Available online at www.sciencedirect.com
ScienceDirect
Journal of Molecular Catalysis B: Enzymatic 49 (2007) 61–68
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Influence of pH and ionic strength on the adsorption, leaching and activity of myoglobin immobilized onto ordered mesoporous silicates

H. Essa, E. Magner, J. Cooney, B.K. Hodnett *

Materials and Surface Science Institute and Department of Chemical and Environmental Sciences, University of Limerick, Limerick, Ireland

Received 22 February 2007; received in revised form 20 June 2007; accepted 6 July 2007
Available online 21 July 2007

Abstract

Myoglobin has been immobilized onto different ordered mesoporous silicates. The effect of the pH on the adsorption, leaching and activity was studied. The results showed that the maximum amount of protein was adsorbed at a pH 6.5, just below the protein isoelectric point (7–7.3). There was no effect of increasing ionic strength on the adsorption profile at different pH values. The adsorption is rationalized in terms of local electrostatic forces acting between the enzyme and the silica surface as well as hydrophobic interactions close to the protein isoelectric point, whereas at low pH the global charges give rise to protein–protein repulsion and at high pH enzyme–silica repulsion. Higher amounts of immobilized myoglobin were leached at a pH 4, while lower amounts were leached at pH 6.5. The catalytic activity of myoglobin immobilized onto SBA-15 showed optimal activity at a pH 6.5 in comparison to a pH of 5 for the free form.

Keywords: Adsorption; Myoglobin; Ordered mesoporous silicates; Leaching; Immobilization

1. Introduction

Enzymes have the well-established capability to catalyse a reaction in neutral aqueous solutions, and at low temperatures and atmospheric pressure. However, under extreme conditions the susceptibility to denaturation and the loss of activity is increased, thus severely limiting their use. Several approaches have been developed in order to improve enzyme stability and create efficient biocatalysts such as immobilization onto polymers and sol–gels [1,2].

Since their disclosure by Mobil researchers in 1992 [3], mesoporous silicates materials (MPS) have been the subject of intense research. They possess uniform pores with specific surface area up to 1500 m^2/g, they are chemically and thermally stable and their surfaces can be modified with different functional groups. Mesoporous silicates can be reused and are considered to be non-toxic safe materials [4].

Diaz and Balkus [5] were the first to immobilize the globular proteins (cytochrome *c*, papain and trypsin) onto MCM-41. Since then, a variety of enzymes have been immobilized onto ordered mesoporous silicates materials [6–8]. To continue the development of mesoporous silicates as an immobilization matrix it is necessary to understand the factors that affect the immobilization behaviour of proteins within mesoporous materials such as the effect of pore diameter, ionic strength, surface characteristics and pH. Recently, Hudson et al. [9] developed a protocol describing a systematic approach to determine the parameters necessary to immobilize enzymes onto mesoporous silicates.

The effect of pH on the adsorption process is very important in terms of obtaining the maximum loading and activity of an enzyme. Han et al. investigated the optimum pH at which chloroperoxidase adsorbed onto mesoporous material while maintaining maximum catalytic activity [10]. The maximum activity of the immobilized enzymes was observed at pH 3.4, which is slightly below the isoelectric point (p*I*) of the enzyme. Vinu et al. [11,12] investigated the adsorption of cytochrome *c* and lysozyme onto SBA-15 and MCM-41 at different pH values and found that the amount adsorbed was strongly influenced

* Corresponding author. Tel.: +353 61202246; fax: +353 61213529.
E-mail address: kieran.hodnett@ul.ie (B.K. Hodnett).

1381-1177/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2007.07.005

by the pH of the solution used. They found that the maximum amount adsorbed was at a pH close to the isoelectric point of the proteins and this could be attributed to the zero net charge on the protein molecules resulting in a size reduction of protein within the pore leading to the high uptake of protein molecules by mesoporous materials.

Myoglobin is a relatively small protein, having molecular dimensions of 4.5 nm × 3.5 nm × 2.5 nm [13]. It is an oxygen-binding protein of muscle cells, which functions to store oxygen and to facilitate oxygen diffusion in rapidly contracting muscle tissue. Myoglobin contains a single polypeptide chain of 153 amino acid residues of known sequence and a single iron protoporphyrin IX as the prosthetic group in a hydrophobic pocket [14].

This study examines the influence of pH on the adsorption, ionic strength and activity of myoglobin adsorbed onto ordered mesoporous silicates in order to improve our understanding of the effect of pH on myoglobin adsorption. Myoglobin adsorption has been reported previously onto SBA-15 and FSM [15–17]. However, the influence of pH and ionic strength on the adsorption, leaching and activity of the myoglobin adsorbed onto mesoporous silicates has not been reported. This study aims at an improved understanding of the forces behind the interaction of myoglobin with various mesoporous silicates.

2. Experimental

2.1. Reagents

Cetyltrimethyl ammonium bromide (CTAB, 99%), myoglobin (horse skeletal muscle, 95–100% pure), 1,2-bis(trimethoxysilyl) ethane (BTMSE, 96%), acetic acid, sodium acetate, potassium hydrogen phosphate, potassium dihydrogen phosphate, sodium hydrogen carbonate, sodium carbonate, tetramethylammonium hydroxide (TMAOH), 1,3,5-trimethylbenzene (TMB) and tris(hydroxymethyl)aminomethane (TRIZMA®), 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) were all obtained from Sigma–Aldrich. Fumed silica (Cab–O–Sil) was obtained from Reidal de Häen. Sodium chloride (NaCl) and ammonium fluoride (NH_4F) were obtained from BDH. Sodium hydroxide was obtained from Lab Pak. Hydrochloric acid (HCl) was obtained from Merck. Sodium dodecyl sulfate (SDS) was obtained from J.T. Backer. Tetraethylorthosilicate (TEOS, 98%) and 2-cyanoethyltriethoxysilane (CEOS, 98%) were obtained from Lancaster. Pluronic P123 ($EO_{20}PO_{70}EO_{20}$) was obtained from BASF. Water was purified (18 MΩ) using an Elgastat spectrum system.

