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MESOSTRUCTURED MATERIALS

Formation of cross-linked glucose oxidase aggregates in mesocellular foams

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Abstract Enzyme immobilization into solid mesoporous inorganic supports is a promising strategy to enable their use in continuous-flow fixed-bed reactors. In this study, the formation of cross-linked enzyme aggregates (CLEAs) of glucose oxidase (GOx) in the pores of mesocellular foams (MCFs) was investigated. The enzymes can enter the ultra-large cavities connected through the smaller windows, where their agglomeration and cross-linking with glutardialdehyde (GA) will take place. After cross-linking, the diameter of the CLEAs is larger than the diameter of the pore entrance and, thus, the enzymes are trapped in the pores of the support. By varying the experimental parameters, the optimum conditions for the preparation of active and stable immobilized biocatalysts were determined with respect to the resulting activity and to the enzyme loading. It is found that the preparation is preferably performed at pH 5.0, with a time delay between GA and GOx addition of 2 h and a n_{GA}/n_{GOx}-ratio of more than 400.

Introduction

In recent years, the contribution of enzymatic catalysis to the field of green and sustainable chemistry is increasing [1]. The mild reaction conditions (namely ambient temperature and pressure as well as physiological pH levels) paired with high activities as well as exceptional chemo-, regio-, and stereo-selectivities offer many new possibilities for different chemical process applications. However, the industrial use of enzymes is often limited by their low operational stability. To overcome this drawback, immobilization of the enzyme on a suitable support is a promising strategy, which not only improves the operational stability of the biocatalyst, but also allows facile separation and reuse [2]. Furthermore, enzyme immobilization on solid (mesoporous) supports is required for their use in continuous-flow fixed-bed reactors. However, leaching from the mesoporous support as well as deactivation of the enzyme are frequently encountered problems [2, 3]. Enzyme encapsulation in the pores of a suitable support suppresses leaching. Silanation or coating with microporous silicas produced via sol–gel routes are often employed approaches to reduce the mesopore opening to about 1 nm after enzyme immobilization. The entrapment of lipases in modified hydrophobic silica matrices prepared from alkyl-substituted silanes, such as methyltrimethoxysilane, propyltrimethoxysilane, and polydimethylsiloxane, has been reported by Reetz et al. [4]. Wang and Caruso adsorbed cytochrome *c*, protease, and catalase in mesoporous silica spheres. Thereafter, a multilayer shell was assembled on the sphere surface by layer-by-layer (LbL) electrostatic assembly of oppositely charged species to encapsulate the enzymes [5]. A "fish-in-net" approach for enzyme encapsulation was reported by Yang et al. [6]. The enzymes were entrapped in macroporous cages connected by uniform mesoporous channels. Ordered mesostructured silica particles were mixed with enzymes in a buffered solution. After the assembly of the enzymes with the mesostructured silica particles, polymerization and condensation of the

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inorganic part occur, which results in the formation of silica spheres encapsulating enzymes in macroporous cages. The formation of cross-linked enzyme aggregates (CLEAs), namely the physical aggregation of enzymes followed by cross-linking, was introduced by Sheldon and coworkers as an elegant method to prepare solid biocatalysts [7–10].

Stucky and coworkers reported the preparation of mesocellular foams (MCFs) which are excellently suited for the encapsulation of CLEAs because of their ultra-large cavities connected by smaller windows [11–14]. Through these windows, enzymes can enter the cavities, where agglomeration and cross-linking with glutardialdehyde (GA) will take place. The diameter of the CLEAs is larger than the diameter of the pore entrance and, thus, leaching is circumvented. Recently, Kim et al. [15] and Lee et al. [16] reported the immobilization of lipase and α-chymotrypsin through cross-linking into hierarchically ordered mesoporous silica. In our previous study, we have shown that deactivation of chloroperoxidase (CPO) from *Calariomyces fumago* immobilized on SBA-15 can be suppressed by in situ H_2O_2 generation via oxidation of glucose to glucono-1,5-lactone by immobilized glucose oxidase (GOx) [17]. Furthermore, we presented evidence for the existence of cross-linked enzymes in mesopore cages by small angle neutron scattering and we clearly demonstrated that the use of cross-linked enzymes in the pores of MCF materials significantly enhances the leaching stability in continuously driven fixed-bed reactors [18].

