# Click chemistry for high-density biofunctionalization of mesoporous silica

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# SUPPORTING INFORMATION

## 1. Experimental section

**1.1 Reagents.** Tetraethyl orthosilicate (TEOS, Fluka, >98%), cetyltrimethylammonium bromide (CTAB, Aldrich, 95%), (3-chloropropyl)trimethoxysilane (CTMS, Fluka, 95 %), mesitylene (Aldrich, 98%), sodium azide (Fluka, 99 %), 4-pentynoic acid (Fluka, 97%), N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC, Fluka, 97%), trypsin from bovine pancreas (Sigma, lyophilized, essentially salt-free, TPCK treated), $N_{\alpha}$-p-tosyl-L-arginine methyl ester hydrochloride (Sigma), Pluronic 123 was received as a gift from BASF company. Doubly distilled water from a Millipore system (Milli-Q Academic A10) was used for all synthesis and purification steps. All solvents and buffer contents were purchased from Sigma-Aldrich. All chemicals were used as received without any further purification.

**1.2 Analytics.** IR spectra were recorded on a Bruker Equinox 55. UV-vis measurements for leaching experiments were performed on a Hitachi U-3501 spectrophotometer. Small angle X-ray scattering was performed on a Scintag XDS 2000. Absorption measurements for BCA assays were performed on a TECAN well plate reader. Enzyme kinetics were recorded on a Genesys 10uv thermospectronic spectrophotometer. SEM micrographs were recorded on a JEOL JSM-6500F. Sorption measurements were performed on a Quantachrome Nova 4000e. For thermogravimetric analysis, a Netzsch STA 449 c was used.

**1.3 Preparation of large pore SBA-15 spherical particles.** Large pore SBA-15 was synthesized according to a published procedure.[1] Pluronic 123 (3.0 g) was dissolved in HCl (60 mL, 1.5 M). CTAB (0.6 g) and mesitylene (0.3 g) were mixed with 25 mL of distilled water. After combining both solutions as the amount of 20 mL ethanol (abs.) was added under

stirring. Subsequently, the amount of 10 mL TEOS was added dropwise. The resulting mixture was vigorously stirred (500 rpm) at 35°C for 45 min before being transferred into a Parr autoclave for hydrothermal treatment at 75°C for 12 hours under static conditions. Subsequently, the mixture was aged at 125°C for another 12 h. The resulting white powder was filtered off, washed with 100 mL of water and 100 mL of ethanol, and dried at 60°C for 12 hours. Removal of the template was performed by calcination at 550°C for 5 hours (heating rate 1°C/min).

**1.2 Preparation of 3-chloropropyl-functionalized large pore SBA-15 (SBA-Cl).** Freshly calcined SBA-15 (200 mg) was dried at 110°C under vacuum conditions for 90 minutes. Afterwards, the amount of 6 mL dry toluene was added under nitrogen atmosphere. After addition of 3-chloropropyltrimethoxysilane (1.5 mmol, 276 µl), the reaction mixture was allowed to stir for 4 hours under reflux conditions. The functionalized SBA-15 (SBA-Cl) was filtered off and washed with each 50 mL of toluene, methanol and water before being dried at 60°C for 12 hours.

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**1.3 Preparation of azide-functionalized SBA-15 (SBA-$N_3$).** The amount of 100 mg SBA-Cl was added to 5 mL of a saturated solution of sodium azide in DMF. The resulting mixture was stirred at 90°C for 3 hours. The material was filtered off and stirred in PBS buffer solution for 3 h in order to remove remaining DMF from the mesopores. After filtration, the material was washed with 50 mL of each water and ethanol before being dried at 60°C for 12 hours.

**1.4 Acetylene-functionalization of trypsin.** Trypsin modified with acetylene groups was synthesized as described in previous publications.[2] The amount of 10 mL of a solution containing trypsin (1 mg/ml) in MES buffer (10 mM, pH 5.5) was prepared. Subsequently, the amount of 500 µl of an aqueous solution of 4-pentynoic acid (0.1 M) was added. The resulting mixture was vortexed for 2 minutes and then stored at 4°C for 15 minutes. Subsequently, the amount of 9.9 mg EDC hydrochloride was added. The resulting mixture was vortexed for 2 minutes. The reaction mixture was stored at 4 °C for 4 h, after which the sample was dialyzed in a cold room at 6 °C against sodium phosphate buffer (10 mM, pH 7.2) for a period of 24 h. The resulting acetylene-functionalized trypsin was used for the click reaction without further purification.

