Adsorption and activity of proteins onto mesoporous silica

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The adsorption and activity of cytochrome c onto two different MCM-41 materials, MCM-41/28 and MCM-41/45 with average pore diameters of 28 and 45 Å respectively, is presented. Nitrogen gas adsorption/desorption isotherms before and after protein adsorption, and peroxidative activity profiles of the adsorbed protein demonstrate that the protein is adsorbed into the mesoporosity and remains active. The adsorption of a range of different proteins onto both MCM-41/28 and 45 shows how protein properties affect adsorption.

KEY WORDS: adsorption/desorption; mesoporous; peroxidative activity.

1. Introduction

Mesoporous molecular sieves (MMS) have been the subject of much interest since they were first described by Beck *et al.* in 1992 [1]. MMS possess large surface areas (up to $1000\,m^2\,g^{-1}$), highly ordered pore structures and very tight pore size distributions (PSD), properties that have made these materials attractive candidates for a wide range of applications in catalysis [2–4]. There have been a number of reports describing the use of MMS to immobilize proteins. Balkus *et al.* [5–7] have shown that the adsorption of proteins is dependent on the pore size of the material. Penicillin acylase (PA) has been adsorbed on to MCM-41 and also by crosslinking to silylated MCM-41 using glutaraldeyde as the crosslinking agent. The activity of the adsorbed PA was more than five times that of the crosslinked enzyme [8]. We have recently shown, by generating adsorption isotherms for cytochrome c (cyt c) onto a range of MMS, that adsorption is dependent on the silicate pore size and that the peroxidative activity of the adsorbed protein is higher than that of the aqueous protein [9]. In addition, spectroscopic studies indicated that the environment of the prosthetic heme group was unchanged [10].

Takahashi *et al.* investigated the immobilization of horseradish peroxidase (HRP) and subtilisin onto FSM-16 (folded sheet mesoporous material), MCM-41 (both synthesized using cationic surfactants) and SBA-15 (synthesized using a non-ionic surfactant) [11]. It was shown that the optimal thermal and catalytic activity of each enzyme was achieved when the average pore size of the MMS was marginally larger than the protein size. This is in marked contrast to results obtained using controlled pore glass, where for successful adsorption the average pore size was significantly larger than that of the protein [12].

Yiu *et al.* have shown that trypsin adsorbed onto MCM-41, MCM-48 and SBA-15 retained its ability to hydrolyze peptides but considerable desorption of the enzyme from MCM-48 occurred (72%) [13]. SBA-15-supported trypsin showed a higher activity than the smaller pore MCM-41. Trypsin supported on porous silica gel was found to give a higher activity than any of the MMS-supported preparations. This was explained by the larger proportion of enzyme adsorbed on the outer surface area of the porous silica gel compared to the MMS. Other studies using SBA-15 functionalized surfaces have shown tailoring of the MMS surface functional groups to be extremely important in enhancing the interactions of the protein surfaces and the MMS surface [14]. With trypsin the presence of thiol, chloride and carboxylate moieties on the MMS surface resulted in increased stability of the adsorbed protein. Phenyl and amine groups on the MMS resulted in less desorption of trypsin (19–25%). In the absence of these groups 48–52% of the protein was desorbed.

More recently Stucky *et al.* [15] have shown that chloroperoxidase (CPO) could be immobilized in an MMS (mesocellular foam) with a 48% decrease in specific activity in comparison to the solution-phase enzyme. Here we present adsorption and peroxidative activity data for the redox protein cyt c adsorbed onto two different MCM-41 materials and also report on the adsorption of other proteins onto MCM-41.

2. Experimental

MCM-41/28 was synthesized as previously reported [9]. MCM-41/45 was prepared following a modified method of Mokaya *et al.* [16]. All materials were characterized by nitrogen gas adsorption/desorption isotherms

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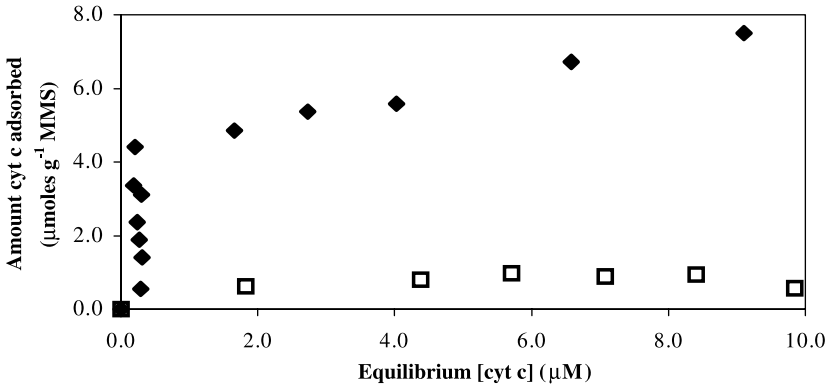

