Trypsin Immobilization on Thiol Functionalized Mesoporous Silicas Prepared Using Castor Oil Template

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Abstract

Mesoporous thiol modified silicas have been prepared using castor oil as a templating agent. The preparation was done through a co-condensation of 3-mercaptopropyltriethoxysilane and tetraethylorthosilicate in a 1:4 molar ratio. Physisorption studies showed that the prepared mesoporous silicas have an average pore diameter of 9 nm, pore volume of 1.0336 cm^3/g and surface areas of up to 605 m^2/g. FTIR confirmed the attachment of thiol groups on the surface of the prepared mesoporous silica as evidenced with an absorption band at around 2600 cm^{-1}. SEM images indicated agglomeration of the prepared particles. The prepared thiol modified silicas were successfully immobilized with trypsin enzyme. The immobilized trypsin enzyme was stable and active for hydrolysis of N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA) substrate. Re-use studies of the immobilized trypsin showed that the enzyme could be reused up to four cycles without significant loss of activity.

Introduction

The advent of ordered mesoporous materials in the early 1990s, separately by Japanese researchers (Yanagisawa et al. 1990) and Mobil's researchers (Kresge et al. 1992, Beck et al. 1992) has attracted enormous research interests in this area (Yu et al. 2004, Gao et al. 2006, Øye et al. 2006, Liu et al. 2007, Yang et al. 2008, Yang et al. 2010, Hamad et al. 2011, Hu et al. 2011, Mubofu et al. 2011, Canlas and Pinnavaia 2012, Deng et al. 2013, Diaz et al. 2013, Si-Han et al. 2013, Velikova et al. 2013, Linares et al. 2014, Gervas et al. 2016, Elimbinzi et al. 2020). Undeniably this discovery has created an amazing amount of interest in the science and technology leading to enormous increase of publications (Kresge and Roth 2013) and thus generating other new materials and knowledge. This explosion in research interest is mainly due to the wide range of applications of mesoporous materials ranging from adsorption, biomedical, catalysis, energy, sensors to separations. The unlimited possibility of modifying the properties of the prepared materials by the inclusion of organic components on the mesopore walls during preparation and hence leading to hybrid organic-inorganic mesoporous materials with desired functionality and applications has further added interests in this field. This flexibility in the preparation of mesoporous materials has generated and is likely to generate more fascinating porous solids with regular mesostructures, along with desired

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properties. The desired properties range from very high specific surface areas, thermal and mechanical stability, highly uniform pore distribution and tunable pore size, high adsorption capacity to other unprecedented materials properties depending on the desired application of the material. As such, this entails that the properties of these materials can easily be modified by changing synthesis conditions such as backbone source (e.g. silica sources), the nature of the template molecule and inclusion of auxiliary compounds. Effects of these conditions on the properties of prepared materials such as thermal, hydrothermal and mechanical stabilities are reported elsewhere (Øye et al. 2006, Liu et al. 2007, Linares et al. 2014). In recent years, some researchers have turned their focus from commercially available fuel-based template molecules to using renewable and bio-based templates (Hamad et al. 2011, Mubofu et al. 2011, Canlas and Pinnavaia 2012). If bio-based templates were to replace the fossil fuel-based templates, then the process of preparing mesoporous materials would be made greener as bio-based templates are renewable and are likely to be biodegradable. Mesoporous materials prepared using some of bio-based template sources such as cashew nut shell liquid and vegetable oils have pore sizes large enough to immobilize biomolecules such as invertase enzymes (Mubofu et al. 2011), immobilize metal catalysts such as copper(II) Schiff base catalysts (Hamad et al. 2011) and for removal of chromium(III) ions from tanneries wastewater (Gervas et al. 2016). The immobilized enzyme on bio-based templated supports have shown high activities and were reused for up to ten cycles. Likewise, the immobilized copper(II) Schiff base catalysts tested for wet oxidation of maleic acid showed excellent catalytic efficiency with a yield of up to 90% and turn over number of about 1000 within ten minutes. Adsorption of up to 87% of Cr(III) ions from tannery wastewater by sunflower oil templated mesoporous materials have also been reported (Gervas et al. 2016).

