Structural Characterization of Proteins Adsorbed at Nanoporous Materials

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A nanoporous material has been applied for the development of functional nanobiomaterials by utilizing its uniform pore structure and large adsorption capacity. The structure and stability of biomacromolecules, such as peptide, oligonucleotide, and protein, are primary factors to govern the performance of nanobiomaterials, so that their direct characterization methodologies are in progress. In this review, we focus on recent topics in the structural characterization of protein molecules adsorbed at a nanoporous material with uniform meso-sized pores. The thermal stabilities of the adsorbed proteins are also summarized to discuss whether the structure of the adsorbed protein molecules can be stabilized or not.

Keywords Nanoporous, mesoporous, protein, adsorption, nanobiomaterials

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1 Introduction

A nanoporous material with uniform mesoporosity has been applied to synthesize functional nanobiomaterials in various fields, such as separation, sensors, catalysis, biofuel cells, energy conversion, drugs, and others.1–23 The strategy for using the nanoporous material is roughly classified to the confinement of biomacromolecules inside the mesosized cavity7–16 and the hierarchical immobilization of biomacromolecules at the

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The confinement of a protein has usually been utilized to improve the stability and activity of a biomolecule.\(^1\)–\(^{16}\) The peptides, oligonucleotides, and proteins at the external material surface have been used for improving the biocompatibility of the nanoporous material, efficient and selective binding of the nanoporous material, and the regulation of mass transfer across the pore entrance.\(^7\)–\(^{16}\) In any application, nanoporous materials with mesoporosity (pore size of 2 to tens of nm) have been used, because the dimensions of biomacromolecules, such as peptides, oligonucleotides, and proteins, are more than a few nm. Hereinafter, we designate a nanoporous material with mesoporosity as a mesoporous material.

In a biocatalytic system based on confined proteins, it has been desired to encapsulate large amounts of biomacromolecules with suitable conformation and molecular orientation. The relationship between the pore size and the dimensions of proteins has been considered to be a critical factor that governs loading of the proteins into the pores and the structure/function of the confined proteins.\(^7\)–\(^{16}\) Its importance was first investigated by Takahashi \textit{et al.}\(^{24}\) They reported that size-matching between the pore size and the dimensions of horseradish peroxidase (HRP) are important for achieving higher enzymatic activity and stability of the confined HRP. Similar results have been reported by various research groups, as summarized in review articles.\(^7\)–\(^{16}\) and size-matching has been considered to be the key concept for designing a stable and efficient biocatalytic system composed of the mesoporous material and proteins. Actually, a formaldehyde sensor with a remarkable storage stability (stable for over 80 days) was developed by the confinement of formaldehyde dehydrogenase (FDH) inside size-matched silica pores (diameter, 8 nm).\(^{25}\)

Size-matching can be achieved by utilizing a mesoporous material with a uniform pore structure. A surfactant-assisted soft-templating approach has hence usually been used to synthesis mesoporous materials, such as mesoporous silica,\(^{24,25}\) mesoporous organosilica,\(^{26,29}\) mesoporous alumina,\(^{30,31}\) mesoporous carbon,\(^{32–34}\) mesoporous metal-organic framework (MOF),\(^{35,36}\) and others.\(^{1–16}\) These mesoporous materials have sometimes been used as a hard-template to synthesize other mesoporous materials, which cannot be simply synthesized by the soft-templating approach.\(^5,6,34\) Mesoporous silica is the most popular mesoporous material because of its ease to synthesize.\(^1–6\) As shown in Fig. 1, mesoporous silica has a narrow pore distribution, and its pore size can be tuned ranging from a few to tens of nm.

In addition to the pore size, the characteristics of the inner pore wall of the mesoporous material is also an important factor to regulate the activity of the confined protein.\(^7\)–\(^{16}\) For example, the lipase lid domain covers its active site, and the closed lid changes to an open conformation by adsorption at a hydrophobic interface.\(^{25,29,37}\) The hydrophobized mesoporous silica and mesoporous organosilica have hence often been used as a rigid host of lipase. Other surface properties, such as acidity and charge, as well as the surface functional groups are also considerable factors that govern the stability and function of the confined proteins.\(^7\)–\(^{16}\)