**1.5 Preparation of trypsin-functionalized large pore SBA-15 (SBA-trypsin).** To a solution containing 7 mg of acetylene-functionalized trypsin (7 mL) in PBS buffer (pH 7.4), the amount of 50 mg SBA-$N_3$ was added. The amount of 1 mg ascorbic acid was added to a freshly prepared aqueous solution of $CuSO_4 \cdot 5\ H_2O$ (1 mM, 5 mL) and the resulting mixture stirred at room temperature for 10 minutes. Subsequently, 12.5 µl of the copper-containing

solution were added to the reaction mixture. The resulting mixture was allowed to stir at 4 °C for 24 h. The trypsin-functionalized SBA was recovered by filtration and washed five times with 20 mL PBS buffer solution (50 mM, pH 7.1).

**1.6 Preparation of 3-chloropropyl-functionalized Cab-o-Sil aerogel (Cab-o-Sil-Cl)**

500 mg of Cab-o-Sil (Grade EH-2) were dried at 100 °C under vacuum conditions for 4 hours. Afterwards, the amount of 15 mL toluene was added. After addition of 3-chloropropyltrimethoxysilane (1.9 mmol, 345 µl), the reaction mixture was stirred for 4 hours under reflux conditions. The functionalized Cab-o-Sil (Cab-o-Sil-Cl) was washed with 50 mL of toluene, methanol and water, respectively, by multiple centrifugation and redispersion steps before being dried at 60 °C for 12 hours.

**1.7 Preparation of azide-functionalized Cab-o-Sil aerogel (Cab-o-Sil-$N_3$)**

The amount of 100 mg Cab-o-Sil -Cl was added to 5 mL of a saturated solution of sodium azide in DMF. The resulting mixture was stirred at 90 °C for 3 hours. The material was filtered off and stirred in PBS buffer solution for 3 h in order to remove remaining DMF. The material was washed with 50 mL of each water and ethanol by centrifugation before being dried at 60 °C for 12 hours.

**1.8 Preparation of trypsin-functionalized Cab-o-Sil aerogel (Cab-o-Sil-trypsin).** To a solution containing 7 mg of acetylene-functionalized trypsin (7 mL) in PBS buffer (pH 7.4), the amount of 50 mg Cab-o-Sil-$N_3$ was added. The amount of 1 mg ascorbic acid was added to a freshly prepared aqueous solution of $CuSO_4 \cdot 5\ H_2O$ (1 mM, 5 mL) and the resulting mixture was stirred at room temperature for 10 minutes. Subsequently, 12.5 µl of the copper-containing solution were added to the reaction mixture. The resulting mixture was stirred at 4 °C for 24 h. The trypsin-functionalized Cab-o-Sil was recovered by centrifugation and washed five times with 20 mL PBS buffer solution (50 mM, pH 7.1).

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## 2. Preparation of buffer solutions

### 2.1 PBS buffer

The amounts of 8.0 g sodium chloride (NaCl), 0.2 g potassium chloride (KCl), 1.44 g disodium hydrogen phosphate ($Na_2HPO_4$), and 0.24 g potassium dihydrogen phosphate ($KH_2PO_4$) were dissolved in 800 mL water. The pH was adjusted to 7.4 using 1 M hydrochloric acid (HCl). The volume was adjusted to 1 L by addition of distilled water.

### 2.2 TRIS buffer

The amounts of 5.57 g tris(hydroxymethyl)aminomethane (TRIS, $C_4H_{11}NO_3$) and 0.169 g calcium chloride dihydrate ($CaCl_2 \cdot 2\ H_2O$) were dissolved in 60 mL of water. The pH was adjusted to 8.1 using 1 M hydrochloric acid (HCl). The volume was adjusted to 100 mL by addition of distilled water.

### 2.3 MES buffer

The amount of 1.95 g 2-(N-morpholino)ethanesulfonic acid (MES, $C_6H_{13}NO_4S$) was dissolved in 500 mL water. The pH was adjusted to 5.5 using 1 M hydrochloric acid (HCl). The volume was adjusted to 1 L by addition of distilled water.

# 3. Protein Assays

### 3.1 BCA Assay for protein quantification

For the quantification of trypsin in solution, a Pierce BCA (bicinchoninic acid) Protein Assay was used. A trypsin stock solution was prepared containing trypsin in PBS buffer solution (2 mg/ml).

