Nanoporous Waveguide Spectroscopy for the Estimation of Enzyme Adsorption on Mesoporous Silica

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Mesoporous silica is considered as promising host material for enzymes due to its uniform pore size of enzyme dimensions and tunable surface chemical properties. In this study, we applied nanoporous waveguide (NPWG) spectroscopy to observe adsorption dynamics of heme proteins with different molecular size within mesoporous silica film modified with different surface functional groups. Since NPWG spectroscopy provides kinetic information and rough quantification of adsorption amount, it is useful to study the adsorption process of enzymes within inorganic nanoporous materials.

Keywords Enzyme adsorption, waveguide spectroscopy, mesoporous silica, adsorption dynamics, heme proteins

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Introduction

An enzyme has highly selective and efficient catalytic abilities under mild reaction conditions, and it has been used as an important biocatalyst for food processing, biosensors, drugs and so on.¹ Since the activity of enzymes dissolved in a bulk aqueous solution is easily degraded, studies have been conducted to achieve long-term stability of their activity. Enzyme immobilization inside inorganic nanopores is a simple and effective way to maintain catalytic activity.²⁻⁴ Surfactant-templated mesoporous silica is a powerful candidate for an ideal host of biomolecules due to its uniform pore size of dimensions of biomolecules and tunable surface chemical properties.⁴⁻⁶ The size-matching between the enzyme and pore size has been proposed to stabilize structure and function of the enzyme immobilized within mesoporous silica. The enzyme within mesoporous silica can be easily recovered from a reaction solution. Until now, various biocatalytic systems based on the mesoporous silica host have been studied.

Adsorption of enzymes into porous media is usually studied by batch process, which is monitored by a decrease in the amount of enzyme in a solution phase.⁷ The batch process can be used to study kinetics of slow adsorption process, while their application for observing rapid adsorption process is difficult. Label-free and high-throughput optical observation techniques have hence been developed for the study of adsorption kinetics of enzymes at porous media. For example, Chen *et al.* applied interferometric spectroscopy for studying adsorption dynamics

of bovine serum albumin, hemoglobin and myoglobin on a porous silicon substrate.⁸ The microscopic method has also been applied for a single-particle system.^{9,10} Optical waveguide spectroscopy is one of the sensitive methods for a label-free and high-throughput method to study rapid adsorption kinetics.¹¹⁻¹⁴ In our previous studies, a mesoporous film on an Al thin layer was used for a planar waveguide, and its waveguiding properties could be used to analyze rapid adsorption kinetics of proteins.^{13,14} Hereafter, optical waveguide spectroscopy based on the nanoporous/aluminum multilayer film is called as nanoporous waveguide (NPWG) spectroscopy.

In the present study, we applied NPWG spectroscopy to characterize the adsorption dynamics of heme proteins (cytochrome *c*, myoglobin, horse radish peroxidase) at a mesoporous silica film with pore size of *ca.* 6 nm. The heme proteins used in this study have different isoelectric point (pI) and molecular size. NPWG spectroscopy utilizes optical waveguide modes excited in mesoporous silica/Al multilayer film, which is observed as a sharp attenuation dip in the reflection spectrum (Fig. 1). Since the position of an attenuation dip corresponds to the change of a refractive index of a mesoporous silica layer, a molecular adsorption process into silica mesopores can be observed by monitoring a shift of the attenuation dip. Herein, the adsorption of these heme proteins at the mesoporous silica layer was studied to discuss the effects of a molecular size and the surface-protein interaction for the adsorption process. In addition, effects of surface modification of mesoporous silica on the adsorption process were also studied.

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Fig. 1 Schematic illustration of a configuration of NPWG spectroscopy and an idealized electric distribution of the TE₀ mode.

Experimental

Reagents and chemicals

Cytochrome *c* (cyt *c*) from equine heart, myoglobin (Mb) from horse heart, horseradish peroxidase (HRP) and non-ionic triblock copolymer Pluronic F127 (F127: PEO₁₀₆ PPO₇₀ PEO₁₀₆) consisting of polyethylene oxide (PEO) and polypropylene oxide (PPO) were purchased from Sigma-Aldrich (Tokyo, Japan). A rectangular cover glass substrate (24 × 45 × 0.3 mm, Matsunami Glass Ind., Ltd., Osaka, Japan) was used for each sample substrates. Al wire (99.99%; Nilaco Co., Tokyo, Japan) was used for the thermal deposition of an Al layer on the glass substrate. Milli-Q water was used for all experiments. Other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Preparation of MS film

Precursor solution for the mesoporous silica (MS) layer was prepared according to our previous report.¹⁴ A mixture of Pluronic F127 (1.38 g), 1 M hydrochloric acid solution (0.30 g), ethanol (26.49 g), and water (2.07 g) was stirred for 1 h at 30°C. Then, tetraethoxysilane (TEOS, 3.0 g) was added to the mixture and the mixed solution was stirred for 2 h at 30°C to give the precursor solution.