The improved properties of proteins confined inside the mesopore have usually been studied by monitoring the activity of the confined proteins.\(^1–23\) On the other hand, the details concerning the improved properties have not been fully realized because a direct experimental method for a structural characterization of confined proteins is limited. The synthetic approaches and significant features of conjugates of biomacromolecule and mesoporous materials have been summarized in other reviews.\(^1–23\) In this review, we focus on recent progress concerning direct experimental methodologies for proteins adsorbed at mesoporous materials. This review includes the characterization of mesoporous materials, because the characteristics of the confined biomacromolecules are significantly affected by the pore structure, features of the inner pore surface, and the properties of the solvent molecules. Furthermore, structural stabilities of the adsorbed proteins are discussed.

\section*{2 Characterization of Mesoporous Materials}

\subsection*{2-1 Mesostructure}

Nitrogen adsorption/desorption isotherm measurement is most popular method to estimate structural parameters of a mesoporous material.\(^{38}\) Isotherm data have usually been analyzed by BET (Brunauer-Emmett-Teller) and BJH (Barrett-Joyner-Halenda) models to estimate the specific surface area (BET surface area) and the pore size distribution (BJH pore distribution).\(^{39}\) X-ray diffraction (XRD) and small-angle X-ray diffraction (SAXS) have been used to analyze the mesostructure.\(^{39,40}\)
Mesoporous materials prepared by the soft-templating approach often have micropores inside the mesoporous framework (intrawall porosity).\textsuperscript{38,41} Since the large protein molecules cannot penetrate into the micropores, the mesopore volume should be estimated in order to discuss the volume fraction of the protein within the mesopores. Simple methods include t-plot and \( \alpha_p \)-plot analysis of the nitrogen adsorption isotherm.\textsuperscript{39-43} A detailed analysis of the mesostructure of a material has been made by using the isotherm and XRD data.\textsuperscript{60}

The non-local density functional theory (NLDFT)\textsuperscript{40,44,45} and quenched-solid density functional theory (QSDFT)\textsuperscript{49} have been developed for structural characterization of mesoporous materials. As results, it has been understood that the BJH method underestimates the pore diameter. For example, Ravikovith \textit{et al.} reported that the BJH analysis of the desorption branch of mesoporous silica underestimated the pore diameter by ca. 20 – 25\%, whereas the pore diameter derived from BJH analysis of the adsorption branch seemed to agree with “real pore diameter” determined by the NLDFT method.\textsuperscript{44} Smith \textit{et al.} reported that underestimations for the BJH analysis of adsorption and desorption branches were respectively 15 and 40\%.\textsuperscript{40} The DFT models can provide the “real” pore size of a mesoporous material.\textsuperscript{39,40} Endo \textit{et al.} reported an empirical equation for pore diameters obtained by NLDFT (d\textsubscript{NLDFT}) and BJH (d\textsubscript{BJH}) methods:
\[ d_{\text{NLDFT}} = d_{\text{BJH}} + 0.48d_{\text{BJH}}^2 + \exp[-0.4d_{\text{BJH}}].\]

In this equation, the BJH pore diameter was calculated from the desorption branch. Although an underestimation of the pore size by the classical BJH method has been proven, the BJH model is still widely used to estimate the pore distribution of various mesoporous materials.

2-2 Solvent and solute molecules inside meso-sized pore

Mesoporous silica materials have often been used to study specific properties of a solvent and solute molecules confined inside the mesopore, because their pore structure can be easily regulated by the synthetic conditions (Fig. 1). In general, there are two surface silanol groups on a silica surface: a geminal (Q\textsuperscript{2}) silanol group and an isolated (Q\textsuperscript{3}) silanol group.\textsuperscript{46-48} Figure 2 shows typical \( ^{29} \)Si CP/MAS NMR spectra of mesoporous silicas prepared by using different template surfactants.\textsuperscript{48} In mesoporous silicas prepared by using neutral surfactants, the relative peak ratio of Q\textsuperscript{2}/Q\textsuperscript{3} sites is around 0.35. This peak ratio is larger than the value of around 0.22 found for mesoporous silicas prepared by using cationic surfactants. The content of the two silanol groups thus depends on the synthetic condition, such as the species of the surfactant, silica source, the pH, and others.\textsuperscript{46-48} Resenholm \textit{et al.}\textsuperscript{49} studied deprotonation of the surface silanol groups in mesoporous silica as a function of the pH of bulk solutions, and reported that the apparent pKa of the Q\textsuperscript{3} silanol was 2 – 4.5, and that of the Q\textsuperscript{2} silanol was around 8.5. These surface silanol groups influence the properties of the solvent water and the solute molecules confined inside the pore.