2.2. Synthesis and characterisation of mesoporous materials

SBA-15, MCF, MSE, SDS/P123, CNS, and MCM/41 were synthesized adopting to previously published procedures [18–23]. All the silicates used were characterized by nitrogen gas adsorption/desorption isotherms at 77 K measured using a Micrometrics Gemini ASAP 2010 system. Samples were pre-heated to 120–150 °C under vacuum for 16 h (to remove bound H_2O). The pore size data were analysed by the thermodynamically based Barret–Joyner–Halenda (BJH) method [24] using adsorption and desorption branches of the nitrogen isotherm. The surface areas were calculated using the Brunauer–Emmet–Teller (BET) method [25]. X-ray diffraction patterns were obtained by a Philips X'pert PRO MPD instrument using the Cu Kα line at 1.542 Å operated at 40 kV and a current of 35 mA. Data were obtained over the range of 2θ from 0.3° to 10°. Isoelectric points were measured using a Malvern Zetasizer 3000HSA, in which samples of mesoporous silicate materials were made up in deionised water at a concentration of 0.5 mg/ml and were sonicated for 15 min before zeta potential measurements were taken. The pH of the solution was manually adjusted by the addition of 0.1 M HCl or 0.1 M NaOH before the zeta potential was measured.

2.3. Adsorption of myoglobin onto mesoporous materials

A suspension of the mesoporous support in a relevant buffer was made up at a concentration of 2 mg/ml, then sonicated for 15 min and stirred for 30 min on a magnetic stirrer to create a dispersed suspension. Equivalent volumes of the protein and the suspension were then mixed together with shaking in a New Brunswisk Scientific C24 incubator shaker (120 rpm, 25 °C for 16–18 h). The protein loading was determined by taking 1 ml of the sample from the reaction vessel, centrifuged, and the supernatant analyzed by UV absorption at 410 nm. The amount of protein adsorbed was determined by taking the difference between concentration before and after adsorption (myoglobin, $\varepsilon_m = 120{,}000\ M^{-1}\ cm^{-1}$) [26]. At the pH values specified below the pH at which adsorption was carried out will be referred to as $pH_{ads.}$. The leaching test was carried out as follows: 2 mg of the loaded material was washed three times with the immobilization buffer, followed by three washes with a buffer of different pH value, and finally three washes with a buffer with higher ionic strength. The pH at which the leaching was used will be referred to as $pH_{lea.}$. Ionic strength studies were performed, by the addition of NaCl of varying concentration (0.2–2 M) to the protein-silicate solution. In all cases the leaching of protein was monitored using absorption at 410 nm.

2.4. Activity assay

Assay of peroxidative activity of myoglobin was carried out by oxidation of ABTS with detection at 405 nm ($\varepsilon_m = 36{,}800\ M^{-1}\ cm^{-1}$) [27]. The assay mixture for the free protein included, myoglobin (4–30 μM), ABTS (0.54 mM) and H_2O_2 (1.2 mM) in a 1 ml cuvette. For the immobilized protein, 2 mg of silicate (4–20 μmol myoglobin/g) were placed in a 1 ml cuvette. Assays were performed at room temperature at pH 4 and 5 (25 mM, citrate buffer), pH 6.5 (25 mM, potassium phosphate buffer) and pH 8 (25 mM, Tris–HCl buffer). Below the pH at which activity was measured is referred to as $pH_{act.}$.

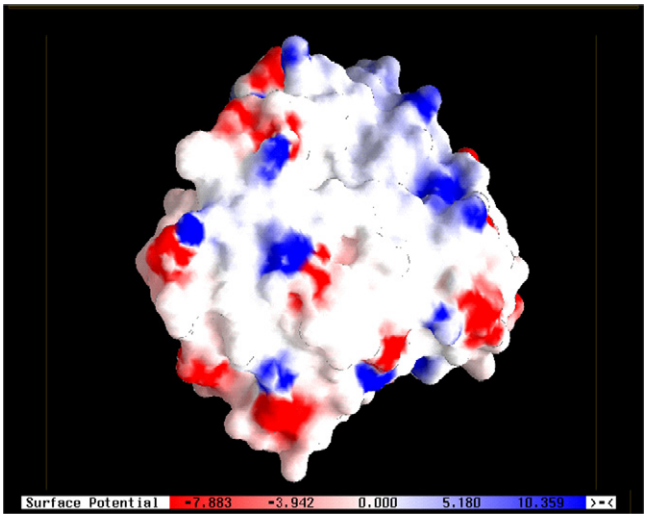

Fig. 1. Poisson–Boltzmann electrostatic potentials of myoglobin.

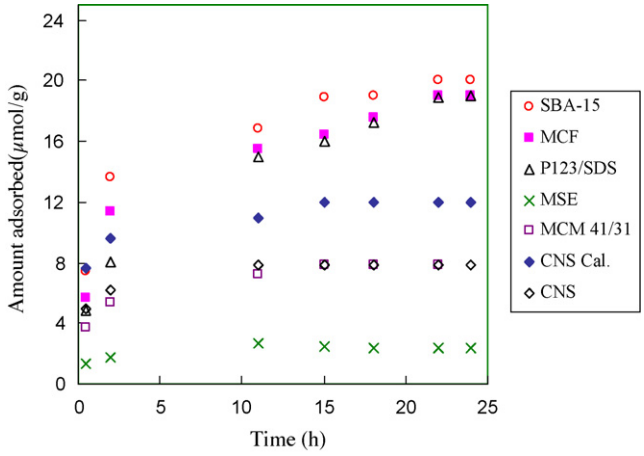

Fig. 2. Kinetics of adsorption of myoglobin onto different mesoporous silicates materials (25 °C, 10 mM phosphate buffer, $pH_{ads.}$ 6.5 with shaking at 120 rpm, initial concentration 20 μM).

