Hemoglobin immobilized on mesoporous silica as effective material for the removal of polycyclic aromatic hydrocarbons pollutants from water†

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Free hemoglobin (Hb) in water at pH 5 is able to oxidize 11 polycyclic aromatic hydrocarbons (PAH) (300 nM each) in the presence of H_2O_2 amounting to 75% PAH removal. PAH are carcinogenic, mutagenic and xenobiotic pollutants found in wastewaters of oil refineries. However Hb is pH sensitive and is effective only at pH 5. In order to use Hb for real wastewater treatment ($6.5 < pH < 8.5$), Hb was immobilized by simple adsorption in selected large pore mesoporous silicas at a loading of 300 mg Hb per g silica. The reaction of Hb with PAH is pseudo-catalytic because an excess of H_2O_2 ($[H_2O_2]/[PAH] > 2500$) and of Hb ($[Hb]/[PAH] > 2$) is necessary to reach an optimal PAH removal. The excess of Hb depends on the PAH physico-chemical characteristics. At pH = 7, the activity of free Hb decreased to 47% of PAH removal, whereas the Hb-immobilized silica allowed 82% of PAH removal. Immobilization of Hb in silicas leads to a protection of Hb towards pH, solvent, temperature and inactivation by H_2O_2. These results open the perspective for a new biotechnology process aimed at cleaning contaminated wastewaters by a reactive adsorption process followed by filtration.

Introduction

Polycyclic aromatic hydrocarbons (PAH) are carcinogenic and mutagenic pollutants found in various places of human industrial activities such as dump sites of coal mines and waste waters of oil refineries.[1] Due to their structure, they are very resistant and thus they accumulate in environment and organisms.[2] Ring juxtaposition results however in a heterogeneous distribution of π electrons over the structure, making some carbon atoms more reactive than others and prone to electrophilic substitution reactions such as oxidation.[3] For several years efforts have been made to degrade PAH. Physical processes such as stripping,[4] adsorption,[5] or ultrafiltration[6] are methods of choice because of their low cost but after these treatments a certain amount of PAH always remains. Chemical and physico-chemical processes such as organic solvent extraction,[7] photo-catalysis[8,9] or Fenton treatments,[10] have also been examined but they display very slow reaction rates (several hours or days) and they are often expensive and ecologically threatening. Moreover, as for physical processes, they are not efficient enough to remove PAH pollutants in trace concentrations. Some biological treatments have been added to the previous methods to improve the overall process.[11] Bacterial baths and trickling filters are the most common processes developed in industry.[12] Effectively, some fungi and bacteria species develop the enzymatic machinery to metabolize and detoxify xeniobiotic compounds such as PAH into simple products (quinone, diol, carboxylic acid[13] and CO_2 in certain cases).[14] Enzymes that can be found in these organisms belong to different families such as oxygenases, oxidases, dehydrogenases, hydrolases, decarboxylases and peroxidases, each one responsible for a reaction step in the long metabolizing chain.[15] These processes are very interesting as they represent a green solution with respect environment but unfortunately, like chemicals processes, these reactions are slow and PAH traces still remain after treatment. Moreover, by using bacteria it is difficult to control the reproducibility of the process because the nature and the amount of the products that are formed as well as the number of bacterial strains and the number of enzymes in each strain may vary from sample to sample.

An alternative way to proceed in biotechnology is to use a single enzyme[16] to oxidize specifically PAH and render them more hydrophilic and more biodegradable by microorganisms. The choice of the biocatalyst is crucial and depends on its activity, stability and its economic impact on the process.[17] Enzymes such as oxygenases and oxidases (P_{450} cytochromes[18] and laccases[19]), which directly use O_2 to oxidize their substrates, as well as peroxidases,[20–22] which need a peroxide to catalyze the oxidation reaction, can be used. The disadvantage to this technology is the high cost of enzymes due to expensive extraction and purification processes that are

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† Electronic supplementary information (ESI) available: Typical chromatograms of the 11 PAH; schematic of the flow reactor used for ABTS test; ABTS tests; Hb leaching; PAH removal at pH 5; inhibition of Hb by H_2O_2. See DOI: 10.1039/c0nj00161a

needed. Vazquez-Duhalt *et al.* have shown in 1995 that human hemoglobin, a non-enzymatic member of the hemoprotein family, was able to oxidize some PAH in a water–organic solvent mixture (20% acetonitrile (v/v)) through a peroxidase-like pathway.[23,24] To envisage a possible industrial application of this biotechnological process, we investigated the use of bovine hemoglobin (Hb), which presents the advantage of being a low cost waste product of the food industry, to remove PAH under conditions analogous to waste water treatment (low amount of co-solvent and low PAH concentrations).

Another challenge for an industrial application is to find a way to increase the stability of hemoglobin towards inactivation by pH variations and to recover the biocatalyst from the medium after reaction. The solution is to heterogenize the biocatalyst by immobilizing it in an inorganic matrix without deactivating it. The resulting solid-biocatalyst can then be easily recovered after a batch process or used in a continuous flow reactor.[25] The immobilization of a biocatalyst in inorganic supports also prevents the contamination of the medium by the protein and, most of the time, enhances its stability in temperature, in storage, in mechanical treatment, in solvent and against inhibitors.[26] Different methods can be used to immobilize enzymes in inorganic supports such as covalent binding, adsorption and encapsulation.[27–30] More and more studies have selected silica as inorganic support[31–34] and have shown that mesoporous silicas are very effective supports to maintain the activity of the biomolecules.[35–37] Hb was immobilized in hexagonal mesoporous silica matrixes such as SBA-15 featuring 5 to 9 nm pore diameters, but not tested in catalysis.[30] The same immobilized in a HMS silica with 4 nm pore diameter for biosensing applications exhibited higher activity towards electrocatalytic H_2O_2 reduction that polymeric organic supports.[38] Other inorganic supports such as layered titanate[39] were used for Hb immobilization and proved promising for catalytic reactions in organic solvent.

In order to develop a simple, cost effective, preparation method, immobilization of Hb to prepare oxidation biocatalysts has been achieved by adsorption. A preliminary screening study (not developed here) of a variety of supports, such as zeolites, carbons, ordered mesoporous silica (HMS, MCM-41, MCM-48, SBA-15), commercial silicas, as well as sol–gel encapsulation, revealed that the best results were obtained using commercially available large pore (>6 nm diameter) mesoporous silicas, belonging to the families of the Davicat silica supports from Grace Davison and LiChrospher from Merck. These materials present the additional advantage of exhibiting well defined homogeneous particle size distributions centered at 50 and 10 μm, respectively, permitting their use in continuous flow processes.

The aim of this paper is to describe in detail the various steps of the preparation and the performances of the biocatalysts obtained from these two families of supports. Usually the choice of the inorganic support depends on the size of the enzyme, and it is recommended to match the size of the pore to the size of the enzyme for a better protection and stabilization of the enzyme in its active conformation. As the size of Hb is 6.5 × 5.5 × 5.0 nm, mesoporous silicas with pore diameters from 6 to 20 nm have been selected. Results show a very high loading capacity of Hb in these silicas, much higher than usually obtained for other enzymes or proteins. The soft immobilization conditions we used allow the protein to keep its conformational structure and thus its biological properties. The catalytic performances of the resulting biocatalysts were evaluated using the ABTS test reaction and the best biocatalyst was used to investigate the removal of a mixture of 11 PAH in water using hydrogen peroxide in a batch reactor. The stability of the immobilized-Hb towards pH, temperature, solvent and H_2O_2 has been explored. Before studying the Hb-immobilized silica, the best conditions of PAH removal from water with the free Hb have been established.

Experimental

Materials

PAH (naphthalene, acenatphtene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, benzo(g,h,i)perylene, indeno(1,2,3,cd)pyrene) and ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonuim salt) were purchased from Sigma-Adrich (98–99.8% purity). Acetonitrile and methanol were of HPLC grade (SDS). Bovine met-hemoglobin (Hb) was obtained from Sigma-Aldrich in a lyophilized powder form (ref. H2625). It was used without further purification and considered as a pure preparation with a molecular weight of 64 500 Da. H_2O_2 was obtained as a 35% solution from Sigma-Aldrich. Chemicals for the buffers were reagent grade (Fluka). Buffers are determined considering their ionic strength (I), which takes into account the concentration of all ions in solution, rather than their molar concentration. For all buffers I = 50 mM. For solutions of pH = 3, 4 and 5 a sodium citrate buffer was used. For buffers of pH = 6, 7 and 8 a phosphate buffer was used. For buffer of pH 9 a glycine buffer was used. The concentration of each salt to obtain I = 50 mM has been determined with free software available on internet (*e.g.* CurTiPot). All solutions were prepared using deionised water from a milli-Q purification system (type I Millipore). Mesoporous silica Davicat Si 1700, Davicat Si 1402, Davicat Si 1404, with mesopores sizes of 20, 10 and 6 nm, respectively, and 50 microns particle size were obtained as a gift from Grace Davison, and will be further noted Dav20, Dav10, Dav6, respectively. LiChrospher 60 with mesopore size of 6 nm and particle size of 10 microns was obtained as a gift from Merck, and will be further noted LiC60. These commercial silicas were used without any modification or treatment.