GOx is a dimeric protein with a molecular weight of 160 kDa, containing one tightly bound ($K_a = 1 \times 10^{-10}$) flavin adenine dinucleotide (FAD) per monomer as cofactor (actually there are two FAD-sites per enzyme). The FAD is not covalently bound and can be released from the holo-protein (a protein with co-factor) following partial unfolding of the protein [19]. The native protein is acidic (p$I = 4.2$) and is active over a broad range of pH values (pH 4–8) [20]. GOx is commonly used in biosensors to determine the glucose concentration by keeping track of the number of electrons passed through the enzyme which is realized by connecting it to an electrode and measuring the resulting charge. GOx is also found in honey and acts as a natural preservative; GOx residing at the surface of the honey reduces atmospheric O_2 to H_2O_2, which acts as an antimicrobial barrier.

In this study, we have explored different approaches for the cross-linking of GOx from *Aspergillus niger* in MCFs. Variation of the experimental parameters, namely the amount of enzyme, the amount of the linker, the succession (linker after enzyme and contrary) and the time interval between the addition of linker and enzyme, allows the preparation of active and stable immobilized biocatalysts with high effective activity.

Materials and methods

Synthesis of the support

The mesocellular foam used in this study has been synthesized according to procedures published by Stucky and coworkers [11–14]. In a typical preparation, the block copolymer P123 (2.0 g, 0.4 mmol) is dissolved in 1.6 M HCl (75 mL, 120 mmol) at 40 °C under stirring. After addition of 1,3,5-trimethylbenzene (TMB; 2.0 g, 17 mmol), the mixture is stirred at 40 °C for 1 h and then tetraethyl orthosilicate (TEOS; 4.4 g, 21 mmol) is added. After further stirring for 24 h at 40 °C, the reaction mixture is aged at 120 °C for 48 h under static conditions. The mixture is allowed to cool to room temperature, then the formed white precipitate is filtered off, dried in air for at least 2 days and finally calcined at 550 °C in air. The obtained materials were characterized by nitrogen sorption at 77 K using a Micromeritics ASAP 2010 instrument.

Determination of GOx activity

The activity of immobilized and native GOx is reliably determined by monitoring the hydrogen peroxide generation during the oxidation of β-D-glucose to δ-D-glucono-1,5-lactone. The acidic buffered reaction mixture contains D-glucose and potassium iodide in excess. During the reaction with D-glucose, the generated hydrogen peroxide oxidizes the potassium iodide to form iodine which reacts to I_3^-. The concentration of produced I_3^- in the potassium iodide solution can be determined photometrically with high precision. The assay is performed at room temperature as follows: To 5 mL of a buffer solution (pH 5.5) containing D-glucose (50 mM) and potassium iodide (100 mM), an aliquot of GOx is added. The experiments were carried out using a Varian Cary 50 UV–vis spectrometer at a detection wavelength of 352 nm [18]. In the experiments performed in a fixed-bed reactor, the D-glucose/potassium iodine solution is pumped through the reactor filled with the GOx–MCF catalyst (flow rate = 0.2 mL/min). At the reactor outlet, the solution is collected for a certain time interval and analyzed photometrically.

Immobilization of GOx

The MCF support, an aliquot of GOx and GA as cross-linker are suspended in 2 mL of a 0.1 mM aqueous citrate buffer or phosphate buffer solution. After shaking the samples in a water bath at 5 °C for several hours, the solid is separated from the supernatant liquid by filtration. The conditions used in the different immobilization runs are stated in the text. The obtained solid material (referred to as GOx–MCF) is washed with citrate buffer until the washing

solution shows no activity and subsequently the solid catalyst is tested for GOx activity. During immobilization experiments, the amount of immobilized GOx was routinely monitored using a Varian Cary 50 photometer operated at $\lambda = 460$ nm.

