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QCM-D as a method for monitoring enzyme immobilization in mesoporous silica particles

Christian Thörn [a,1], Hanna Gustafsson [b,*,1], Lisbeth Olsson [a]

[a] Industrial Biotechnology, Department of Chemical and Biological Engineering, Chalmers University of Technology, SE-412 96 Gothenburg, Sweden
[b] Applied Surface Chemistry, Department of Chemical and Biological Engineering, Chalmers University of Technology, SE-412 96 Gothenburg, Sweden

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ABSTRACT

Enzyme immobilization in mesoporous materials is a field of great interest, with applications in biocatalysis and biosensing. However, the actual immobilization process is not well understood and has mainly been studied by indirect measurements. The present work demonstrates a direct method for real time study of enzyme immobilization in mesoporous silica particles using quartz crystal microbalance with dissipation monitoring (QCM-D). Silica-coated sensors were grafted with amine groups followed by adsorption of small (40 nm), spherical mesoporous silica particles, after which the enzyme immobilization into the mesoporous particles could be studied in real time. The influence of pH on the immobilization efficiency was studied using two different enzymes; lipase from *Rhizopus oryzae* and feruloyl esterase from *Fusarium oxysporum*. The results showed that the silica particles adsorbed readily to the amine-grafted surface. The QCM-D measurements indicated that considerably more enzyme was immobilized into mesoporous silica particles than to non-porous silica particles and to a flat silica surface. The viscoelastic effect of the immobilized enzymes was visualized by plotting the frequency shift against the corresponding dissipation. It was observed that the immobilization into the porous particles can be divided into two regimes where the first regime is suggested to represent adsorption to the outer surface and pore openings and the second regime represents further adsorption inside the pores. In summary, we demonstrated QCM-D as a novel method for understanding enzyme immobilization in mesoporous particles in real time and the approach may be of general use for studies of entrapment of molecules into porous particles.

1. Introduction

Immobilization of enzymes into mesoporous materials has been studied extensively during the last decade. Enzyme loaded nanoparticles have broad applications within biocatalysis and biosensing [1–4]. The porous structure has the advantage of providing a very large surface area, which allows for a high enzyme loading. In previous work on enzyme immobilization into mesoporous materials the adsorption process has been studied by indirect measurements, analyzing the protein concentration or the enzyme activity in the bulk solution surrounding the porous particles, rather than directly monitoring the adsorption of the enzyme into the porous particles [1,5]. Since the adsorption of the enzyme into the pores occurs within minutes, these indirect methods give poor accuracy and do not allow studies of the adsorption kinetics. A direct and continuous way of monitoring how the enzyme becomes immobilized into the pores is therefore of interest. To the best of our knowledge, this work constitutes the first description of such a direct monitoring of the enzyme immobilization process.

Quartz crystal microbalance with dissipation monitoring (QCM-D) is a surface sensitive analytical technique based on piezoelectric properties of quartz [6]. Upon applying an alternating potential between the two plates, AT-cut crystals will oscillate in the shear mode. Resonance is built up in an alternating circuit with a frequency corresponding to the resonance frequency of the crystal (referred to as sensor). The technique is capable of measuring, in real time, mass changes in the nanogram range while at the same time monitoring the viscoelastic properties when analytes dispersed in a liquid adsorb to the sensor surface. The adsorbed mass is related to changes in resonance frequency and the viscoelastic properties are related to changes in dissipation. Dissipation is detected as damping or decay of vibrations in the film adsorbed to the sensor, thus softer and more flexible films result in increased dissipation. For a thin and rigid film evenly covering the sensor, the mass adsorbed is linearly proportional to the frequency shift. The characterization of soft films requires more advanced modeling.

QCM-D has previously been used to study immobilization of enzymes onto surfaces. Horseradish peroxidase immobilized on

* Corresponding author. Tel.: +46 317722844.
E-mail address: hanna.gustafsson@chalmers.se (H. Gustafsson).
[1] These authors equally contributed to this work.

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poly(ethylene-*co*-acrylic acid) (PEAA) films via covalent linkages was found to form a rigid film with low dissipation whereas the same enzyme when physically adsorbed resulted in a highly dissipative structure [7]. Porous films have been shown to increase the sensitivity of QCM-D due to the larger surface area that allows more molecules to be adsorbed [8–11]. Mesoporous surface modified silica particles have been attached onto a gold film and subsequent adsorption of platinum ions was monitored by surface plasmon resonance. A 2–3 fold increase in signal strength compared to a flat silica surface was observed [12].

In the present work we present a direct method for real time monitoring of the immobilization of enzymes into solid mesoporous silica particles deposited onto a QCM-D sensor. Small (40 nm) mesoporous silica particles were adsorbed onto silica coated sensors modified with amine linkers and the particle-coated surface was subsequently used for immobilization of either lipase from *Rhizopus oryzae* and feruloyl esterase from *Fusarium oxysporum*. The stability of both particle adsorption and enzyme immobilization was evaluated by rinsing procedures. The influence of pH on the immobilization efficiency was studied for both enzymes. To the best of our knowledge, enzyme immobilization into mesoporous particles has not been previously studied using QCM-D.