Immobilization of Hb in mesoporous silica

Mesoporous silica materials (1 g) were suspended in various volumes (4.0, 3.5, 3.0 and 2.5 ml for Dav20, Dav10, Dav6 and LiC60, respectively) of citrate buffer pH = 5 (I = 50 mM) containing various quantities of Hb (50, 100, 250, 500, 1000 mg). The aim was to obtain a paste rather than a suspension to force the protein to enter into the pores. The mixture was gently stirred for 30 min. The resulting paste was dried by lyophilization to fix the protein into the pores. The resulting powder was then washed 5 times with 40 ml citrate buffer pH = 5 (I = 50 mM) with a fast stirring to ensure the removal of non-immobilized proteins and to keep only

strongly adsorbed Hb into the materials. Each washing step of 2 min shaking (Vortex) in buffer solution was followed by a 10 min centrifugation at 5000 rpm to recover the material. An aliquot of the supernatant solution of each five centrifugations was kept to determine indirectly the amount of Hb effectively adsorbed by subtracting the amount of Hb in the supernatants from the total amount of Hb used for immobilization (see below). The final product was dried by lyophilization and stored at 4 °C until characterization and catalysis study.

Determination of Hb content in Hb-silica

The amount of Hb immobilized into the silica supports has been determined by subtracting the Hb content in the supernatant after centrifugation to the total amount engaged for the immobilization. The Hb content in the supernatant was measured by UV-Vis spectrophotometry. Different supernatants have been obtained during Hb immobilization on silica. After the first drying step by lyophilization, 40 ml of buffer citrate (pH = 5; I = 50 mM) were precisely added and let to react for 2 min and then centrifuged. This first supernatant was sampled. Then 4 washing steps were performed followed by centrifugation and an aliquot of each supernatant was sampled. The concentration of Hb in the different aliquots of the 5 supernatants has been determined by measuring the absorbance at the wavelength of 280 nm and by comparing it to a calibration curve established with a known concentration of Hb in citrate buffer (pH = 5; I = 50 mM). The quantities of Hb determined in the 5 supernatants were then summed up and subtracted from the initial amount of Hb used for the immobilization.

Nitrogen adsorption/desorption isotherms of the materials

Nitrogen sorption isotherms were used to define more precise the pore size and the shape of the commercially available silica materials and to localize Hb in the silica supports: inside the pore or on the external surface. Nitrogen sorption isotherms at 77 K were recorded with an ASAP 2020 apparatus from Micromeretics. Before measurement, the Hb-immobilized silica were outgassed for at least 12 h under vacuum at 50 °C and at 250 °C for the native silica supports. The BET equation was applied to determine the surface area of the materials and the corresponding C_{BET} parameter to evaluate the polarity of the surface and the surface roughness. Average pore diameters of the materials were determined from the nitrogen desorption branch according to the Broekhoff and De Boer (BdB) method,[40] as this method has been demonstrated to be one of the more accurate method to evaluate mesopore size of materials.[41] The method to evaluate more precisely the pore size and shape of disordered silica materials with constricted pores will be explained in the text below, as well as the method of estimating the fraction of Hb present in the interior of the pores and on the external surface of the materials.

PAH removal conditions

Studies of PAH removal were conducted in a 100 ml reaction mixture containing various quantity of free Hb (0.15 to 15 μM) or 17 g l^{-1} of Hb-silica, 300 nM of each PAH, citrate buffer pH = 5 (I = 50 mM) or phosphate buffer pH = 7 (I = 50 mM) and 1% (v/v) acetonitrile. H_2O_2 concentration has been studied from 0.3 μM to 6 mM, corresponding to various $[H_2O_2]/[HAP]$ ratios. The kinetics of the PAH removal were followed by UltraFast Liquid Chromatography (UFLC). Evolution of PAH content within time was calculated from the decreasing area of chromatogram peaks (ESI, Fig. S1†). The maximum amount of PAH removal was determined after 15 min for free Hb and 1 h for Hb-immobilized silica, which corresponds to the time where the reaction stops. With UFLC method, the detection limit for PAH is 15 ± 0.75 nM. Reactions were performed in flasks protected by an aluminium foil to avoid PAH photo-oxidation cross-reaction. To ensure reproducible results, after reaction and before UFLC analysis, samples have been diluted in 50% (v/v) acetonitrile. For Hb-immobilized silica, an additional filtration step has been performed on a regenerated cellulose membrane syringe filter from Macherey-Nagel (25 mm, 0.2 μm pore diameters) to eliminate solid particles and to stop the reaction. It is to notice that without dilution, PAH is adsorbed on the filter. The dilution step in acetonitrile was necessary to stabilize aqueous PAH samples until UFLC analysis and to allow stopping the enzymatic reaction. All reactions were repeated three times. Blank reactions have been performed with silica only, with silica and H_2O_2, with Hb-silica without H_2O_2 and with free Hb without H_2O_2.

pH stability

The removal of pyrene by Hb-immobilized silica in presence of H_2O_2 has been performed at different pHs in the range 3 to 9 and compared to the results obtained with free Hb. Typically the experiments were conducted in a 100 ml reaction mixture containing 3 μM of free Hb or 60 mg of Hb-silica, 3 μM pyrene and 7.5 mM H_2O_2. For solutions of pH = 3, 4 and 5, a sodium citrate buffer was used with a molar concentration of 0.107, 0.032, and 0.011 M, respectively corresponding to I = 50 mM. For buffers of pH = 6, 7 and 8, a phosphate buffer was used with a molar concentration of 0.038, 0.017, 0.009 M, respectively, corresponding to I = 50 mM. For pH = 9, a glycine buffer was used with a molar concentration of 0.029 M, corresponding to I = 50 mM. Analyses were performed by UFLC as described above. Each experiment was repeated three times.

Leaching study

The amount of Hb released from the Hb-silica solid in the reaction conditions has been followed during 20 h by UV-Vis spectrophotometry at pH 5. The experiment was conducted in a 100 ml reaction mixture containing 17 g l^{-1} of Hb-immobilized silica in a citrate buffer (pH = 5; I = 50 mM) and 1% (v/v) acetonitrile. At various times (5, 15, 30, 60, 120, 240, 480 and 1200 min) a sample was filtered on a regenerated cellulose membrane syringe filter (25 mm, 0.2 μm pore diameters) from Macherey-Nagel to ensure a supernatant free of biocatalyst material, then the absorbance of the solution was measured at 280 nm and compared to a calibration curve done with known concentrations of Hb in citrate buffer (pH = 5; I = 50 mM).

ABTS activity

The ABTS oxidation reaction is commonly used to characterize the oxidation activity of different enzymes in presence of H_2O_2 (peroxidase) or in absence of H_2O_2 (oxigenases, phenol oxidases, laccases). The ABTS test is not specific for peroxidase activity but it brings the advantage of using a unique substrate and therefore to minimize diffusion limitations inside the solids, compared to more specific peroxidase tests which usually use two substrates.[42] For free Hb, 100 µl of ABTS solution with a concentration of 75 mM and 100 µl of Hb solution with a concentration of 0.1 g l^{-1} were mixed with 2.7 ml of phosphate buffer pH = 6 in a spectrophotometric cell. The reaction was started by adding 100 µl of H_2O_2 with a concentration of 75 mM. A blank reaction was performed without H_2O_2. The apparition of the blue-green colored product of oxidized ABTS was detected at 414 nm. For the Hb-immobilized silica biomaterials a home-made continuous flow cell (ESI, Fig. S2†), consisting of a peristaltic pump, a mechanical stirrer and a bottom opened flask equipped by a fritted glass was used. Typically, 19.6 ml of phosphate buffer pH = 6 (I = 50 mM) were loaded to the flask together with 700 µl of ABTS solution at a concentration of 75 mM and 20 mg of Hb-silica. The pump and the stirrer were activated and the reaction started by adding 700 µl of H_2O_2 at a concentration of 75 mM. The formation of the colored product (oxidized ABTS) was followed at 414 nm with an UVIKON 930 spectrophotometer from Kontron Instrument polystat 22/86602 Bioblock integration software.

