Journal of Materials Chemistry

Cite this: *J. Mater. Chem.*, 2011, **21**, 6321

www.rsc.org/materials

PAPER

Functionalised mesoporous silica: a good opportunity for controlled peptide oligomerisation†

Gilles Subra,*[a] Ahmad Mehdi,*[b] Christine Enjalbal,[a] Muriel Amblard,[a] Luc Brunel,[a] Robert Corriu[b] and Jean Martinez[a]

Received 22nd December 2010, Accepted 14th February 2011
DOI: 10.1039/c0jm04492j

In this paper, mesoporous organosilicas functionalised with aminopropyl groups have been successfully used for peptide oligomerisation. For this purpose, three mesoporous silica SBA-15 type containing different amounts of aminopropyl groups were prepared by direct synthesis and using 3-*tert*-butyloxycarbonylamino propyltriethoxysilane. Thanks to amino groups and under well selected experimental conditions, amino acids *N*-carboxyanhydride-polymerisation has been achieved within the pores with control of the physical properties of peptide functionalised hybrid materials. NCA of alanine, side chain protected glutamic acid and methionine were used for this study. For the first time, direct LDI-MS analysis was successfully performed on the resulting covalently bound supported oligomers. To demonstrate the potential application of this class of hybrid bio-organic–inorganic material as supported catalysts, one of the methionine-functionalized OMSs was used to promote disulfide bond formation in a model peptide.

Introduction

Organic–inorganic hybrid materials based on silica and obtained by sol–gel process have attracted considerable attention during the last decades, as they constitute a fascinating class of materials combining the properties of organic moieties and inorganic matrix.[1–3]

Since the discovery of ordered mesoporous silicas,[4] many investigations have focused on their functionalization to render them suitable for applications such as in catalysis, separation, chemical sensors, *etc.*[5] Two main approaches have been used to anchor organic groups onto the inner pore surface of mesoporous silicas: grafting method (post-synthesis) and co-condensation method (direct synthesis). Post-synthesis grafting of an organotrialkoxysilane $RSi(OR')_3$ onto the pore surface of the mesoporous silica was the first method established for functionalization.[6] This method is generic and allows the incorporation of many R groups including bulky ones. However, neither the loading control nor the distributions of the functional groups, which depend on several parameters such as the number of the residual silanol (SiOH) groups at the surface as well as their accessibility, are possible.[7] A one step alternative approach, overcoming the main restrictions of the post-synthesis method, has been developed.[8,9] It consists of the copolymerisation of tetraethoxysilane (TEOS) and an organotrialkoxysilane $RSi(OR')_3$ in the presence of a structure-directing agent. In this case, the functional groups of the resulting materials were regularly distributed on the pores' surface.[10] Among organic–inorganic hybrid materials, those prepared from ordered mesoporous material (OMS) are of special interest. Indeed, silica can easily be functionalised through trialkoxysilane chemistry, providing functional groups used as anchoring points for covalent immobilization of organic moieties. Moreover the synthesis of OMS which proceeds in the presence of surfactants, enables the tunable control of this structure at the nanometric scale yielding pore diameters from 2 to 30 nm. Compared to other materials of undefined porosity such as silica gels, the use of OMS greatly rationalizes the inclusion of organic compounds in a non-covalent or covalent way. Large proteins such as enzymes and heme proteins[11] were recently covalently immobilized on OMS. More recently, nanocomposite materials were prepared from an OMS obtained by post-synthetic grafting of 3-(trimethoxysilyl)propan-1-amine and *N*-carboxyanhydride (NCA) of alanine and side chain protected lysine.[12]

In this paper, we report the preparation of hybrid oligopeptide–silica material starting from well defined aminopropyl functionalised silica of the SantaBarbara Amorphous type (SBA)[13] and NCA of alanine, protected glutamic acid and methionine. The pore size and aminopropyl group loading

[a]*Institut des Biomolécules Max Mousseron (IBMM), UMR5247 CNRS, Universités Montpellier 1 et 2, 15 avenue Charles Flahault, 34000 Montpellier, France. E-mail: gilles.subra@univ-montp1.fr; Fax: +33 4 67 54 86 54*
[b]*Institut Charles Gerhardt, Chimie Moléculaire et Organisation du Solide, Université Montpellier 2, cc 1701, Place E. Bataillon, 34095 Montpellier Cedex 5, France. E-mail: ahmad.mehdi@univ-montp2.fr; Fax: +33 4 6714 38 52; Tel: +33 4 67 14 38 32*

† Electronic supplementary information (ESI) available: See DOI: 10.1039/c0jm04492j

(mmol g^{-1}) of each OMS sample were carefully controlled thanks to the direct synthesis approach consisting of the hydrolysis and co-condensation of variable amounts of TEOS and 3-*tert*-butyloxycarbonylaminopropyltriethoxysilane in the presence of surfactant self-assemblies under acidic conditions.