Results and discussion

Characterization of the MCF support

The powder X-ray diffraction pattern exhibits a distinct broad peak at $2\theta = 0.37°$ ($d = 23.8$ nm) confirming the ordered structure of the MCF support employed in this study (not shown). The nitrogen sorption isotherm recorded at 77 K is in close agreement with those published previously [11–14] and exhibits a large H1 hysteresis loop, which suggests that the MCF material possesses cage-type mesopores connected by smaller windows. The diameter of the cages as well as the window size can been determined in a good approximation from the nitrogen sorption isotherm employing the BJH formalism [21, 22]. For the material employed in this study, the cage diameter calculated from the adsorption branch is about 37 nm, while the entrance diameter determined from the desorption branch amounts to 18 nm. The specific surface area is estimated to 482 m^2/g using the BET model (Fig. 1).

Optimization of the immobilization procedure

Variation of the enzyme loading

The initial experiments were carried out in order maximize the GOx loading and the activity of the MCF material loaded with GOx. Therefore, 20 mg MCF were suspended

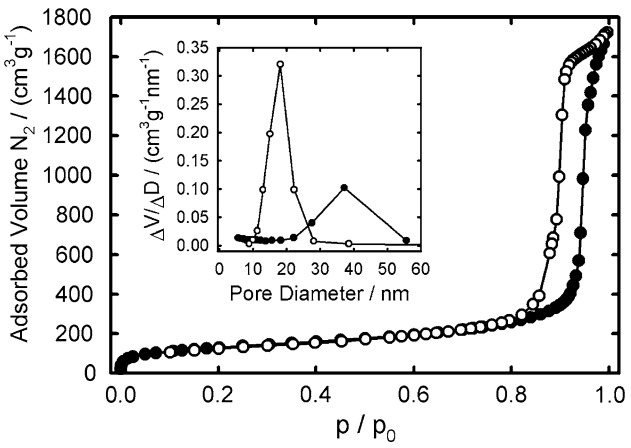

Fig. 1 Nitrogen sorption isotherm at 77 K of the MCF support (*filled symbols*: adsorption branch; *unfilled symbols*: desorption branch). Inset: BJH pore size distribution

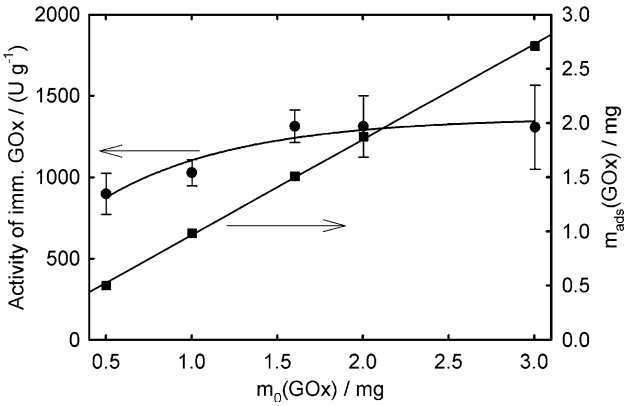

Fig. 2 Activity of GOx-MCF (*filled circle*) and the immobilized amount of GOx (*filled square*) as function of the added amount of GOx (20 mg MCF, 2 mL buffer (pH 4), 0.1 mmol GA, after 3 h addition of GOx)

in 2 mL of buffered solution (pH 4.0). Three hours after the addition of 0.1 mmol of the cross-linker GA, an aliquot of GOx was given to the mixture. The pH was fixed to 4.0, because we have shown in our previous paper that physisorption of GOx on siliceous materials at this pH results in catalysts exhibiting high activities [18]. The amount of immobilized GOx increases proportional to the amount of GOx added (Fig. 2), while the resulting activity of GOx–MCF reaches its maximum of 1,300 U g^{-1}, when about 2 mg of GOx were added. A further increase in the amount of GOx added does not result in a further increase of activity, probably due to the limited availability of the enzymes for the substrate. Initially, the pore cavities, which are connected to the outer surface, are filled. Thereafter, the enzymes diffuse further into the porous network and thus, the uptake increases, while the availability is reduced and the averaged activity reaches its limiting value.

