Spatially Controlled Synthesis of Protein/Inorganic Nano-assembly: Alternate Molecular Layers of Cyt *c* and TiO_2 Nanoparticles

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A molecular layer of Cytochrome *c* was self-assembled on two-dimensionally accumulated TiO_2 nanoparticles, that have been deposited by hydrolysis of titanium hexafluoride in water. Repeated cycles of TiO_2-deposition and cyt *c*-adsorption from aqueous phases afforded multilayered nano-assemblies, in which native spectral characteristics of cyt *c* was preserved.

Nacre of shells are composed of multilayered nanostructures of proteins and inorganic substances, which are periodically deposited in living organisms under physiological conditions.[1] Though immobilization of functional proteins in inorganic matrices has been attracting considerable interests,[2-6] missing among them is an ability to control such a spatial organization. Fabrication of nano-lattices from redox active proteins and TiO_2 nanoparticles is of particular interest, since it leads to protein assemblies whose function can be regulated by photo-induced electron transfer processes.

One of the authors previously reported a method to prepare ultrathin layers of metal alkoxide gels by sequential chemisorption and activation processes (surface sol-gel technique).[7,8] Alternate layers of TiO_2-based gel and hydroxyl polymers were also formed by alternate adsorption of polyhydroxyl compounds and $Ti(O^nBu)_4$.[9] In addition, N-protected amino acids were molecularly imprinted in these TiO_2-based gels.[10] However, extension of these sol-gel approaches to prepare protein/TiO_2 nano-lattices is hindered, due to the use of organic solvents that may induce protein denaturation.

In this study, we describe a novel approach to fabricate multilayered nano-assemblies consisting of cyt *c* and TiO_2. Cyt *c* is a cationic electron transfer protein (Mw = 12380, isoelectric point, pI = 10.1) that plays essential roles in bioenergetics.[11] The TiO_2 layer was deposited from aqueous solution by boric acid-promoted hydrolysis of hexafluorotitanate ion (liquid phase deposition process, LPD).[12] The LPD technique enables preparation of anatase TiO_2 without employing organic solvents.

Au-coated quartz crystal microbalance (QCM) resonators used (9 MHz, USI system, Fukuoka) were hydroxylated by mercaptoethanol as described previously.[7,8] TiO_2 layer was first deposited on the Au-coated QCM electrode by dipping into an aqueous solution containing $(NH_4)_2TiF_6$ (0.1 M) and boric acid (0.5 M) for 1 h. This QCM electrode was rinsed in pure water, dried with nitrogen gas, and frequency shift was measured. This procedure was repeated for three times. The TiO_2-deposited QCM electrode obtained was then immersed in a phosphate buffer solution of cyt *c* (1×10^{-5} M, pH 7) for 0.5 h, rinsed with pure water, and its frequency was measured after drying with nitrogen gas. This cycle of TiO_2-deposition and cyt *c*-adsorption was further repeated as schematically shown in Figure 1a.

The frequency decrements ($-\Delta F$) of the QCM at each deposition steps are shown in Figure 1b (filled circles for TiO_2 and open circles for cyt *c*). The essentially linear frequency

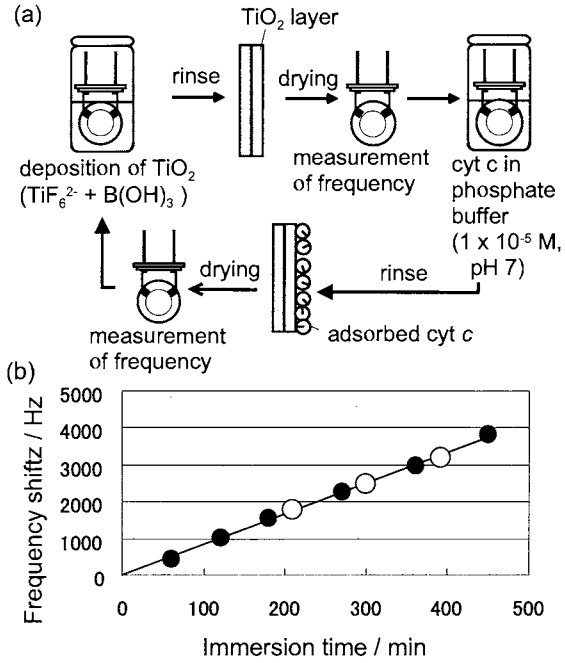

Figure 1. Schematic representation of aqueous phase deposition of TiO_2 and cyt *c* adsorption process (a), and QCM frequency shifts ($-\Delta F$) (b). (●) $[(NH_4)_2(TiF_6)] = 0.1$ M, $[B(OH)_3] = 0.5$ M, 20 ℃. (○) [cyt *c*] = 1×10^{-5} M, phosphate buffer (pH 7), 20 ℃.

