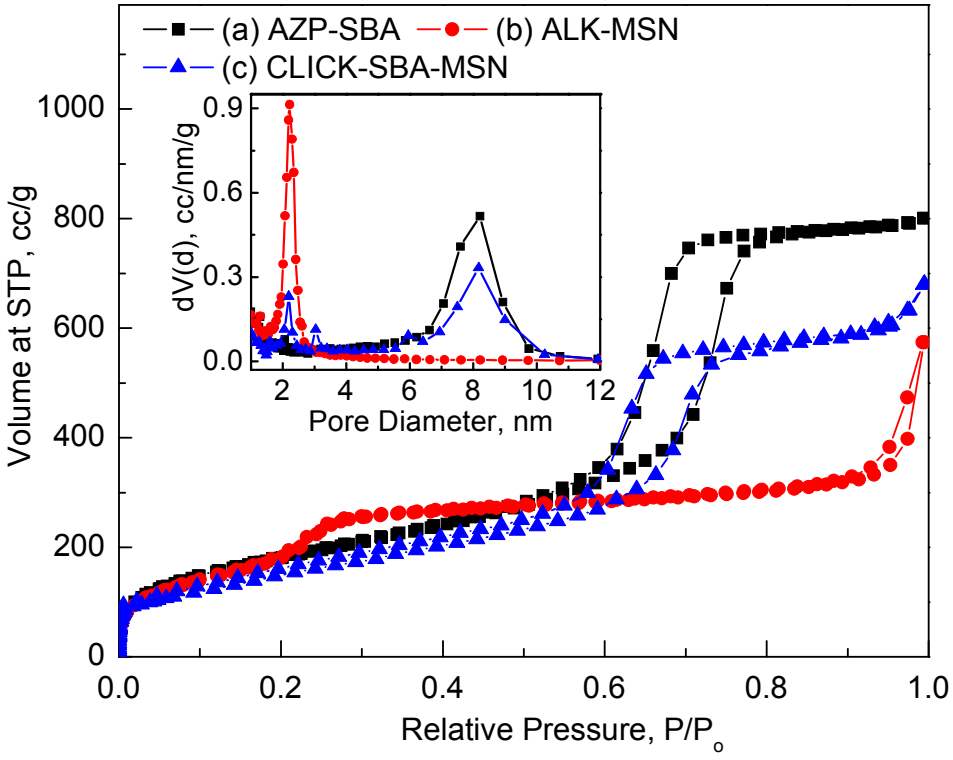


Figure S6. Nitrogen adsorption-desorption isotherms of (a) AZP-SBA, (b) ALK-MSN and (c) CLICK-SBA-MSN: Nitrogen adsorption-desorption studies of all the samples showed type IV isotherm, characteristic of mesoporous materials. The BJH pore-size distribution (PSD) analysis shows very narrow PSD. Physical properties of both MSN materials are listed in table S1. These values are consistent with the other organo-functionalized mesoporous silica materials reported earlier.[4-7] BJH pore size distribution of CLICK-SBA-MSN shows presence of hierarchical pore structure having pore sizes 8.18 nm and 2.2 nm consistent with pore sizes of parent mesoporous materials used for the synthesis of hierarchical mesoporous material.

Table S1. Physical properties of various mesoporous silica materials

Sample Name	Multi point BET (m^2/g)	Pore Diameter (nm)	Pore Volume (cm^3/g)
AZP-SBA	666	8.2	1.24
ALK-MSN	587	2.2	0.84
CLICK-SBA-MSN	542	8.18 and 2.2	0.96