Figure 1. Cytochrome c adsorption isotherms onto MCM-41/28 (□) and MCM-41/45 (◆).

at 77 K measured using a Micromeritics Gemini ASAP 2000 system. Samples were pretreated at 150 °C for 1 h. The pore size data were analyzed by the thermodynamic-based Barrett–Joyner–Halenda (BJH) method [17] using the desorption branch of the isotherm and surface areas were measured using the Brunauer–Emmett–Teller (BET) method [18].

The protein adsorption experiments were carried out at 25 °C in 25 mM phosphate buffer (pH 6.5), without stirring as previously described [9]. The other proteins were adsorbed as described elsewhere [10]. Adsorption was allowed to proceed for 16 h for both MCM-41 materials, after which the adsorbed protein was washed with buffer (four times). The peroxidative activity of the adsorbed protein was determined as previously reported using 2,2′-azino(bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) in the presence of H_2O_2 [9].

3. Results and discussion

We previously reported adsorption isotherms and peroxidative activity profiles for cyt c adsorbed onto a range of different MMS materials [9]. Here we compare the adsorption of the protein onto MCM-41/28 with the results obtained with a similar material, MCM-41/45. The only major difference between these two materials is their average pore size; the surface areas of both are very similar and their isoelectric points are identical (see table 1).

In figure 1 the adsorption isotherms for cyt c onto both MCM-41/28 and MCM-41/45 are presented. It can be seen that the affinity of the protein for the MCM-41/45 material is significantly greater than for the MCM-41/28 material. From structural data cyt c has a unit cell of dimensions $a = b = 58.34$ Å, $c = 41.83$ Å [19] and a hydrodynamic radius of 30 Å [20], giving the protein an average radius of ~30 Å. The MCM-41/45 material thus has pores that are just larger than, or approaching, the diameter size of the protein. It is intriguing that the protein penetrates into the mesopore network in spite of the fact that the pore size is similar to that of the protein. It has been previously reported that the protein size is a limiting factor for adsorption to occur [5,7]. The pores of MCM-41/45 appear to have a strong attraction for cyt c as shown in figure 1.

The peroxidative activity of cyt c adsorbed onto MCM-41/45 is compared to that of MCM-41/28 in figure 2. The same trend of higher activity at lower levels of adsorbed protein is evident, with the activity approaching that of the aqueous protein at higher protein loadings (i.e. >0.0045 mmol g^{-1}). For MCM-41/28, cyt c adsorption occurs initially on the outside of the silicate (at lower protein concentrations), followed by the appearance of multiple layers as the protein concentration increases. Access to the redox center of cyt c is more readily achieved at the lower levels of adsorbed protein, accounting for the higher turnover frequencies (TOF, μmoles of reduced ABTS produced per second per μmole of adsorbed cyt c) that are observed in comparison to the values at the higher protein loadings [9]. The adsorbed protein which has a higher TOF in comparison to the aqueous protein indicates that diffusion

Table 1
Physicochemical characteristics of mesoporous silicates

Sample	Pore diameter (Å)	BET surface area ($m^2 g^{-1}$)	Pore volume ($cm^3 g^{-1}$)	Isoelectric point
MCM-41/28	28	1000	0.31	3.6
MCM-41/45	45	970	1.14	3.6
MCM-41/45 (0.11 μmol cyt c g^{-1} MCM-41)	35	581	0.52	–
MCM-41/45 (4.23 μmol cyt c g^{-1} MCM-41)	23	509	0.30	–