The calibration curve was measured using the trypsin concentrations outlined in Table S-1.

**Table S-1.** Samples measured for the BCA assay calibration curve

| trypsin concentration [µg/mL] | 0 | 5 | 10 | 15 | 20 | 25 |
|---|---|---|---|---|---|---|
| µL PBS | 100 | 97.5 | 95 | 92.5 | 90 | 87.5 |
| µL trypsin stock | 0 | 2.5 | 5 | 7.5 | 10 | 12.5 |

The amount of 5 µl of the investigated protein solution was diluted in 95 µL PBS buffer ($0.1 < c_{max} < 2$ mg/mL).

BCA-Kit solutions B and A were mixed in a ratio of 1:50. The amount of 500 µL of the resulting BCA assay mixture was added to each sample. The samples were incubated at 60°C for 30 minutes in a thermocycler. 150 µL of every sample were transferred into a well plate, and the absorption at 590 nm was measured using a TECAN well plate reader.

By using the linear equation determined from linear regression of the calibration curve, the concentration of each investigated sample was determined. The obtained calibration curve can be found in Figure S-1.

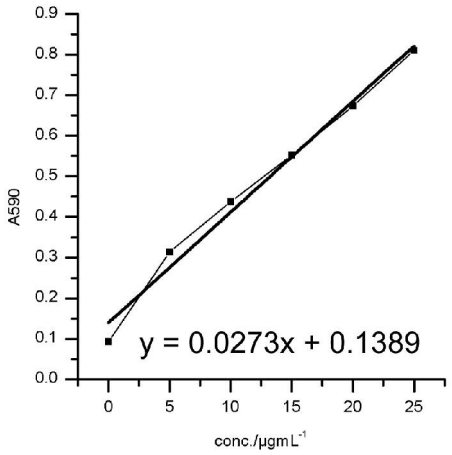


**Figure S-1.** BCA assay calibration curve

The supernatant of reaction 1.7 was analyzed in order to determine the amount of enzyme that reacted with SBA-$N_3$.
In both reactions 1.7 and 1.10, the amount of 7.0 mg protein was used. The presence of 1.0 mg protein in the supernatant in reaction 1.7 implies that 6.0 mg protein have bound to 50 mg of SBA-$N_3$. This value confirms the results from TGA of the sample SBA-trypsin (12%).
In reaction 1.10, no protein could be detected by the BCA method. The applied amount of enzyme was completely attached to the surface. This value was also confirmed by TGA.

**3.2 Trypsin activity determination**

The activity of trypsin, acetylene-modified trypsin (sp-trypsin), and silica supported trypsin (SBA-trypsin) was quantified by the release of $N_\alpha$-p-tosyl-L-arginine from the substrate $N_\alpha$-p-tosyl-L-arginine methyl ester hydrochloride (TAME) according to a procedure developed by Hummel et al.[3]. The reaction was monitored photometrically by measuring the increase of extinction at 247 nm.

In the following, one unit is defined as the amount of enzyme releasing 1 µmol $N_\alpha$-p-tosyl-L-arginine per minute under the denoted conditions.

A TAME stock solution is prepared containing 189 mg TAME in 50 mL water. The enzyme is dissolved in 0.001 n HCl with an enzyme concentration of 5.5 µg/mL.
For the activity determination, a 1.5 mL cuvette with 75 µL TAME solution, 200µl enzyme solution (1.1 µg enzyme), and 1225 µl Tris buffer was prepared.
For the sample SBA-trypsin, the amount of 55 µg sample containing 6.6 µg of enzyme (data from TGA and BCA assay) was dispersed in a mixture of 75 µl TAME solution, 100 µl 0.001 n HCl, and 1325 µl Tris buffer.
For the sample Cab-o-Sil-trypsin, 14 µg solid sample containing 2 µg of enzyme (data from TGA and BCA assay) was dispersed in the mixture described for SBA-trypsin.
The amount of enzyme attached to the solid materials was determined by TGA and confirmed by quantification of remaining enzyme in the supernatant of the synthesis solution using the BCA assay. The extinction at 247 nm was acquired at intervals of one minute. Between each acquisition, the SBA-containing suspension was homogenized by shaking. The enzyme activity was determined from the slope of the obtained linear curve (eq. 1).

$$\frac{E_{247}}{0.54 \cdot E_w} \cdot 1.5 \quad \textbf{(eq.1)}$$

$E_{247}$: Increase in extinction at 247 nm
0.54: Extinction of 1 µmol $N_\alpha$-p-tosyl-L-arginine
$E_w$: Initial weight of enzyme in mg per 0.1 mL solution
1.5: Total volume of sample in mL

The curve measured for trypsin can be found in Figure S-2. The resulting slope of the linear regression is 0.0091 $min^{-1}$.