Stability of Hb towards ethanol

Free Hb loses up to 95% of its peroxidase activity in a 50% ethanol medium. The ABTS activity of the free Hb and the Hb-immobilized silica in a mixture ethanol/aqueous buffer (pH 5) at 50/50 in volume has been compared to the activity in pure aqueous solution at pH 5. Typically, 1 ml of free Hb (0.1 mg l^{-1}) or Hb-immobilized silica (20 g l^{-1}) in citrate buffer (pH = 5; I = 50 mM) were mixed with 1 ml of ethanol for 15 min. Then the activity of Hb over ABTS oxidation has been tested has described above. Each experiment was repeated three times.

Inactivation of Hb by H_2O_2

The stability of free Hb and Hb-immobilized silica over H_2O_2 inactivation has been examined by incubating a solution of 0.1 g l^{-1} of free Hb (15 µM) or 0.312 g l^{-1} of Hb-immobilized silica in citrate buffer (pH = 5; I = 50 mM) with various H_2O_2 amounts. After 3 h, when the reaction was completed, an aliquot of 100 µl for free Hb and 700 µl of Hb-immobilized silica was used to measure ABTS activity. The decrease of linear activity rates of each sample was compared to a blank reaction without H_2O_2. Each experiment was repeated three times.

Thermal stability of Hb

The stability of free Hb and Hb-immobilized silica over temperature has been compared by measuring the loss of activity toward ABTS oxidation reaction. Typically, 1 ml of free Hb (0.1 mg l^{-1}) or Hb-immobilized silica (20 g l^{-1}) in citrate buffer (pH = 5; I = 50 mM), were incubated at 50, 60, 70 and 90 °C for 15 min. After cooling in an ice bath, the activity over ABTS oxidation has been measured as described above. Each experiment was repeated three times.

Results and discussion

Textural properties of the silica supports

Mesoporous silicas with different textural characteristics (Table 1) have been selected to investigate the immobilization of Hb by adsorption (Fig. 1): Davicat silicas with surface areas around 320 m^2 g^{-1} and pore sizes, determined by the supplier, of 6, 10 and 20 nm featuring a particle size of 50 microns (corresponding to an external surface area of around 15–30 m^2 g^{-1}), and LiChrospher 60 with a surface area of 750 m^2 g^{-1}, a pore size of 6 nm (determined by the supplier) and a particle size of 10 microns (corresponding to an external surface area of around 15 m^2 g^{-1}). These silica supports have been chosen in relation to the size of Hb (5.5 × 5 × 6.5 nm).

However, the mean pore diameter determined by the BdB[40] method from the desorption branch of the nitrogen isotherms is slightly different from the one given by the suppliers. Actually, LiC60 features a mean diameter of 5.3 nm, which should therefore be too small for Hb adsorption, and Dav6, 10 and 20 reveal mean pore diameters of 8.1, 12.3 and 19 nm, respectively. Furthermore, by careful analysis of the nitrogen isotherms (Fig. 2) it appears that there is some delay in the desorption branch of the isotherms indicating that the

Table 1 Pore volumes (per g of biomaterial and per g of silica), surface area, C_{BET} parameter, pore diameter of Hb-silica biomaterials prepared with an initial solution corresponding to 500 mg Hb per g silica in comparison to bare parent silicas supports. The total amount of Hb in the biomaterials ($m_{Hbtotal}$) and the amount of Hb in the pore volume (m_{Hbpore}) are also reported

Silica	$m_{Hbtotal}$/ mg per g Si	m_{Hbpore} [b]/ mg per g Si	S_{BET}/ m^2 g^{-1}	C_{BET}	D_{mean} [c]/ nm	$D_{opening}$ [d]/ nm	D_{cavity} [e]/ nm	V/ ml g^{-1}	$V_{Hb/Si}$ [a]/ ml g^{-1}_{Si}
LiC60	0	0	744	96	5.3	4.1	7.0	0.73	0.73
	79	24	695	73	5.0	4.0	6.5	0.58	0.69
Dav6	0	0	397	122	8.1	5.2	10.4	0.77	0.77
	49	24	380	85	8.1	5.1	10.4	0.69	0.73
Dav10	0	0	326	133	12.3	7.2	14.6	0.90	0.90
	69	42	298	81	11.9	7.0	14.4	0.70	0.77
Dav20	0	0	275	135	19.0	10.4	30	1.58	1.58
	319	291	220	58	17.5	10.1	30	0.73	1.09

[a] $V_{Hb/Si} = V/(1 - m_{Hbtotal})$. [b] $m_{Hbpore} = 594.7 \times (V_{parent\ silica} - V_{Hb/Si})$. [c] D_{mean}: determined by BdB method applied to the desorption branch. [d] $D_{opening}$: determined by BdB method applied to the desorption branch at the lower closing point of hysteresis. [e] D_{cavity}: determined by BdB desorption applied to the adsorption branch.

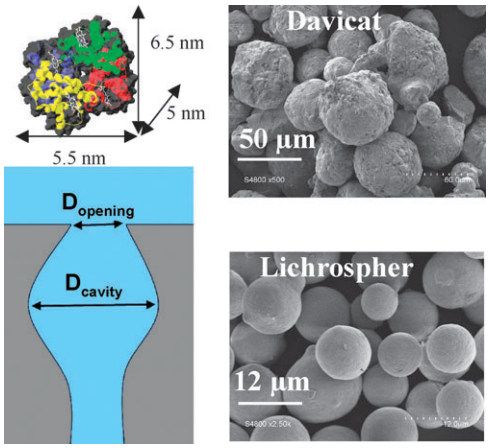

Fig. 1 SEM images of silica supports of the LiChrospher 60 and Davicat series. Schematic representation of a constricted pore in these silicas with a diameter of pore opening and pore cavity (bottom left). Dimensions of hemoglobin (top left).

porosity of these silica supports corresponds more precisely to pores with constrictions (cavities and pore openings) (Fig. 1) rather than to regular cylindrical pores. For materials with constricted pores, we have previously shown by simulation[43,44] that a good estimation of the cavity size is obtained by applying the desorption BdB[40] values to the adsorption branch of the isotherm and that the pore opening size corresponds to the lower closing point of the hysteresis to which the desorption BdB method is applied. Therefore the pores of commercial silicas are described by a diameter of cavity and a diameter of pore opening (Table 1): LiC60 has pore openings of 4.1 nm and cavities of 7 nm, Dav6 has pore openings of 5.2 nm and cavities of 10.4 nm, Dav10 has pore openings of 7.2 nm and cavities of 14.6 nm and Dav20 has pore openings of 10.4 nm and cavities of 30 nm. Consequently, by considering the pore openings of these silicas, Hb should be able to penetrate completely into Dav20, slightly into Dav10, but not into Dav6 and LiC60 (Fig. 2).

Adsorption of Hb on the silica supports

Solutions containing different amounts of Hb have been prepared in order to achieve mixtures corresponding to 50, 100, 250, 500 and 1000 mg Hb per g silica. The pH of immobilization has been set at 5, which corresponds to the optimal pH for the peroxidase-like activity of hemoglobin.[45] It leads to a negatively charged surface of the silica (isoelectic point: pH = 2) and a positively charged surface of Hb (isoelectric point: pH = 6.8). Due to strong interactions between the positively charged Hb and the negatively charged silica, a favorable loading of Hb is expected in these conditions. The amounts of Hb immobilized into the different mesoporous silica supports in function of the initial amount of Hb in solution are shown in Fig. 3. Increasing the pore size in the Davicat series (Dav6, Dav10, Dav20) leads to an increase of the amount of immobilized-Hb in the silica supports equal to 49, 69 and 319 mg Hb per g silica, respectively.

There is a real threshold in Hb adsorption for Dav20 featuring 10.4 nm pore openings compared to Dav6 and Dav10 with 5.2 and 7.2 nm pore openings, respectively. Materials with pore openings much larger than the size of Hb are needed to immobilize large quantities of Hb. The maximum amount of Hb immobilized in silica is obtained using an impregnation solution containing 500 mg Hb per g silica. Higher amounts of Hb in the solution (1000 mg Hb per g silica) do not lead to an increase of the Hb content in the materials suggesting a maximum loading threshold (Fig. 3). The same observation holds for LiC60, with a maximum amount of immobilized Hb of 79 mg Hb per g silica. This value is twice that observed for the Dav6 featuring similar but larger pore openings compared to LiC60 (5.2 *vs.* 4.1 nm, respectively) where Hb is not supposed to enter into the pore. This behavior will be explained below.