Variation of the amount of glutardialdehyde added

Another set of experiments was conducted in order to study the influence of the amount of cross-linker added. While the amount of GOx added was fixed to 2 mg, the amount of GA was varied between 0.01 and 0.5 mmol. With increasing amount of cross-linker added, the amount of GOx immobilized reaches a limit slightly below 2.0 mg, which corresponds to almost 97% of the initial amount of GOx in solution (Fig. 3). The maximum uptake of GOx is already reached after addition of about 0.1 mmol of GA, while the activity of GOx–MCF increases further and reaches 1,800 U g^{-1} after addition of 0.5 mmol GA. In order to explain this finding, two effects have to be considered: (i) the remaining activity of the immobilized enzymes and (ii) their availability for the substrate. In the case of decreasing enzyme activity with increasing amount

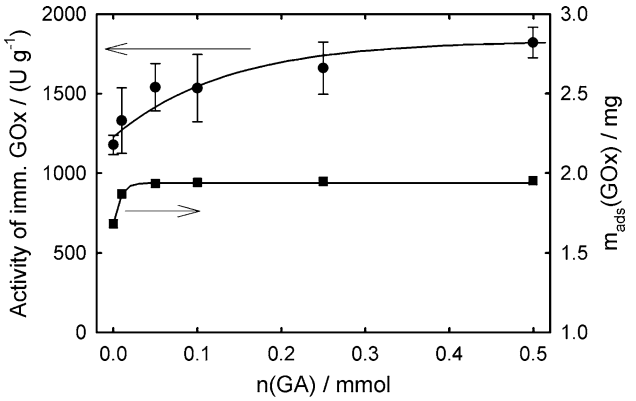

Fig. 3 Activity of the cross-linked GOx in MCF (*filled circle*) and the immobilized amount of GOx (*filled square*) as function of the amount of GA added (20 mg MCF, 2 mL buffer (pH 4.0), 2.0 mg GOx, after 3 h addition of an aliquot of GA)

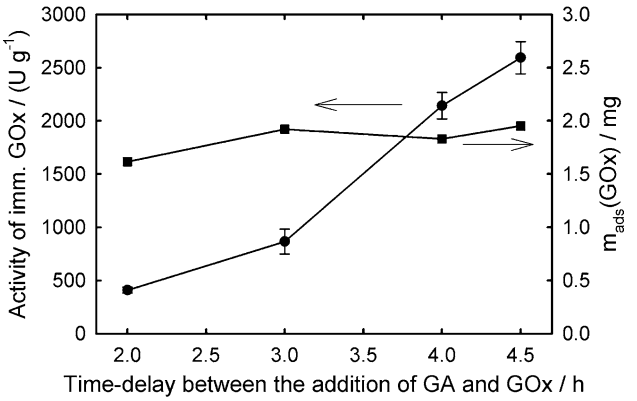

Fig. 4 Activity of the cross-linked GOx in MCF (*filled circle*) and the immobilized amount of GOx (*filled square*) as function of time delay between the addition of cross-linker and enzyme. 0.05 mmol GA were added to a suspension of 20 mg MCF in 2 mL buffer (pH 4.0). After a certain time delay, 2.0 mg GOx were added to the mixture

of cross-linker added, it has to be assumed that the linkages between the enzymes are numerous thereby affecting the tertiary structure. Contrary, an increase in activity of GOx–MCF loaded with the same amount of GOx with increasing amount of GA added indicates a higher substrate availability. As a consequence of the higher GA concentration, the cross-linking is fast and the enzymes do not diffuse deep into the porous network. Therefore, it is assumed that the cages which are close to the outer surface of the particles are the preferred sites for the CLEA formation. Thus, for the same amount of immobilized enzyme the specific activity is increased, because the cross-linked enzymes in these cages are more accessible for the substrate. In addition to the enhanced availability, a higher dispersion of cross-linked GOx in the porous network as a result of the formation of smaller CLEAs may also contribute to the observed increase in activity.

