Characterisation of SBA-15 and adsorption of Human Lysozyme.

SBA-15 is a silica-based ordered mesoporous structure. Its characterisation by means of ATR-FTIR, SAXS and N_2 adsorption/desorption isotherms is reported in Figure 1.

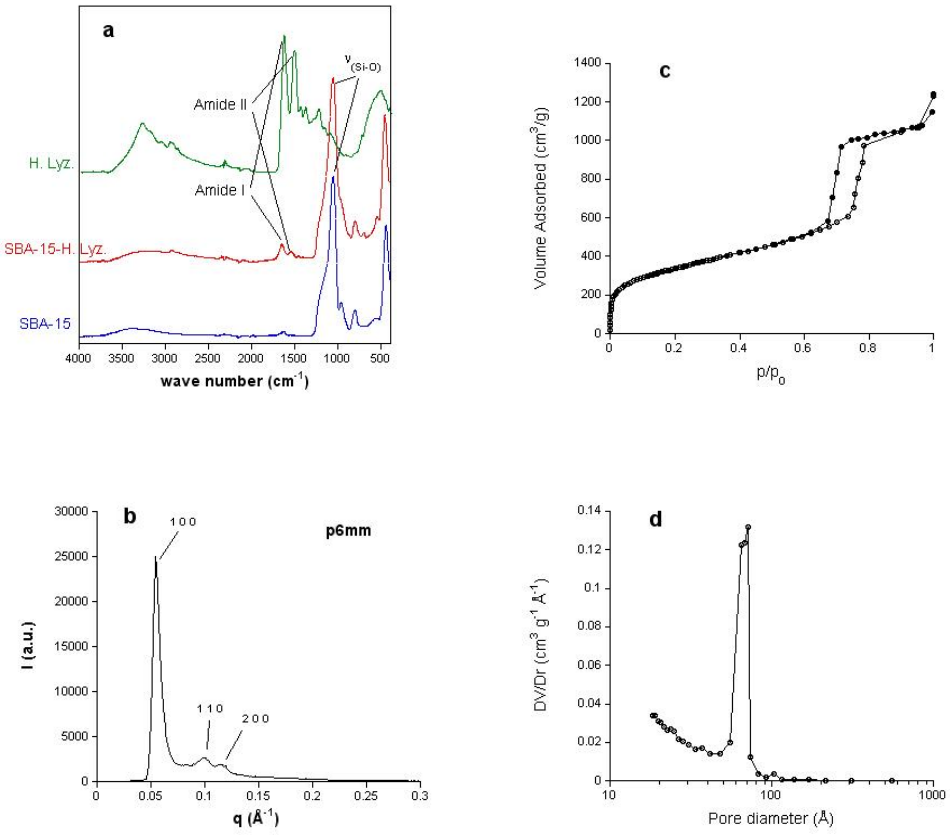

Figure 1. (a) ATR-FTIR spectra of SBA-15, before and after lysozyme loading; (b) SAXS Pattern of SBA-15; (c) N_2 adsorption/desorption data; (d) pore size distribution.

The ATR-FTIR spectrum of SBA-15 (Fig.1a) presents an intense band at 1070 cm^{-1} due to Si-O stretching, one less intense at 450 cm^{-1} due to Si-O-Si bending, and also a band at 950 cm^{-1} due to silanols. SAXS analysis (Fig. 1b) shows the typical pattern of a hexagonal phase where a strong peak due to (1 0 0) plane and other two weak peaks due to (1 1 0) and (2 0 0) planes occur. This characterisation allowed to determine the lattice parameter of SBA-15, a = 11.7 nm. The N_2 adsorption/desorption isotherms of the SBA-15 (Fig. 1c) sample is of type IV, with a steep increase of the adsorption branch at a relative pressure around 0.74-0.78. The presence of a hysteresis cycle, defined by IUPAC as H1 type, is closely associated to the presence of channel-like mesopores.