Location of Hb in the silica supports

To determine whether Hb is immobilized inside the pores or on the outer surface of the silica supports we have performed some calculations based on a quantitative analysis of the nitrogen adsorption isotherms of the resulting Hb-immobilized silica materials. The presence of Hb in the pores results in a decrease of the pore volume of the Hb-immobilized silica materials compared to the pore volume of the bare silica support. However, direct subtraction of pore volumes is not possible as nitrogen isotherms are presented in volume per g of material for Hb-immobilized silica materials and per g of silica for the bare silica support. To compare the two nitrogen adsorption isotherms a mass correction by the amount of Hb immobilized in the material is needed to express the pore volume of the Hb-immobilized silica per g of silica ($V_{Hb/Si}$ in Table 1). If $V_{Hb/Si}$ of the Hb-immobilized silica material is equal to the pore volume of the parent silica, this indicates that all Hb is located on the outer surface of the silica support. By doing this mass correction (Fig. 2, Table 1), it appears that the pore volume per g of silica ($V_{Hb/Si}$) for all Hb-immobilized silica is inferior to the pore volume of the parent silicas. Therefore Hb proteins are occluded inside the pores or at the entrance of the pores for all silica supports. By attributing the decrease of pore volume to the volume occupied by Hb proteins, an estimation of the maximum amount of Hb into the pores can be obtained by using the following equation:

$$m_{Hbpores}\ (\text{mg per } g_{silica}) = [(V_{silica} - V_{(Hb/Si)}) \times M_{Hb}]/(v_{Hb} \times N_A)$$

with V_{silica}, pore volume of bare silica; N_A, Avogadro number; M_{Hb} = 64 000 Da; Hb volume, v_{Hb} = 6.5 × 5.5 × 5 = 178.75 nm^3 and numerically corresponds to (for pore volumes expressed in ml g^{-1}):

$$m_{Hbpores}\ (\text{mg per } g_{silica}) = 594.7 \times (V_{silica} - V_{Hb/Si})$$

It should be noted that location of Hb at the entrance of the pore will lead to an overestimation of the amount of Hb inside the pores. These amounts (Table 1, 2nd column) have been calculated for all Hb-immobilized silica prepared with solutions containing 500 mg Hb per g_{silica} corresponding to the maximum content of Hb in the silica supports. For LiC60, Dav6, Dav10 and Dav20, the maximum amounts of Hb inside the pores or at the entrance of the pore are 24, 24, 42 and 291 mg Hb per g silica, respectively for a total Hb content of 79, 49, 69, 319 mg

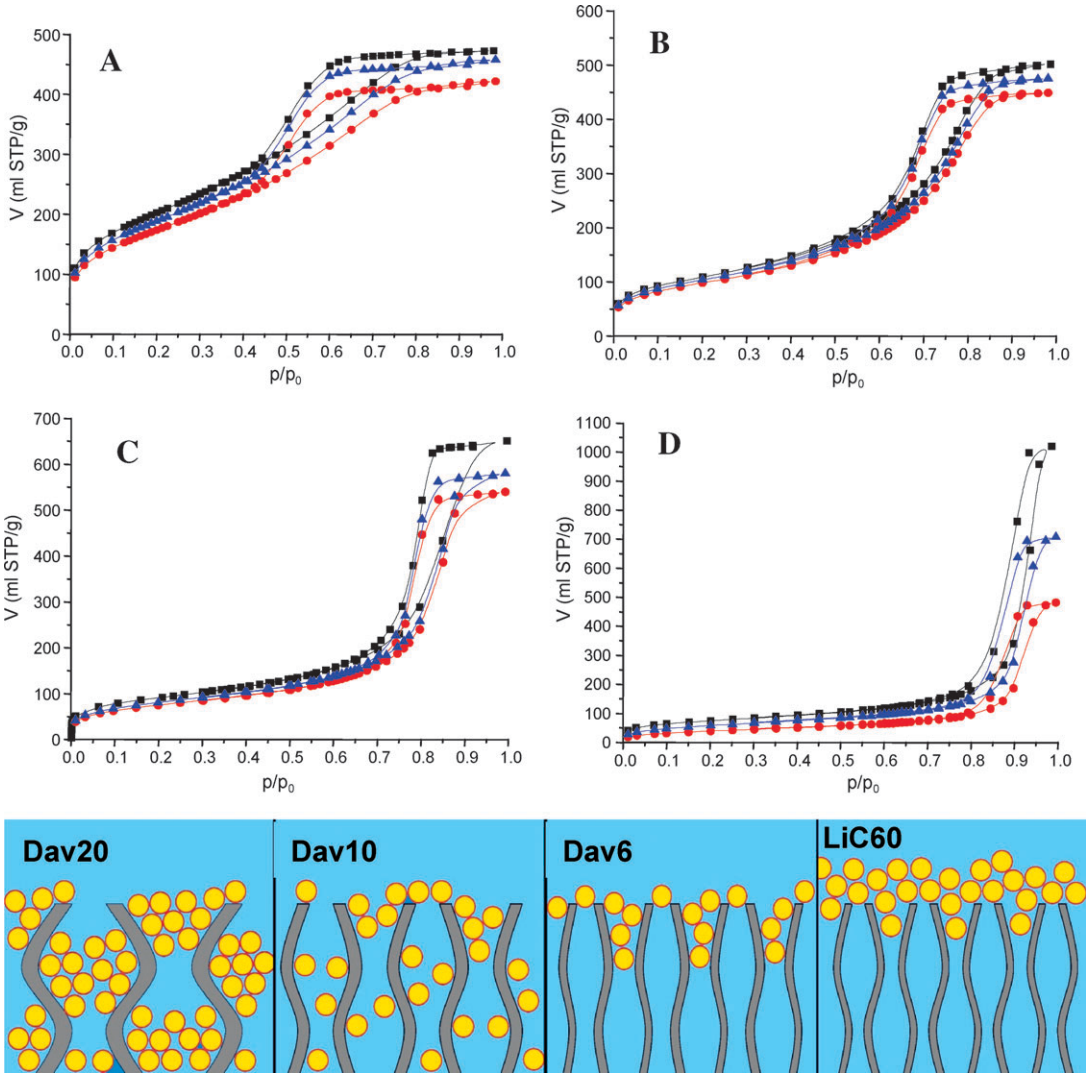

Fig. 2 N_2 adsorption-desorption isotherms at 77 K for (square) initial silica, (circle) Hb-immobilized silica prepared with 500 mg Hb per g silica considering the volume adsorbed per g of material and (triangle) Hb-immobilized silica considering the volume adsorbed per g of silica. Silica supports are (A) LiChrospher 60, (B) Davicat 6 nm (C) Davicat 10 nm and (D) Davicat 20 nm. Schematic representation of the localization of Hb in the different silica (bottom).

Hb per g silica, respectively. These results indicate that the majority of Hb is on the outer surface of the silica for LiC60 and Dav6 supports, that around half of Hb proteins are inside the pore for Dav10 and that the majority of Hb is inside the pore for Dav20 silica support (Fig. 2).

ABTS catalytic activity of Hb-silicas

To rapidly analyze the catalytic properties of the different Hb-immobilized silica, oxidation tests have been performed using the ABTS substrate (Table 2). This test is also helpful to confirm the localization of Hb in the silica supports by comparing the specific activity to the relative activity of the material.

For the ABTS test, different behaviors have been observed depending on the silica support for materials prepared with less than 500 mg Hb per g silica (ESI, Fig. S3†). For LiC60 and Dav6, the specific catalytic activity (expressed in micromol oxidized-ABTS/min per g material) remains constant, at a value ~15 μmol oxidized-ABTS/min per g material, whereas the amount of Hb in the silica support is increasing. This is also accompanied by a decrease of the relative activity of Hb (activity compared to free Hb) from 70 to 20%. This suggests that multilayers of Hb proteins are forming at the surface of these silica supports. Indeed, additional Hb proteins mask the first adsorbed Hb proteins, which are no longer able to participate in the reaction. This confirms that Hb is located on the external surface for LiC60 and Dav6, and that an aggregation of Hb occurs at the surface of these supports. The amount of Hb inside the pores (24 mg Hb per g silica) reported in Table 1 for these two silica supports corresponds therefore to an overestimation of Hb inside the pore and Hb is blocking the entrance of the pores. For Hb immobilized in Dav10, its specific activity is also constant with the increase of Hb content (~15 μmol oxidized-ABTS/min per g material) as for Dav6 and LiC60, but its relative activity remains constant at 20% instead of decreasing. This suggests that all Hb

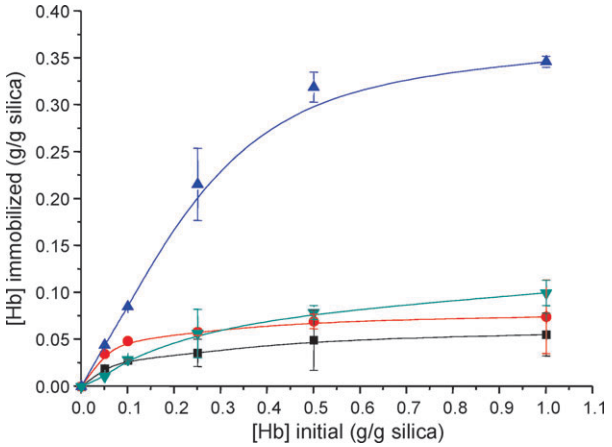

Fig. 3 Amount of Hb immobilized on LiC60 (down triangle), Dav6 (square), Dav10 (circle) and Dav20 (up triangle) as a function of the amount of Hb contained in the impregnation solution. Values have been determined indirectly by subtracting the Hb amount in the supernatant found by UV-Vis spectrophotometry to the total amount of Hb used for the immobilization.