Differential scanning calorimetry (DSC) and FTIR have been applied to study solvent water confined inside a cylindrical mesopore channel within mesoporous silica.\textsuperscript{55,56-58} The freezing/melting behaviors of water confined inside the pore (pore water) are sensitive to the shape and size of the pores, and DSC melting/freezing peaks of the pore water have been analyzed by the Gibbs-Thomson equation. A schematic model of the pore water proposed by DSC studies is shown in Fig. 3(A). Water in the vicinity of the channel wall is regarded as an unfreezeable water layer. The thickness of the unfreezeable water layer was reported to be 0.38 to 0.7 nm,\textsuperscript{46,50-52} which corresponds to first and second water layers at the inner pore surface. On the other hand, pore water in the interior of the pore channel is regarded as freezeable water. An X-ray diffraction study indicated that the water molecules in the first layer bound to the surface silanol groups via strong hydrogen bonding.\textsuperscript{53} The existence of a distorted tetrahedral-like hydrogen-bonded network of water in the interior of the pore channel was also suggested.

A quasi-elastic neutron scattering study of water confined inside mesoporous silicas (pore diameter, 2.1 to 3.7 nm) indicated that the transrotational and rotational diffusion of the confined water is slower than those of bulk water.\textsuperscript{54} Recently, similar slow diffusivity of the confined water was examined by NMR spectroscopy (silica pore diameter, 2 to 57.7 nm)\textsuperscript{55} and nonlinear IR spectroscopy (silica pore diameter, 2.5 to 5.4 nm).\textsuperscript{56} These studies predicted that the water/silica interaction is a critical factor for the slow diffusivities of confined water. This prediction was supported by fact that the diffusivities of water confined inside hydrophilic mesoporous silica pores were slower than those inside hydrophobic mesoporous organosilica pore (pore diameter: 3.0 nm).\textsuperscript{57} The strong binding of water molecules at the inner pore surface of mesoporous silica was also reported by a solid state-state NMR study.\textsuperscript{58} The viscosity of water confined inside amin-functionalized mesoporous silica pores (pore diameter, 3.1 nm) was reported to be smaller than that of bulk water.\textsuperscript{59} The density of water confined inside mesoporous silica pores (pore diameter, 1.4 to 2.4 nm) at room temperature was reported to be almost the same as that of bulk water.\textsuperscript{60,62}

The mobility and diffusivity of a small solute confined inside mesoporous silica pores are slower than those in a bulk solution.\textsuperscript{63,64} The slow dynamics of the solute has been explained by the interactions between the solute and the inner pore surface, intermolecular hydrogen bonding network of the solvent molecules, and the solvation shell around the solute. The properties of the solvent and solute molecules confined inside
the mesopores are thus different from those in bulk phase, and they will influence the overall biocatalytic reaction of a protein confined inside the pores.

3 Adsorption of Proteins at Mesoporous Materials

3-1 Protein adsorption

The distribution of biomacromolecules at a mesoporous material is the most important factor for the development of functional nanobiomaterials. The mesoporous material possesses both an outer surface and internal pore surfaces, and both can be regarded as the adsorption sites. In a drug delivery system based on a mesoporous nanoparticle, the external surface of the nanoparticle is modified with biomacromolecules such as DNA, antigen/antibody, and protein. These biomacromolecules are utilized for improving the biocompatibility, specific binding, and controlled release of drug molecules stored at the pore interior region. An artificial biocatalytic system has been synthesized by the adsorption of biomacromolecules, particularly enzymes, into the pore interior region.7–16 The adsorption of biomacromolecules at the mesoporous material is generally carried out by immersing the mesoporous material in a solution containing biomacromolecules. In this adsorption scheme, changes in the concentration of the biomacromolecules in the supernatant solvent phase have usually been utilized to estimate the total amount of biomacromolecules adsorbed. From the total adsorption amount, it has been demanded to distinguish biomacromolecules adsorbed on and in mesoporous materials. The distribution of biomacromolecules affects the performances of the drug delivery and the biocatalytic systems.