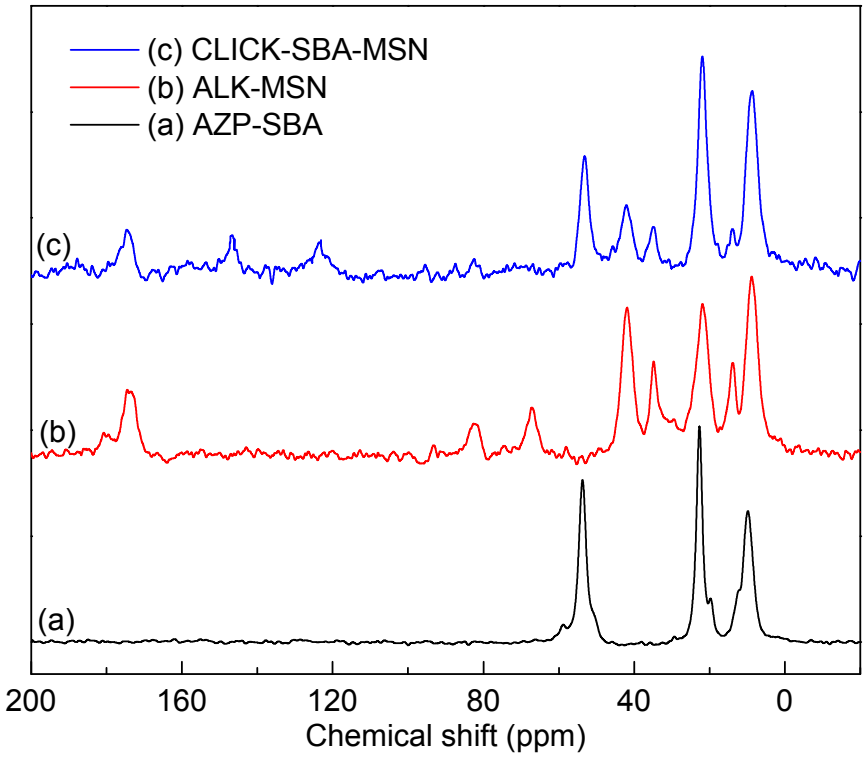


Figure S7. ^{13}C CP-MAS spectra of various silica materials: (a) three peaks at ~9 ppm, 23 ppm, 54 ppm represent the three C-atoms of the azido-propyl chain of AZP-SBA. (b) peaks at 67 ppm and 83 ppm represent acetylene C-atoms in ALK-MSN where as peak at ~ 175 ppm is due to presence of amide carbonyl carbon atom. All other peaks of aliphatic C-atoms are also present in the region 9 ppm to 42 ppm. (c) two new peaks at 124 ppm and 147 ppm in CLICK-SBA-MSN material arised by the formation of triazole in CuAAC reaction all other peaks due to presence of other C-atoms were also observed.