Experimental

Chemicals

Pluronic P123 triblock copolymer ($PEO_{20}PPO_{70}PEO_{20}$ with PEO = poly(ethylene oxide), PPO = poly(propylene oxide) and M_{av} = 5800), 3-aminopropyltriethoxysilane (APTES), tetraethoxysilane and di-*tert*-butyl dicarbonate were purchased from Aldrich and used as supplied.

Synthesis

3-*tert*-Butyloxycarbonylaminopropyltriethoxysilane 1. Product **1** was prepared by mixing 11.30 g (51.1 mmol) of 3-aminopropyltriethoxysilane and 12.50 g of di-*tert*-butyl dicarbonate (57.3 mmol) in 50 ml of ethanol. The resulting mixture was stirred overnight at room temperature. The solvent was removed under vacuum and the residual liquid was distilled to afford 12.10 g of **1** (37.3 mmol, 73%) as a colourless liquid (bp 95 °C at 0.05 Torr). ^{1}H NMR (δ ppm, 200 MHz, $CDCl_3$): 0.60 (m, 2H, CH_2Si), 1.20 (t, 9H, $^3J_{HH}$ = 6.90 Hz, OCH_2CH_3), 1.41 (s, 9H, $C(CH_3)_3$), 1.54 (m, 2H, $CH_2CH_2CH_2$), 3.09 (m, 2H, CH_2N), 3.79 (q, 6H, $^3J_{HH}$ = 7.00 Hz, OCH_2), 4.76 (s, 1H, NH). ^{29}Si NMR (δ ppm, 40 MHz, $CDCl_3$): −45.40 MHz.

Aminopropyl functionalised mesoporous silica at 0.8 mmol g^{-1}. 4.0 g of triblock copolymer [$EO_{20}PO_{70}EO_{20}$ with PEO = poly(ethylene oxide) and PPO = poly(propylene oxide)] Pluronic P123 as surfactant were dissolved in an aqueous HCl solution (160 ml, pH ≈ 1.5). This solution was poured into a mixture of TEOS (8.86 g, 42.6 mmol) and 0.72 g (2.2 mmol) of 3-*tert*-butyloxycarbonylaminopropyltriethoxysilane **1**, at ambient temperature. The mixture was stirred for 2 h giving rise to a microemulsion. After heating this perfectly transparent solution at 60 °C, a small amount of NaF (75.4 mg) was added under stirring to induce the polycondensation. The mixture was left at 60 °C under stirring for 48 h. The resulting solid was filtered and washed with ethanol and ether. The surfactant was removed by hot ethanol extraction in a Soxhlet apparatus for 24 hours. After filtration and drying at 60 °C under vaccum, 3.10 g (95%) of a white solid were obtained.

The obtained solid was introduced into a one-neck round bottom flask. The flask was heated at 160 °C under vacuum for 12 hours. The resulting solid was washed with ethanol and ether. After filtration and drying at 60 °C under vacuum, 2.4 g of aminopropyl functionalised mesoporous silica were obtained as a white solid.

NCA oligomerisation (exp. 1). 200 mg of OMS (0.4 mmol g^{-1} of free amino groups) were placed in 12 ml plastic syringe equipped with frit. 8 ml of freshly prepared 0.1 M Ala NCA (92 mg, 0.8 mmol) solution in dry DMF were added to the syringe. The syringe was placed for 3 hours on an orbital shaker under gentle agitation. The syringe was percolated and the functionalised OMS was washed 3 times with 10 ml of DMF and 3 times with the same volume of DCM.

