

## Covalent Immobilization of Metal-binding Motifs of Enzymes on Quartz Surface. [Ni(Cys-X<sub>2</sub>-Cys)<sub>2</sub>]<sup>2-</sup> of Hydrogenases

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The peptide ligand, Cys-Pro-Leu-Cys, was covalently immobilized on the surface of quartz via (3-aminopropyl) triethoxysilane (APTES) by the general peptide-synthesis method; then, Ni<sup>2+</sup> was introduced into this surface to construct the [Ni(S-Cys)<sub>4</sub>]<sup>2-</sup> unit contained in the active site of hydrogenases.

Attention has recently been directed toward the development of enzyme-<sup>1</sup> and (oligo)peptide-immobilized<sup>2</sup> transparent surfaces. In this context, the covalent immobilization of the metal peptides that correspond to the metal-binding motifs of metal enzymes on the surface of transparent substrate, such as SiO<sub>2</sub>, TiO<sub>2</sub>, and ITO, may provide a new means of developing a model chemistry for enzymes, and at the same time, provide a new class of supported catalysts. It enables us to carry out spectroscopic and electrochemical studies on such transplanted active sites when the desired metal peptides are successfully immobilized on transparent substrata.

As a target of this approach, we selected the nickel (Ni) center of hydrogenases. Ni ions in hydrogenases are surrounded by two Cys-X<sub>2</sub>-Cys residues forming a [Ni(S-Cys)<sub>4</sub>]<sup>2-</sup> coordination sphere.<sup>3</sup> Cys-X<sub>2</sub>-Cys is a short metal-binding motif that is widely found in Fe, Ni, and Zn-containing proteins.<sup>4</sup> In this study, we examined the immobilization of [Ni(CPLC)<sub>2</sub>]<sup>2-</sup> (CPLC = Cys-Pro-Leu-Cys) on a quartz surface via (3-aminopropyl)triethoxysilane (APTES).<sup>5</sup>

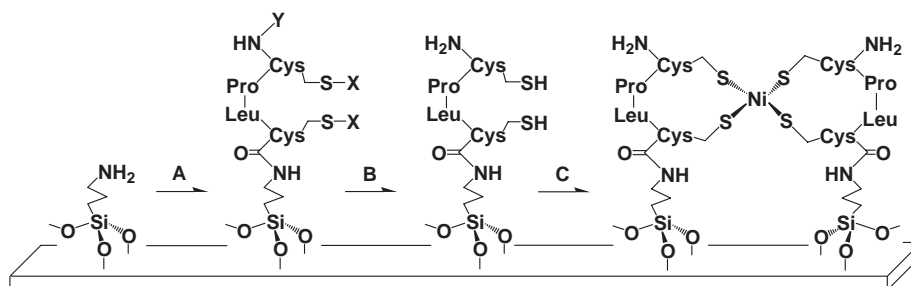
There are three main routes for immobilizing peptides on solid surfaces via APTES (Scheme 1): use of *N*-carboxyanhydrides,<sup>6</sup> use of maleimide,<sup>7</sup> and use of the general peptide coupling method.<sup>8</sup> The former two are predominantly used at present; however, they are limited for amino acid sequence; therefore, we adopted the general peptide coupling between APTES and the protected forms of CPLC: Boc-Cys(Tacm)-Pro-Leu-Cys(Tacm)-OH (Boc, tertialybutoxycarbonyl; Tacm, trimethyl acetoamidomethyl) and Fmoc-Cys(Acm)-Pro-Leu-Cys(Acm)-OH (Fmoc, 9-fluorenylmethoxycarbonyl; Acm; acetoamido-

methyl), which are synthesized by the Boc liquid-phase method and Fmoc solid-phase method, respectively. The former was used in the Ni introduction experiments, while the latter was used for the measurement of the surface coverage of the CPLC-immobilized quartz.

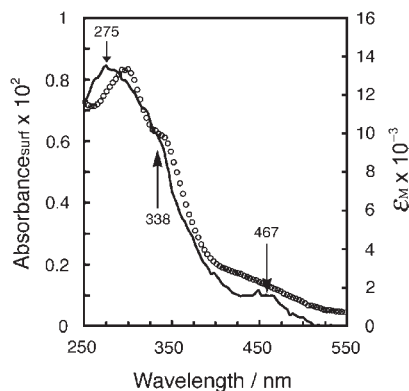
After the silanization of a quartz substratum (10 × 10 mm<sup>2</sup>), in which the reaction time for APTES modification (30 min) was optimized by monitoring the absorbance of the Schiff base formed between APTES and *p*-nitrobenzaldehyde ( $\lambda_{\max}$  282 nm;  $\epsilon = 1.56 \times 10^4$  cm<sup>2</sup>·mmol<sup>-1</sup>),<sup>9</sup> the coupling reaction between APTES and CPLC was performed in DMF using DIC/HOBt (DIC, 1,3-diisopropylcarbodiimide; HOBt, 1-hydroxybenzotriazole) as coupling reagent in a dry and dust-free Ar atmosphere.<sup>10</sup> The surface concentration of immobilized Fmoc-Cys(Acm)-Pro-Leu-Cys(Acm)-OH ( $\Gamma_{\text{CPLC}} = 4.6 \times 10^{-7}$  mmol·cm<sup>-2</sup>) was estimated from Fmoc absorbance ( $\lambda_{\max}$  264 nm;  $\epsilon = 2012.3$  cm<sup>2</sup>·mmol<sup>-1</sup>). The Acm and Tacm of Cys side-chain-protecting groups were eliminated by CF<sub>3</sub>SO<sub>3</sub>Ag,<sup>11</sup> and the Cys residues were reduced by using dithiothreitol to give Cys-SH. The quantity of the Cys-SH group was estimated using DTNP (2,2'-dithiobis(5-nitropyridine),  $\lambda_{\max}$  314 nm). Finally, Ni<sup>2+</sup> was introduced to the CPLC-modified quartz using NiCl<sub>2</sub>·6H<sub>2</sub>O in HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethane sulfonic acid) buffer solution (pH = 7.5).