In this paper, the catalytic activities of trypsin enzyme immobilized on castor oil templated mesoporous silica are reported. Castor oil is a naturally occurring resource, inexpensive and environmentally friendly triglyceride composed of glycerol and three fatty acids in which about ninety percent of fatty acid chains are ricinoleic acid (Figure 1). The oil is obtained by extracting it from castor seeds of *Ricinus communis* plant of the family Eurphorbiacae (Ogunniyi 2006, Nielsen et al. 2011).
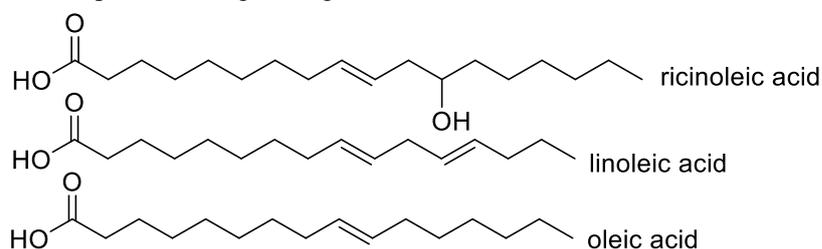
Figure 1: Major components of castor oil fatty acids.

Castor oil is an amphiphilic molecule with qualities to form micelles in solution (Canlas and Pinnavaia 2012). Because of the amphiphilic nature of castor oil, when dissolved in aqueous ethanol above critical micellar concentration (CMC), it self-

aggregates into supramolecular structures (micelles) and thus making it a good templating agent for the synthesis of mesoporous materials.

Materials and Methods
Material and reagents
Tetraethylorthosilicate (TEOS, 98%), dodecylamine (assay > 98%), ethanol (assay 98%), *n*-hexane, sodium fluoride, potassium bromide, and 3-mercaptopropyltriethoxysilane (MPTES) were all purchased from Sigma Aldrich. Castor oil beans were collected from Ludewa district, Njombe region, Tanzania. All purchased chemicals were used as received without further purification.

Solvent extraction of castor oil
Castor oil was solvent (*n*-hexane) extracted from ground castor seeds using Soxhlet apparatus following the procedure reported by Nielsen et al. (2011). In a typical experiment, *n*-hexane (200 mL) was poured into a round bottomed flask followed by the insertion of the thimble containing crushed castor beans (10 g) at the centre of the extractor. The mixture in the Soxhlet apparatus was boiled at 60 °C, allowing the vapour to rise through the vertical tube into the condenser at the top. The condensate dripped into the thimble containing the castor paste to be extracted. The oil seeped through the pores of the thimble down into the round bottomed flask. At the end of the extraction, the oil was concentrated in a rotary evaporator at 40 °C to recover the solvent.

Preparation of castor oil mesoporous silica
The synthesis of pure castor oil mesoporous silica (CO-MTS) was done according to the procedure reported by Mubofu et al. (2011) with some modification. In a typical experiment, castor oil (2.5 g) was added into aqueous ethanol (53 mL water, 47 mL ethanol) and stirred for one hour at ambient temperature and atmospheric pressure. To this stirred mixture, TEOS (8.3 g) was added followed by addition of a little amount of sodium fluoride catalyst (0.1 g). The reaction mixture was stirred for 24 hours at 40 °C. The wet solid product obtained was refluxed by Soxhlet apparatus using ethanol as extracting solvent for 10 hours to remove the template. The final solid was dried in an oven at 100 °C for 8 hours and then stored in sealed bottles in desiccators for further modification and application. For comparison purposes (in catalytic studies), a commercially available surfactant; dodecylamine, DDA (5 g) was also used to prepare mesoporous materials using similar procedure.

Synthesis of the thiol-functionalized MTS (SH-CO-MTS)
The synthesis of the thiol-functionalized MTS (SH-CO-MTS) materials was done using similar procedure as for pure samples; by co-condensation of 3-mercaptopropyltriethoxy silane and TEOS in a 1:4 mole ratio. In a typical experiment, castor oil (2.5 g) was added into aqueous ethanol (53 mL water, 47 mL ethanol) and stirred for one hour at ambient temperature and atmospheric pressure. To this stirred mixture, TEOS (8.3 g) and 3-mercaptopropyltriethoxysilane (2.377 g) were added separately, but simultaneously and rapidly. Sodium fluoride catalyst (0.1 g) was added and the reaction mixture was stirred for 24 hours at 40 °C. The wet solid obtained was refluxed by Soxhlet apparatus using ethanol as extracting solvent for 10 hours to remove the template. The final solid was dried in an oven at 100 °C for 8 hours and then stored in sealed bottles in desiccators for characterization and immobilization of trypsin enzyme.