Table 2 Results of the ABTS test in aqueous medium for biomaterials of Table 1 identified by their parent silica supports

Biomaterials	mg Hb per g	Activity/g biomat. (μmol min^{-1}/ g biomat.)	Activity/g Hb (μmol min^{-1}/ g Hb)	Relative activity (%)[a]
LiC60	79	15	182	18
Dav6	49	12	243	24
Dav10	69	16	226	22
Dav20	319	71	222	22

[a] Relative activity = (Activity/g Hb)/(Activity free Hb); Activity of free Hb = 1015.6 μmol min^{-1} g^{-1} Hb.

proteins in Dav10 interact with the solid on the same manner and that no aggregation of Hb proteins occurs. Hb proteins in Dav10 are located at the external surface of the solid and in the pore at the entrance without aggregation. For Dav20, a different behavior is observed compared to the other silica supports. The specific activity of the Hb-immobilized in Dav20 increases linearly with the increase of Hb content to reach a maximum activity of 70 μmol oxidized-ABTS/min per g biomaterial. The relative activity of the materials remains constant at 20%. These results confirm that Hb is immobilized inside the pore of Dav20 with a similar interaction of all Hb with the solid for all Hb contents. A special behavior is obtained for the materials prepared with the initial solutions corresponding to 1000 mg Hb per g silica. The specific activities for LiC60, Dav6 and Dav10 increase drastically from 15 to 40 μmol oxidized-ABTS/min per g material and the relative activities of these materials increase as well suggesting a rearrangement of Hb to a state closer to the free Hb, so with less interaction with the solid. This is not the case for Hb in Dav20, which expresses the same activity for materials prepared with 500 or 1000 mg Hb per g silica. From these results it appears clearly that Hb-Dav20 prepared with a solution of 500 mg Hb per g silica is the most active biocatalyst and the most suitable for our further study of PAH removal.

Stability of Hb towards solvent in Hb-immobilized silica

ABTS tests have also been performed in a medium defined as "organic medium" (Table 3) by adding 50% of ethanol in volume to water.

This amount of ethanol was sufficient to strongly inhibit the activity of the free Hb, which decreased from 1016 to 50 μmol oxidized-ABTS/min per g Hb, corresponding to 5% of its original activity. This test in organic medium allows to probe the stabilization effect of the silica support for Hb and at the same time to refine the amount of Hb on the outer surface of the materials. In this organic medium the activity of Hb immobilized in all Davicat silicas decreases with respect their activity in aqueous medium, but remains higher (2 to 3 times) than the activity of the free Hb, demonstrating the protecting effect of the Davicat silica supports. A similar effect was reported in the case of Hb immobilized in layered titanate[39] with a 2 to 5 fold enhancement of activity (depending on the solvent) for the oxidation of o-phenylenediamine using H_2O_2 as the oxidant. This is supposed to be due to the ability of organic solvents to distort the essential water layer on the Hb molecular surface and therefore to the protection of the channel water molecules against the attack of organic solvents. By contrast, for Hb adsorbed on LiC60 where Hb is essentially immobilized on the external surface, the activity of the protein is affected to the same extent than the free form, indicating no protecting effect of the support. The comparison between the aqueous and the "organic" media allows to quantify the protective effect of the supports against solvent inactivation (Table 3 and ESI, Fig. S4†). The percentages of remaining activity in organic media are 24, 33, 47 and 66% for Hb immobilized in LiC60, Dav6, Dav10, Dav20, respectively. These values correspond to the percentages of Hb protected by the solid, so mainly in contact with the pores. This result highlights again the superiority of the Dav20 silica support. From these figures we can recalculate the amount of Hb immobilized inside the pores (or at their entrance). It is found that 19, 16, 32, 210 mg Hb per g silica are protected inside the pores for LiC60, Dav6, Dav10, Dav20, respectively. These values are consistent with the maximum amount of Hb located into the pores determined by nitrogen adsorption (Table 1). The amount of Hb on the external surface of the silica supports is then deducted by subtracting these values to the total amount of Hb and correspond to 60, 33, 37 mg Hb per g silica

Table 3 Results of the ABTS test in organic medium (50% EtOH/ 50% buffer pH 5) for biomaterials of Table 1 identified by their parent silica supports

Biomaterials	mg Hb per g	Activity/g biomat. (μmol min^{-1}/ g biomat.)	Activity/ g Hb (μmol min^{-1}/ g Hb)	Relative activity (%)[a]	Org/Aq. Activity (%)[b]
LiC60	79	3	43	86	24
Dav6	49	4	80	160	33
Dav10	69	7	107	215	47
Dav20	319	47	148	296	66

[a] Relative activity = (Activity/g Hb)/(Activity free Hb); Activity of free Hb = 49.8 μmol min^{-1} g^{-1} Hb. [b] Org/Aq. activity = (Activity/ gHb)org (Table 3)/(Activity/gHb)aq (Table 2).

for LiC60, Dav6, Dav10, respectively. The density of Hb per external surface area has then been calculated for these supports by the following formula:

$$\mathrm{Hb}/(100\ \mathrm{nm}^2) = [(m_{\mathrm{Hb}} \times N_{\mathrm{A}})/(M_{\mathrm{Hb}} \times \mathrm{S} \times 10^{18})] \times 100$$

where m_{Hb} is the amount of Hb in the material (g Hb per g silica); M_{Hb} the molecular weight of Hb (64 000 Da); N_{A} the Avogadro number and S is the external surface.

The external surface areas of the silicas calculated by *t*-plot analysis of the nitrogen adsorption isotherm are 30 $m^2\ g^{-1}$ for Davicat silicas and 14 $m^2\ g^{-1}$ for LiC60. Therefore the densities of Hb on the external surface of the materials are 1.5 and 2.1 Hb/100 nm^2 for Dav6 and Dav10, respectively, showing a non-dense Hb monolayer at the surface of these supports. Indeed, taking into account the size of Hb, a density of 3 Hb/100 nm^2 is expected for a total coverage of the surface. For LiC60, the density of Hb on the external surface is 5.3 Hb/100 nm^2, which is above the monolayer coverage, confirming the aggregation of Hb on the outer surface of this support as suggested above. For Dav20, Hb is located mainly inside the pores. The amount of Hb inside the pore (210 mg Hb/ g silica) corresponds to an internal density of 0.8 Hb/100 nm^2 (the surface S in the previous formula is replaced by S_{BET}) and the other remaining 109 mg Hb per g silica are therefore on the external surface and at the entrance of the pores with a density of 3.4 Hb/100 nm^2, corresponding to a densely packed Hb layer on the total outer surface. All Hb in Dav20 is in strong interactions with the silica surface.

Leaching study of Hb in Hb-immobilized silica

Prior to perform PAH removal experiments, leaching tests of Hb from Dav20 have been realized. Recent literature results concerning Hb immobilization into SBA-15 mesoporous silica materials show that depending on the experimental conditions a leaching of Hb as high as 50% could occur.[30] It was shown that the highest binding capacity of Hb with silica together with the lowest leaching rate were obtained at pH = 5, a value which corresponds to the pH of Hb immobilization in the present work. At a higher pH value, the positive charge of Hb decreases. The interactions between Hb and the silica become repulsive for pH = 7 or 8, leading to an increase of the leaching in the final materials. Another interesting result of this literature report is that when pore sizes increased from 5.2 to 8.9 nm the Hb loading increased linearly from 70 to 350 mg Hb per g silica. This is in accordance with the results we obtained for the Davicat silicas series (the Hb loading increased from 55 to 320 mg Hb per g silica when pore openings increased from 5.2 to 10.4 nm). The same authors note, in addition, that the leaching rate was increased from 3 to 27% with the increase of pore size and that larger pore size SBA-15 (11.4 nm) did not allow a higher Hb loading (300 mg Hb per g) but led to a higher leaching rate corresponding to 50%. This last result is difficult to explain on the sole basis of the size of the pores. Such a behavior could be the result of a change of surface roughness of SBA-15 depending on the size of the pores.[46,47] Indeed SBA-15 with large pores (>9 nm) present a smoother surface compared to SBA-15 with smaller pores. SBA-15 with pores from 5 to 9 nm exhibit "micropores" on their pore surface which can be also called surface roughness due to the small depth of the micropores, ranging from 1 × 1 nm (for SBA-15 with 5-6 nm pore size) to 0.5 × 0.5 nm (for SBA-15 with 7–8 nm).[48] This roughness could contribute to anchor favorably Hb on the surface. Larger pore SBA-15 (>9 nm) do not feature micropores (or surface roughness), a situation that could explain the higher leaching of Hb in these materials. From the above considerations, it can be anticipated that the presence of a rough surface in silica supports should be favorable to anchor Hb and to minimize its leaching.