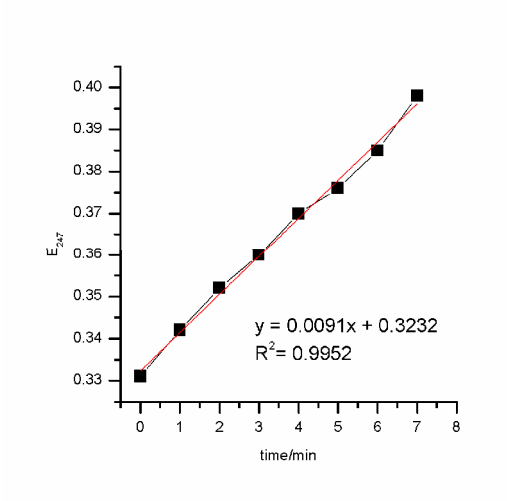


**Figure S-2.** Activity determination of trypsin

By using equation 1, an activity of 346.3 units was found for natural trypsin.
The curve measured for sp-trypsin is shown in Figure S-3. The resulting slope of the linear regression is 0.0088 $min^{-1}$, which corresponds to 334.9 units for sp-trypsin.

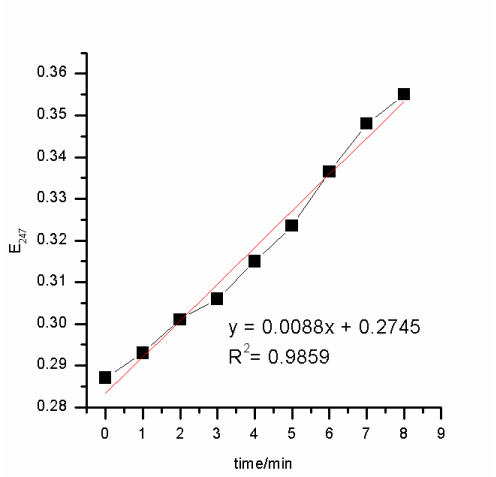


**Figure S-3.** Activity determination of sp-trypsin

The curve measured for SBA-trypsin is shown in Figure S-4.

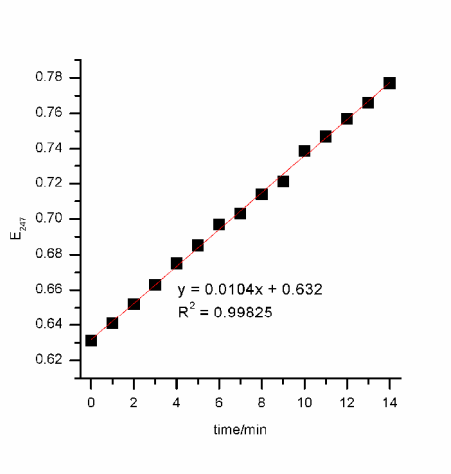


**Figure S-4.** Activity determination of SBA-trypsin

The slope of 0.0104 in Figure S-4 and a loading of 123 mg enzyme per 1 g SBA-15 (TGA and BCA quantification, see **8**, **3.1**) correspond to an enzyme activity of 65.6 units per mg enzyme.

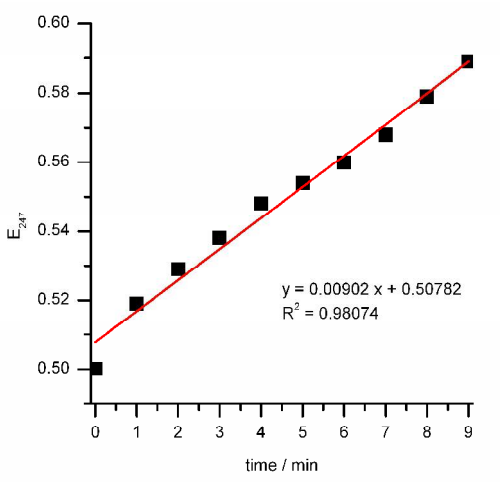


**Figure S-5.** Activity determination of Cab-o-Sil-trypsin

With the slope of 0.00902 in Figure S-5 and the determined enzyme loading of 141 mg enzyme per 1 g of Cab-o-Sil, the remaining enzymatic activity can be calculated to 84 u/mg.