Solids characterization

The solid-state CP-MAS ^{13}C NMR spectra were recorded on a BRUKER FTAM 300 by using the TOSS technique. The repetition time was 5 seconds with contact times of 3 milliseconds. The duration of the ^{1}H pulse was 4.2 microseconds and the MAS rate was 10 kHz. Chemical shifts (δ, ppm) were referenced to Me_4Si. The nitrogen adsorption isotherms were measured at liquid temperature (77 K) using a Micromeritics Tristar 3000 analyser. Before the measurements, the samples were out gassed under vacuum for 12 h at 100 °C. The specific surface areas were calculated by the Brunauer–Emmett–Teller (BET) method (using 74 points and starting from 0.01 as the value of the relative pressure) and the pore size distributions were determined by the BJH method applied to the adsorption branch. Elemental analyses of Si, N, S, and P were performed by the Service Central d'Analyse (CNRS, Vernaison, France).

Mass spectrometry analyses

A MALDI-ToF/ToF apparatus (Ultraflex III mass spectrometer, Bruker Daltonics, Germany) was used for all MS analyses (three sets of experiments according to the sample deposit protocol on the MALDI target). The source was operated in the positive mode. An acceleration voltage of 25.0 kV (IS1) was applied for a final acceleration of 21.95 kV (IS2). The ToF mass analyzer was set in the reflectron mode (voltages of 26.3 kV and 13.8 kV). A pulsed Nd : YAG laser at a wavelength of 355 nm was operated at a frequency of 100 Hz with a delayed extraction time of 50 ns. Data were acquired with the Flex Control software and processed with the Flex Analysis software. External calibration was performed with a commercial peptide mixture (calibration peptide standard 2, Bruker Daltonics, Wissembourg, France). Mass spectra were acquired from 250 laser shots, the laser fluence being adjusted for each studied sample. Ions were detected over a mass range from *m*/*z* 200 to 5000. No deflection was applied in heterogeneous deposit conditions whereas a deflection up to *m*/*z* 500 was used for homogeneous deposit to remove matrix ions.

Sample deposit protocols on the MALDI target. Every sample was analysed with all 3 deposit protocols.

1 Direct deposit on MALDI steel plate without matrix. The silica (1 mg) was suspended in a solution (1 ml) of water–acetonitrile–trifluoroacetic acid (70/30/0.1 v/v/v) and thoroughly sonicated. 0.5 µl of such a suspension was then rapidly aliquoted and directly deposited on the steel plate. The deposit was allowed to air-dry.

2 Direct deposit on MALDI steel plate with matrix. HCCA (α-cyano-4-hydroxycinnamic acid) was chosen as the matrix at a concentration of 20 mg ml^{-1} in water–acetonitrile–trifluoroacetic acid (70/30/0.1 v/v/v). 0.5 µl of the matrix was deposited first on the steel plate. The same protocol was then applied for the silica except that the aliquoted suspension (0.5 µl) was

directly mixed with 0.5 μl of the matrix solution and then allowed to air-dry.

3 Silica solubilization and sample deposit on MALDI steel plate with matrix.[12] Silica solubilization was performed in a mixture (1 : 1 v : v) of aqueous fluorhydric acid (48% w) and hexafluoroisopropyl alcohol. Trihydroxyacetophenone (THAP) and HCCA were chosen as the matrix for two sets of experiments. A solution in water–acetonitrile (70 : 30 v : v) was mixed with the silica solution (1 : 1 v : v). 0.5 μl of such a mixture was deposited on the MALDI steel plate and air-dried.

Results and discussion

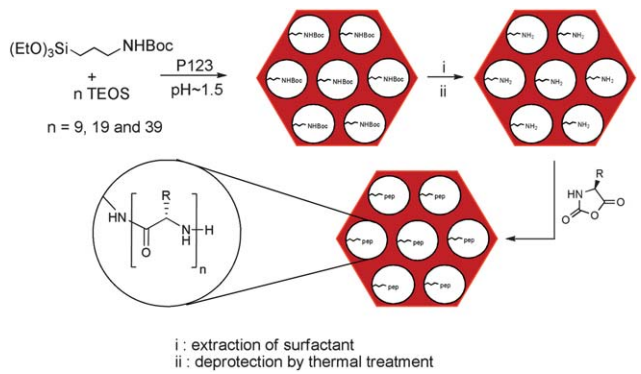
Three amino functionalised mesoporous silicas with different loadings (0.4, 0.8 and 1.6 mmol g^{-1}) were prepared by direct synthesis following the method already described.[14] For this study, SBA-15 mesoporous silica containing variable amounts of *tert*-butyloxycarbonylamino (NHBoc) groups were prepared (Scheme 1). The synthesis of these materials was achieved by co-polymerization of **1** and different amounts of tetraethoxysilane (TEOS) in the presence of P123 as structure directing agent (see Experimental).