Characterization of the prepared mesoporous materials
Diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS, Perkin

Elmer BX FT-IR spectrophotometer) was used to identify the anchored functional groups on the prepared materials. This technique generates data about chemical bonds and functional groups of samples based on their characteristic absorption of infrared radiation in vibrational modes. Prior to the DRIFT measurements, samples were diluted using KBr at a ratio of 9:1. The diluted samples were ground using mortar and pestle to ensure thorough mixing and were dried overnight in an oven at 80 °C. Potassium bromide (KBr) was used as both the background and matrix. Typically, 130 scans were acquired for each spectrum with the resolution of 4 cm^{-1}. The FTIR spectra were collected in the 4000–500 cm^{-1} region range and results were obtained by subtracting the KBr background spectrum from the sample spectra. The spectra were reported in absorbance and wave numbers (cm^{-1}).

Nitrogen physisorption studies were done using Quantachrome instrument Nova 4200 multi-station nitrogen sorption at 77 K. The specific surface area and pore size distribution of porous materials were assessed through construction of adsorption-desorption isotherms. The Brunauer-Emmett-Teller (BET) method for *p/po* between 0.05-0.3 was used to determine the specific surface area from the adsorption data whereas the pore size distribution was determined from the desorption branches of isotherm using the Barrett-Joyner-Halenda (BJH) (Brunauer et al. 1938, Barrett et al. 1951, Sing 2001). Prior to adsorption measurements, samples were dried in the oven overnight and outgassed at 120 °C for 3 h. The aim of outgassing was to reach a well-defined intermediate state by removing physisorbed molecules. Outgassing also helps to avoid any drastic change as a result of surface modification or ageing (Sing 2001).

Field emission gun scanning electron microscopy (FEGSEM) with an accelerating voltage of 10 kV was used to assay the morphology of the prepared materials. The powdered solid materials for analysis were placed on a conductive carbon tape previously mounted on the aluminium stub. Prior to SEM analysis, samples were coated using Quorum coater (Model Q150TE). FEGSEM is the electron microscopic technique that produces high-resolution image of the material by scanning it with a focused beam of electrons. These electrons (with energies of up to 40 keV) are directed to a specimen, which interact with atoms in the sample to produce signals that contain information about the surface topography of the sample.

Catalytic activities of trypsin-immobilized mesoporous materials were determined via hydrolysis of the substrate, N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA). The rate of hydrolysis was obtained by measuring the absorbance of the reaction mixture using a UV-Vis spectrophotometer SPECORD PLUS (Analytikjena, German) at 405 nm due to the formation of p-nitroaniline, which imparts yellow colour.

Immobilization of trypsin on the mesoporous material

Trypsin was immobilized onto mesoporous materials following the procedure reported by Yiu et al. (2001) with some modifications. In a typical experiment, 0.04 g of trypsin in 10 mL of 0.05 M tris–HCl buffer, pH = 6.0 with 0.02 M CaCl$_2$ (to reduce enzyme auto-digestion) was stirred with 0.25 g of carrier for two hours at 4 °C. Then, the supernatant was separated from the particles by centrifugation. The amount of free enzyme not immobilized (trypsin remaining in the supernatant) was calculated from the differences in UV spectra at 280 nm. The

amount of trypsin immobilized was, therefore, calculated by subtracting this figure from the blank. The blank was prepared by mixing tris-HCl buffer with mesoporous materials, then centrifuged to separate the supernatant from the solids. The supernatant solution was then employed as a blank in determining the amount of trypsin in solution by UV spectrophotometer. Finally, the trypsin immobilized mesoporous material was washed with tris–HCl buffer solution (pH 6.0), air-dried and stored at 4 °C. To assess leaching, the mesoporous silica with immobilized trypsin was resuspended in 5 mL tris-HCl buffer (pH = 8.0) solution and stirred for 2 hours. The suspended solids were allowed to settle for 2 hours. The solution left above the solid was placed in a UV sample bottle and the spectrum of the solution was measured by UV spectrometer which enabled to calculate the amount of enzyme leached out from the support.