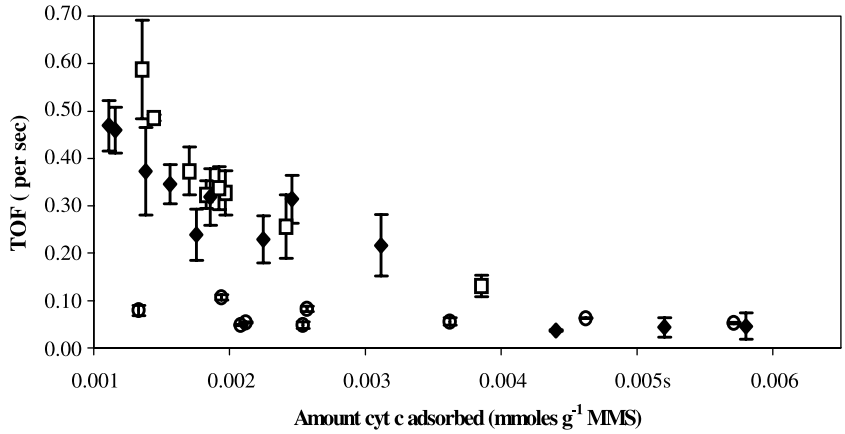

Figure 2. Peroxidative activity profiles for cytochrome c adsorbed onto MCM-41/28 (□), MCM-41/45 (◆) and aqueous cyt c (○).

of the substrate to the adsorbed protein (on the external surface and within the pores) is not rate limiting.

Figure 3 shows nitrogen gas adsorption isotherms (BJH method) of MCM-41/45 both before and after adsorption (see summary in table 1). The pore volume decreases sharply upon adsorption of protein demonstrating that adsorption is occurring in the mesopore network. The protein does not seem to penetrate deeply into the mesopore network. After adsorption of a relatively small amount of protein ($0.11\,\mu mol\,g^{-1}$, approximately 1.5% of the total binding capacity of MCM-41/45 for the protein) the mesopore volume decreased sharply from 1.15 to $0.52\,cm^3\,g^{-1}$. When the loading was increased to $4.23\,\mu mol\,g^{-1}$ the mesopore volume was further reduced to $0.30\,cm^3\,g^{-1}$, but the relative percentage decrease is not significantly higher (74%) compared with 55% for the lower loading. This implies that only a small amount of protein is needed to block the pores. The protein therefore seems to penetrate into the mesopore network but only partially travels through the mesopores before blocking them. In the presence of higher protein loadings the protein starts to pack into the mesopores, effectively filling the pores. Increasing the loading significantly does not result in further significant decreases of the surface area, the pore volume, or the pore diameter. The reduction in activity of the adsorbed protein compared to that observed for the protein in aqueous solution at higher loadings of protein also suggests that the protein is being packed into the pores and that a significant amount is inaccessible to the substrate. The protein adsorption isotherm onto MCM-41/45 (figure 1) also supports this hypothesis. There is a strong affinity for the protein at low equilibrium concentrations, but above protein loadings of $\sim 4.30\,\mu mol\,g^{-1}$ the rate of increase in the amount of adsorbed protein decreases, due to the relatively slow diffusive packing/blocking of the protein into the pores.

Table 2 presents the physicochemical characteristics of a range of different proteins and their adsorption onto MCM-41/28 and MCM-41/45. Protein adsorption isotherms were generated for each of these and the amounts of protein adsorbed at an equilibrium concentration of $\sim 1.00\,\mu M$ for MCM-41/28 and $1.66\,\mu M$ for MCM-41/45 (see values in parentheses) are presented. All of the proteins were adsorbed at a pH where the protein is either neutral or bears a positive charge. It can be seen that the relatively large glucose oxidase (GOx) and pepsin do not adsorb onto either MCM-41. Myoglobin, which has a similar size to cyt c, does not

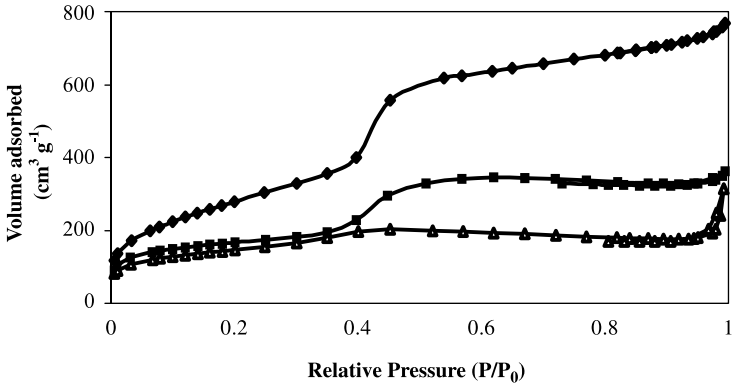

Figure 3. Nitrogen gas adsorption/desorption isotherms of MCM-41/45 (◆), MCM-41/45 with a cytochrome c loading of $0.11\,\mu mol\,g^{-1}$ (■), MCM-41/45 with a cytochrome c loading of $4.23\,\mu mol\,g^{-1}$ (△).