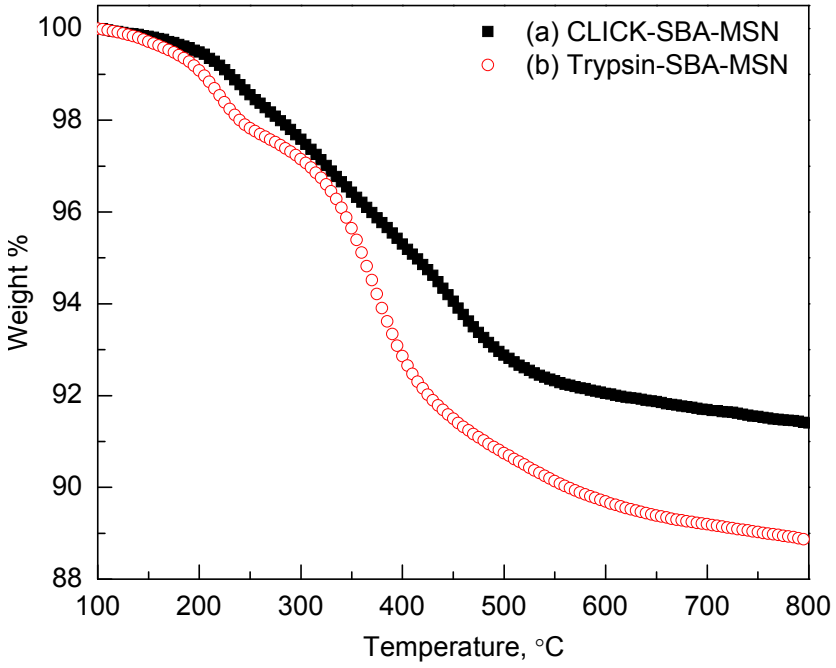


Figure S8. TGA graphs of (a) CLICK-SBA-MSN (b) Trypsin-SBA-MSN

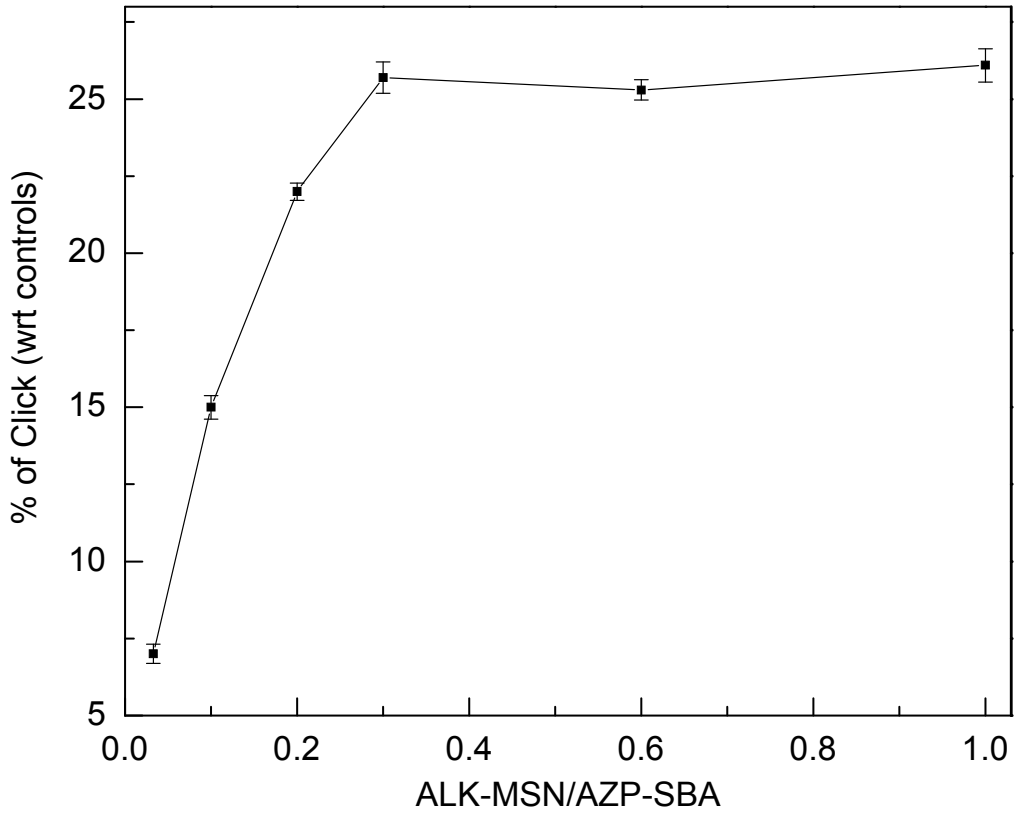


Figure S9. Loading curve for AZP-SBA and ALK-MSN: In order to find out optimum ratio of AZP-SBA to ALK-MSN for the formation of hierarchical mesoporous material. CuAAC reaction between AZP-SBA and ALK-MSN with various ratios as presented in the graph. Percentage of click reaction was estimated by FT-IR spectroscopy by comparing the decrease in the integrated intensity of $\nu_{as}(N_3)$ at 2100 cm^{-1} with respect to control samples without click reaction prepared with same ratio of AZP-SBA and ALK-MSN.