3-2 Distribution of protein molecules at mesoporous particle

The nitrogen adsorption/desorption isotherm (nitrogen isotherm) measurement is the most popular method to examine the distribution of biomacromolecules, particularly proteins. The adsorption of proteins at the internal pore surface of mesoporous particles induce decreases in the surface area, mesopore volume, and mesopore size.70 The decreases in these parameters can be calculated by the nitrogen isotherm. The requirement for large sample weights, however, often prevents applying this technique to adsorption studies of novel protein samples extracted from biological cells. The sample weight for the nitrogen isotherm measurement of a structured protein can be calculated from the protein crystalline structure. On the other hand, the structure of the denatured and aggregated protein molecules adsorbed at the mesoporous material is difficult to determine. Therefore, the decrease in the mesopore volume and the mesopore size cannot be used to quantify the amount of proteins adsorbed at the internal pore surface.

Recently, a differential scanning calorimetry (DSC) method was proposed to quantify the amounts of protein molecules adsorbed on and in particulate mesoporous materials. The DSC method relies on observing the decrease in the mesopore volume upon the adsorption of protein molecules at the internal pore surface of mesoporous particles.48,71,72 When suspension of particulate mesoporous material is applied for DSC measurement, the melting peak of the water inside the pores (pore water) appears to be far below the melting temperature of bulk water (273 K). The melting peak area (heat of fusion) of the pore water is proportional to the mesopore volume, and the change in the mesopore volume upon the adsorption of protein molecules is used to estimate the amount of protein molecules located inside the pores. The sample weight required for the DSC measurement is below ten mg. Since the temperature for the DSC method is below room temperature, thermal denaturation of the protein molecules adsorbed at the mesoporous particles hardly occurs during the DSC measurement. This means that the well-known molecular volume of a folded protein can be used to quantify the amount of protein molecules inside the pores from the DSC measurement.

An analysis of the DSC melting peak area requires various factors, such as mesopore size and volume, thickness of the unfreezable water layer at the inner pore surface, the density of pore water, the melting enthalpy of the pore water, and the interfacial free energies.71 In addition, the thickness of the hydration shell around the confined protein is also a considerable factor for analyzing the DSC melting peak (Fig. 3(B)).48,71,72 The former parameters have been extensively studied for mesoporous silica materials,48,71,72 but those for other mesoporous materials are still unclear. The DSC method hence requires some assumption on those parameters to analyze the DSC data. However, its availability was demonstrated for the adsorption of myoglobin at mesoporous silicas with different pore sizes.71,72 Figure 4 shows the total amount of myoglobin adsorbed at mesoporous silica particles (A<sub>tot</sub>) and the amount of myoglobin

![Figure 4](image-url)

**Fig. 4** Comparison of the total amount of adsorbed myoglobin (A<sub>tot</sub>) and the amount of myoglobin inside pores (A<sub>pore</sub>) for mesoporous silicas with (A) 6.9 nm and (B) 3.9 nm in pore diameters.71
located inside the pores ($A_{pore}$) as a function of the equilibrium concentration of myoglobin in a bulk solution.\textsuperscript{71} For the adsorption of myoglobin (diameter: 3.52 nm) at mesoporous silica with 3.9 nm in BJH pore diameter, the $A_{tot}$ values almost agree with $A_{pore}$. This agreement indicates that almost all myoglobin molecules adsorbed are located inside the pore, of which the diameter is matched to the dimensions of myoglobin. On the other hand, the deviations between $A_{tot}$ and $A_{pore}$ are recognized for the adsorption of myoglobin at mesoporous silica with large pores (BJH pore diameter of 6.4 nm), indicating that the myoglobin molecules adsorb on and in the mesoporous silica with a large pore diameter. The DSC method is thus useful to estimate the amount of protein molecules adsorbed inside the pores.