To estimate the surface roughness of the silica supports used in the present study, we have calculated the C_{BET} parameters of the BET equation of the nitrogen adsorption isotherms. C_{BET} is correlated to the adsorption energy of the nitrogen molecules with the solid surface and it has been observed that a smooth hydroxylated silica surface gives a value of $C_{\mathrm{BET}} \sim 100$. Higher C_{BET} parameters or negative values are an indication of the presence of micropores, surface roughness or strong adsorption sites. As shown in Table 1, LiC60 exhibits a C_{BET} of 96, characteristic of a smooth surface and Davicat silicas feature higher C_{BET} values, around 130, indicating a rough surface. It is noteworthy that special care should be taken to calculate C_{BET} parameters that are dependent on the range of pressure used for the linearization of the BET equation. For a more accurate determination we have used the Rouquerol test[49] by plotting $V(1 - p/p_0)$ as a function of p/p_0. For LiC60 and Dav6 a pressure range of ($0.06 < p/p_0 < 0.30$) was used and for Dav10 and Dav20 a lower pressure range was found ($0.06 < p/p_0 < 0.20$) indicating again a higher surface roughness for these two supports. Therefore, Davicat silicas are expected to be suitable supports for a minimum leaching of Hb. The leaching of Hb has been followed for the Hb-immobilized into Dav20 prepared with 500 mg Hb per g silica. This Hb-Dav20 has been put in an aqueous solution at a pH of 5 under vigorous stirring for 20 h. After 1 h of stirring, which is the time corresponding to that of the following PAH removal experiment (see below), only 4% of Hb is leached from the solid (ESI, Fig. S5) and after 20 h a maximum of 15% of Hb has been removed from the solid. In the case of SBA-15, in the work referred to above,[30] 50% of Hb was leached from a SBA-15 support with 11.4 nm pore size. Our result on Dav20 featuring 10.4 nm pore openings and 30 nm cavities, highlights the beneficial effect of the roughness of the silica surface against leaching.

PAH removal with free Hb

Before using the Hb-Dav20 biocatalyst for PAH removal, we have optimized the reaction using the free Hb in aqueous solution at pH 5. The peroxidase activity of Hb follows a bi-bi ping-pong mechanism, where H_2O_2 binds first to the iron (Fe^{III}) of the Hb porphyrin which is activated into $Fe^{IV}{=}O^{+\bullet}$. PAH reacts then with the newly formed activated oxygen species to give an oxidized form of PAH (Fig. 4).

In a previous study of anthracene removal with free Hb,[45] we have shown that the peroxidase-like activity of Hb is in competition with a catalase-like activity of Hb, leading to the dismutation of H_2O_2 into O_2 and H_2O (Fig. 4) and with the inactivation of Hb by H_2O_2 (Hbi in Fig. 4). The production of

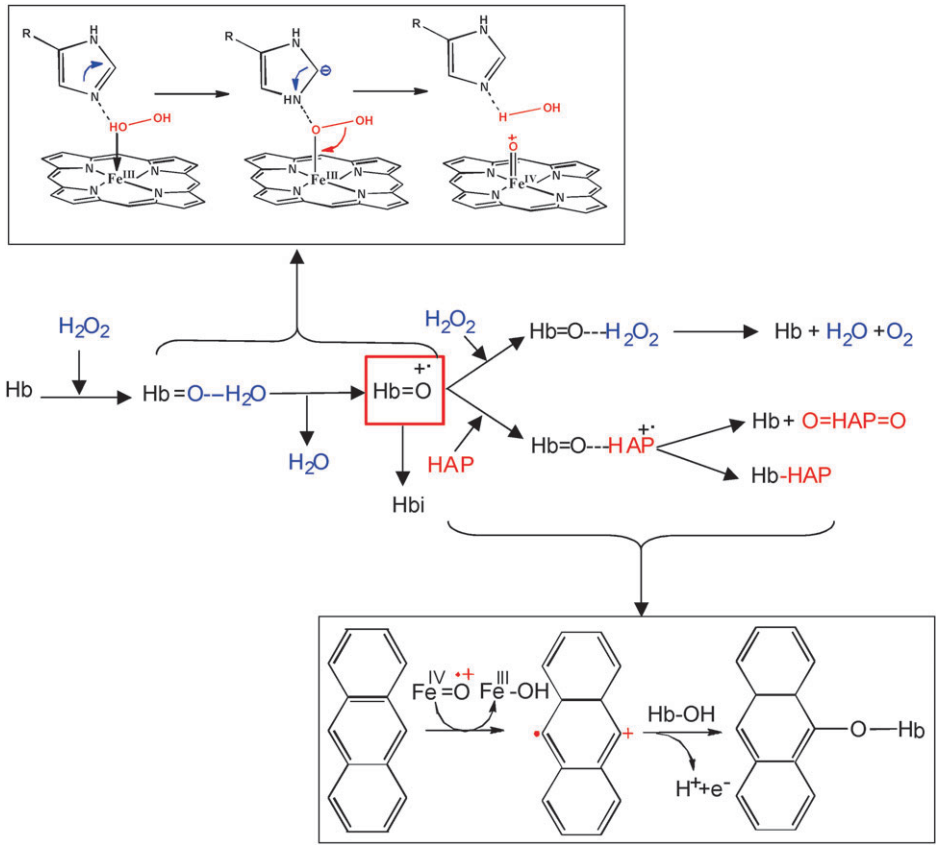

Fig. 4 Schematic representation of the three competitive reactions of Hb with H_2O_2 of PAH: (1) the suicide inactivation of Hb by H_2O_2 leading to an inactive form of the protein (Hbi), (2) the catalase activity of Hb forming O_2 and H_2O and (3) the peroxidase activity of Hb producing oxidized PAH covalently grafted on Hb and some free oxidized PAH.

O_2 by Hb is uncommon but has been previously observed in other conditions.[50,51] Total anthracene removal has been reached at pH = 5 (the optimum pH for the peroxidase activity of the free Hb)[45] in only 15 min for a very large molar excess of H_2O_2 with respect to Hb (H_2O_2/Hb > 1000) and a two-fold molar excess of Hb compared to anthracene, qualifying the reaction as "pseudo-catalytic".

The conditions described above have been applied to a mixture of 5 PAH (anthracene, fluoranthene, pyrene, naphthalene, phenanthrene): 300 nM for each PAH, 3 μM for Hb and 3 mM for H_2O_2 corresponding to molar ratios of $[Hb]/[PAH]_{tot} = 2$ and $[H_2O_2]/[PAH]_{tot} = 2000$.

As shown in Fig. 5, Hb is able to react with the the five PAH, though anthracene remains the easiest to remove. The rate of all PAH removal is very fast and the reaction stops after 15 min. It is to notice that PAH are removed at different degrees, in the following order: anthracene > pyrene > phenanthrene > fluoranthene > naphthalene, with 95, 90, 40, 40, 5% (±5%), respectively. This PAH removal sequence is in accordance with theoretical calculations of Niu *et al.*[52] based on the molecular structural characteristics of PAH predicting their aptitude to be oxidized by Hb. To find the best oxidation conditions for all PAH, the influence of different parameters has been studied, such as the molar ratio [Hb]/[PAH] which was varied between 0.5 and 25 (Fig. 6) for a concentration of 300 nM for each PAH (phenanthrene and anthracene) at a ratio $[H_2O_2]/[Hb] = 1000$. The maximum of

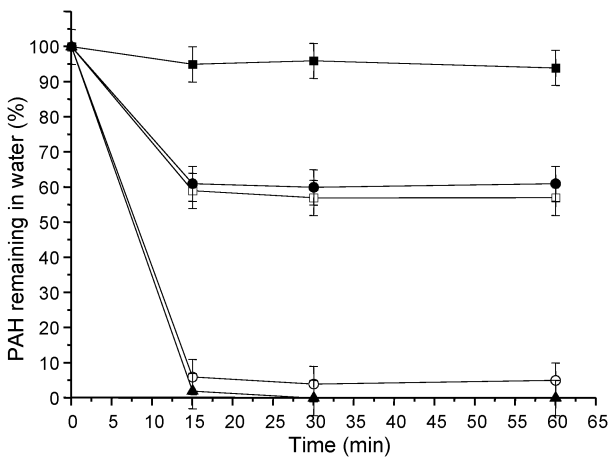

Fig. 5 PAH removal in non-optimized conditions as a function of time for 5 PAH using free Hb at pH 5: naphthalene (plain square), phenanthrene (empty square), anthracene (plain triangle), fluoranthene (plain circle), pyrene (empty circle). [ANT] = [PYR] = [NAP] = [PHE] = [FLT] = 300 nM; [Hb] = 3 μM; $[H_2O_2]$ = 3 mM. Analyses were done by UFLC.