The surfactant was removed by washing to give the *tert*-butyloxycarbonylaminopropyl functionalised material in high yield. It is worth noting that no solid was obtained under the same conditions by using APTES instead of **1**. This result demonstrates the importance of the approach with protected amino groups.

Thermal treatment of the material containing NHBoc at 160 °C under vacuum gave rise quantitatively to mesoporous silica containing aminopropyl groups.

^{13}C CP-MAS NMR technique was used to demonstrate the efficiency of the non-ionic template extraction procedure, as well as to control the organic incorporation within the materials.

The spectrum of OMS containing 0.8% of aminopropyl groups (see ESI 1†) indicates the complete removal of the block copolymer surfactant. The resonances at 9.3, 24.5 and 42.7 ppm refer respectively to the carbons in α, β and γ positions of the silicon atom, settling the incorporation of $-(CH_2)_3NH_2$ groups in the material. It is also worth noting that the observed signals at 58 and 17 ppm were assigned to residual $-SiOCH_2CH_3$ groups.



Scheme 1 General way for the preparation of controlled oligopeptide modified SBA.

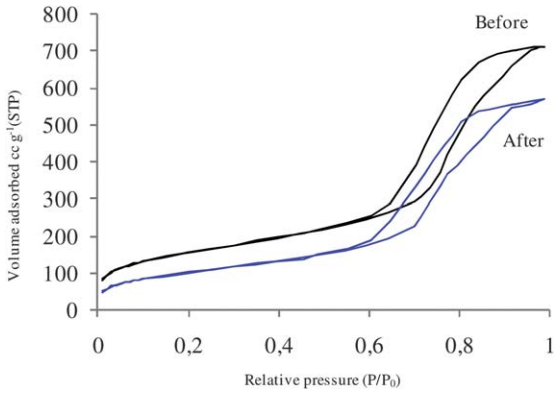


Fig. 1 N_2 adsorption–desorption isotherms of OMS containing 0.8% of aminopropyl groups before and after peptide oligomerisation.

The N_2 adsorption–desorption measurements at 77 K for all aminopropyl groups functionalised OMS showed type IV isotherms (see Fig. 1 as example) with H1-type hysteresis loop at relatively high pressure characteristic of mesoporous materials with high surface areas. The BET surface areas were in the range of 400 to 700 $m^2\ g^{-1}$, the total pore volumes are from 0.60 to 1.30 $cm^3\ g^{-1}$ with regular pore size distributions.

In this way, the influence of the physical properties of the starting aminopropyl functionalised OMS as well as the concentration and the equivalents of NCA used for oligomerization was examined. Hybrid materials were characterized by several spectroscopic and spectrometric measurements. Notably, we first demonstrated that direct laser desorption ionization mass spectrometry (LDI-MS) analysis of these organic–inorganic hybrid materials is feasible without prior dissolution of such silica-based samples in HF aqueous solution. In contrast to

Table 1 Functionalised OMS prepared with NCA

Exp	OMS loading (mmol of free $NH_2\ g^{-1}$)	NCA	Eq. of NCA	[NCA]	Exp. average oligomer length
1	0.4	Ala	5	0.1	4.0
2	0.4	Ala	10	0.2	6.3
3	0.4	Ala	20	0.4	10.9
4	0.8	Ala	5	0.1	2.9
5	0.8	Ala	10	0.2	4.0
6	0.8	Ala	20	0.4	9.7
7	1.6	Ala	5	0.1	1.2
8	1.6	Ala	10	0.2	1.7
9	1.6	Ala	20	0.4	4.8
10	0.4	Met	5	0.1	4.5
11	0.4	Met	10	0.2	4.2
12	0.4	Met	20	0.4	5.3
13	0.8	Met	5	0.1	2.4
14	0.8	Met	10	0.2	2.2
15	0.8	Met	20	0.4	2.2
16	1.6	Met	5	0.1	0.1
17	1.6	Met	10	0.2	0.1
18	1.6	Met	20	0.4	0.1
19	0.8	Glu(OBzl)	5	0.1	0.7
20	0.8	Glu(OBzl)	10	0.2	0.4
21	0.8	Glu(OBzl)	20	0.4	0.4
22	0	Ala	20	0.1	–
23	0	Glu(OBzl)	20	0.2	–
24	0	Met	20	0.4	–

literature data based on the latter tedious and potentially dangerous analytical protocol,[12,15] each of the prepared functionalised mesoporous silica was suspended in water, sonicated and an aliquot was deposited on the MALDI steel plate either directly as such (LDI analysis) or mixed with an organic MALDI matrix (MALDI analysis).