Various fluorescence microscopies have been applied to visualize the distribution of the fluorescence and fluorescence-labelled proteins adsorbed at a mesoporous particle.\textsuperscript{73,74} The penetration depth of the adsorbed protein can be estimated from the fluorescence intensity profile of a cross-section of a single mesoporous particle. Fluorescence microscopies are thus powerful methods for direct determinations of the penetration depth of the adsorbed proteins, but its application is limited for relatively large particles (more than a few micrometers).\textsuperscript{73} The diffraction limits for conventional wide-field fluorescence microscopies are a few hundred nanometers. Recently, stochastic optical reconstruction microscopy (STORM) was applied to analyze the depth profile of proteins within small mesoporous silica particles (particle diameter: 900 nm).\textsuperscript{74} As shown in Fig. 5, the spatial resolution of the STORM image is higher than those of wide-field fluorescence microscopy images.\textsuperscript{74} From an analysis of high-resolution STORM images, the penetration depths for various proteins were estimated to be 202 to 395 nm. Further progress in high-resolution fluorescence microscopy will improve the accuracy of the penetration depth profile.

3-3 Distribution of protein molecules at mesoporous film

Spectroscopic ellipsometry (SE), X-ray reflectometry (XRR), and neutron reflectometry (NR) have been applied to characterize protein adsorption at various mesoporous films.\textsuperscript{75–79} These methods rely on changes in the polarization state and/or the interference of light upon reflection from the mesoporous film on a solid substrate. The above methods provide information on the thickness, refractive index or scattering length density (SLD), and roughness of each layer. The theory and data analysis for the above methods are described in detail elsewhere.\textsuperscript{80,81}

Figure 6 shows typical NR profiles for glucose oxidase (GOD) adsorbed at mesoporous aluminum oxide (MAO) film on a Si disc.\textsuperscript{78} The mesoporous aluminum oxide film possesses perpendicularly oriented cylindrical mesopore channels.\textsuperscript{82} An analysis of the NR profile revealed that a majority of GOD molecules were located at the external surface and/or pore entrance region. In addition, the amount of GOD at the MAO surface was estimated to be $8.0 \times 10^{-13}$ mol cm$^{-2}$, which corresponded to 0.2 of the surface coverage of GOD. Isaksson et al. applied NR to characterize the conformation of transmembrane protein human aquaporin 4 (hAQP4) within a lipid bilayer deposited on a mesoporous silica film (Fig. 7).\textsuperscript{79} In this study, it was determined that the water-soluble domain of hAQP4 protruded into the mesoporous silica pore (7.2 ± 1.0 nm). SE was also used to examine the amount and depth profile of human serum albumin (HSA) adsorbed at porous silicon films.\textsuperscript{75} The NR and SE can thus be used for a precise determination of various structural parameters of protein adsorbed at a mesoporous film. In addition, several spectroscopic methods utilizing optical interference and the waveguide mode have been applied for kinetic studies on protein adsorption at mesoporous films.\textsuperscript{83–86}

4 Structural Characterization of Protein Adsorbed at Mesoporous Material

4-1 Reaction center and secondary structure

The structure of proteins adsorbed at a mesoporous material is a primary factor to govern the activity of the adsorbed protein. Until now, the reaction centers in several metallo proteins have been examined by spectroscopic methods. Particularly, hemes in myoglobin, metmyoglobin, hemoglobin, and cytochrome c were extensively studied by UV-vis absorption and circular-dichroism (CD) spectroscopies,\textsuperscript{87–90} because the position and shape of the Soret absorption band of heme strongly depend on the oxidation state of ion, environment around the heme, binding, and tertiary structure of the heme protein. Other spectroscopic method for characterizing the reaction center is electron paramagnetic resonance (EPR).\textsuperscript{91–93} Kao et al. examined cytochrome c immobilized at mesoporous silicas by EPR.\textsuperscript{94} They observed the EPR signal ($g = 6.01$) of high-spin Fe(III), which arises from replacing Met-80 ligand of heme Fe(III) by water or the surface silanol group. The high-spin state is attributed to a fully open heme groove and accessible active center. They hence concluded that the formation of high-spin Fe(III) was due to an enhanced activity of cytochrome c
immobilized at mesoporous silica. On the other hand, such a replacement of the amino acid residue was not observed for blue copper protein Pseudoazurin (PAz) confined inside mesoporous silicas. Since EPR spectra for the confined PAz were essentially the same as that for free PAz, it was reported that the confinement did not induce replacement of ligands of Cu(II). Shifts in the spin Hamiltonian parameters ($g$ and $A_z$) by confinement, on the other hand, indicated that confinement induced a slight change in the copper coordination sphere toward a more axial geometry. This slight change in the copper coordination sphere predicted that the confined PAz has a lower reduction potential than free PAz. In addition to the metal coordination in proteins, EPR or ESR has been applied to examine the molecular orientation, hydration, and catalytic reaction of proteins confined inside mesoporous materials.