Variation of the time between GA and GOx addition

In this set of experiments, 0.05 mmol of GA were added to a suspension of 20 mg of MCF in 2 mL buffer (pH 4.0). After a certain time interval, 2 mg of GOx were added to the mixture. For a time delay of 2 h, the amount of GOx immobilized amounts to 1.6 mg, while the activity was determined to 400 U g^{-1} (Fig. 4). While the amount of immobilized GOx varies only slightly with an increasing time delay between GA and GOx addition, a strong increase in activity up to 2,600 U g^{-1} is observed. The uptake of enzymes which amounts to about 95 % of the concentration in solution is not affected by the time-delayed addition, but the resulting activity is strikingly different. This set of experiments was carried out in order to optimize the filling of the cages close to the outer surface of the MCF particles thereby aiming at a good availability of the CLEAs for the substrate. Although the adsorption kinetics of GA is fast (not shown), an increase in the time delay between the GA and GOx addition results in an increased activity of GOx–MCF. Consequently, it can be assumed that the already adsorbed GA is better dispersed. Thus, the enzymes are trapped and cross-linked directly after entering the porous network by the already adsorbed cross-linker. A shorter time interval between the addition of GA and GOx results in a less efficient cross-linking process, which presumably allows the enzymes to diffuse deeply into the pore network. Therefore, the increase in activity observed for longer time intervals is a result of increased GOx availability at an almost constant loading.

Influence of the pH

In a further step, cross-linking of GOx into MCF was studied at different pH values in order to find the optimum pH for immobilization. In Fig. 5, the activity and the amount of the immobilized GOx are plotted versus the pH. Both the activity and the adsorbed amount are maximal at pH 5.0. It has to be assumed that two pH-dependent processes interplay during CLEA formation: (i) the adsorption of the enzyme into the porous network and (ii) the formation of the cross-linked enzymes. The maximum activity of physisorbed GOx is observed when the sample is prepared at pH 3.8, which is slightly below the isoelectric point of GOx ($pI = 4.2$). For the PZC of mesoporous silica, different values between 2.2 and 3.7 are reported depending on the sample used [3, 23]. Thus, at pH 3.8, the electrostatic attraction between the slightly positively charged enzyme and the negatively charged silica framework is dominant. On the other hand, Schoevaart et al.

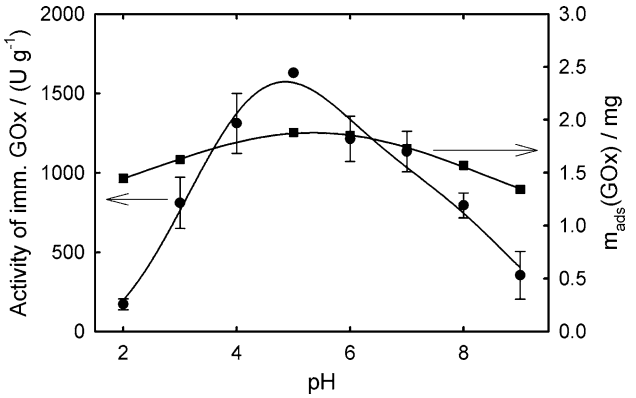

Fig. 5 Resulting activity of GOx-MCF (*filled circle*) and the immobilized amount of GOx (*filled square*) as function of the pH level (20 mg MCF, 2 mL buffer, 0.1 mmol GA, after 3 h addition of 2.0 mg GOx)

have reported that the preparation of CLEAs of GOx is ideally performed at pH 7.3 in a phosphate buffered ethylacetate solution [9]. The optimum pH level for the formation of CLEAs in our mesoporous MCF silica is found to be around 5.0, because both adsorption and cross-linking proceed conjointly. Furthermore, not only the uptake of GOx for catalysts prepared at pH 5.0, but also the resulting activity of GOx–MCF prepared under these conditions is maximal. This indicates that the formation of cross-linked GOx in MCF is preferably performed at pH 5.0. The immobilized amount of GOx drops from 95% to about 70% under acidic as well as basic conditions. The activity decreases from 1,630 U g^{-1} for GOx immobilized at pH 5.0 to 170 and 350 U g^{-1} for GOx–MCF catalysts prepared at pH 2.0 and 9.0, respectively. For a tentative explanation two effects have to be taken into account: (i) under acidic conditions, the enzymes are strongly cross-linked, while (ii) under basic conditions cross-linking occurs very slowly. The formation of imino groups is acid catalyzed, thus, at pH levels below 5.0 a multitude of amino functions of the enzymes are inter- and intra-enzymatic cross-linked. These linkages strongly affect the tertiary structure of the enzymes, resulting in denaturation and a concomitant decrease in activity while a high enzyme loading is maintained. In preparations at pH >5.0, the formation of the imino linkages is slow. Therefore, the enzymes are allowed to diffuse deeper into the porous network before cross-linking takes place and consequently the activity is significantly reduced due to lower availability of the enzymes for the substrate, while the enzyme loading is only slightly lower. Furthermore, from nitrogen sorption measurements after immobilization (not shown) it is deduced that the structural properties of the MCF support are not altered during the immobilization procedure.