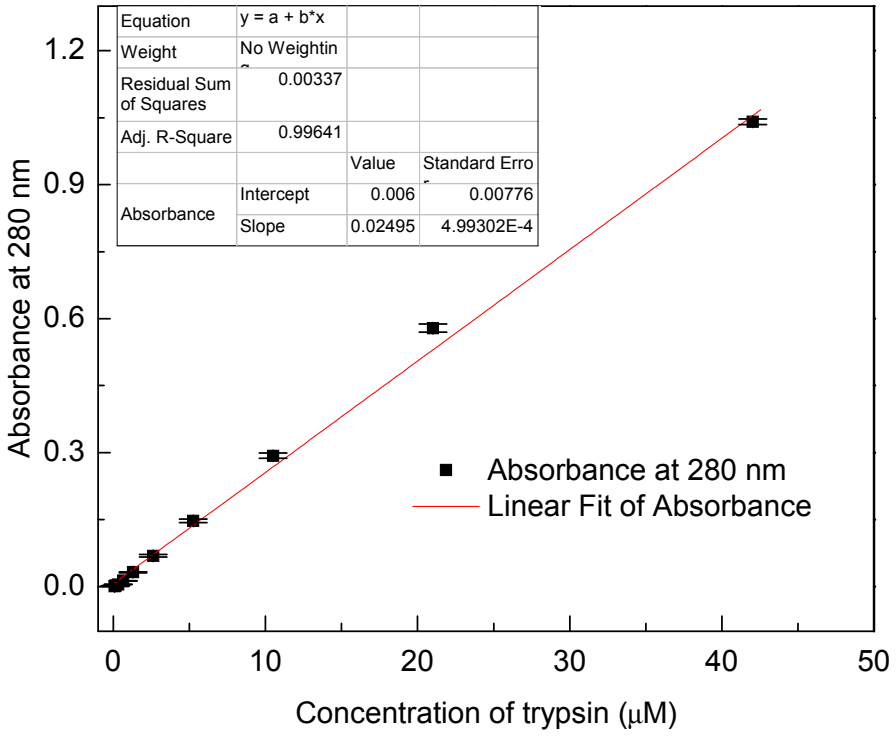


Figure S10. Calibration curve for tyrpsin in 100 mM, pH 7 phosphate buffer

Amount of trypsin immobilized on PHY-SBA-MSN was calculated according to the equation

$y = a + b*x$ where $a = 0.006 \pm 0.0078$, $b = 0.02495 \pm 0.000499$ and $R^2 = 0.9964$ from Figure S10.

Total volume	Absorbance at 280 nm	Trypsin present in 2.1 mL (μmol)	% of trypsin immobilization	% of trypsin present in solution
2.1 mL	0.04339	3.15×10^{-3}	88	12

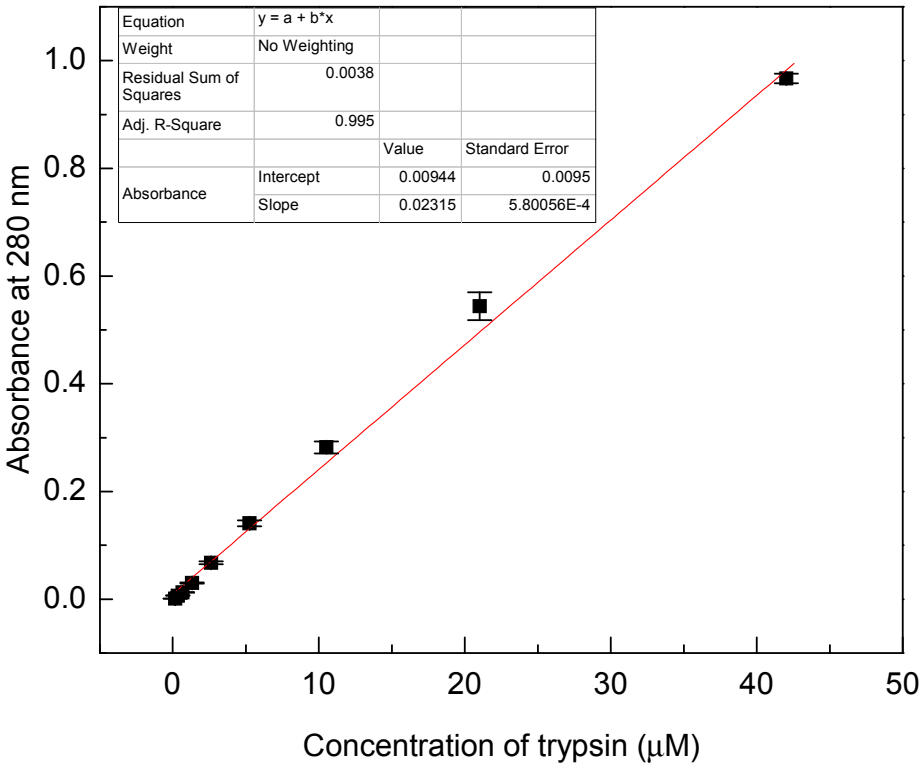


Figure S11. Calibration curve for tyrpsin in 10% PEG solution prepared in 50 mM, pH 8 tris buffer

Amount of trypsin leached from PEG-SBA-MSN during PEG solution treatment was calculated according to the equation y = a + b*x where a = 0.00944 ± 0.0095, b = 0.02315 ± 0.00058 and R^2 = 0.995 from Figure S11.