3. Results

Myoglobin has an isoelectric point (p*I*) between 7 and 7.3 [26]. A Poisson–Boltzmann electrostatic surface calculation for myoglobin shows that in agreement with reported p*I* values for the molecule, the surface is relatively uncharged (indicated by white, Fig. 1). Small localised patches of charge (indicated by red for negative electrostatic potential and blue for positive) are also apparent.

The physicochemical properties of the seven MPS materials used in this study are listed in Table 1. SBA-15, MSE, MCF and P123/SDS were synthesized from the same non-ionic poly(alkylene oxide) triblock copolymer surfactant P123. While MCM-41, CNS and CNS-cal were synthesized from the cationic surfactant CTAB, resulting in a higher p*I* than those synthesized with P123. It was previously reported that MSE had a p*I* value (4.9) higher than SBA-15 (3.8) due to the presence of ethylene groups (–CH_2CH_2–) on the surface of MSE [9]. SBA-15 and MSE exhibit type IV adsorption isotherms (IUPAC definition), where the volume of nitrogen adsorbed increases with increasing relative pressure (over range of 0.6–0.8), with a sharp rise in adsorption due to capillary condensation in the mesopores. The mesopore volume was calculated from the increment in volume of nitrogen adsorbed in the sharp rise in the adsorption/desorption isotherm. P123/SDS shows a sharp capillary condensation step at high relative pressures (0.6–0.8) and an H_1 hysteresis loop. MCF, CNS and CNS calcined have hysteresis loops in the region (0.8–1). MCF has a large hysteresis loop due to the addition of TMB and NH_4F to the aqueous solution of P123, leading to the material possessing large sized spherical cells that are interconnected by windows of uniform size.

3.1. Adsorption study

The kinetics of adsorption of myoglobin onto different mesoporous silicates materials are presented in Fig. 2. Maximum adsorption was achieved with SBA-15 after 20 h (20 μmol myoglobin/g MPS). This corresponds to adsorption of all the available myoglobin. Both MCF and P123/SDS adsorbed the same amount of myoglobin (19 μmol/g) after 20 h, with CNS cal. (12 μmol/g), CNS (7.8 μmol/g) and MCM-41 (7.8 μmol/g) the maximal adsorption of myoglobin was lower and was completed after 15 h. The results suggest that for adsorption to occur it is not just sufficient to have a protein of a smaller diameter

Table 1
Physicochemical properties of MPS materials

Materials	Pore diameter (Å) (from adsorption branch)[a]	Pore diameter (Å) (from desorption branch)[b]	S_{BET} (m^2/g)	Total pore volume (cm^3/g)[c]	Mesopore volume (cm^3/g)[c]	Isoelectric points (p*I*)	Wall thickness (Å)[d]	Structure
MCM-41	32	31	927	1.0	0.45	4.0	22	Hexagonal
SBA-15	80	62	827	1.0	0.47	3.8	32	Hexagonal
MSE	76	60	1005	1.1	0.42	4.9	42	Hexagonal
P123/SDS	65	51	921	1.3	0.60	3.7	31	Cubic
CNS	220	147	357	1.2	0.84	4.6	–	Disordered
CNS cal.	225	154	464	1.3	0.86	4.9	–	Disordered
MCF	260	140	486	2.0	1.60	3.8	–	Disordered

[a] Calculated from desorption branch of nitrogen isotherm.
[b] Calculated from adsorption branch of nitrogen isotherm.
[c] Volume of liquid nitrogen at STP.
[d] Calculated by lattice parameter–pore diameter ($a^o = 2d_{100}/\sqrt{3}$).

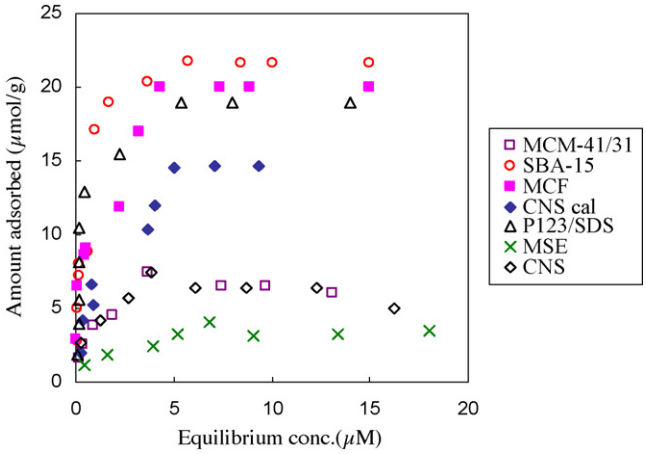

Fig. 3. Adsorption isotherms of myoglobin onto different mesoporous silicates (10 mM phosphate buffer, $pH_{ads.}$ 6.5 with shaking 120 rpm, at 25 °C for 16 h).

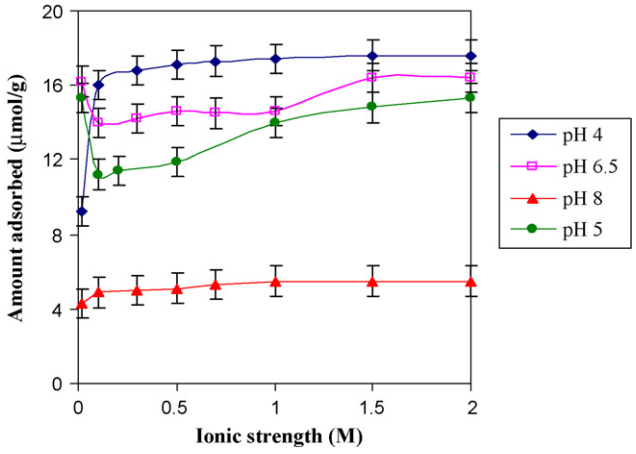

Fig. 4. Adsorption of myoglobin onto SBA-15 with varying ionic strength (10 mM citrate buffer, $pH_{ads.}$ 4 and 5), (10 mM phosphate buffer, $pH_{ads.}$ 6.5) and (10 mM Tris–HCl buffer, $pH_{ads.}$ 8), with shaking 120 rpm for 16 h at 25 °C, initial concentration of myoglobin 18 μM).

than the mesoporous materials pore diameter. The p*I* and surface charges of the protein and mesoporous silicates also must be taken into account [28]. It is interesting to observe that both MCM-41 with a pore diameter of 31 Å and CNS with a pore diameter of 220 Å adsorbed the same amount of myoglobin. A pore diameter of 31 Å is too small to allow access of myoglobin into the pore. MSE however only adsorbed (3 μmol/g). The low amounts adsorbed onto MSE and CNS can be attributed to the presence of polar cyano groups on the surface of CNS and ethylene groups on MSE, which may limit the adsorption of myoglobin onto the surface.