Effective activity of the immobilized GOx

An optimized immobilization process not only aims for high resulting loadings and activities, but also the efficiency of the process and the resulting biocatalyst should be considered. Efficiency of immobilization involves that a certain (not necessarily a large) amount of enzyme is immobilized on the porous material and shows an activity close to the same amount of native enzyme, which means that the immobilized enzymes remain active as well as available for the substrate. Therefore, effective activities, namely the resulting activity per immobilized amount of enzyme, were calculated and compared with the effective activity of the native enzyme (Fig. 6). The native enzyme in solution shows an effective activity of 40.5 U mg^{-1}, which is the upper achievable limit. The comparison between the effective activity of the immobilized and the native GOx is expressed in Fig. 6 as the percentage of the enzymes which remain active during the immobilization process and are available for the substrate. For the immobilized GOx, an effective activity closed to 40.5 U mg^{-1} is desirable; however, a dramatic decrease in effective activity from 35.8 U mg^{-1} (88%) to 9.6 U mg^{-1} (24%) is observed with increasing amount of GOx (Fig. 6a). This is due to the fact that the activity increases only slightly up to a limit of 1,300 U g^{-1}, while the immobilized amount increases directly proportional to the amount of GOx added. While the amount of cross-linker added affects the effective activity only slightly, an increase of the time

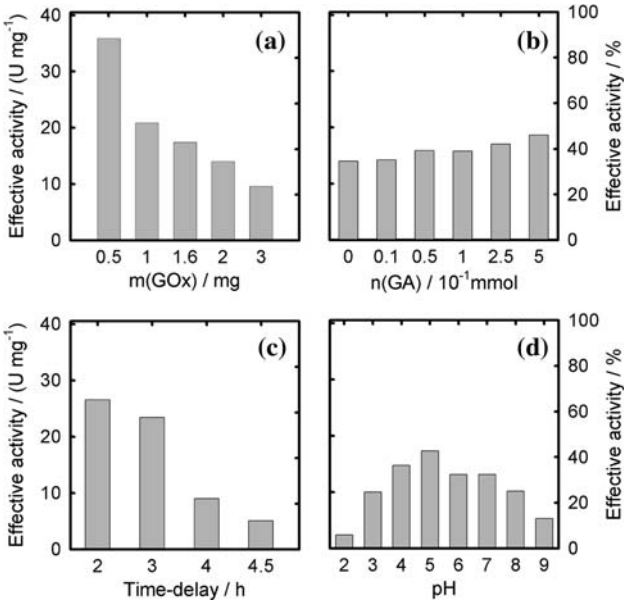

Fig. 6 Comparison of the effective activity between the samples prepared under different conditions: **a** variation of the amount of GOx added, **b** variation of the amount of GA added, **c** time delay between GOx and GA addition, and **d** pH of the enzyme solution

delay between addition of cross-linker and enzyme from 2 to 4.5 h results in a significant decrease in effective activity from 26.5 U mg^{-1} (66 %) to 5.1 U mg^{-1} (13%; Fig. 6b). The largest effective activity is observed for the GOx–MCF catalyst prepared at pH 5.0, which exhibit an effective activity of 17.3 U mg ^{1} (43%; Fig. 6d).