The glass substrate was sonicated in acetone, methanol, and then Milli-Q water for 5 min each. Then it was immersed in concentrated nitric acid and boiled for 75 min to remove organic species adsorbed on the substrate surface. Finally, its surface was etched by immersing it in a 10 wt% hydrofluoric acid solution for 20 s. An aluminum layer (12 nm) was deposited on the one side of the cleaned-glass substrate using an Ulvac model VPC-1100 vacuum deposition system. The MS layer was dip-coated on the Al-deposited glass substrate using the precursor solution of mesoporous silica. The sample substrate with the MS/Al multilayer film was heated at 70°C overnight, and then, it was calcined at 420°C for 4 h to remove the template surfactant. We designated the mesoporous silica layer after calcination as Cal-MS. Thickness of Cal-MS was 320 nm.

The inner pore surface of Cal-MS was modified with ethoxy group or octadecylsilyl (ODS) group to study the effect of surface moiety on adsorption dynamics of enzymes. Surface treatment of mesoporous silica with ethanol to modifying ethoxy group on silica surface was according to the literature.¹⁵ Briefly, Cal-MS/Al was refluxed in dry ethanol (20 mL) at 80°C for 16 h, and then dried under vacuum for 4 h to obtain ethoxy-modified MS/Al (Ethoxy-MS/Al). ODS modified mesoporous silica film (ODS-MS) was obtained by refluxing Cal-MS/Al in toluene (20 mL) containing octadecyldimethylchlorosilane for 1 h, followed by end-capping of residual silanol groups with trimethylchlorosilane.¹⁴

NPWG spectroscopy

The details of the experimental setup for the OWG spectroscopy were described in our previous report.¹⁶ In brief, s-polarized white light from a Xe lamp (Hamamatsu Photonics; L8254, 150W) was irradiated on the MS/Al sample substrate and its reflection spectra were measured by using a multichannel analyzer (Hamamatsu Photonics; PMA-11). A fluidic channel (total volume, 2.8 μL; channel width, 1.5 mm; channel depth, 0.14 mm) was fabricated using a PDMS substrate that was placed on the MS/Al/glass sample substrate. A syringe pump (Harvard apparatus; Model 11 plus) was used to deliver the sample solution into the fluidic channel and the OWG spectroscopy measurements were performed with continuous flow of enzymes in a phosphate buffer solution (pH 5.0, 6.0 or 7.0) at 15 μL min⁻¹. Ionic strength of each buffer solution was set as 0.01 M.

Results and Discussion

The position of the attenuation peak shifts toward longer wavelength due to the adsorption of enzymes within MS layer. The wavelength shift of the attenuation peak ($\Delta\lambda_{\text{OWG}}$) in the reflection spectrum is approximately proportional to the amount of adsorbent within a mesoporous film as given in Eq. (1),¹⁴

$$\Delta\lambda_{\text{OWG}} = aq_{\text{ads}} \quad (1)$$

where a is a proportional constant and q_{ads} is the amount of adsorbent. $\Delta\lambda_{\text{OWG}}$ thus can be used to monitor the relative amount of enzyme adsorbed within the MS layer. The increase and decrease of $\Delta\lambda_{\text{OWG}}$ are associated with adsorption and desorption of enzymes, respectively. On the basis of Fick's law, time-course shift of λ_{OWG} , $\Delta\lambda_{\text{OWG}}(t)$, can be described as follows:¹⁴

$$\Delta\lambda_{\text{OWG}}(t) = \Delta\lambda_{\text{OWG}}(\infty) \left(1 - \frac{8}{\pi^2} \sum_{n=0}^{\infty} \frac{\exp[-(2n+1)^2 D_{\text{app}} t / l^2]}{(2n+1)^2} \right) \quad (2)$$

where $\Delta\lambda_{\text{OWG}}(\infty)$, D_{app} , and l are the $\Delta\lambda_{\text{OWG}}$ value at equilibrium, the apparent diffusion coefficient (in cm² s⁻¹) of enzyme inside the mesopores, and the thickness of the porous layer (in cm), respectively. The Eq. (2) was used to analyze the adsorption kinetics of enzymes.