Although peptide analyses by LDI-MS from various inert supports, including silica (of non-controlled porosity), has been previously investigated in the laboratory,[16,17] the peptides were not covalently attached to the material, but only adsorbed onto the surface. Suppression of the solubilization step allowed gaining in analysis safety, robustness and speed. For all the studied organic–inorganic hybrid materials, covalent bond cleavage was achieved upon laser irradiation in the MALDI mass spectrometer ion source, liberating in the gas phase positively charged oligopeptide ions.

Each amino functionalised OMS reacted three hours with different concentrations of NCA solution (0.1, 0.2 and 0.4 M) in dry dimethylformamide yielding an excess of 5, 10 and 20-fold respectively (Table 1). Silica was filtrated and washed thoroughly with DCM and DMF. Aliquots were dried, weighed and analyzed by various methods. Firstly, transmission FTIR was used to check the appearance of the two characteristic carbonyl vibration bands at 1658 and 1541 cm^{-1} witnessing the formation of peptide bonds on the silica. The strong silyl ether band was observed at 1061 cm^{-1}. Due to the direct synthesis of amino OMS, no silanol band at 3450 cm^{-1} was observed (see ESI 2†).

Secondly, the average polymer weight was determined by weighing freeze-dried materials after polymerization. At a fixed concentration and fixed number of equivalents of NCA, the length of the polypeptide chain decreased when the loading of the OMS increases (*i.e.* comparing exp. 1, 4, 7 and exp. 10, 13, 16). This can be interpreted as an effect of steric hindrance generated by the proximity between two close amino groups of the OMS that did not allow the peptidyl chain to grow longer. Moreover, the pore size (initially ~5 nm) changed during oligomerization process and the NCA monomers probably react more easily with the *N*-terminus of the growing peptide chains in the less functionalised OMS material. When comparing oligo-OMS **4** (Ala), **13** (Met) and **19** (Glu), one can note that polymerization was highly influenced by the nature of the amino acid side chain. The more bulky the side chain was (*i.e.* benzyl ester protected glutamic acid) the less polymerization occurred. Moreover, when non-hindered Ala NCA was used, for a given OMS, increasing the number of equivalents of NCA resulted in an increase of the polymer chain. As an example, when comparing oligo-OMS **1**, **2**