Materials and methods

Chemicals. Chemicals for the synthesis and modification of SBA-15, Tetraethylorthosilicate (TEOS, 98%), Pluronic copolymer 123 ($EO_{20}PO_{70}EO_{20}$), and buffer components, Na_2HPO_4 (99%) and NaH_2PO_4 (99%) were purchased from Sigma-Aldrich. Human Lysozyme (92717 U/mg) and LR Gold resin were purchased from Fluka. Rabbit polyclonal antibody specific for human Lysozyme was purchased from DAKO. Gold-labeled goat anti-rabbit IgG was from Auroprobe EM, Amersham International PLC (Little Chalfont, UK).

Synthesis and characterization of SBA-15. SBA-15 Mesoporous silica was synthesized and activated according to the methods reported in refs 2 an 6. SBA-15 was characterized with the following techniques. SAXS patterns were recorded (2400 s) with a S3-MICRO SWAXS camera system (HECUS X-ray Systems, Graz, Austria). Cu Kα radiation of wavelength 1.542 Å was provided by a GeniX X-ray generator, operating at 50 kV and 1 mA. A 1D-PSD-50 M system (HECUS X-ray Systems, Graz, Austria) containing 1024 channels of width 54.0 μm was used for detection of scattered X-rays in the small-angle region. Textural analysis was carried out on a Thermoquest-Sorptomatic 1990, by determining the N_2 adsorption/desorption isotherms at 77 K. Before analysis the sample was out-gassed overnight at 40 °C. The specific surface area, the total pore volume and the pore size distribution were assessed by the BET and BJH methods respectively. ATR-FTIR studies were carried out by a Bruker Tensor 27 spectrophotometer equipped with a diamond-ATR accessory and DTGS detector. A number of 64 scans at resolution of 4 cm^{-1} was averaged from wavenumber 4000 to 400 cm^{-1}. Opus spectroscopic software was used for data handling.

Loading of lysozyme. To load lysozyme on SBA-15, 0.2 g of the glutharaldhyde activated[6] mesoporous sample were suspended in 10 mL of aqueous solution of lysozyme (10 mg/mL) prepared in 10 mM phosphate buffer pH 7.0, and soaked for 24 hours with shaking at 100 rpm and 25°C. The concentration of lysozyme was measured by a UV-Vis spectrophotometer (Varian Cary 50) at λ = 280 nm. The amount of lysozyme loaded onto the samples was determined according to the change of

concentration before and after the adsorption process reached the equilibrium. After loading, the powders were quickly and thoroughly washed with water and dried under vacuum. The loading of lysozyme - L_{Lyz} (mg/g) – on mesoporous material was calculated according to the following formula:

$$L_{Lyz} = \frac{[Lyz_i]V - [Lyz_r]V - [Lyz_w]V_w}{m_{SBA15}} \quad (1)$$

Where, $[Lyz_i]$ is the lysozyme concentration in the initial solution ($mg_{Lyz}/mL_{solution}$); $[Lyz_r]$ is the residual concentration of lysozyme in solution ($mg_{Lyz}/mL_{solution}$); $[Lyz_w]$ is the lysozyme concentration determined in the washing solution ($mg_{Lyz}/mL_{solution}$); V is the volume of the lysozyme solution (mL), V_w is the washings volume (mL), and m_{SBA15} is the mass of SBA-15 (g).

Electron microscopic embedding procedure. Samples of human Lysozyme loaded SBA-15 and samples of unloaded SBA-15 were infiltrated with LR Gold resin rotating at -20 °C for three days with changes of fresh resin each day. Then they were transferred to gelatin capsules or flat polyethylene molds, filled with fresh resin and polymerized in a UV (long wave, 365 nm) chamber at -20°C for 3 days. Ultrathin sections (~ 60-80 nm thick) were cut from the embedded samples with a MT2-B ultramicrotome and collected on Formvar-coated nickel grids. In order to verify the ultrastructural integrity of the mesoporous materials undergoing embedding procedures, some sections were observed in a JEOL 100S transmission electron microscope (TEM).

Immunochemistry. In this study we have used the postembedding immunogold staining (IGS) method that consists mainly of consecutive incubation steps of the sections with specific antibodies. In general, a first incubation is performed with the unlabelled primary antibody specific to the protein followed by the incubation with secondary colloidal gold conjugated antibodies.