### 3.3 Reusability - activity during multiple recovery cycles

Both enzyme-carrying materials SBA-trypsin and Cab-o-Sil-trypsin were compared in terms of reusability. After one catalytic use cycle, both materials were centrifuged off (43500 rcf) and stored at 4 °C for 2 h. Subsequently the hosts were re-suspended and the enzyme activity experiments were repeated. As can be seen in Figure S-6, SBA-trypsin retains 72 % of its initial catalytic activity after 4 recovering steps. In contrast, Cab-o-Sil-trypsin retains only 6 percent of its initial activity.

This experiment clearly demonstrates the stabilizing role of the SBA-15 host for the encapsulated enzyme.

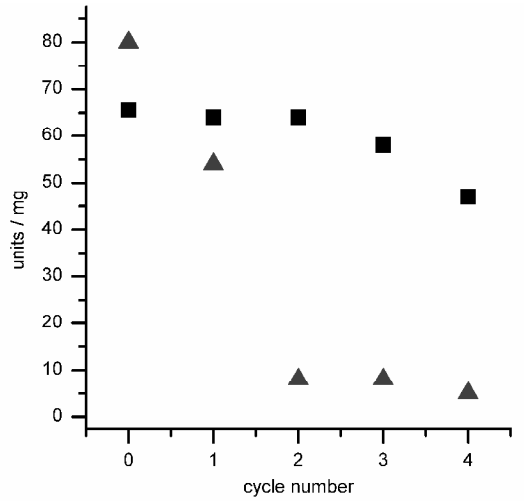


**Figure S-6.** Reusability of the samples (■) SBA-trypsin and (▲) Cab-o-Sil-trypsin

## 4. Leaching

### 4.1 Experimental procedure

The sample SBA-trypsin was resuspended in Tris-buffer solution for 4 hours. The supernatant and the powder were then separated by centrifugation. Then, the absorption at 279 nm of the protein in the supernatant was measured A previously acquired calibration curve (Fig. S-5) was used to quantify the amount of enzyme leached out the pores.

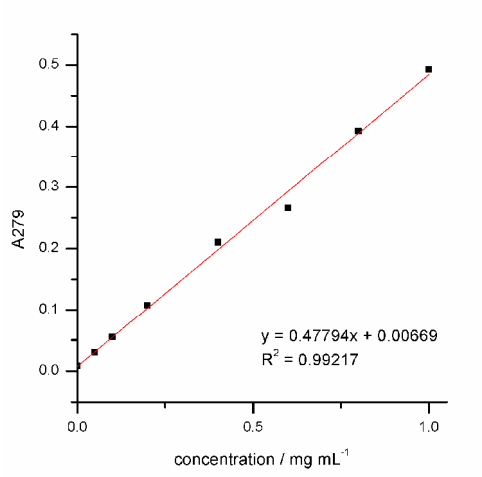


**Figure S-7.** Calibration curve for the determination of trypsin concentrations in the performed leaching experiment

### 4.2 Leaching calculation of supernatant of sample SBA-trypsin

The amount of 1.1 mg SBA-trypsin was allowed to stir in 2 mL Tris buffer solution. Then, the solid support was separated from the solution by centrifugation (5000 rpm for 10 min). Subsequently, the absorption of the supernatant solution was determined by UV-vis spectroscopy at 279 $cm^{-1}$. The absorption value of 0.00673 implies that trypsin leaching has not occurred. This result was confirmed by BCA assay using 150µl of the supernatant of the leaching experiment described above. As a complementary approach, the catalytic activity of the supernatant was determined as described in **3.2**. The obtained curve is shown in Figure S-6. The resulting slope of zero implies that the measured solution does not contain any trypsin.