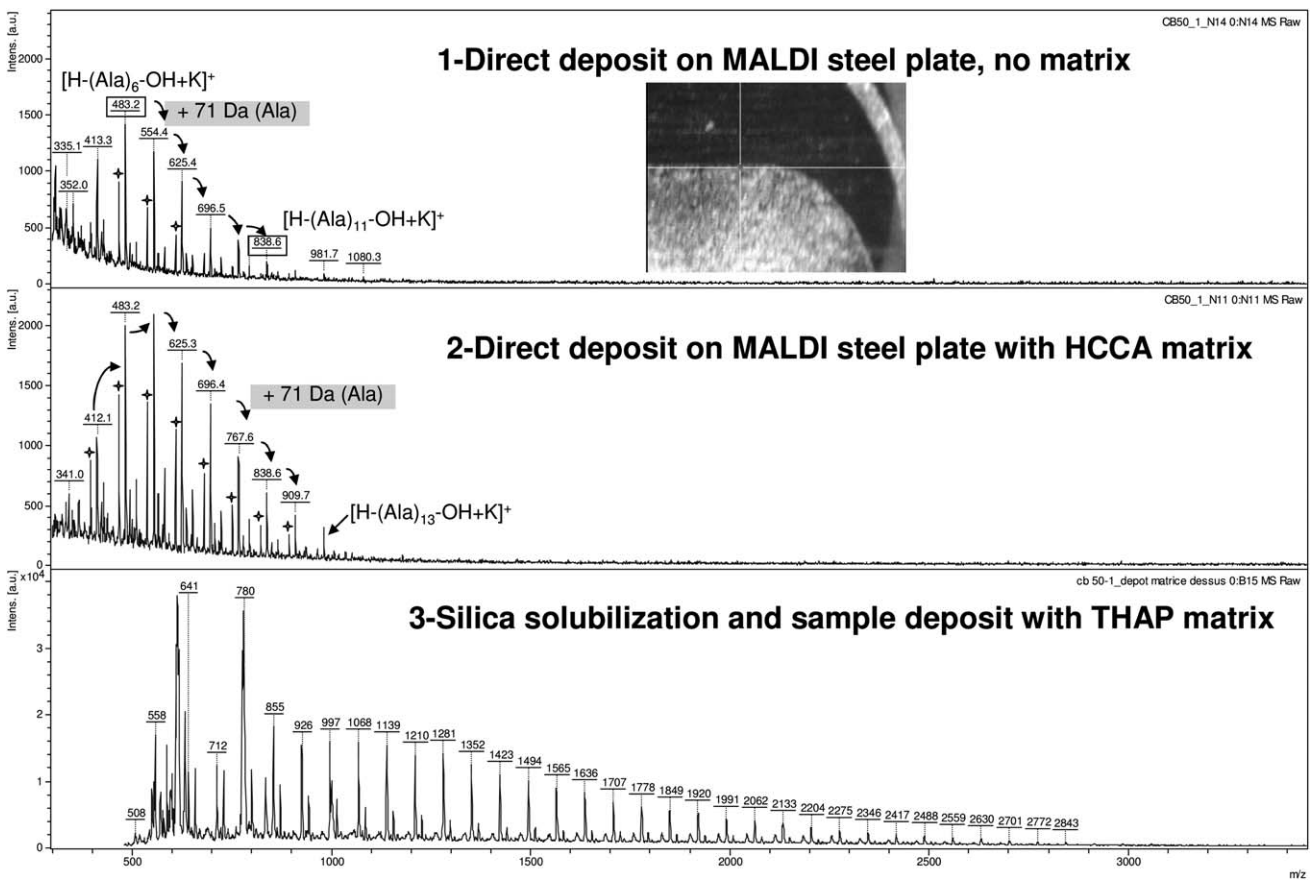


Fig. 2 LDI MS spectra of oligoAla-OMS material (exp. 4) acquired under three different conditions: 1, direct deposit on the MALDI steel plate (heterogeneous deposit as shown in the inset, no matrix); 2, direct deposit on the MALDI steel plate with a prespotted HCCA matrix solution; 3, OMS solubilization and sample deposit on MALDI steel plate with THAP matrix.[12] Ions were produced as cationized species (sodiated ions indicated by a star and potassium adducts representing the most intense polymeric distribution).

and **3** obtained from 0.4 mmol g^{-1} OMS, the average number of Ala residues increased from 4.0 to 10.9 while the equivalents of NCA increase from 5 to 20. On the contrary, whatever excess of bulkier Met NCA was used, it has almost no influence on polymer length. Thirdly, three sets of mass spectrometry analyses were carried out from solid samples (heterogeneous sample deposit conditions with and without MALDI matrix) and from HF solubilized silicas serving as analytical controls (homogeneous sample deposit conditions with a MALDI matrix).

In the case of alanine (exp. 1–9), the expected polymeric distributions were observed with an increased intensity and wider mass range detection in the presence of an organic matrix mixed with the silica particles. The same results were obtained in standard homogeneous conditions from solubilized materials indicating that the described analytical method is not only efficient but above all, simple, safe, rapid and ready to use in laboratories equipped with a conventional MALDI mass spectrometer. As an illustration, the mass spectra recorded for exp. 4 are displayed in Fig. 2. The LDI MS data of exp. 6 corresponding to the same polymerization with 4 times more equivalents of Ala NCA can be found in ESI 3†. Compared to alanine, glutamic acid derivatives provided shorter peptides that failed to be identified after solubilization in aqueous HF according to the published protocol.[12] Only direct MS characterization upon heterogeneous LDI conditions allowed the detection of few ions separated by the mass of Glu(OBzl) *i.e.* 221 Da (exp. 19–21) (see ESI 4†). Methionine used in exp. 10–18 provided MS spectra that were more difficult to interpret, no obvious polymeric distribution being clearly detected whatever be the used method. Thus, for this residue transmission FTIR, weighing freeze-dried materials and elemental analysis of sulfur content were preferred.