Table 2
Physicochemical characteristics of proteins used in adsorption studies. The values in parenthesis refer to the equilibrium protein concentrations at which the amounts adsorbed were taken. N/A refers to not available

Protein	Molecular weight (daltons)	Isoelectric point	Hydrophobic index [23]	pH adsorption	MCM-41/28 ($\mu mol\,g^{-1}$)	MCM-41/45 ($\mu mol\,g^{-1}$)
Pepsin	33 000	1.0	85.5	1.6	0.00 (1.00)	0.00 (1.66)
Glucose oxidase (GOx)	160 000	4.3 [24]	81.5	4.0	0.00 (1.00)	0.00 (1.66)
Myoglobin	17 600	7.0 [25]	89.4	6.5	0.00 (1.00)	5.32 (1.66)
Horseradish peroxidase (HRP)	44 000	7.3 [21]	84.8	4.0	0.32 (0.86)	0.05 (1.59)
Trypsin	22 000	8.5	76.9	6.5	N/A	6.10 (1.68)
Cytochrome c (cyt c)	12 300	10.6 [26]	59.1	6.5	0.13 (0.66)	4.85 (1.66)

adsorb onto MCM-41/28 but substantial amounts adsorb onto MCM-41/45 which are comparable with cyt c (see figure 4). The lack of adsorption of myoglobin onto MCM-41/28 can be attributed to the less electrostatic and more hydrophobic nature of myoglobin, i.e. cyt c's hydrophobic index is significantly smaller than that of myoglobin and the latter protein bears a slight positive charge at pH 6.5 (i.e. pH of adsorption). Nevertheless myoglobin exhibits a similar high affinity for MCM-41/45.

Trypsin has a large affinity for MCM-41/45, the amounts adsorbed being similar to the cyt c loadings (see figure 4 and table 2). Both proteins have similar isoelectric points and trypsin's hydrophobic index is closer to cyt c's than any of the other proteins studied here. Indeed MCM-41/45 adsorbs significantly more trypsin than cyt c at similar equilibrium concentrations. Trypsin has a unit cell of $a = 63.33$, $b = 63.33$, $c = 130.61$ Å [21] suggesting that protein size is not the only important factor for adsorption; protein surface chemistry is also important.

Horseradish peroxidase (HRP) adsorbs onto both MCM-41 materials but has a higher affinity for MCM-41/28. HRP has a unit cell of $a = 159.17$, $b = 159.17$, $c = 114.27$ Å [22] and is too large to penetrate into the pores of either material. The amount adsorbed onto both MCM-41 materials is significantly lower than the smaller proteins used (i.e. cyt c, myoglobin and trypsin). This is a consequence of both HRP's larger size but also its higher hyrophobic index, especially in comparison with cyt c and trypsin. HRP has a relatively high isoelectric point comparable with the smaller proteins used but HRP has an extensive glycosylation pattern on its surface (an extensive network of polymerized sugars). These sugars are absent from the surfaces of cyt c and trypsin and therefore HRP's surface chemistry is fundamentally different from these proteins.

The overall structures of the MCM-41 catalysts that have been loaded with cyt c are dependent on the pore size of the material and the loading of protein. MCM-41/28 allows for adsorption only on the outside of the silicate surface and multilayer adsorption occurs when protein concentration is above monolayer values. The redox center of the protein is only therefore accessible at the top layers of protein in such cases. For MCM-41/45 the protein enters the pores and partially travels through the mesopore network before blocking the pores. At loadings $<0.003\,mmol\,g^{-1}$ MMS the catalyst activity is higher than the aqueous protein. As the protein loading is increased further the activity drops to aqueous levels due to the packing of protein molecules into the mesoporosity. Thus at high protein loadings onto MCM-41/45 a substantial amount of protein is inaccessible to the substrate.

4. Conclusions

Cyt c has a remarkably high affinity for MCM-41/45 despite the fact that its size approaches that of the diameter of the MCM-41 pores. The protein penetrates the mesoporosity but only partially travels down the pore channels. The adsorbed cyt c exhibits analogous peroxidative activity behavior to that adsorbed on the smaller pore size MCM-41. Protein size, isoelectric

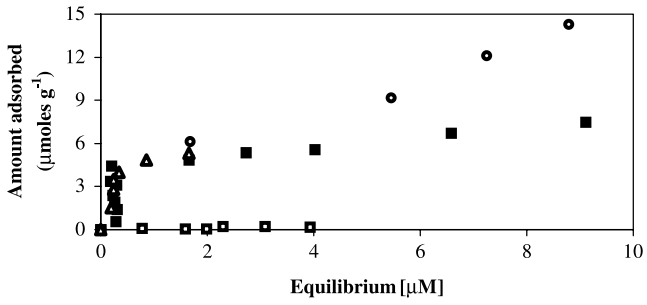

Figure 4. Adsorption isotherms for proteins adsorbed onto MCM-41/45: horseradish peroxidase (HRP, □), myoglobin (△), cytochrome c (cyt c, ■), trypsin (○).

point and also protein surface chemistry are important parameters, which influence adsorption.