Catalytic activity measurements of native and immobilized trypsin

The catalytic activity of free and immobilized trypsin was measured via hydrolysis of N-α-benzoyl-DL-arginine-4-nitroanilide (BAPNA) in 0.05 M tris-HCl buffer (pH 8.0). BAPNA (21.7 mg) was dissolved in DMSO (1 mL), and diluted with tris–HCl (0.05 M, pH = 8) buffer to prepare a 1 mM BAPNA. 20 mL of 1 mM BAPNA solution (pH = 8) was added to 0.25 g immobilized enzyme sample and stirred at 25 °C. Samples were then withdrawn in regular time intervals (15, 30, 45, 60 and 90 min) and catalytic activities were measured by measuring the absorbance of the mixture at 405 nm using a UV spectrophotometer. The blank was prepared by stirring a mixture of 4 mL of 1 mM BAPNA solution and 0.05 g mesoporous materials, then centrifugated to separate the supernatant from the solids. The supernatant solution was then employed as a blank in determining the amount of *p*-nitroaniline by UV spectrophotometer. Measurements were also made using free trypsin and SH-DDA-

MTS immobilized enzyme. The catalytic performance of both free and immobilized enzymes was expressed as µmoles nitroaniline produced per milligram of protein. Initial rates were calculated over the first 15 minutes of the reaction. The catalyst used in the hydrolysis reaction was filtered and washed with a pH = 8 buffer solution for reuse. The reuse capability of immobilized trypsin was studied by measuring the residual activity up to four operational cycles. Each time, immobilized trypsin was washed and centrifuged four times with 0.05 M tris-HCl buffer (pH = 6) at 25 °C.

Results and Discussion
Nitrogen adsorption-desorption isotherms

Nitrogen adsorption-desorption isotherms of the unmodified and modified CO-MTS materials are shown in Figure 2. The nitrogen isotherms of these samples are of the type IV with clear hysteresis loop at relative pressures from 0.5 to 0.9. They are typical for mesoporous solids (Kruk and Jaroniec 2001) which at low relative pressure (less than 0.1) show a gradual increase in the amount of adsorbate with relative pressure. The initial part of the adsorption-desorption isotherms of the materials show a gradual increase of nitrogen uptake at a low relative pressure (< 0.1) which indicates that there is formation of monolayer of adsorbate and hence there are stronger interactions between adsorbate and adsorbent, followed by multilayer formation (Brunauer et al. 1938). Above 0.1 relative pressures, the isotherm form a plateau followed by another sharp rise of nitrogen uptake starting from about 0.5 relative pressure. The high uptake of nitrogen at relative pressures between 0.6-1.0 corresponds to capillary condensation of nitrogen indicative of the uniformity of the pores (Brunauer et al. 1938, Dai et al. 2010). Thiol modified MTS materials showed a slightly low nitrogen uptake when compared to the unmodified MTS materials at the same relative pressure. This implies that

mercaptopropyltriethoxysilane occupied very   small part of the CO-MTS pore volume.
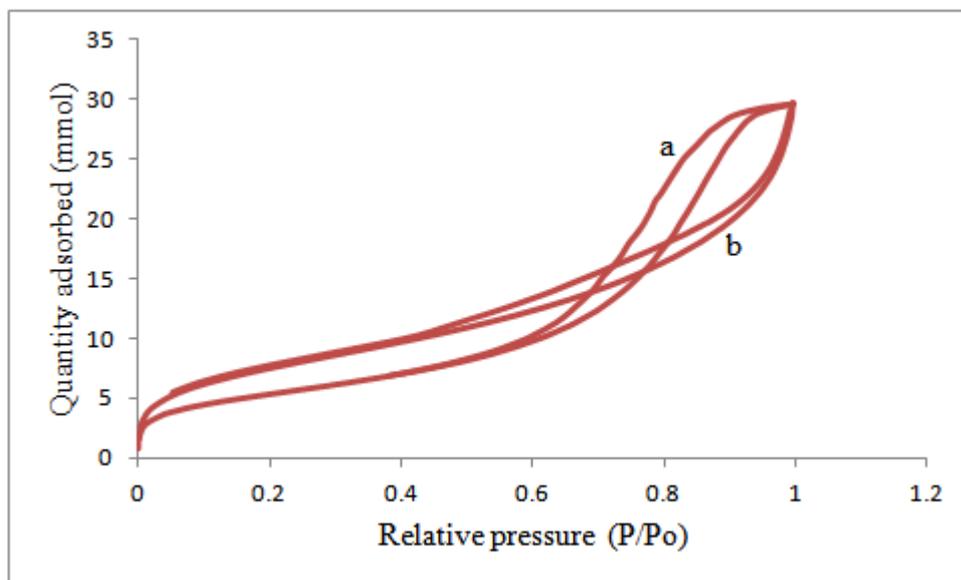
Figure 2: Nitrogen adsorption-desorption isotherms for (a) CO-MTS and (b) SH-CO-MTS.