Total volume	Absorbance at 280 nm	Trypsin present in 1.5 mL (µmol)	% of trypsin leached (wrt 0.51 mg in AZP-SBA)
1.5 mL	0.305	0.0191	89

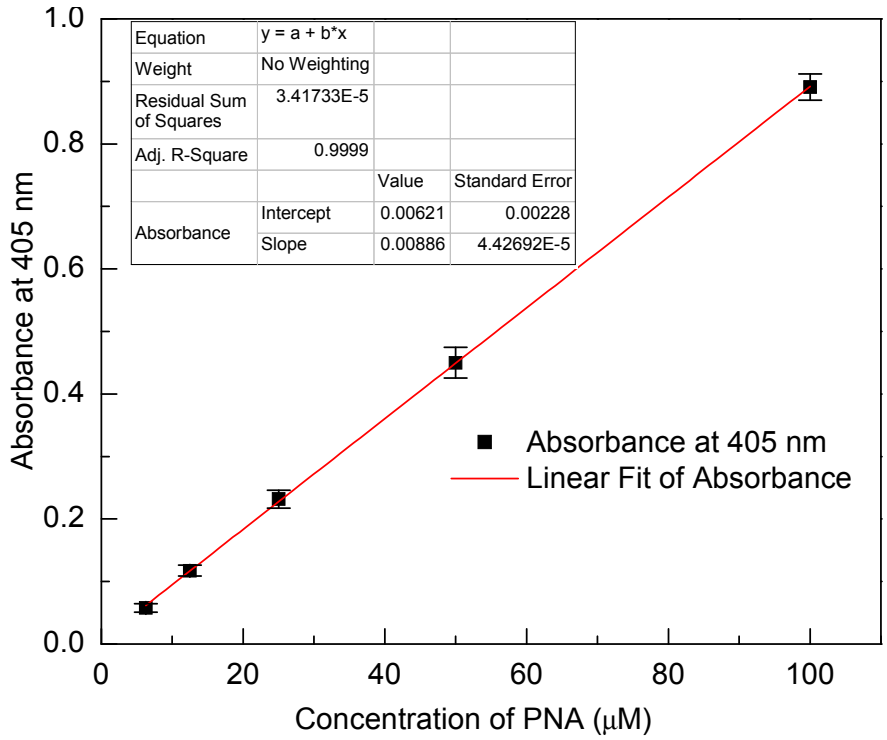
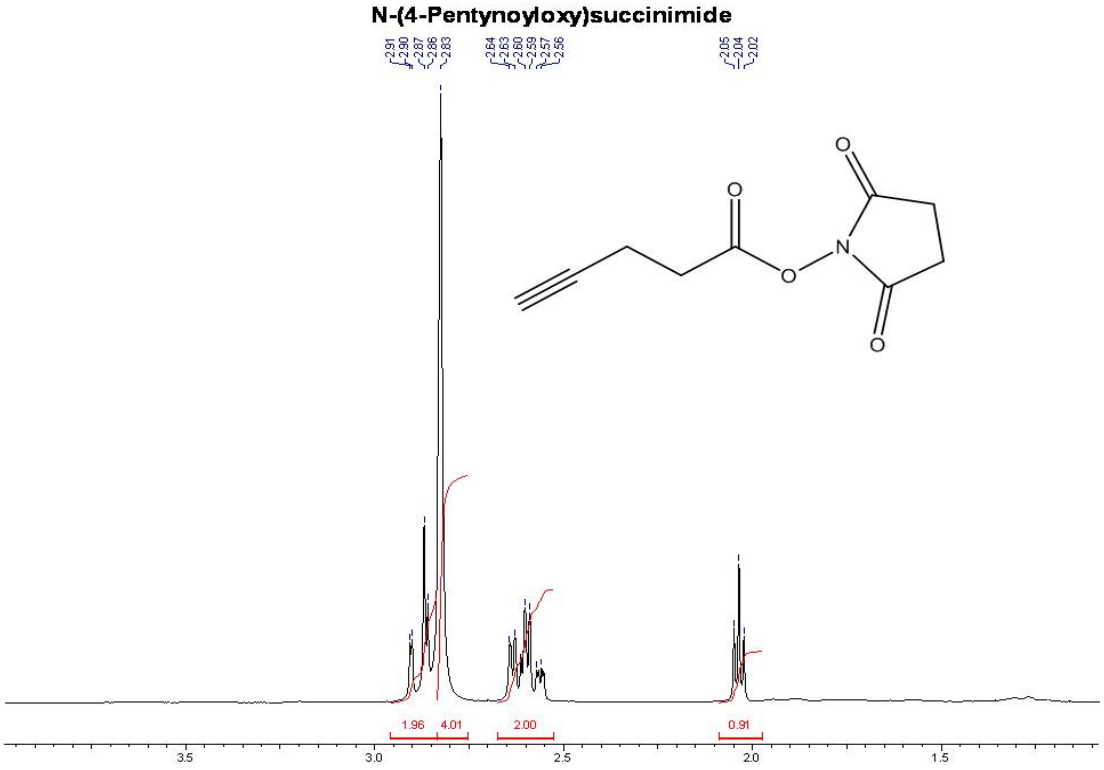
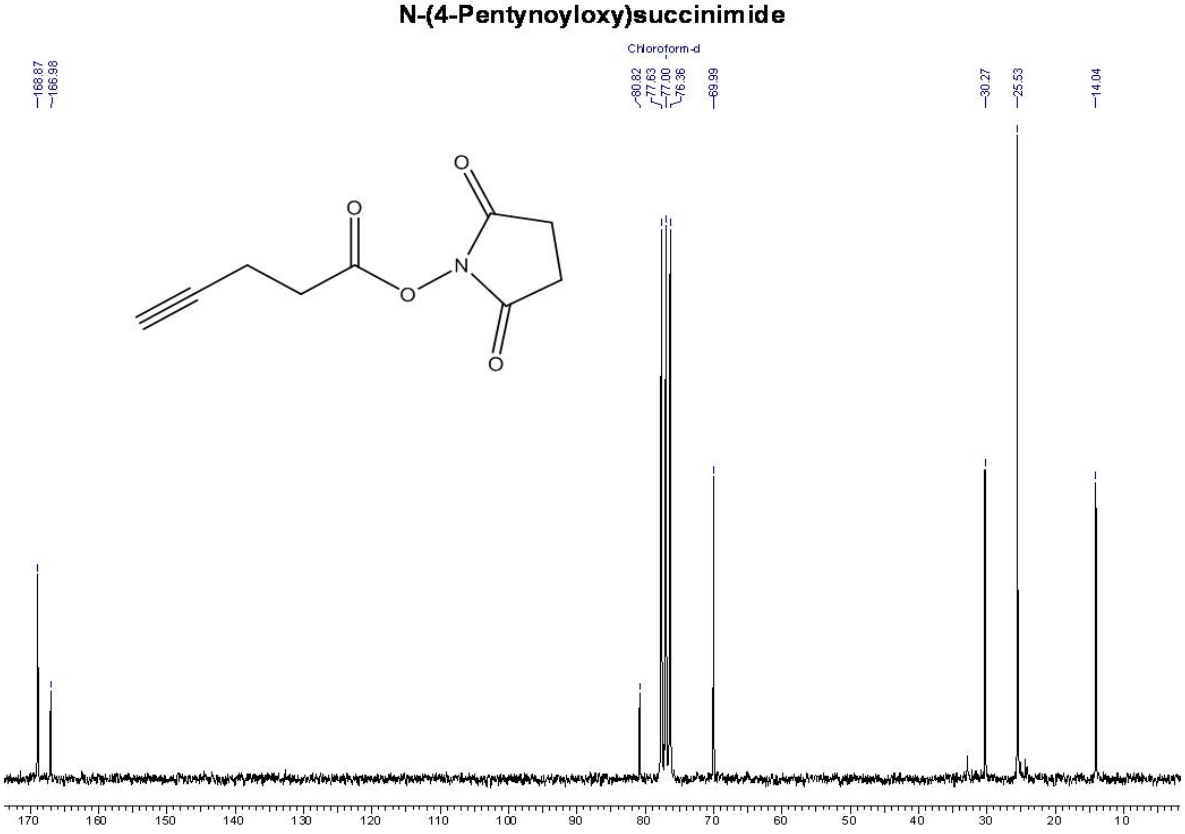


Figure S12. Calibration curve for p-nitroaniline (PNA) in 50 mM, pH 8 tris buffer.

(xi) ^{1}H-NMR and ^{13}C-NMR Characterization of N-(4-Pentynoyloxy)succinimide





(xii) References

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