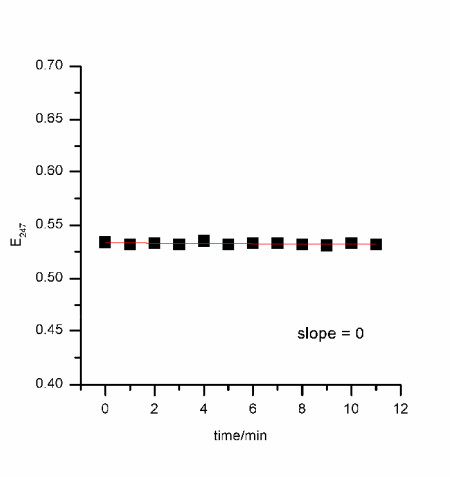


**Figure S-8.** Obtained catalytic curve of the supernantant of the performed leaching experiment

## 5. SEM micrographs of large pore SBA-15

The recorded SEM-micrographs of the synthesized large pore SBA-15 are shown in Figure S-7.

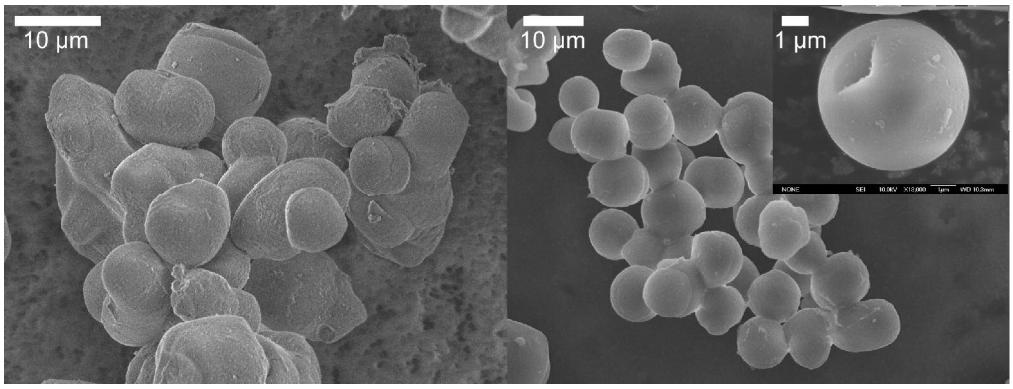


**Figure S-9.** SEM-micrographs of the sample SBA-15

## 6. Small angle X-ray scattering (XRD)

The obtained XRD diffraction patterns of the samples SBA-15 and SBA-Cl are shown in Figure S-8.

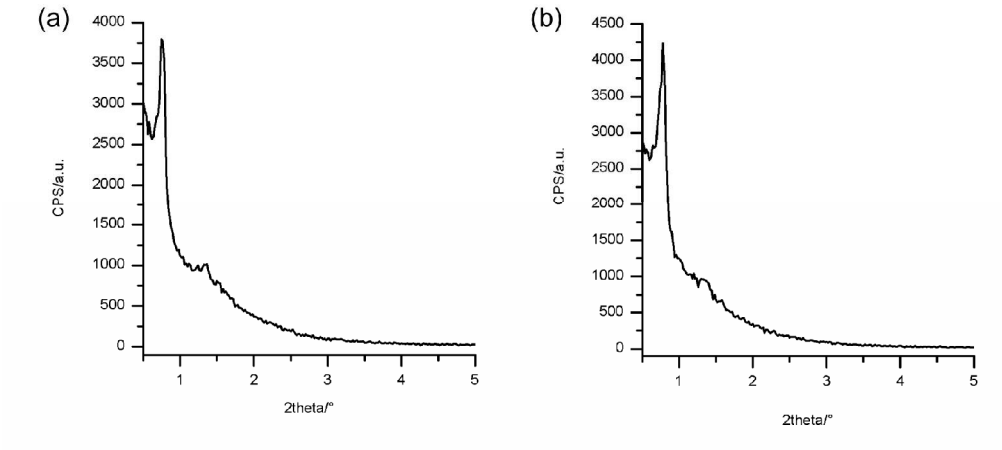


**Figure S-10.** XRD patterns of the samples (a) SBA-15 and (b) SBA-Cl

## 7. Nitrogen sorption isotherms

The obtained isotherms of the synthesized samples are shown in Figure S-9, the corresponding NLDFT pore size distributions are shown in figure S-10.