The average pore diameter, the BET surface areas and the total pore volume display the porosity properties of a porous material. Table 1 summarizes these parameters for the materials synthesized in this work. It is clearly observed that the introduction of thiol functional group did not have notable effects on the porosity characteristics of the mesoporous materials. Generally, the CO-MTS materials and their derivatives had wide pore size distribution ranging from 5 to 20 nm with the average pore diameters of about 9 nm (Table 1).

Table 1: Textural properties of the mesoporous silica materials (CO-MTS, and SH-CO-MTS)

Material	Average pore diameter (nm)	BET surface area (m^2/g)	Total pore volume (cm^3/g)
CO-MTS	8.9	428	1.1
SH-CO-MTS	8.5	605	1.0

Thiol modified materials had slight narrow pore size distribution when compared to the unmodified CO-MTS material (Figure 3).
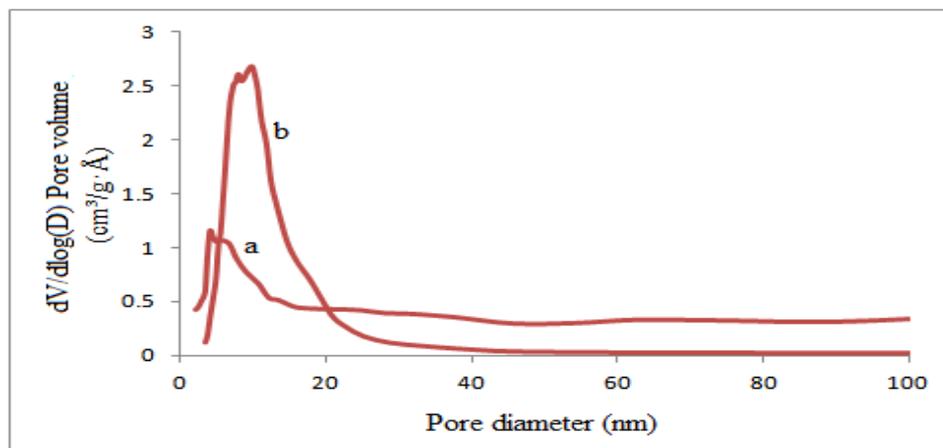
Figure 3: Pore size distribution of SH-CO-MTS (a) and CO-MTS (b).

The pore sizes of these materials are sufficiently large enough to support enzymes and chemical catalysts as well as in heavy metal removal and separation purposes. Jang et al. (2006) reported the successful immobilization of trypsin enzyme of mesoporous materials (prepared using non-ionic block copolymer surfactants) with pore diameters in the range 5-10 nm. The immobilized trypsin was catalytically active. This entails that castor oil can be used in its natural form to produce mesoporous materials of up to 9 nm pore diameter which are capable of immobilizing small enzymes like trypsin and hence making the whole synthesis process green.

Drifts of CO-MTS and SH-CO-MTS materials

Notable differences between the two spectra (Figure 4) gave clear evidence that a new functional group(s) have been introduced on the unmodified MTS material. The IR spectra of modified samples showed the presence of organosilanes as identified by the methylene stretching bands of the propyl chain in the region of 2950-2850 cm^{-1}, with their deformation bands at 1455–1410 cm^{-1}. This is further evidence that the modification

of mesoporous silica with mercaptopropyltriethoxysilane was successful.

The introduced thiol functional group was associated with the absorption bands that appeared at around 2600 cm^{-1} (Figure 4 a). Other regions of the spectra are common to both modified and unmodified spectra such as 3600–3000 cm^{-1} which is assigned to hydrogen bonded silanol species. A strong and broad band with two peaks in the region 1450 cm^{-1} to 900 cm^{-1}, and two strong bands at 960 cm^{-1} and 800 cm^{-1} all assigned to Si-O lattice vibrations are also common to both spectra.