From the experiments performed so far, the following strategy with respect to the preparation of the GOx CLEAs in the pores of MCF possessing maximal effective activity can be derived. The preparation should be executed at pH 5.0, with a time delay between GA and GOx addition of 2 h, while minimizing the amount of GOx added. The amount of GA added plays a minor role, but should be adjusted to the amount of GOx employed (ratio n_{GA}/n_{GOx} = 400–4,000).

Leaching test

In order to test the resistance of GOx cross-linked in the mesopores of MCFs to leaching, the activity assay solution was pumped through a fixed-bed reactor filled with the GOx–MCF catalyst. The performance of cross-linked GOx in MCF under continuous conditions in the fixed-bed reactor is similar irrespective of the sample preparation provided that the initial activity is equal. Thus, the leaching behavior of cross-linked GOx in MCF is compared to a catalyst prepared by physisorption of GOx onto the widely used enzyme support SBA-15 since physical adsorption of GOx on MCF result in a catalysts that exhibits a complete loss of activity after about 12 h time-on-stream. In the large cage-type MCF pores, GOx is more susceptible to leaching because the large pore cages and entrances are significantly larger than the enzyme, while for SBA-15 pore and enzyme diameter are similar. Figure 7 shows the relative activity of cross-linked GOx in MCF compared to physisorbed GOx on SBA-15 under continuous-flow in a fixed-bed reactor. The decrease in activity after time-on-stream is given as percentage of the initial activity. The activity of the CLEA catalyst drops to around 70% of the initial activity within 23 h. For the catalyst prepared by physisorption, the activity decreases to 36% after the same time. Nevertheless, the formation of GOx CLEAs in the pores of MCFs allows the preparation of active heterogeneous biocatalysts, which are significantly more stable against leaching than conventional catalysts prepared by physical adsorption. Similar conclusions were drawn in a similar study devoted to chloroperoxidase encapsulation in MCFs [20].

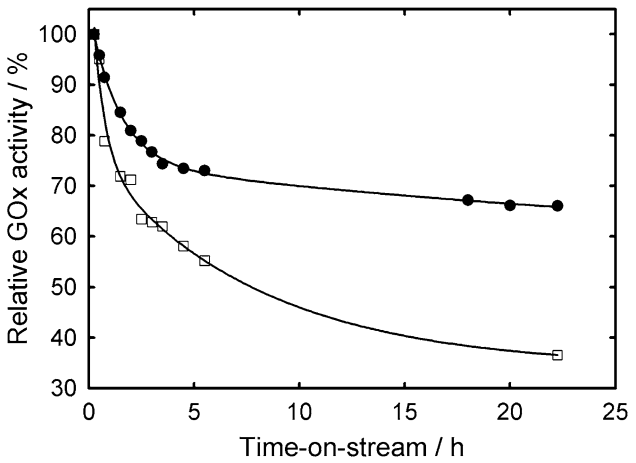

Fig. 7 Relative activity of GOx adsorbed onto SBA-15 (*unfilled square*) and cross-linked in the pore cages of MCF (*filled circle*). The biocatalysts were filled in a fixed-bed reactor and the assay solution (D-glucose (50 mM) and potassium iodide (100 mM), pH 5.5) was pumped through the fixed-bed (flow rate = 0.2 mL/min)

Conclusions

The immobilization of the enzyme GOx into MCFs was investigated. Variation of the immobilization conditions including the amount of GA and GOx added, the time delay between enzyme and cross-linker addition and the pH of the enzyme solution result in catalysts with different activity. A maximum activity of 2,600 U per gram MCF can be achieved; however, the effective activity is only 5.1 U per mg GOx (compared to 40.5 U per mg GOx in solution). Under optimized conditions, an effective activity of 38.8 U per mg GOx is achieved, i.e., about 96% of the immobilized enzymes are still active and available for the substrate. Furthermore, it is concluded that immobilized cross-linked GOx exhibits high activity, if (i) denaturizing during the cross-linking process is avoided and (ii) the cross-linked enzymes are easily available for the substrate in the porous network. Finally, it is confirmed that leaching is significantly reduced by the formation of GOx–CLEAs in the pores of the MCF support.

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