In details, the ultrathin sections, previously collected on Formvar-coated nickel grids, were floated section-side down on phosphate-buffer solution (PBS) for 5 min and transferred to small drops (30 µl) of PBS containing 1% bovine serum albumin (BSA) and 5% normal goat serum (NGS) for 30 min at room temperature to block non-specific binding of antibodies. The sections were incubated in a humidified chamber overnight at 4°C with a rabbit polyclonal antibody specific for human Lysozyme.

1wt% BSA and 5wt% NGS were included in incubation medium in order to protect the antibodies. After rinsing with PBS, the grids were incubated for 60 min at room temperature with the secondary antibody, gold-labeled goat anti-rabbit IgG, diluted 1:50 in 1% BSA–PBS. The grids were washed with PBS and distilled water and finally observed and photographed in the TEM. Appropriate controls were performed in order to establish the specificity of the labeling (Figure 2). They included: a) omission of the primary antibodies from the labeling sequence, b) substitution of non-immune serum for the primary antibody, c) treatment of unloaded SBA-15 mesoporous silica with the above primary and secondary antibodies. The presence of few Au particles is likely to be due to the non-quantitative removal of antibody-conjugated-gold nano particles during sample washing.

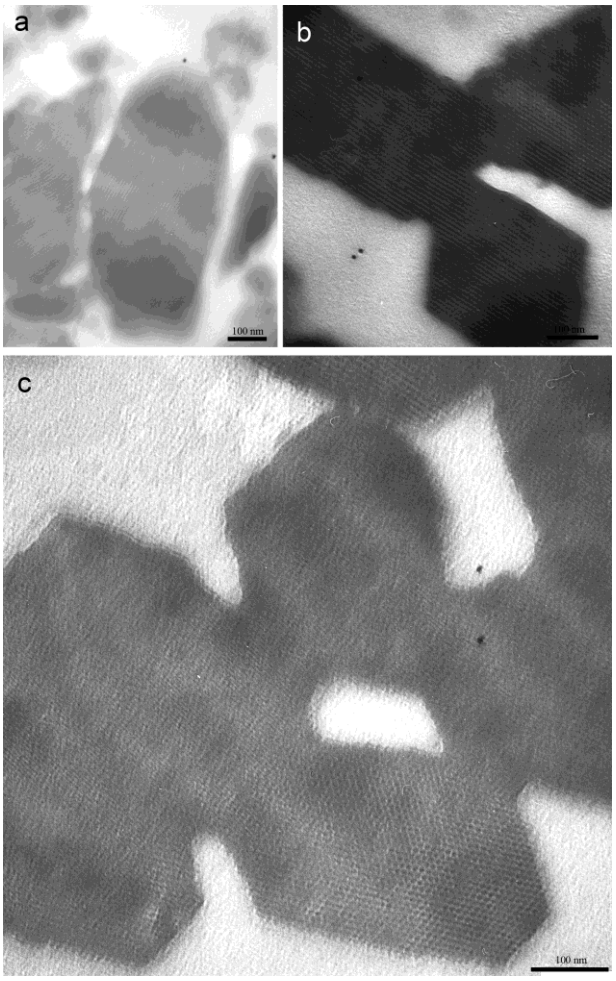

Figure 2. TEM micrographs of controls performed in order to establish the specificity of the labeling. a) Omission of the primary antibodies from the labeling sequence; b) Substitution of non-immune serum for the primary antibody; c) Treatment of unloaded SBA-15 mesoporous silica with the above primary and secondary antibodies.

Three dimensional (3D) reconstruction of Lysozyme distribution in SBA-15 from serial ultrathin sections. Serial ultrathin sections (~ 60-80 nm thick) were cut from a block of embedded SBA-15, collected on nickel grids and treated for postembedding immunogold staining (IGS) procedures as described above. The same specific SBA-15 region was selected on each section and photographed in a Jeol Transmission Electron Microscope. A total of 7 serial micrographs were taken at initial magnification of 30000 X and processed using the free editor software for serial section microscopy reconstruction. All section images were mounted and correctly realigned, whereas the magnification value of the image data was calibrated by drawing bars of known size. Boundary of the mesoporous silica material region and gold particles, present on each section, were drawn manually and finally grouped together using the 3D scene function to form the three dimensional (3D) visualization of both mesoporous silica material and gold particle distribution.