SEM of CO-MTS and SH-CO-MTS materials

The particle morphology obtained from SEM analysis show that they are in amorphous shape for the CO-MTS, while they are micro spherical shaped for SH-CO-MTS. In both SEM images (Figure 5), particles seem to be highly agglomerated. This is likely due to some castor oil surfactants remaining adsorbed in the particles which can cause particles to stick together.
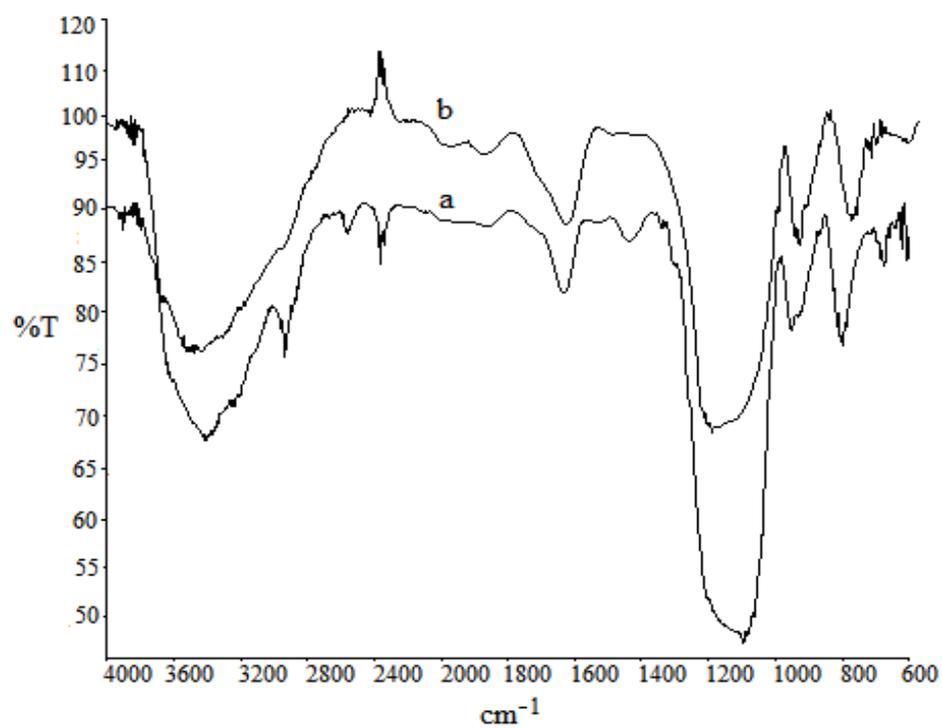
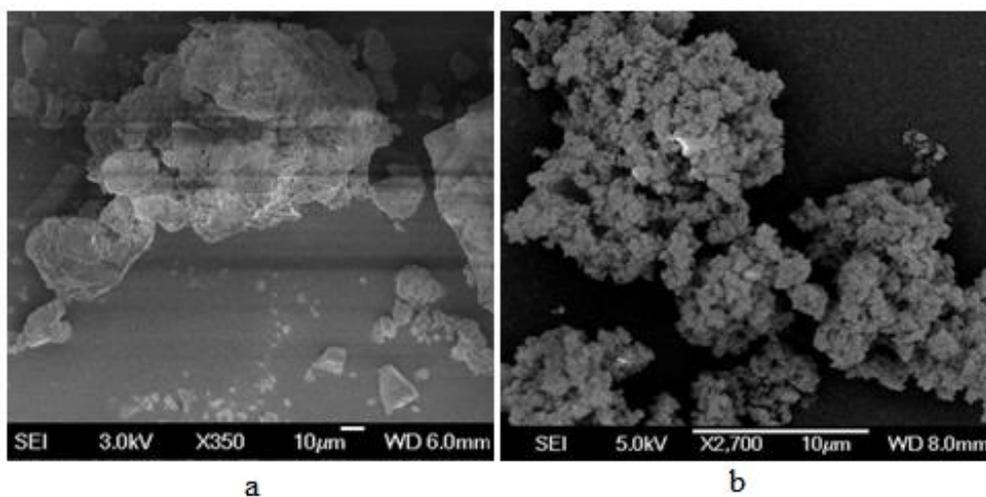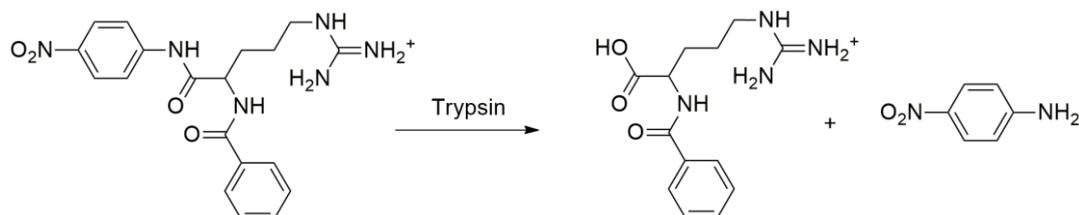**Figure 4:** FTIR spectra for SH-CO-MTS (a) and CO-MTS (b).