PAH removal has been obtained for a two-fold molar excess of [Hb]/[PAH] for anthracene and a molar excess of 25 for phenanthrene. The amount of Hb should be therefore adjusted in function of the PAH, a higher amount of Hb is necessary to oxidize the less reactive PAH.

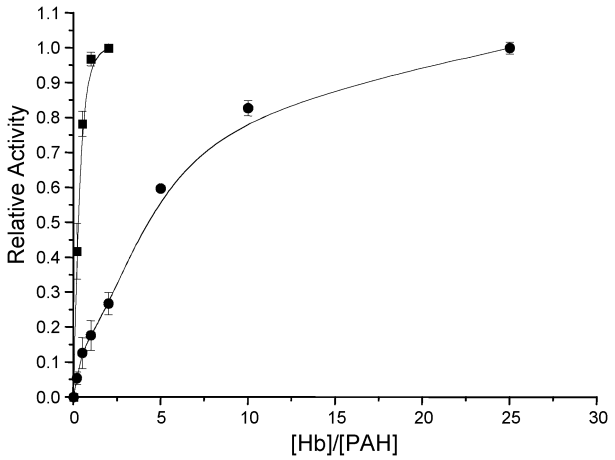

Fig. 6 Relative activity of free Hb towards anthracene (square) and phenanthrene (circle) removal as a function of the molar ratio [Hb]/[PAH]. The maximum PAH removals, corresponding to a relative activity of 1, are 95% and 65 ± 5% for anthracene and phenanthrene, respectively. The conditions were 300 nM PAH; $[H_2O_2]/[Hb] = 1000$; pH = 5. Analyses were done by UFLC.

In the conditions used in Fig. 6, the maximum removal rate, normalized to a relative activity of 1 for phenanthrene and anthracene, were 65 and 95 ± 5%, respectively. In order to follow the effect of the experimental conditions for PAH removal, phenanthrene with an intermediate reactivity has been selected instead of the highly reactive anthracene. For phenanthrene, the increase of Hb amount from [Hb]/[PAH] = 2 (Fig. 5) to [Hb]/[PAH] = 25 leads to an increase of its percentage of removal from 40 to 65%. The optimization of the amount of H_2O_2 with $[H_2O_2]/[PAH]$ varying between 100 and 10 000 (Fig. 7) has been performed with phenanthrene. Results show that, whatever the amount of Hb ([Hb]/[PAH] = 1, 5, 25), the maximum phenanthrene removal is reached for $[H_2O_2]/[PAH] \geq 2500$. In the conditions used in Fig. 7, the maximum phenanthrene removal (corresponding to a relative activity of 1) with [Hb]/[PAH] = 25 was 66%. These results

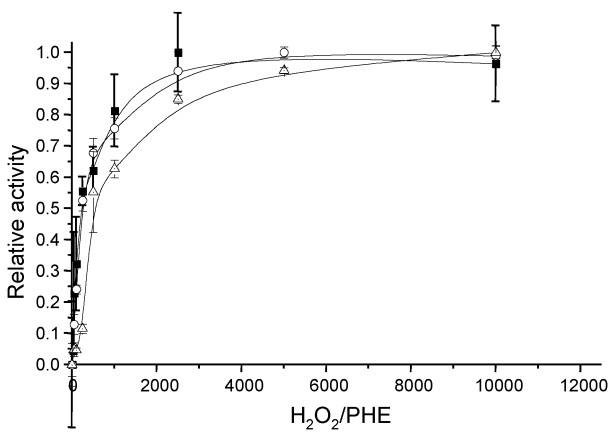

Fig. 7 Relative activity of Hb towards phenanthrene removal with free Hb as a function of the molar ratio $[H_2O_2]/[PAH]$. The maximum PAH removal, corresponding to a relative activity of 1, are 12, 36 and 66% ± 5, for 0.3 (square), 1.5 (circle) and 7.5 (triangle) μM Hb, respectively. Reactions were performed with 300 nM PHE, pH = 5. Analyzes were done by UFLC.

show that the amount of H_2O_2 has to be optimized in function of PAH concentration rather than of Hb concentration. These optimized conditions for free Hb have been then applied to a solution containing 11 PAH at a concentration of 300 nM for each PAH with 1% (v/v) of acetonitrile, at room temperature, with [Hb] = 16.5 μM (corresponding to [Hb]/[PAH] = 55 for each PAH and $[Hb]/[PAH]_{tot} = 5$ for the total amount of PAH) and $[H_2O_2]$ = 8.25 mM (corresponding to $[H_2O_2]/[PAH]_{tot} = 2500$). Results (ESI, Fig. S6†) show that at pH 5 after 15 min of reaction, when the reaction stops, PAH have been removed from the aqueous solution at different levels. Three PAH families show similar activities: (i) acenaphtene, anthracene, pyrene and benzo(a)pyrene have been totally removed (considering the detection limit of the analytical apparatus), (ii) phenanthrene, benzo(k)fluoranthene, fluoranthene benzo(b)fluoranthene, indeno(1,2,3,cd)pyrene and benzo(g,h,i)-perylene have been removed at 60–80% and (iii) naphthalene is removed at around 50%. The total PAH removal at pH 5 for free Hb corresponds therefore to 75 ± 5%. These data correspond to an initial total concentration of PAH of 3.3 μM and to a final concentration of PAH of 0.8 μM. However if the same reaction is performed at pH = 7, corresponding to the pH value of the industrial wastewater, only 47% of the 11 PAH have been removed (Fig. 8).

By UFLC no products were detected, excepted a small amount of anthraquinone (0.1 μM). As previously shown in literature for *in vivo* activated PAH,[53–55] the oxidized-PAH remain covalently linked to Hb. For other substrates (styrene, stilbene), it is supposed that the oxidized-substrates are linked to the tyrosine groups of Hb close to the hemic group[51,56] and could be, in the case of PAH, at the origin of an ether bond between the protein and the oxidized PAH (Fig. 4). It should be noted that the identification of PAH oxidation products linked to the protein and their localization on the protein after

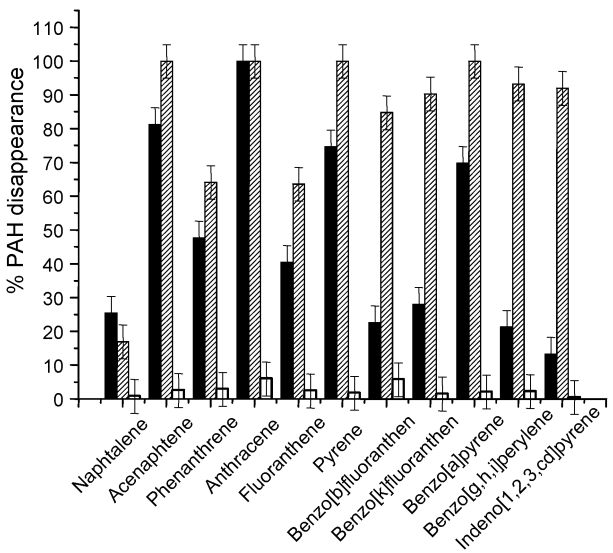

Fig. 8 Percentage of removal at pH 7 of 11 PAH using (black) free Hb, (striped) Hb-Dav20 containing 320 mg Hb per g silica and (white) blank reaction with H_2O_2 only, without Hb. Conditions are: [PAH] = 300 nM each, $[H_2O_2]$ = 8.2 mM, 25 °C. For free Hb, the reaction was performed with [Hb] = 16.5 μM and for Hb-Dav20, with 17 g material per l. Analyses were done by UFLC.

a peroxidase-like reaction is not easy to realize and is still under study. Harsh hydrolysis conditions did not allow to hydrolyze Hb-PAH binding until now.