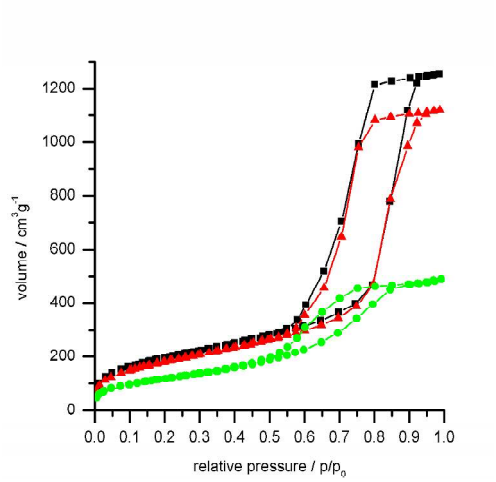


**Figure S-11.** Nitrogen sorption isotherms of the samples SBA-15 (black), SBA-Cl (red) and SBA-trypsin (green)

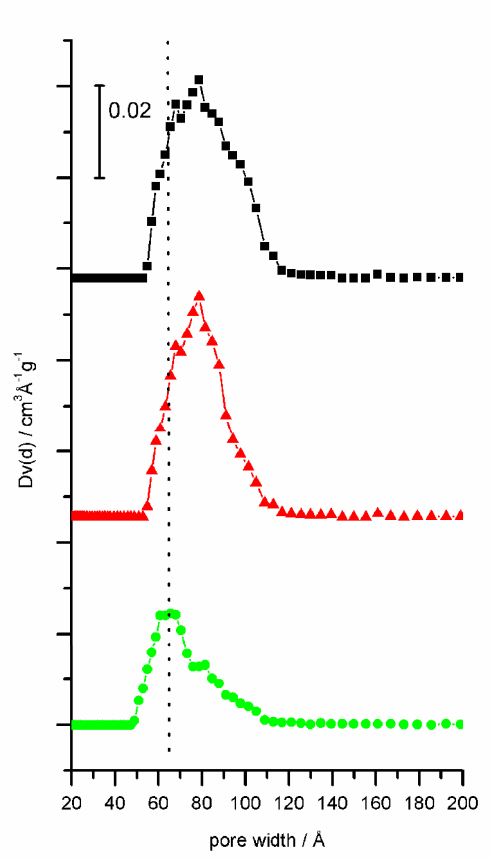


**Figure S-12.** NLDFT pore size distributions of the samples SBA-15 (black), SBA-Cl (red) and SBA-trypsin (green)

# 8. TGA calculations

### 8.1 Investigated samples

The samples investigated by TG analysis are outlined in Table S-2. The minor weight loss in sample SBA-15 was attributed to silanol condensation of the unfunctionalized surface at high temperatures.

**Table S-2.** Samples investigated by TGA

| sample | mass loss [%] | organic residue(s) | M (org. residue) [gmol$^{-1}$] |
|---|---|---|---|
| SBA-15 | <1% | ---------- | ---------- |
| SBA-Cl | 5.03 % | chloropropyl | 77.53 |
| SBA-trypsin | 15 % | trypsin | 23800 |
| Cab-o-Sil-Cl | 6 % | chloropropyl | 77.53 |
| Cab-o-Sil-trypsin | 17 % | trypsin | 23800 |

### 8.2 Calculation for SBA-Cl

The mass loss of 5.03 % is attributed to the loss of the chloropropyl groups during the heating process.

→ 1 g of SBA-Cl contains 50.3 mg of chloropropyl

$$\frac{0.0503g}{77.53gmol^{-1}} = 6.48 \cdot 10^{-4} mol$$

50.3 mg corresponds to 0.648 mmol chloropropyl residues per g SBA-Cl
This value was divided by 0.9497 (100%-5.03%) to get the amount of chloropropyl-residues bound to SBA-15.

$$\frac{6.48 \cdot 10^{-4} mol}{0.9497} = 6.82 \cdot 10^{-4} mol$$

→ The amount of 0.682 mmol chloropropyl residues has bound to the surface of 1g SBA-15
→ This value corresponds to 52.9 mg organic residues bound to the surface of 1g SBA-15.

**8.3 Calculation for SBA-trypsin**

1 g SBA-trypsin contains 150 mg of organic residues attached to the surface.

$$\frac{0.15g}{0.85g} = 0.176g$$

→ 1 g of SBA-15 contains 176 mg of organic residues attached to the surface.

→ 0.176 g- 0.0529 g = 0.123 g of trypsin attached to 1 g of SBA-15

**8.4 Calculation for Cab-o-Sil-Cl**

The mass loss of 6 % is attributed to the loss of the chloropropyl groups during the heating process.