Circular dichroism (CD) spectroscopy is a common method to characterize the secondary structure of protein and polynucleotide. The CD spectrum at the UV region is generally used to characterize the secondary structures of those biomacromolecules in a bulk solution. UV light scattering by mesoporous particles distort the CD spectrum of biomacromolecules adsorbed at mesoporous particles. Small mesoporous particles (particle size, below hundreds of nm) have hence often been used for the quantitative characterization of secondary structures. For example, the CD spectroscopy was applied to determine the secondary structure of DNA, and the secondary structure content of proteins and peptides. Attenuated total reflectance–Fourier transform infrared (ATR-FTIR) spectroscopy also provides quantitative information on the secondary structure of the biomacromolecules adsorbed at mesoporous particles. It can be applied for large mesoporous particles, because the scattering of infrared light by the mesoporous particles does not disturb the ATR-FTIR spectrum. As typically shown in Fig. 8, curve-fitting of the amide I and/or amide II bands of a protein provides a fraction of the protein secondary structure.

### 4-2 Tertiary structure

The tertiary structure of a protein in bulk solution has been studied by various spectroscopic methods, but those methods cannot be simply applied for a protein adsorbed at a mesoporous material. Small angle X-ray scattering (SAXS) is a powerful tool to determine the precise structure of a protein in bulk water,
but its application for the adsorbed protein is difficult. Figure 9(A) shows SAXS profiles for mesoporous silica particles with and without adsorbed myoglobin. The Bragg diffraction peaks due to the hexagonal arrangement of silica mesopores were not affected by the myoglobin adsorption. This no significant change is due to larger X-ray scattering length density (SLD) of silica (SiO2) than water and myoglobin, which are mainly composed of light elements (C, H, N, and O). In contrast, neutron SLDs of silica, water, and myoglobin are remarkably different. The adsorption of myoglobin hence induces a significant change in the small angle neutron scattering (SANS) profiles (Fig. 9(B)). SANS is thus useful method for the structural characterization of protein molecules adsorbed at mesoporous materials.97,102,103

The solvent contrast variation in SANS is a useful technique for structural characterization in multicomponent systems.104 In a multicomponent system composed of silica, protein molecules, and a mixed H2O/D2O solvent, the SLDs of individual components can be selectively matched to the solvent (Fig. 10), because the average SLDs of protein molecules and silica materials for neutrons are located between those of H2O and D2O.104 The scattering signal from silica-phase or protein molecules can thus be separately observed by tuning the H2O/D2O ratio. The scattering signal from only protein molecules can be used to characterize the protein size and structure. In a contrast-variation SANS study,102 it was reported that the globular structure was preserved by confinement inside pores of mesoporous silicas, which were synthesized by neutral surfactants (see Fig. 3(B)). On the other hand, denaturation of the secondary and tertiary structure of the confined myoglobin was observed for mesoporous silica synthesized by a cationic surfactant.

The fluorescence of tryptophan and tyrosine residues in the polypeptide chain of protein is sensitive to the polarity around those amino acid residues.105 Since the polarity around those amino acid residues change by unfolding of the protein’s tertiary structure, the fluorescence of tryptophan and tyrosine can be used for a qualitative discussion about the denaturation of protein confined inside a mesoporous material.105 A polarity indicator dye, such as 1-anilinonaphthalene-8-sulfonate (ANS), is also available to study protein denaturation within a mesoporous material.97

5 Structural Stability of Adsorbed Protein

Mesoporous silica has a uniform pore structure, and its surface can be easily modified with various functional groups. It hence has been often used to study the effects of pore size and surface properties for the structural stability of an adsorbed protein. The changes in secondary and tertiary structures of proteins upon adsorption at mesoporous silica were examined by CD, ATR-FTIR, and fluorescence spectroscopies. These studies discussed the effects of surface properties and pore size for the structure of the adsorbed protein.