We observed adsorption behaviors of heme proteins under a continuous flow of each protein solution (100 μM, pH 6.0). Adsorption profiles of cyt *c*, Mb and HRP at Cal-MS are shown in Fig. 2. These profiles could be well-fitted to Eq. (2), and the values of $\Delta\lambda_{\text{OWG}}(\infty)$ and D_{app} are summarized in Table 1. It is clearly recognized that both $\Delta\lambda_{\text{OWG}}(\infty)$ and D_{app} values are lower for larger protein; cyt *c* > Mb > HRP. Since pI values of Mb and HRP are almost the same, it can be considered that Mb and

Fig. 2 Adsorption profiles of *cyt c* (open square), Mb (filled square) and HRP (filled triangle) under the flow of a 100 μ M solution (pH 6.0) at silica mesopores. The solid lines are the results of the best fit using Eq. (2).

Table 1 Physical properties of each enzymes and calculated values of $\Delta\lambda_{\text{OWG}}(\infty)$ and D_{app} molecular size of enzymes are based on existing literature⁵

Protein	Size/ nm	Mw/ kDa	pI	$\Delta\lambda_{\text{OWG}}(\infty)/$ nm	$D_{\text{app}}/$ $\text{cm}^2 \text{ s}^{-1}$
<i>cyt c</i>	$2.6 \times 3.2 \times 3.3$	12	10.7	134	15×10^{-13}
Mb	$2.1 \times 3.5 \times 4.4$	17	7.0 – 7.3	96	5.1×10^{-13}
HRP	$4.0 \times 4.4 \times 6.8$	44	7.2	17	0.6×10^{-13}

HRP exhibited similar protein-protein interaction and protein-surface interaction. The lowest D_{app} for HRP is hence supposed to be due to the spatial restriction. The longest axis of HRP is 6.8 nm, which is almost consistent with the pore diameter of Cal-MS (*ca.* 6 nm). This size-matching would induce the slowest diffusivity of HRP inside the silica mesopore. Since *cyt c* and Mb are smaller than the mesopore diameter, less contribution of the spatial restriction for the diffusion of those proteins is expected.

The diffusion of small proteins (*cyt c* and Mb) was further studied by adsorption dynamics depending on solution pH. Figure 3(a) shows adsorption profiles of *cyt c* and Mb observed under different pH conditions. The $\Delta\lambda_{\text{OWG}}$ and D_{app} values estimated from those adsorption profiles are summarized in Table 2. For *cyt c*, $\Delta\lambda_{\text{OWG}}$ and D_{app} tend to be larger as pH increased, suggesting that the increase in the adsorbed amount of *cyt c* was related to the acceleration of *cyt c* mobility inside the pore. Keeping in mind the pI of *cyt c* (10.7), positive surface charge of *cyt c* was almost the same at pH 5.0 – 7.0. By contrast, dissociation constants of surface silanol group are 4.5 and 8.5,¹⁷ where the negative surface charge of the inner pore surface significantly increased with increasing pH from 5.0 and 7.0. It can be considered that the electrostatic interaction between *cyt c* and the silica surface became larger with increasing pH.¹⁸ The strong electrostatic interaction led to the increase in the initial concentration of *cyt c* at the top surface of Cal-MS layer accompanied by rapid diffusion into the pore interior by a concentration gradient. In addition, an electrostatic repulsion between charged Mb molecules might contribute to the diffusivity of Mb inside the pore. $\Delta\lambda_{\text{OWG}}(\infty)$ and D_{app} values of Mb show relatively high value at pH 6.0. Since Mb is neutral or weakly charged in this pH region, the obtained result is

Fig. 3 Adsorption profiles of (a) *cyt c* and (b) Mb under the flow of a 100 μ M solution with various pH conditions at silica mesopore ($\circ$, pH 5.0; $\times$, pH 6.0; $\diamond$, pH 7.0). The solid lines are the results of the best fit using Eq. (2).