2. Materials and methods

2.1. Chemicals and enzymes

Citrate and Na_2HPO_4 were purchased from Sigma and Merck, respectively. All citrate–Na_2HPO_4 buffers (pH 4.0–8.0) were prepared to 0.1 M concentrations. D_2O (99.9%) was purchased from Larodan Fine Chemicals. Cetyltrimethylammonium bromide (CTAB, ⩾99%), tetraethylorthosilicate (TEOS, ⩾99%), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AIBA, 97%), L-lysine (⩾98%), aminopropyltrimethoxysilane (APTMS), sodium dodecyl sulphate (SDS), toluene (99.8%), ethanol (99.5%), nitric acid and acetone (99.8%) were all purchased from Sigma. The non-porous silica particles (Bindzil 50/80) were a gift from Eka Chemicals AB (Bohus, Sweden). The feruloyl esterase FoFAEC was received as a gift from Dr. Evangelos Topakas, National Technical University of Athens, Greece [13]. Lipase from *R. oryzae* (ROL) was purchased from Sigma. Before the adsorption, the enzyme solutions were washed with citrate–phosphate buffer using spin filters (Amicon Ultra – 0.5 ml 10 K ultracel), leading to a final protein concentration of 0.03 mg/ml.

2.2. Synthesis of mesoporous silica particles

The mesoporous silica particles were synthesized by a method adapted from a previously published protocol [14] which was adapted from Nandiyanto et al. [15]. See Supplementary materials for a detailed description of the synthesis and particle characterization (Fig. S1).

2.3. Rehydroxylation

Rehydroxylation of calcined porous silica particles was performed by simply boiling the particles in Milli-Q water. The silica particles were dispersed in Milli-Q water (2% v/v) and heated under reflux for 24 h. The temperature was lowered to 25 °C and HNO_3 was added until a pH <1 was reached. The rehydroxylated particles were washed with Milli-Q water until a neutral pH was obtained followed by rinsing with acetone. Finally, the material was dried in vacuum (CHRIST RCV 2-18) for 2 h. The rehydroxylated particles were used only when explicitly stated.

2.4. Cleaning protocol for silica coated QCM-D sensors

Cleaning of the silicon dioxide coated sensors (QSX 303, Q-Sense AB, Gothenburg, Sweden) was performed according to a standard protocol supplied by Q-Sense. In short: UV/ozone treatment for 20 min followed by washing in a 2% solution of sodium dodecyl sulphate for 30 min and rinsing 4 times with Milli-Q water. The sensors were dried with nitrogen gas and exposed to UV for another 10 min before finally dried with nitrogen gas.

2.5. Binding of silica particles to the QCM-D sensor

The QCM-D experiments were run on a Q-Sense E4 instrument equipped with a QAFC 301 axial flow chamber (Q-Sense). Both the porous and the non-porous silica particles were attached to the QCM-D sensor using aminopropyltrimethoxysilane (APTMS) as a linker. The silanization protocol was based on previous work [16], but with major modifications. The clean sensors were immersed in a toluene solution with 1% (v/v) of APTMS for 40 min followed by sonication 2×5 min in pure toluene and 2×5 min in 99.5% ethanol. The sensors were cured in Milli-Q water at 60 °C for 30 min, rinsed in Milli-Q water and dried with nitrogen gas. The porous and the non-porous particles were dispersed in 0.01 M HCl (pH 2) and the rehydroxylated particles were dispersed in citrate–Na_2HPO_4 buffer (pH 4). Finally, the sensors were mounted into the QCM-D chamber and a suspension of 0.4% (w/w) of silica particles was flowed (25 μl/min, 21 °C) through the system. Milli-Q water (H_2O) and pure deuterium oxide (D_2O) were used to calculate the pore volume of the materials. During the subsequent enzyme immobilization, all settings remained the same. For the enzyme loadings at different pH, to be comparable, the data was normalized according to frequency shifts during the binding of porous particles to the sensor (the average binding of porous particles showed a frequency shift of 464 Hz with a standard deviation of 61.4). The particle distribution on the QCM-D sensor was studied with scanning electron microscopy (SEM; Leo Ultra 55 FEG SEM).

2.6. Analysis and modeling of QCM-D data

All frequency shifts presented are based on data recorded at the 5th overtone, if not specified differently. Q-tools software (Q-Sense) was used for viscoelastic modeling of the particle adsorption and the enzyme loading. The solution with porous particles (0.4% v/v) was approximated to have the same density and viscosity as pure water (0.998 g/cm^3 and 0.001 kg/ms).

3. Results and discussion

3.1. Adsorption of silica particles to the flat silica surface

In order to study enzyme immobilization on porous and non-porous silica particles with the QCM-D technique, the particles were first attached to a silica coated sensor. For the particles to bind, a silane linker containing an amino group (APTMS) was first grafted onto the silica coated sensor leading to an amino-terminated surface (Fig. 1a). The APTMS modified sensor was mounted in the QCM-D chamber and the adsorption of the silica particles was monitored (Fig. 1b). Exposure of the modified silica surface to suspensions of the particles (porous or non-porous) in 0.01 M HCl (pH 2) gave a significant decrease in the measured frequency. This is an indication of adsorption of the negatively charged particles to the positively charged sensor surface at pH 2 (Fig. 2). SEM images confirmed that the sensors had been covered with particles (Fig. 3). The particle size and the sensor coverage were quite