3.2. Myoglobin adsorption isotherms

Fig. 3 presents the adsorption isotherms for all mesoporous silicates materials used in this study. In agreement with the kinetic studies, in which SBA-15 displays a higher affinity for myoglobin (22 μmol/g), the isotherm exhibits a sharp initial rise and finally reaches a plateau (L-type). Slightly less myoglobin was adsorbed onto MCF (20 μmol/g). As MCF has a pore diameter of 260 Å compared to 80 Å for SBA-15, the relative binding is not likely to be limited by the access to the pores but more related to the available total surface area of mesoporous material. MCF has a much reduced surface area of 486 m^2/g compared to that of SBA-15 with surface area of (827 m^2/g). It can be seen from Fig. 3 that myoglobin also adsorbs strongly onto P123/SDS (19 μmol/g). The CNS cal. has a lower affinity towards myoglobin (14.5 μmol/g). The p*I* of CNS cal. material is 4.9 whereas that of SBA-15 is 3.8 (Table 1). These adsorption experiments were all performed at pH 6.5. At this pH the mesoporous silicate materials with a higher isoelectric point value will be somewhat less negatively charged (MCM-41, MSE, CNS and CNS cal.). With myoglobin will have a positive charge. Irrespective of other parameters this grouping constantly adsorbed lower amounts of myoglobin 7.5, 4, 7.4 and 14.5 μmol/g, respectively, indicating that the charge characteristics of the mesoporous silicates has a large influence on the adsorption. Furthermore, despite having similar p*I* values, pore sizes and mesopore volume CNS adsorbed less myoglobin than with CNS cal., suggesting that the presence of cyano groups on the surface of CNS decrease the affinity for myoglobin adsorption. CNS cal. has no cyano groups on the surface due to their removal by calcination. MSE adsorbed the lowest amount of myoglobin (4 μmol/g) and this could be attributed to the lower density of silanol groups (SiOH) on the surface due to the presence of $–CH_2CH_2–$ groups [20]. The charge on the surface of the mesoporous silicates is important for adsorption of myoglobin, as is the chemical character.

3.3. Influence of ionic strength at different pH values

The influence of pH and ionic strength on the adsorption of myoglobin onto SBA-15 was examined. As seen in Fig. 4, at pH 4 the amount adsorbed increased from 9.2 μmol/g in buffer to 16 μmol/g at 2 M NaCl. At pH 8 the loading was enhanced slightly from 4.3 μmol/g in buffer to 5.5 μmol/g at (2 M) NaCl. A different effect was noticed at both pH 5 and 6.5 in which the loading decreased from 15.3 and 16.2 μmol/g to 11.2 and 14 μmol/g, respectively as ionic strength increased (0.1 M), and then subsequently as ionic strength increased the loading increased. At pH 4 the surface charge of SBA-15 will be at least weakly negative as its p*I* is 3.8. Thus, electrostatic attraction of myoglobin to the surface will be low. The protein however will be strongly positively charged and intermolecular repulsion will be high. This supported by the low adsorption of myoglobin (Fig. 4) with no additional NaCl. The presence of NaCl will provide counter ions to these charges and thus potentially mask them, or lower the electrical double layer thickness of both the proteins and support, making adsorption easier. This observation is supported by the fact that at pH 8 the protein will carry a net negative surface charge and the SBA-15 will be strongly negatively charged, thus very low adsorption occurs and the addition of Na^+ counter ions did little to improve the situation.

At intermediate pH of 5 and 6.5, there is an opposite global surface charges character between the protein and MPS. At these pH values, increasing the NaCl concentration initially reduces

the adsorption of the protein onto SBA-15. This is consistent with electrostatic interactions, which is expected at these pH values. At higher ionic strength the protein will be dehydrated due to the hydrated effect of salt molecules surrounding the protein allowing the hydrophobic interactions to contribute.

3.4. Effect of pH on adsorption of myoglobin onto SBA-15

Adsorption of myoglobin onto SBA-15 was investigated at different pH values 4–10 (Fig. 5). The amount adsorbed increased from pH 4 to 7 and then gradually decreased as the pH increased from 7 to 10. The maximum amount adsorbed (17.3 μmol/g) was at pH 6.5, which is below the report isolelectric point of myoglobin of 7.3. The isoelectric point of SBA-15 is 3.8 and therefore is negatively charged at pH above this value. On the other hand Fig. 5 clearly shows that at low and high pH values, low amounts of protein are adsorbed as the overall charge of both the protein and SBA-15 are the same. However, in the zone p*I* (SBA-15) the p*I* (myoglobin), the charges are complementary and adsorption occurs. Miyahara et al. [29] studied the effect of pH on adsorption of cytochrome *c* and myoglobin, both proteins showed the maximum adsorption capacity near their isoelectric points. The maximum absorption capacity for cytochrome *c* was 40 μM around pH 10, which is the same result obtained by Hudson et al. [9], while for myoglobin was 31 μM around pH 7.