Figure 5: SEM images of CO-MTS (a) and SH-CO-MTS (b).

Trypsin immobilization

Immobilization of trypsin to the thiol-functionalized mesoporous silica takes advantage of the presence of S-S groups (Yiu et al. 2001) in the trypsin structure. The S-S groups interact with a thiol group of the mesoporous silica to form stronger chemical bonds. Thus, less leaching of immobilized trypsin by this type of bonding is expected compared to immobilization by physical adsorption with pure mesoporous silica (Jang et al. 2006). Measurements by a UV spectrophotometer at 280 nm indicated that 94% of trypsin was successfully immobilized in SH-CO-MTS support. Assessment for leaching (by stirring the mesoporous silica immobilized with trypsin in tris-HCl buffer for two hours) showed that 7% trypsin had leached out of the SH-CO-MTS support. This percentage is less compared to those reported elsewhere for physical adsorption (Jang et al. 2006). These results agree with the expectation that trypsin forms strong bonds with silica supports using its S-S groups and the silicas thiol group.

Catalytic activity studies on the immobilized and free trypsin enzyme

The catalytic activities of immobilized trypsin were monitored using the substrate BAPNA at 25 °C. For comparison purposes, the catalytic activities of free trypsin were measured under similar conditions. Hydrolysis of BAPNA by trypsin yields *p*-nitroaniline (Figure 6), a yellow product which is easily measured in a UV-VIS spectrophotometer.

**Figure 6:** Hydrolysis of BAPNA substrate for enzymatic activity assay of trypsin.

Trypsin and the substrate BAPNA were stirred while varying the stirring period from 15 to 90 minutes in order to ascertain the optimum stirring time (for both free and immobilized trypsin). It was observed that the amount of *p*-nitroaniline produced from hydrolysis of BAPNA by both free and immobilized trypsin increased with increasing stirring time reaching maximum at 15, 50 and 60 minutes for Enz-SH-DDA-MTS, Enz-SH-CO-MTS and free trypsin, respectively (Figure 7).
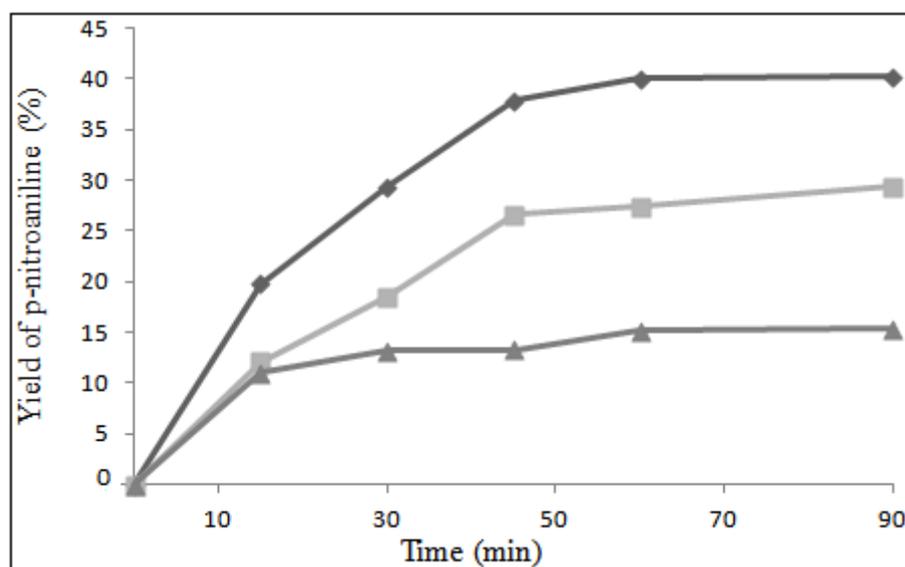
Figure 7: BAPNA hydrolysis catalyzed by trypsin enzymes; ♦ free trypsin, ■ trypsin immobilized on SH-CO-MTS, ▲ Trypsin immobilized on SH-DDA-MTS.