→ 1 g of SBA-Cl contains 60 mg of chloropropyl

$$\frac{0.06g}{77.53gmol^{-1}} = 7.74 \cdot 10^{-4} mol$$

60 mg corresponds to 0.774 mmol chloropropyl residues per g Cab-o-Sil-Cl
This value was divided by 0.94 (100%-6%) to obtain the amount of chloropropyl-residues bound to Cab-o-Sil.

$$\frac{7.74 \cdot 10^{-4} mol}{0.94} = 8.06 \cdot 10^{-4} mol$$

→ The amount of 0.806 mmol chloropropyl residues was bound to the surface of 1g Cab-o-Sil

→ This value corresponds to 62.5 mg organic residues bound to the surface of 1g Cab-o-Sil.

**8.3 Calculation for Cab-o-Sil-trypsin**

1 g Cab-o-Sil-trypsin contains 170 mg of organic residues attached to the surface.

$$\frac{0.17g}{0.83g} = 0.2g$$

→ 1 g of SBA-15 contains 200 mg of organic residues attached to the surface.

→ 0.2 g- 0.0625 g = 0.137 g of trypsin attached to 1 g of Cab-o-Sil.

# 9. Analytics of the Cab-o-Sil-trypsin reference experiment

## 9.1 IR-Spectroscopy

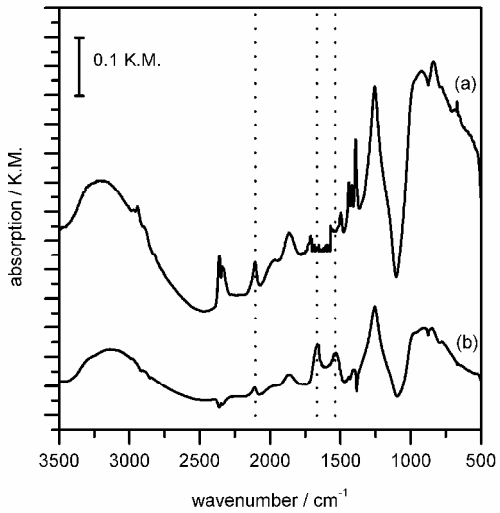


**Figure S-13.** IR data of the samples (a) Cab-o-Sil-$N_3$ and (b) Cab-o-Sil-trypsin

The covalent attachment of trypsin on the surface of Cab-o-Sil aerogel is clearly shown by the reduction of intensity of the azide bond (Figure S-12b , 2106 $cm^{-1}$) in the sample Cab-o-Sil-trypsin in comparison to the sample Cab-o-Sil-$N_3$ (Figure S-12a). As in the samples based on SBA-15, the two typical amide absorption bands of the attached enzyme emerge at 1667 $cm^{-1}$ and 1531 $cm^{-1}$.

**9.2 TGA**

The TGA curves of the samples Cab-o-Sil, Cab-o-Sil-Cl, and Cab-o-Sil-trypsin can be found in Figure S-14

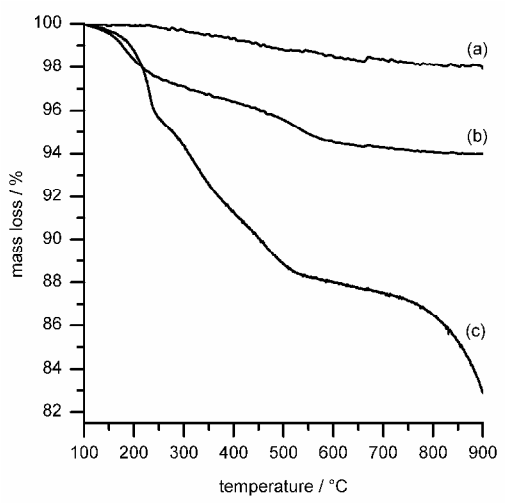


**Figure S-14.** TGA-data of the samples (a) Cab-o-Sil, (b) Cab-o-Sil-Cl, and (c) Cab-o-Sil-trypsin

## 10. References


1. Katiyar, A.; Yadav, S.; Smirniotis, P. G.; Pinto, N. G., *Journal of Chromatography, A* **2006,** 1122, 13-20.
2. Gole, A.; Murphy, C. J., *Langmuir* **2008,** 24, 266-272.
3. Hummel, B. C. W., *Canadian Journal of Biochemistry and Physiology* **1959,** 37, 1393-9.