3.5. Leaching tests (stability tests)

Leaching of the protein/enzyme immobilized on the support through physical adsorption methods is unavoidable due to the weak bonds (non-covalent nature of the interaction) between the protein/enzyme and the support [30]. Myoglobin was adsorbed onto SBA-15 at pH 4, 5, 6.5 or 8. For each adsorption pH, leaching tests were performed at pH 4, 5, 6.5 or 8. Fig. 6A shows the amount of leaching observed for myoglobin that had been adsorbed at pH 4 and tested for leaching at pH 4, 5, 6.5 or 8. At

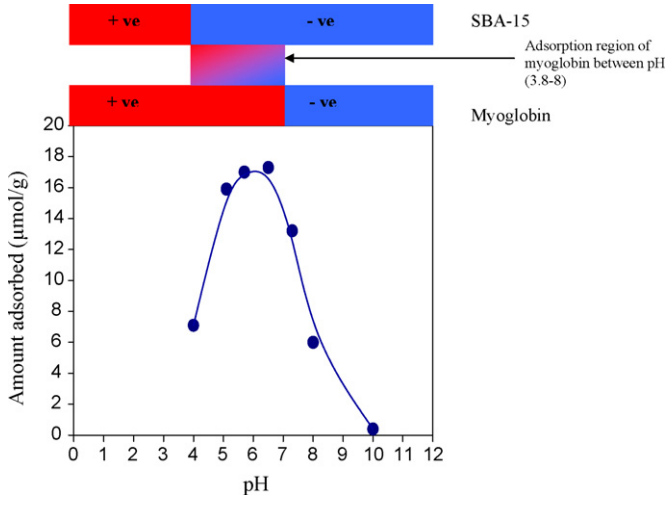

Fig. 5. Amount of myoglobin adsorbed onto SBA-15 at different pH values (10 mM citrate, phosphate, Tris–HCl and carbonate buffers, $pH_{ads.}$ 4–10, 25 °C) (initial concentration 20 μM).

pH 4, 6% of the myoglobin adsorbed at pH 4 was leached by washing with a fresh aliquot of the same buffer (10 mM phosphate buffer) used in the adsorption cycle, washing at pH 6.5 removed 3% and washing at pH 8 (10 mM Tris–HCl buffer) removed 80% of the adsorbed myoglobin. This leaching test was repeated for myoglobin adsorbed onto SBA-15 at pH 5 (Fig. 6B) and (Fig. 6C). In all of these tests the amounts of leaching were very small, indicating that once the myoglobin was adsorbed at pH values between 5 and 8 that a strong interaction was established that resisted leaching. An important point is that at pH 6.5 (Fig. 6C) just 2% leaching occurred upon each further washing even at higher loading (21 μmol/g), indicating that the interaction between myoglobin and SBA-15 is much stronger at pH 6.5 than pH 4 and that due to weak repulsive forces between individual myoglobin molecules and also between myoglobin molecules with SBA-15 at pH 6.5 which is near the p*I* of myoglobin resulting in strong interaction. When the leaching test was performed for myoglobin adsorbed at pH 8 (Fig. 6D) the amount of myoglobin removed by washing with the same pH buffer, pH 6.5 and pH 4 increased to 13% per each washing pH and these results could be attributed to the negatively charged surface of myoglobin and SBA-15 which is higher than the p*I* of both protein and the support. Thus, SBA-15 immobilized myoglobin shows highest loading and lowest leaching at pH 6.5.

3.6. Activity assay

It is necessary that the immobilized enzymes should retain their activity when immobilized in porous materials. Recently Itoh et al. [17] found that metmyoglobin (the oxidized form of myoglobin) exhibited peroxidase like activity for two substrates ABTS and guaiacol when immobilized onto FSM material. The immobilized metmyoglobin showed higher activity with guaiacol than ABTS. In this study we investigated the peroxidative activity of myoglobin immobilized onto SBA-15 because of its high order and higher adsorption capacity for myoglobin molecules. It can be seen clearly from Fig. 7 that the activity was enhanced for immobilized myoglobin in comparison with the free protein. Higher k_{cat} (μmol of oxidised ABTS produced per second per μmol of adsorbed myoglobin) values were obtained at lower loadings and decreased significantly as the amount adsorbed increased. This could be attributed to pore blocking by myoglobin, leading to the decrease in the amount of myoglobin available for the reaction with ABTS. Similar results have been reported with cytochrome *c* immobilized onto SBA-15, in which the activity enhancement was nearly five times that of the native form at low loadings [9].

3.7. Influence of pH on the activity assay

The maximum activity of immobilized myoglobin on SBA-15 and the native protein was determined at different pH values. As seen in Fig. 8 the maximum activity of the immobilized myoglobin was observed at pH 6.5 ($0.40\ s^{-1}$), while for the native myoglobin it is observed at pH 5 ($0.46\ s^{-1}$). At pH 4 and 8 both the immobilized and the free form showed negligible