It was reported that formaldehyde dehydrogenase preserved its secondary structure upon adsorption at mesoporous silica, whereas its secondary structure changed upon adsorption at hydrophobized mesoporous silica.106,107 Similar structural changes upon adsorption at hydrophobized mesoporous silicas were reported for myoglobin,100 lysozyme,100 and hemoglobin.88,89 No significant changes in the cytochrome c secondary structure upon adsorption at hydrophilic unmodified mesoporous silica was reported.108 These results imply that the structure of a water-soluble protein with a hydrophilic surface is perturbed at a hydrophobic interface. This hydrophobic/hydrophilic effect is not however clearly confirmed because several water proteins changed their structure upon adsorption at hydrophilic mesoporous silicas.100,102,109 In addition to the hydrophobic/hydrophilic effect, functional groups at the silica surface also affect the structures of the adsorbed proteins.97,100,106,110–112 The acidity of the silica surface is another considerable factor to influence the structures of adsorbed proteins.48,102

The effect of pore size for the protein structure was examined for myoglobin and lysozyme adsorbed at mesoporous silicas.97,100,102 In an ATR-FTIR study on protein adsorption at mesoporous silicas with relatively large pore diameters (NLDFT pore diameter, 5.9 to 11 nm),100 an analysis of the FTIR spectra indicated that the adsorbed myoglobin was less structured in mesoporous silica with wider pores. In contrast, adsorbed lysozyme was more structured and the extent of the structural change increased with increasing pore size. On the other hand, opposite pore size effects were reported for lysozyme adsorbed at amine-functionalized mesoporous silicas (BJH pore diameter,
2.5 to 5.6 nm).97 The secondary and tertiary structures of myoglobin were also examined by ATR-FTIR and SANS.102 In this study, no significant structural changes were observed for the myoglobin adsorbed at the mesoporous silicas with relatively small pore diameters (BJH pore diameter, 3.9 to 7.5 nm). The myoglobin adsorbed at the mesoporous silicas preserved its globular structure and its radius (3.5 nm) almost agreed with that of free myoglobin. Different conclusions were thus reported for a study on the pore size effect for the structure of the adsorbed protein.

The thermal stability of a protein adsorbed at a mesoporous material has been examined by DSC and spectroscopic methods (Table 1). The first experimental evidence on the enhanced thermal stability of the adsorbed protein was reported for RNase A adsorbed at MCM-type mesoporous silica.113 The enhanced thermal stability of the adsorbed RNase A was confirmed by a significant change in the denaturation temperature (ΔTm) upon adsorption. The enhanced stabilities were also reported for lysozyme,97 FDH,106 and subtilisin112 adsorbed at mesoporous silicas. On the other hand, no significant changes in the protein thermal stability upon adsorption were reported for water-soluble HSA88 and OPH,110 as well as membrane protein LH2.114

Recently, it was reported that the thermal stability of myoglobin became lower upon adsorption at mesoporous silicas.102 Enhanced thermal stabilities of proteins adsorbed at mesoporous silicas were likely explained by the configurational restriction and increased strength of the hydration of a protein molecule surrounded by a rigid pore wall,113 hydrogen bonding between a protein molecule and the inner pore surface,97 and heat transfer from matrices of a pore wall to a protein.97,106,112 In contrast, the destabilization of myoglobin adsorbed at mesoporous silicas was ascribed to myoglobin and silica interactions, particularly the electrostatic interaction.102 It is thus considered that the interaction between a protein and a pore wall induces not only stabilization but also destabilization of a protein adsorbed at a mesoporous material. Although the hydration of a protein was proposed for the enhanced thermal stability of the adsorbed protein,113 a recent ESR study on polypeptides in mesoporous silica pores suggested that the secondary structure of the polypeptide was insensitive to the hydration properties.94 Additional systematic studies are required to realize thermal stability of the adsorbed protein.