The incorporation of Ni<sup>2+</sup> onto the quartz surface was demonstrated by UV-vis spectroscopy. Ni-introduced quartz shows absorption bands at 275, 338 (Ni-S LMCT), and 467 nm (d-d transition) (Figure 1). The absorption maxima are similar to those of the Ni-cyclopeptide ( $\lambda_{\max}$  295, 335, and 420 nm).<sup>12</sup> The estimated surface concentration of the Ni/CPLC was  $\Gamma_{\text{Ni/CPLC}} = 2.0 \times 10^{-7}$  mmol·cm<sup>-2</sup> (about half the  $\Gamma_{\text{CPLC}} = 4.6 \times 10^{-7}$  mmol·cm<sup>-2</sup> calculated from the Ni-S LMCT band absorbance).<sup>13</sup>

The Ni<sup>2+</sup>/Cys-Pro-Leu-Cys system on the quartz surface was also analyzed by XPS (Figure 2). The S2p<sub>3/2</sub> XPS approximately exhibits a single peak at 163.5 eV (Figure 2a) indicating



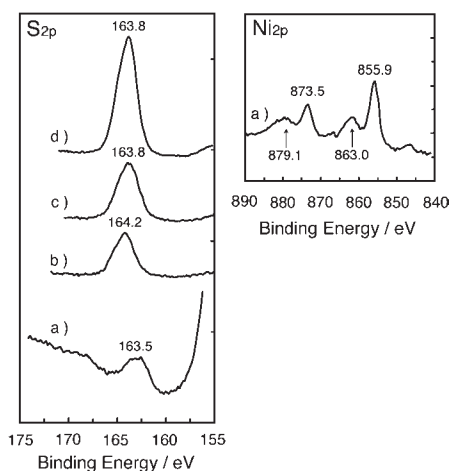
**Scheme 1.** Schematic drawing of the peptide modification and complexation on a quartz surface. (A) Modification of protected peptide ligand to silanized quartz surface (X = Acm or Tacm, Y = Boc or Fmoc). (B) Elimination of side-chain-protective groups (Acm and Tacm) by CF<sub>3</sub>SO<sub>3</sub>Ag and N-terminal (Boc)-protective group by trifluoroacetic acid. (C) Complexation of Ni ion.



**Figure 1.** UV-vis spectra of  $\text{Ni}^{2+}$ /Cys-Pro-Leu-Cys immobilized on a quartz surface (solid line, plotted on left axis) and  $[\text{Ni}(\text{S-Cys})_4]^{2-}$  metalloctapeptide (circles, plotted on right axis).<sup>12</sup>

that the sulfur atom predominantly forms a single species. Its binding energy is lower than those of the protected oligopeptide and cystine (163.8–164.2 eV), and close to that of  $\text{NiS}_4$  complexes (163.7 eV).<sup>14</sup> On the other hand, the Ni-introduced substratum shows two peaks at 855.9 and 873.5 eV in the  $\text{Ni}^{\text{II}}2p_{3/2}$  and  $\text{Ni}^{\text{II}}2p_{1/2}$  regions. Considering that  $\text{NiS}_4$  and  $\text{NiS}_2\text{N}_2$  complexes usually show  $\text{Ni}^{\text{II}}2p_{3/2}$  and  $\text{Ni}^{\text{II}}2p_{1/2}$  XPS peaks at 854.5–855.8 and 871.0–871.2 eV, respectively;<sup>14,15</sup> the observed Ni2p energies indicates that the Ni ion is bound to CPLC on the surface of the quartz.<sup>16</sup> This is supported by the result for  $\text{S}2p_{3/2}$  XPS mentioned above. Consequently, XPS experiments indicate that the Ni ion introduced onto the CPLC-immobilized quartz surface forms  $\text{NiS}_4$  species. These XPS results and the UV-visible result indicate that the  $\text{Ni}^{2+}$  introduced onto the CPLC-immobilized quartz surface forms a  $[\text{Ni}(\text{S-Cys})_4]^{2-}$  coordination sphere.

Much effort is now devoted to the model synthesis of the active sites of  $[\text{NiFe}]$  hydrogenases. A widely adopted strategy for hydrogenase modeling is to react  $\text{Fe}(\text{CN})_x(\text{CO})_y$  units with  $\text{NiS}_4$



**Figure 2.** XPS spectra (normalized by using  $\text{N}2p$  peak area and corrected with  $\text{C}1s$  285.0 eV) of the  $\text{S}2p$  (left),  $\text{Ni}2p$  (right), and  $\text{Fe}2p$  (right bottom) binding energy regions of (a)  $\text{Ni}^{2+}$ /Cys-Pro-Leu-Cys-immobilized quartz surface, and powder samples of (b) Fmoc-Cys(Acm)-OH, (c) Boc-Cys(Tacm)-Pro-Leu-Cys(Tacm)-OH, and (d) cystine.

or  $\text{NiS}_2\text{N}_2$  compounds.<sup>17</sup> Considering that the model chemistry of hydrogenases requires electrochemical detection in the future, the introduction of  $\text{Fe}(\text{CN})_x(\text{CO})_y$  to the  $[\text{Ni}(\text{CPLC})_2]^{2-}$ -immobilized ITO will be the basis of the next-step.

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