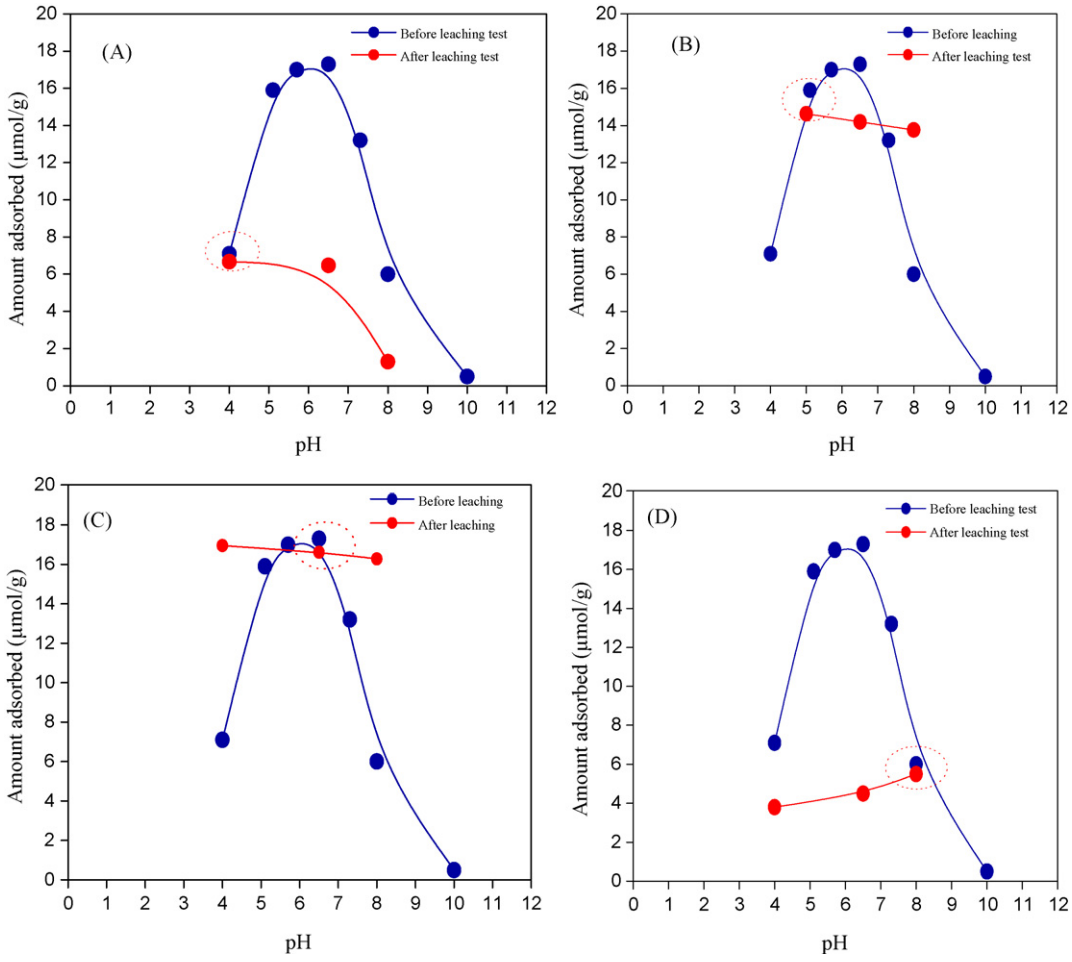

Fig. 6. (A) pH 4 $_{ads.}$ ($pH_{lea.}$ 4, 6.5 and 8), (B) pH $5_{ads.}$ ($pH_{lea.}$ 5, 6.5 and 8), (C) $pH_{ads.}$ 6.5 ($pH_{lea.}$ 4, 6.5 and 8) and (D) $pH_{ads.}$ 8 ($pH_{lea.}$ 4, 6.5 and 8).

activity. Thus, it can be noted that the pH of maximum activity of both free and immobilized myoglobin is more than pH 4 and less than pH 8. There have been several reports suggesting the occurrence of both a shift of optimum pH and a change in pH activity. For instance, Hartmann and Streb [31] reported that the maximum activity of chloroperoxidase (CPO) immobilized onto SBA-15 with a pore diameter of 13 nm was at a pH between 4 and 7, while for free CPO was at a pH between 5

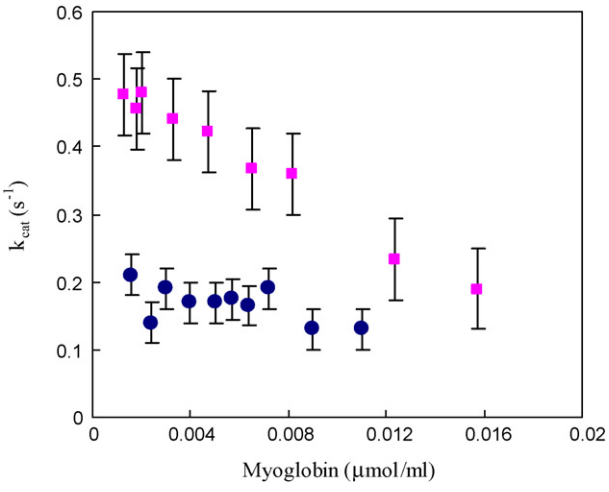

Fig. 7. Peroxidative activity (ABTS assay) profiles for free (●) and myoglobin adsorbed (■) onto SBA-15, at 25 °C, $pH_{ads.} = pH_{act.} = 6.5$, [ABTS] = 0.54 mM and $[H_2O_2] = 1.2$ mM.

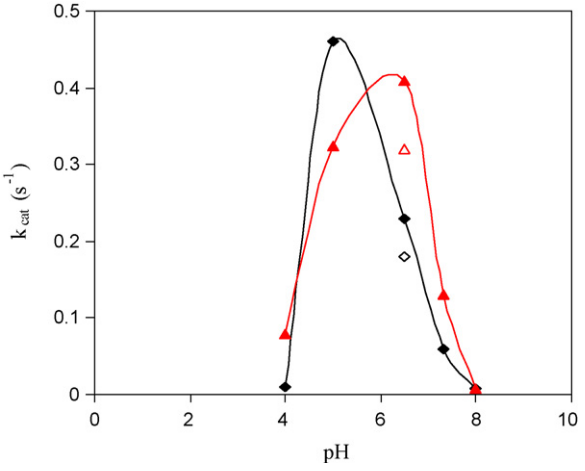

Fig. 8. Peroxidative activity (ABTS assay) profiles for free (♦), free with 1 M NaCl (◊) and adsorbed myoglobin (▲), adsorbed myoglobin with 1 M NaCl (△) (0.003 µmol/ml) onto SBA-15, at 25 °C myoglobin, [ABTS] = 0.54 mM and $[H_2O_2] = 1.2$ mM.

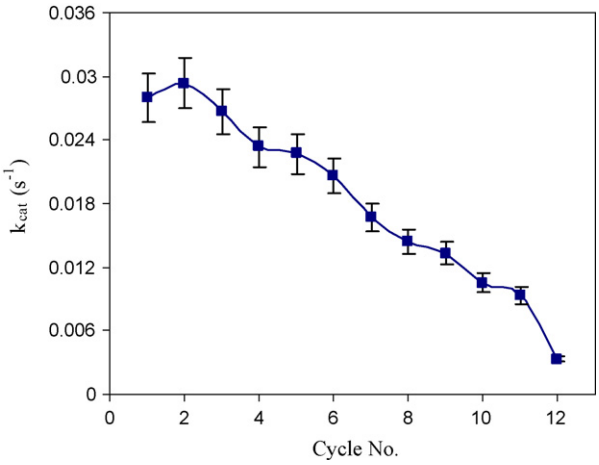

Fig. 9. Reusabilty of myoglobin immobilized onto SBA-15 (loading 0.003 μmol/mg) at 25 °C, (25 mM phosphate buffer, $pH_{ads.} = pH_{lea.}$ 6.5).