References

[1] J.S. Beck, J.C. Vartuli, W.J. Roth, M.E. Leonowicz, C.T. Kresge, K.D. Schmitt, C.T. Chu, D.H. Olson, E.W. Sheppard, S.B. McCullen, J.B. Higgins and J.L. Schlenker, J. Am. Chem. Soc. 114 (1992) 10834.

[2] B.F.G. Johnson, S.A. Raynor, D.S. Shephard, T. Mashmeyer, J.M. Thomas, G. Sankar, S. Bromley, R. Oldroyal, L. Gladden and M.D. Mantle, Chem. Commun. (1999) 1167.

[3] Z. Zhang, J. Suo, X. Zhang and S. Li, Chem. Commun. (1998) 241.

[4] J.M. Thomas and W.J. Thomas, *Principles and Practices of Heterogeneous Catalysis* (Wiley VCH, 1997), p. 16.

[5] J.F. Diaz and K.J. Balkus Jr., J. Mol. Cat. B: Enz. 2 (1996) 115.

[6] L. Washmon-Kriel, V.L. Jimenez and K.J. Balkus Jr., J. Mol. Cat. B: Enz. 10 (2000) 453.

[7] Y.-J. Han, G.D. Stucky and A. Butler, J. Am. Chem. Soc. 121 (1999) 9897.

[8] J. He, X. Li, D.G. Evans, X. Duan and C. Li, J. Mol. Cat. B: Enz. 11 (2000) 45.

[9] J. Deere, E. Magner, J.G. Wall and B.K. Hodnett, Chem. Commun. (2001) 465.

[10] J. Deere, E. Magner, B.K. Hodnett and J.G. Wall, J. Phys. Chem. B 106 (2002) 7340.

[11] H. Takahashi, B. Li, T. Sasaki, C. Miyazaki, T. Kajino and S. Inagaki, Micro. Meso. Mater. 44–45 (2001) 755.

[12] H.H. Weetall in: *Methods in Enzymology* (1976), pp. 134–148.

[13] H.H.P. Yiu, P.A. Wright and N.P. Botting, Micro. Meso. Mater. 44–45 (2001) 763.

[14] H.H.P. Yiu, P.A. Wright and N.P. Botting, J. Mol. Cat. B: Enz. 15 (2001) 81.

[15] Y.-J. Han, J.T. Watson, G.D. Stucky and A. Butler, J. Mol. Cat. B: Enz. 671 (2001) 1.

[16] R.J. Mokaya, W. Zhou and W. Jones, Chem. Commun. (2000) 51.

[17] E.P. Barrett, L.G. Joyner and P.P. Halenda, J. Am. Chem. Soc. 73 (1951) 373.

[18] S. Brunauer, P.H. Emmett and E. Teller, J. Am. Chem. Soc. 60 (1938) 309.

[19] G.W. Bushnell, G.V. Louie and G.V. Brayer, J. Mol. Biol. 1990 (1990) 585.

[20] P.L. Altman and D.S. Dittmer, in: *The Biology Data Book* (Federation of American Societies for Experimental Biology, Bethesda, MD, 1972).

[21] M. Gajhede, D.J. Schuller, A. Henriksen, A.T. Smith and T.L. Poulos, Nat. Struct. Biol. 4 (1997) 1032.

[22] U. Rester, W. Bode, M. Moser, M.A. Parry, R. Huber and E. Auerswald, J. Mol. Biol. 292 (1999) 93.

[23] Hydrophobic indexes were calculated using Compute pI/MW program on the web site http://expasy.hcuge.ch/

[24] R. Wilson and A.P.F. Turner, Biosensors Bioelectron. 7 (1992) 165.

[25] E. Antonini and M. Brunori, *Hemoglobin and Myoglobin in their Reaction with Ligands*, North-Holland Research Monographs, Frontiers in Biology (North-Holland, Amsterdam/London, 1976).

[26] R.A. Scott and A.G. Mauk, *Cytochrome c*, *A Multidisciplinary Approach* (University Science Books, Sausalito, CA, 1996).