Table 2 Calculated values of $\Delta\lambda_{\text{OWG}}(\infty)$ and D_{app} of *cyt c* and Mb at various pH

pH	<i>cyt c</i>		Mb	
	$\Delta\lambda_{\text{OWG}}(\infty)/$ nm	$D_{\text{app}}/$ $\text{cm}^2 \text{ s}^{-1}$	$\Delta\lambda_{\text{OWG}}(\infty)/$ nm	$D_{\text{app}}/$ $\text{cm}^2 \text{ s}^{-1}$
5.0	91	3.0×10^{-13}	69	4.7×10^{-13}
6.0	134	15×10^{-13}	96	5.1×10^{-13}
7.0	150	18×10^{-13}	65	3.4×10^{-13}

supposed to be due to the complementary effect of the decrease in electrostatic repulsion between Mb-Mb and electrostatic attraction between Mb-silica. Diffusivity of Mb was remarkably smaller than that of *cyt c* at above pH 6.0, whereas the relation is reversed at pH 5.0. If the molecular size governs the diffusivity of proteins inside the silica mesopore, diffusivity of small *cyt c* would be larger regardless of the solution pH. Therefore, it is considered that the diffusivity of those heme proteins is likely governed by the protein-surface electrostatic interaction rather than the molecular size.

The influence of hydrophobicity of the inner pore surface on the adsorption process was examined by comparing adsorption behaviors of Mb at Cal-MS, Ethoxy-MS, and ODS-MS layers. Figure 4(a) shows typical reflection spectra for the Ethoxy-MS layer before/after Mb adsorption. The adsorption profiles of Mb at each MS layers are summarized in Fig. 4(b). The order of $\Delta\lambda_{\text{OWG}}(\infty)$ is Ethoxy-MS > Cal-MS > ODS-MS, indicating that

Fig. 4 (a) Reflection spectra of Ethoxy-MS before (open triangle) and after (shaded triangle) under the flow of a 100 μM Mb solution (pH 6.0) and calculated spectra (lines) obtained by Fresnel calculation. (b) Adsorption dynamics of 100 μM Mb solution (pH 6.0) at Ethoxy-MS (triangle), Cal-MS (circle) and ODS-MS (cross). The solid line is the result of the best fit using Eq. (2).

the ethoxylation of the silica surface enhanced the Mb adsorption into the silica mesopore.

The volume fraction of Mb (f_{Mb}) within the Ethoxy-MS was estimated from analysis of the reflection spectra by 4-layer Fresnel calculation.¹⁹ As typically shown in Fig. 4(a), the reflection spectra could be well-fitted to the calculation model, and the estimated f_{Mb} values for Cal-MS and Ethoxy-MS are 24 and 50%, respectively. Miyahara *et al.* reported that maximum pore occupation of Mb in columnar silica mesopore of SBA-15 (pore diameter: 9.2 nm) is 48% based on the adsorption model of a sphere-shaped protein.²⁰ Hence, it can be considered that the ethoxylation of the inner pore surface is an effective way for the adsorption of Mb.

In contrast to the efficient adsorption of Mb into the hydrophobic ethoxylated pore, the adsorption of Mb into the ODS-modified pore was significantly suppressed (Fig. 4(b)). One plausible reason for the suppression is the decrease of effective pore size by modification of long alkyl-chains of ODS on inner pore wall. However, since PEG-modified Mb can adsorb in the ODS-modified silica mesopore,¹⁴ it can be considered that the effective pore diameter of the ODS-MS layer is larger than the Mb size. The strong hydrophobicity of the ODS layer would be responsible for the suppression of the Mb adsorption into the pore.

In summary, we applied NPWG spectroscopy for studying adsorption behaviors of heme proteins (cyt c, Mb and HRP) into silica mesopores. The analysis of NPWG response provided apparent diffusion coefficient of a protein inside the mesopore.

The comparison of the apparent diffusion coefficients for heme proteins suggested that the diffusion of HRP inside the size-matched pore (pore diameter = *ca.* 6 nm) was restricted due to the steric hindrance. When the protein size is smaller than the pore diameter, on the other hand, the electrostatic interaction between the protein and inner pore surface was proposed as a key factor to determine the protein diffusion. When influence of the surface hydrophobicity on the Mb adsorption was examined, it was confirmed that the maximum adsorption of Mb into the pore could be achieved by the ethoxylation of the inner pore surface. Since the NPWG spectroscopy provided kinetic information and rough quantification of the adsorption amount, it was useful to study adsorption of enzymes within inorganic nanoporous materials.

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