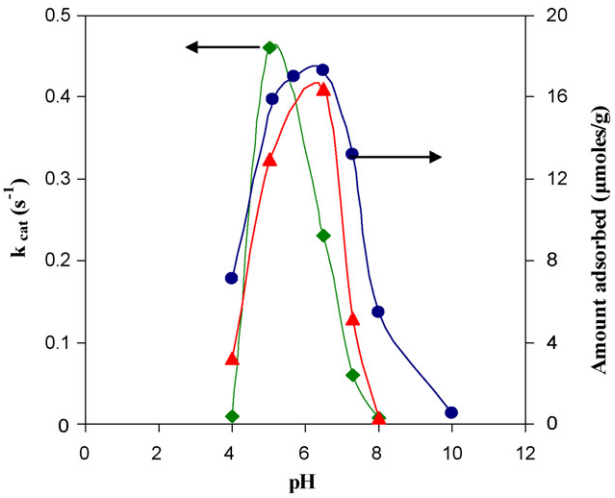

Fig. 10. Influence of pH on the activity of free myoglobin (♦) and immobilized myoglobin onto SBA-15 (▲), and on the amount adsorbed of myoglobin onto SBA-15 (●).

and 6. It was also of interest to examine the activity of free and immobilized myoglobin in the presence of higher ionic strength. Fig. 8 shows that there is no significant effect of increasing the ionic strength of the solution to 1 M for both free and immobilized enzyme with a 10–20% reduction in activity recorded for both.

3.8. Reusability of the immobilized protein

One of the most important requirements for immobilized enzymes is the achievement of prolonged use in comparison to the free form. The reuse of immobilized myoglobin onto SBA-15 was tested, by measuring the oxidation of ABTS over 12 cycles of 8 min each (see Fig. 9). The observed decrease in activity could be attributed to desorption of myoglobin by three times washing with adsorption buffer after each cycle in order to remove residual substrates. However, immobilized myoglobin was found to retain about 70% of its activity after six uses.

3.9. Discussion

This discussion will focus on the influence of pH on the amount adsorbed and also the activity of the immobilized myoglobin. As seen in Fig. 10, the maximum amount of myoglobin adsorbed was found at pH 6.5 that is just below its p*I* (7–7.3). In these conditions the globular charge on myoglobin is positive so that repulsion between myoglobin molecules must still be a factor. On the other hand even at the isoelectric point, at the local level the enzyme surface will still carry electrostatic charges. The overall charge on the silica surface at pH 5.5 will be negative, but there will also be zones of positive charge localised on the pore surface. In terms of electrostatics the maximum adsorption observed at pH 6.5 must represent an optimum in terms of minimising the enzyme–enzyme interactions and maximising the enzyme–silica interactions. The other important result presented here is that high ionic strength solutions do not cause the desorption of myoglobin from the silica surface as seen in Fig. 4. Indeed changing the pH shift also did not cause desorption (Fig. 10). This finding is consistent with a significant amount of hydrophobic interactions between local zones on the enzyme and the silica surface. Clearly when high ionic strength solutions are introduced to the adsorbed myoglobin–silica system, the hydrophobic interactions are significantly strong to retain the myoglobin on the silica surface.

The amount of myoglobin adsorbed at pH 8 (≈5.5 μmol/g) is attributed to the strong electrostatic repulsion between the SBA-15 surface and myoglobin, since both protein and the support have a strong global negative charges. This effect is similar to that reported previously [32], in which myoglobin molecules showed lower affinities towards ultrafine silica particles at pH 8. In these conditions the globular opposing surface charges overcome the effect of local electrostatic and hydrophobic effects.

The activity test is necessary for the choice of optimum pH at which the protein can bind to the support and obtain the highest activity at the same time. The data in Fig. 10 clearly shows that at pH 4 no activity was obtained for both free and immobilized myoglobin, indicating that myoglobin are inactive at pH 4. This is due to a reversible pH induced conformational change in the protein at low pH. At pH 5 the activity of free myoglobin reached a maximum activity of k_{cat} (0.46 s^{-1}), while for immobilized protein it was 0.26 s^{-1}. Interestingly, at pH 6.5 the activity rate for the immobilized protein increased to 0.40 s^{-1} in comparison with 0.23 s^{-1} for free form. At pH 7.3, the decrease in the activity can be observed with both free and immobilized myoglobin but activity of the immobilized form is still higher than the free form. The shift in the pH-activity profile upon immobilization is due to the conformational changes that occurred for the protein near the p*I*, in which the electrostatic repulsive forces between protein molecules and the effect of the support can play an intimate role. Such an effect was noticed previously for immobilized chloroperoxidase within MCF [10]. Fig. 10 presents a comparison of myoglobin adsorption onto SBA-15 with the corresponding activity results and the activity of the free myoglobin. It should be noted that the k_{cat} values for the

adsorbed myoglobin are corrected for the loading factor shown in Fig. 7. A clear shift in maximum activity with adsorbed myoglobin can be observed, but there is a clear coincidence between adsorption amount and activity. One point to note is that the myoglobin adsorbed at pH 8 is inactive. A clear requirement for a successful biocatalyst is the ability to achieve a substantial loading of the enzyme in a pH range in which the enzyme is active. The other requirement is a matching of the isoelectric points of the enzyme and support in active pH range.