PAH removal by Hb-immobilized silica

PAH removal from aqueous solution has been investigated for a solution containing 11 PAH at a concentration of 300 nM each and 1% (v/v) acetonitrile with Hb immobilized in Dav20 prepared with 500 mg Hb per g silica. As seen above, an amount of 16.5 μM of free Hb was necessary to obtain a maximum activity for PAH removal. In order to use the same amount of active Hb in the case of the Hb-Dav20, we have considered the percentage of active Hb present in the material, which corresponds to the relative activity determined by the ABTS test (Table 2) and equals 22%. Therefore an amount of 17 g of Hb-Dav20 per l is needed to obtain the maximum PAH removal. The same amount of H_2O_2 (8.25 mM) as for the study with free Hb was used as H_2O_2 quantities depends on the PAH concentration and not on Hb amount. As for free Hb, PAH removal with Hb-Dav20 was performed at pH = 5 (ESI, Fig. S6†) and at pH = 7 (Fig. 8) as the latter is more representative of wastewater treatment. The reaction of PAH with Hb-Dav20 is slightly slower than with free Hb and shows a rapid PAH removal during the first 45 min, which stops after 1 h, whereas with free Hb the reaction was stopped within 15 min. This is probably due to a diffusion limitation inside the solid and/or to a higher protection of Hb against H_2O_2. After 1 h of treatment over Hb-Dav20, the reaction mixture has been analyzed. At both pH values, a higher degree of PAH removal has been obtained with Hb-Dav20 as compared with free Hb. Indeed, 81% and 82% ± 5% of PAH removal have been reached for Hb-Dav20, at pH = 5 and at pH = 7, respectively, whereas 76% and 48% were obtained with free Hb.

The higher removal efficiency obtained with Hb-Dav20 compared with free Hb could results from the higher stability of Hb towards H_2O_2 and pH in the Davicat support (see section below). The stabilization effect could arise from the presence of water trapped in the rugosity of the silica (identified by the high C_{BET} parameter for Dav20) which could contribute to maintain a good protein conformation and the essential water layer on the Hb molecular surface as suggested by Wang *et al.*[39] by locally diluting acid, base, H_2O_2 or solvent. It should be noted that PAH do not adsorb on silica or on Hb-Dav20 alone. Indeed series of blank reactions have been realized with H_2O_2 alone (Fig. 8 and ESI, Fig. S6†), Hb without H_2O_2, silica alone, Hb-Dav20 alone without H_2O_2. These control experiments lead to a PAH removal rate lower than 5% in 4 h with an accuracy of the measurements of 2.5%. For the treatment performed at pH = 5, the higher activity of the Hb-immobilized silica compared to free Hb may be rather due to a better stability towards H_2O_2 inactivation. At pH = 7 the Hb-immobilized silica maintains its activity contrary to the free Hb. One can easily notice that Hb-Dav20 is a very effective material for the removal of 11 PAH.

Different reactivities of the PAH have been noticed with respect to the reaction with free Hb. Considering the results obtained at pH = 5, for high molecular weight PAH, such as benzo(a)pyrene, benzo(k)fluoranthene, benzo(b)fluoranthene, benzo(g,h,i)perylene and indeno(1,2,3,cd)pyrene, the PAH removal rate is enhanced with Hb-Dav20 compared to the free Hb. By contrast for low molecular weight PAH, like phenanthrene, fluoranthene and naphthalene, the PAH removal rate is decreased. This phenomenon can be explained by an increase of the hydrophobicity of the material when Hb is immobilized on the silica material (supported by lower C_{BET} parameter in Table 1) which facilitates the diffusion of the more hydrophobic PAH to the active site of the protein.

No products of PAH oxidation by Hb-Dav20 were observed except some small amounts of anthraquinone (0.08 μM) resulting from the conversion of 28% of the anthracene, as was the case with free Hb. All oxidized products remain therefore covalently linked to the protein inside the material. The material, together with the oxidized PAH products can be easily removed from the solution by simple filtration and the latter destroyed by calcination. The resulting calcined silica support can be then reused for further immobilization of Hb.

It should be noted that we have used a much higher concentration of PAH ([PAH] = 300 nM) in the present study than the ones present in actual wastewaters of oil refineries ([PAH] = 1–10 nM) because the actual HPLC instruments could not allow to use lower concentrations of PAH. Therefore the amount of H_2O_2 to add in a real (industrial) process should be around 1–10 ml H_2O_2 (35%) /m^3 of water to be treated, which is acceptable for an industrial process.

Stability of Hb in Hb-Dav20

The pH stability of Hb is a crucial parameter from an application point of view, wastewaters to be processed usually feature pH values in the range 6.5 and 8.5. At these pH values, the free Hb suffers a decrease of activity with only 60% of its optimal peroxidase activity remaining at pH 7 (Fig. 9).

On the contrary, Hb immobilized in Dav20 maintained 90% of its activity at pH 7, as shown in Fig. 9 for pyrene oxidation. At higher pH (pH = 8.5), immobilized-Hb in Dav20 maintained 65% of its activity, whereas only 25% of the activity of free Hb remained at this pH. This makes therefore Hb-Dav20 suitable for oxidation reactions in wastewater at neutral or near alkaline pH values. Stability *versus* the H_2O_2 inhibitor of free Hb and Hb-Dav20 has been tested (ESI, Fig. S6†). H_2O_2 is a suicide substrate and it inactivates irreversibly the protein.[57] Free Hb is totally inactivated by H_2O_2 after 3 h for a molar ratio $[H_2O_2]/[Hb]$ = 30 (ESI, Fig. S7†). For Hb immobilized in Dav20, this ratio increases by almost a factor of two, the total inactivation of the material is observed after 3h in a solution containing an equivalent of $[H_2O_2]/[Hb]$ = 50. Thermal stability of Hb in Hb-Dav20 has been compared to the thermal stability of free Hb by analyzing two solutions of free Hb (0.1 mg l^{-1}) and Hb-Dav20 (20 g l^{-1}) in a buffer solution at pH 5 at different temperatures for 15 min. After cooling the samples, the ABTS test was performed at 25 °C. Free Hb loses its activity for a temperature higher than 60 °C with 17 and 8% of remaining activity at 70 and 80 °C, respectively, whereas Hb immobilized in Dav20 maintains 40% of activity at these temperatures. These tests

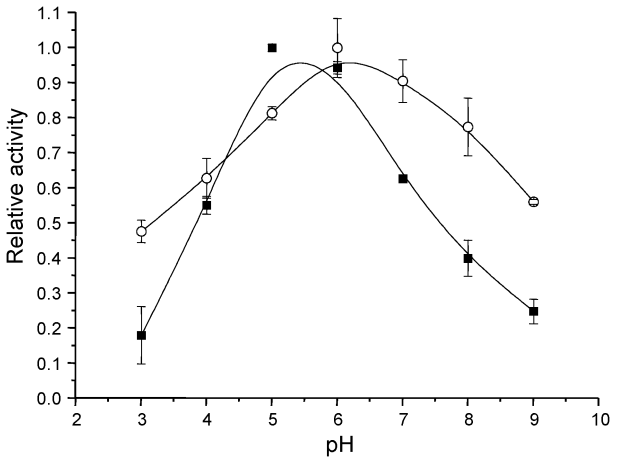

Fig. 9 Relative activities for pyrene removal in function of pH for (square) free Hb and (circle) Hb-Dav20 (320 mg Hb per g silica). The maximum activities for free Hb and Hb-Dav20 were 92% and 73% pyrene (PYR) removal, respectively. Reactions were performed with [PYR] = 3 μM; [Hb] = 6 μM; [H_2O_2] = 7.5 mM. Analyses were performed by UFLC.

of stability towards pH, temperature and H_2O_2 illustrate the protecting effect of the Dav20 silica support for Hb.

Conclusion

Bovine hemoglobin is a cheap and efficient biocatalyst for oxidation reaction using aqueous H_2O_2. Hb is readily and efficiently immobilized in commercially available mesoporous silica supports by simple adsorption. The selected supports bring the additional advantage of an adequate shape and morphology for continuous flow operation of a biocatalytic process. Immobilization of Hb in large pore (20 nm) mesoporous silica leads to a protection of Hb against unfriendly environments generated by variations of pH, temperature, H_2O_2 and solvent. The simple immobilization of Hb into silica results in an enhanced catalytic activity in water compared to that of native Hb. The enhanced catalytic activity of the confined Hb into the pores may be due to a protecting effect of the water confined in the mesopores towards the essential water layer at the Hb surface. The electrostatic interaction between Hb and the silica surface is strong enough to resist to the leaching. These properties suggest that the immobilization of Hb in large pore silica (Davicat® silicas) would have wide application as a simple and economical way of preparing biocatalysts for the removal of pollutants from aqueous or organic media by an oxidation/extraction process.

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