The thermal stabilities of simple secondary structures of DNA confined inside amine-functionalized mesoporous silica pores were examined.115-117 The results indicated that short-DNA duplexes were stabilized by confinement inside amine-functionalized cylindrical silica pore channels (BJH pore diameter, 1.6 to 7.4 nm).115,116 Particularly, short-DNA duplexes confined inside the size-matched pores (BJH pore diameter: 1.6 and 2.3 nm) were significantly stabilized; the hybridization equilibrium constants found for the size-matched pores were 2 orders of magnitude larger than those for larger pores (≥3.5 nm). Since significant stabilization was observed for not only complementary but also single-mismatched DNA duplexes,115 spatial confinement of the DNA duplex inside the size-matched pore was suggested for the enhanced duplex stability. In contrast, the hairpin structure of DNA (hairpin (CGG)) was destabilized upon confinement inside amine-functionalized cylindrical silica pore channels (BJH pore diameter: 3.5 and 5.7 nm).117 This destabilization was explained by a conformational strain of the internal loop in the hairpin (CGG) by electrostatic interaction at the inner pore surface. These studies on DNA stabilities implies that the interfacial interaction between the internal loop in protein and a pore wall is one of critical factors concerning the thermal stability of the adsorbed protein.

Up to now, the thermal stability of protein adsorbed at a mesoporous material has been studied by various spectroscopic and thermal analysis methods. In those studies, results on the thermal stabilities were usually discussed by assuming that the protein molecules were located inside a pore region, of which size is larger than the protein dimensions. On the other hand, recent studies have indicated that such pore adsorption does not always take place.71,72,78 Myoglobin (3.5 nm) molecules are effectively adsorbed inside a size-matched pore of mesoporous silica (BJH pore diameter, 3.9 nm), whereas they are preferentially adsorbed at the external surface of mesoporous silica with large pores (BJH pore diameter, 6.4 nm).71 Even for protein molecules that are located inside the pore region, their distribution inside mesoporous material would not be unity.74 These inhomogeneous protein distributions may be related to stacking and/or aggregation of protein molecules.75 For further studying the structural characterization of a protein adsorbed at a mesoporous material, determining the protein distribution would be necessary to clarify any confinement effect for the structure and the function of a protein.

### 6 Summary

The structure/function relationship of a protein adsorbed at a mesoporous material is a key issue for the development of functional nanobiomaterials by utilizing specific properties of the adsorbed protein. Recently, the structural characterization of protein molecules adsorbed at mesoporous materials have been made by using spectroscopic and thermal analysis methods. As results, an opposite tendency concerning the thermal stability of the adsorbed protein has been reported. The thermal stability of the adsorbed protein depends on not only the pore size and surface properties of the mesoporous material but also on the intrinsic properties of the protein itself. Further systematic studies will be required to obtain detailed insights concerning the structure of the adsorbed protein.

Protein molecules can adsorb on and in a mesoporous material,

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**Table 1** Thermal stabilities of proteins adsorbed at mesoporous silicas

| Protein   | Surface group | Pore diameter/ nm | ΔGΔm (kJ mol⁻¹) | Ref. |
|-----------|---------------|-------------------|-----------------|-----|
| RNase A   | -OH           | 2.5               | 30°C            | DSC | 113 |
|           | -Fh           | 12 nm             | 6°C             | DSC | 106 |
| Lysozyme  | -NH₂          | 2.5               | 28°C            | DSC | 97  |
|           | -OH           | 5.4               | 24°C            | CD  | 97  |
| Subtilisin| -OH           | 5.3               | 8°C             | DSC | 112 |
|           | -Ethyl        | —                 | -4°C            |     |     |
|           | -SH           | —                 | -2°C            |     |     |
| Myoglobin | -OH           | 3.9 - 7.5         | 40 - 88 kJ mol⁻¹| Söret⁷| 102 |
| Hemoglobin| -OH           | 7.5               | No              | Söret⁷| 88  |
| LH2       | -OH           | 2.4 - 10.6        | No              | BChl⁷| 114 |
| OPH       | -COOH         | 30                | No              | Fi⁷  | 110 |

### a. ΔTm and ΔGΔm are change in denaturation temperature and apparent denaturation energy, respectively.

### b. Absorption of Söret band.

### c. Absorption of B800 and B850 bands of BChl.

### d. Fluorescence anisotropy of tryptophane.
and the distribution of the adsorbed protein is one of important factors for analyzing the protein structure. The spatial confinement effect can be considered for protein molecules located inside the pore interiors. Recently, thermal analysis and high-resolution fluorescence microscopy methods have been applied to estimate the amount and penetration depth of protein molecules adsorbed inside mesoporous silica pores. Using these methods will facilitate the structural characterization of a protein adsorbed at a mesoporous material.

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