4. Conclusions

The adsorption of myoglobin onto a variety of MPS materials was investigated. The MPS materials were characterized using different methods such as N_2 gas adsorption isotherm, and XRD. SBA-15 is preferred because of its high order and higher adsorption capacity for myoglobin molecules, as indicated from the adsorption isotherm studies. The adsorption studies showed that the maximum amount adsorbed was 21 μmol/g with SBA-15 and the minimum amount adsorbed was 4 μmol/g with MSE at pH 6.5. At low pH global net positive charges on the protein and silica do not facilitate adsorption and protein–protein repulsion is also important in these conditions. At high pH global net negative charges on the protein and silica, similarly lead to repulsion. Close to the isoelectric point of the protein localised surface charges can interact with similarly localised charges on the silica. In addition there will be patches of zero charge on the protein and silica which will facilitate hydrophobic interactions. The effect of pH on adsorption was also investigated, and the maximum amount adsorbed was found at pH (5–7). Leaching tests showed that a higher amount of myoglobin leached off at pH 4 as a result of weak electrostatic interactions between SBA-15 and myoglobin in which SBA-15 having slightly negatively charges and myoglobin having positive charges, while the least amount leached off at pH 6.5. The activity assay at various pH profiles was investigated. Free myoglobin displayed highest activity at pH 5, while the immobilized myoglobin at pH 6.5. There was no activity obtained at pH 4 and 8. The reusability test indicated that myoglobin immobilized onto SBA-15 could be used up to six times. Finally this study gives further details for the nature of the interactions between myoglobin and MPS and the effect of the pH profile on this interaction.

Acknowledgements

This research was funded by Enterprise Ireland (grant no. SC/2003/0159). BASF is thanked for the gift of pluronics.

References

[1] E.P.S. Piletsky, A. Bossi, N. Turner, A. Turner, Biotechnol. Bioeng. 82 (2003) 86.
[2] I. Gill, Chem. Mater. 13 (2001) 3404.
[3] J.S. Beck, J.C. Vartuli, W.J. Roth, M.E. Leonowicz, C.T. Kresge, K.D. Schmitt, C.T.W. Chu, D.H. Olson, E.W. Sheppard, t.S.B. McCullen, J.B. Higgins, J.L. Schlenkert, J. Am. Chem. Soc. 114 (1992) 10834.
[4] M. Vallet-Regí, L. Ruiz-González, I. Izquierdo-Barba, J.M. González-Calbet, J. Mater. Chem. 16 (2006) 26.
[5] J.F. Diaz, K.J. Balkus, J. Mol. Catal. B: Enzym. 2 (1996) 115.
[6] L. Washmon-Kriel, V.L. Jimenez, K.J. Balkus, J. Mol. Catal. B: Enzym. 10 (2000) 453.
[7] H.H.P. Yiu, P.A. Wright, N.P. Botting, Micropor. Mesopor. Mater. 44–45 (2001) 763.
[8] M. Hartmann, Chem. Mater. 17 (2005) 4577.
[9] S. Hudson, E. Magner, J. Cooney, B.K. Hodnett, J. Phys. Chem. B 109 (2005) 19496.
[10] Y.-J. Han, J.T. Watson, G.D. Stucky, A. Butler, J. Mol. Catal. B: Enzym. 17 (2002) 1.
[11] A. Vinu, V. Murugesan, O. Tangermann, M. Hartmann, Chem. Mater. 16 (2004) 3056.
[12] A. Vinu, V. Murugesan, M. Hartmann, J. Phys. Chem. B 108 (2004)7323.
[13] M.A. Bos, Z. Shervani, A.C.I. Anusiem, M. Giesbers, W. Norde, J.M. Kleijn, Colloids Surf. B: Biointerfaces 3 (1994) 91.
[14] D.L. Nelson, M.M.C. Lenin, Principles in Biochemistry, Worth Publishers, New York, 2000, p. 175.
[15] H.H.P. Yiu, C.H. Botting, N.P. Botting, P.A. Wright, Phys. Chem. Chem. Phys. 3 (2001) 2983.
[16] A. Katiyar, L. Ji, P. Smirniotis, N.G. Pinto, J. Chromatogr. A 1069 (2005) 119.
[17] T. Itoh, R. Ishii, T. Ebina, T. Hanaoka, Y. Fukushima, F. Mizukami, Bioconjug. Chem. 17 (2006) 236.
[18] D. Zhao, Q. Huo, J. Feng, B.F. Chmelka, G.D. Stucky, J. Am. Chem. Soc. 120 (1998) 6024.
[19] P. Schmidt-Winkel, W.W. Lukens, P. Yang, D.I. Margolese, J.S. Lettow, J.Y. Ying, G.D. Stucky, Chem. Mater. 12 (2000) 686.
[20] X.Y. Bao, X.S. Zhao, X. Li, P.A. Chia, J. Li, J. Phys. Chem. B 108 (2004) 4684.
[21] D. Chen, Z. Li, C. Yu, Y. Shi, Z. Zhang, B. Tu, D. Zhao, Chem. Mater. 17 (2005) 3228.
[22] A. Doyle, B.K. Hodnett, Micropor. Mesopor. Mater. 58 (2003)255.
[23] R. Mokaya, Chem. Commun. (2001) 933.
[24] E.P. Barrett, L.G. Joyner, P.P. Halenda, J. Am. Chem. Soc. 73 (1951)373.
[25] S. Brunauer, P.H. Emmett, E. Teller, J. Am. Chem. Soc. 60 (1938) 309.
[26] T. Arai, W. Norde, Colloids Surf. 51 (1990) 1.
[27] C.U. Carlsen, I.M. Skovgaard, L.H. Skibsted, J. Agric. Food Chem. 51 (2003) 5815.
[28] J. Deere, E. Magner, J.G. Wall, B.K. Hodnett, J. Phys. Chem. B 106 (2002) 7340.
[29] M. Miyahara, A. Vinu, K.Z. Hossain, T. Nakanishi, K. Ariga, Thin Solid Films 499 (2006) 13.
[30] Y. Zhu, W. Shen, X. Dong, J. Shi, J. Mater. Res. 20 (2005) 2682.
[31] M. Hartmann, C. Streb, J. Porous Mater. 13 (2006) 347.
[32] A. Kondo, J. Mihara, J. Colloid Interface Sci. 177 (1996) 214.