Compared to free and covalently CO-MTS immobilized trypsin, the trypsin immobilized on DDA-MTS showed low activity which started to decrease after 15 minutes of stirring. This may be explained by the small pore diameter of the DDA-MTS which disfavours the uptake of the enzyme during immobilization, hence fewer number of active sites possibly on the surface are accessed by the substrate. On the other hand, free trypsin exhibited the highest catalytic activity compared to covalently CO-MTS and DDA-MTS immobilized trypsin. This is due to the larger amount of the free enzyme involved in the hydrolysis reaction than the immobilized enzyme. The decrease in activity of trypsin after immobilization on CO-MTS seems to be a major disadvantage of the immobilization process. However, it should be noted that the immobilized trypsin was capable of being reused up to four cycles with only slight decrease in catalytic activity (Figure 8). Focussing on this reusability capacity, the implication is that the overall efficiency of immobilized trypsin is higher than the free trypsin enzyme under the same reaction conditions.
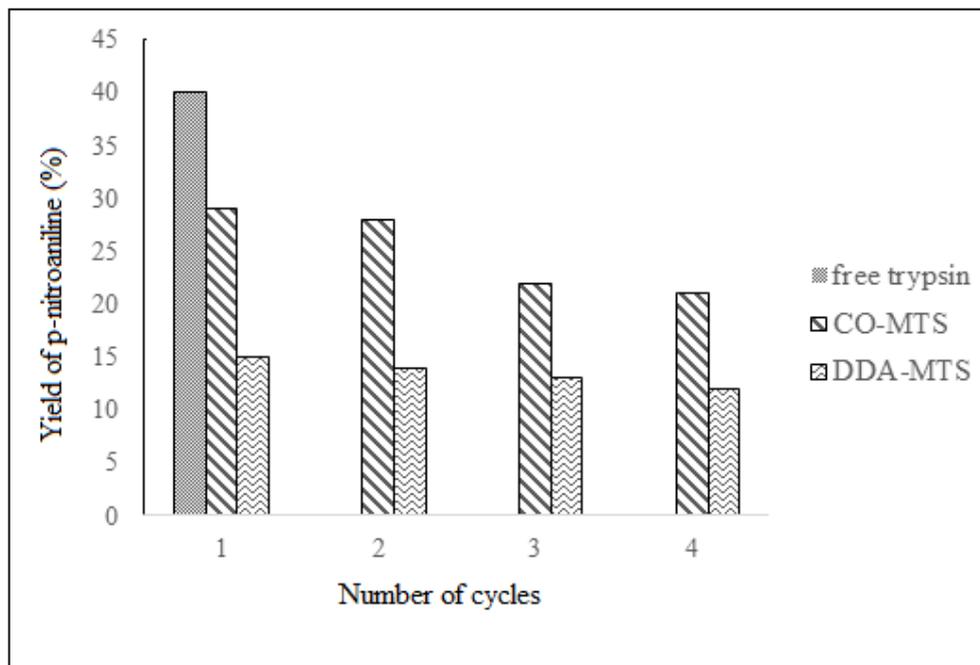
Figure 8: Reusability of the CO-MTS and DDA-MTS immobilized trypsin enzyme.

The slight loss of activity of the immobilized trypsin during reuse is a common phenomenon and may be due to mechanical loss of the enzyme during separation (Yamada et al 2003). In this study, the immobilized trypsin was washed thoroughly (four times) with tris-HCl buffer before reuse.

Conclusions
Castor oil has been employed as a template for the synthesis of mesoporous silicas in the presence of sodium fluoride catalyst. Characterizations of the prepared materials indicated that the prepared materials were mesoporous organosilica materials with pore diameters slightly above 8 nm. This pore size is sufficient for immobilization biomolecules as a method of biocatalysts stabilization. Trypsin enzyme immobilized onto thiol functionalized samples was active and stable upon reuse.

Conflict of Interest
The author declares that there is no conflict of interest.

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