The nitrogen adsorption–desorption isotherm of functionalised OMS (exp. 4) material after peptide oligomerisation (Fig. 1) shows a similar type of isotherm to that of the starting material with 375 $m^2\ g^{-1}$ of surface area, 0.8 $cm^3\ g^{-1}$ of pore volume. This diminution of surface area (from 700 to 375 $m^2\ g^{-1}$) and pore volume (from 1.3 to 0.8 $cm^3\ g^{-1}$) demonstrated that the oligomers developed within the pores of mesoporous materials.

Fig. 3 shows the powder X-ray diffraction pattern of OMS (exp. 4) which exhibits an intense diffraction Bragg peak corresponding to d_{100} spacing with 11.2 nm as lattice parameter a_o.

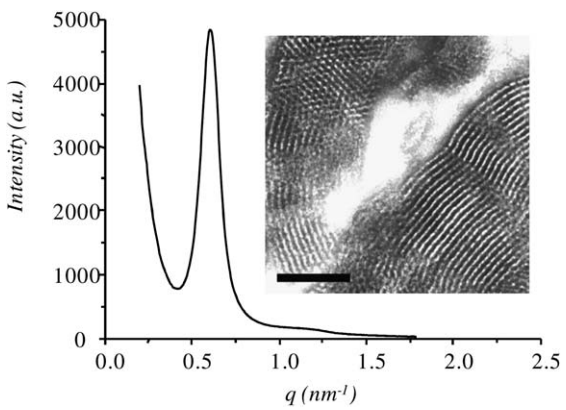
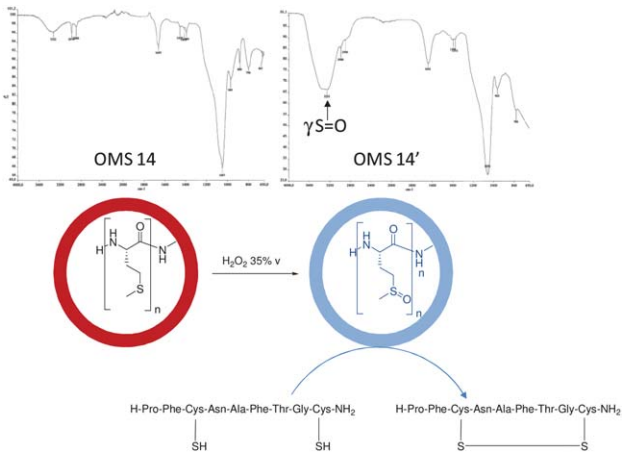


Fig. 3 XRD pattern of OMS containing 0.8% of aminopropyl groups after peptide oligomerisation. The inset shows TEM image for the same material. Scale bar = 100 nm.



Scheme 2 Preparation of oligoMet[O]-OMS and its use for disulfide bond formation in crustacean cardioactive peptide (CCAP).

In addition, transmission electron microscopy (TEM) micrograph (see inset of Fig. 3) confirms that the material exhibits local hexagonal symmetry.

It is worth noting that non-functionalised OMS (exp. 22–24) neither displayed any characteristic peptide bands in transmission FTIR, nor ions in the MALDI mass spectra, nor gained significant weight after NCA reaction.

As a first application, we proposed to prepare an OMS supported reagent. Supported oligomers of methionine sulfoxide were efficient in promoting intramolecular disulfide bond formation in peptides containing two or more cysteine residues.[18] To that purpose oligoMet-OMS **14** was treated with hydrogen peroxide to yield oligoMet[O]-OMS **14′** (Scheme 2). The presence of sulfoxide was controlled by FTIR showing the appearance of a broad band at 3350 cm^{-1}.

OligoMet[O]-OMS **14** was mixed in water with 5 mM solution of purified (free sulfhydryl) form of crustacean cardioactive peptide (CCAP) obtained by classical microwave assisted Fmoc/tBu SPPS.[19] After 30 minutes stirring, the oligoMet[O]-OMS **14** was filtered and the solution was analysed showing <90% disulfide bond formation. After 3 hours, only the oxidized form of CCAP was detected (see ESI 5†).

Conclusions

In this work, we described the synthesis and characterization of new organic–inorganic hybrid materials functionalised with peptide units. We demonstrated that physical properties of peptide–OMS hybrid materials can be tuned by experimental conditions used for NCA-polymerisation within the pores. Direct LDI-MS analysis can be performed on the resulting covalently bound supported oligomers. Gathering the structural features of OMS and peptide properties, this class of hybrid bio-organic–inorganic material could find other applications in separation and molecular recognition.

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