shifts indicate regular film growth on the electrode during consecutive adsorption cycles. The frequency change in the initial TiO_2 deposition was 465 Hz, while it was increased to 520 Hz and to 570 Hz in the next two steps. Such an increase in the beginning of the TiO_2-deposition process is characteristic of the boric acid-promoted deposition process.[13] Using the bulk density (3.90 g cm^{-3}) of anatase TiO_2,[13] the observed ΔF value of 520 Hz corresponds to a thickness increase of ca. 73 Å from eq. 1.[7]

$$2d\,(\text{Å}) = -\Delta F\,(\text{Hz}) / 1.83\ \rho\,(\text{g cm}^{-3}) \qquad (1)$$

Deposition of TiO_2 layer under the present conditions was also examined by UV-vis absorption spectroscopy and scanning electron microscopy (SEM, instrument, Hitachi S-900). When a quartz plate was used as a substrate, visible absorption with a peak at 240 – 250 nm and an edge extending to ca. 340 nm was observed after deposition. This is ascribed to the band gap absorption of TiO_2,[12] and its intensity was increased in proportion to the absorption time. In SEM observation, the surface of the deposited film (immersion time, 5 h) was densely covered with TiO_2 nanoparticles with diameters of 10 to 20 nm.

In the adsorption step of cyt *c*, ΔF values of 218, 213 and 240 Hz were observed for three adsorption cycles, respectively (Figure 1b, open circles). By assuming a density of 1.3 g cm^{-3}

for proteins,[14] the thickness of the adsorbed cyt *c* layer is estimated to be 47 ± 2.8 Å. This value is comparable to the molecular dimension of cyt *c* (ca. 25 x 25 x 37 Å),[15] indicating formation of a molecular layer on TiO_2. In Figure 2a, UV spectra for a TiO_2/ cyt *c* film at consecutive steps of assembly is shown. These multilayers are formed on both sides of a quartz plate, and together with the increased absorbance of TiO_2, absorbance at the Soret band increased linearly with the number of cyt *c* layers in the film (Figure 2b). The maximum of the Soret band was observed at 409 nm, which is identical with that of cyt *c* observed in phosphate buffer (pH 7). Therefore, ternary structure of cyt *c* must be well reserved in the multilayered assembly. The average increase in absorbance, ΔA at 409 nm, is ca. 3.1 x 10^{-3} for one adsorption step, and this value is 73% of that estimated for a closely packed monolayer of cyt *c* (ΔA, ca. 4.2 x 10^{-3}).[16,17]

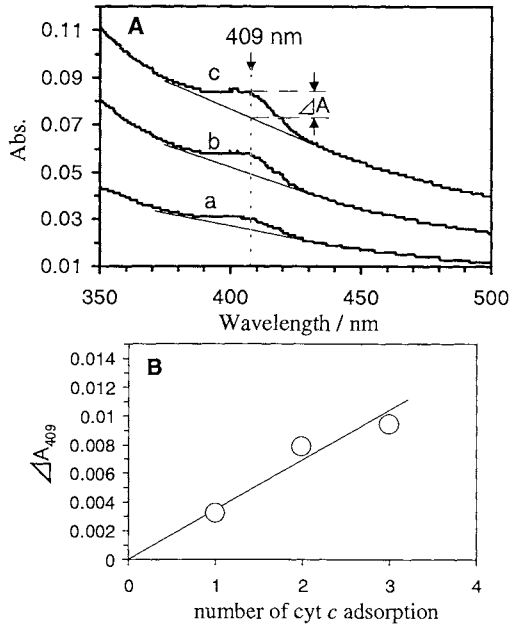

Figure 2. A. UV-Vis adsorption spectra of the layered TiO_2 - cyt *c* superlattice : (a) one cyt *c* layer (b) two cyt *c* layers (c) three cyt *c* layers. B. Dependence of ⊿A (at 409 nm) on number of cyt *c* adsorption.

These QCM and absorption data indicate that cyt *c* is adsorbed nearly as a monolayer on the surface of aligned TiO_2 nanoparticles. The repeated cycles of adsorption/deposition process provided a smooth film surface, as can be seen in a scanning electron micrograph of the cross section of the cyt *c*/TiO_2 multilayer formed on a gold-coated QCM resonator (Figure 3). Electrostatic interaction between cationic cyt *c* and anionic TiO_2 surface (surface pKa, ca. 6.0)[18] seems to be responsible for the absorption process, since anionic protein, ferritin (Mw., 444.000, pI = 4.8) was not efficiently adsorbed to the deposited TiO_2 surface (ΔF value of ca. 100 - 200 Hz by QCM).

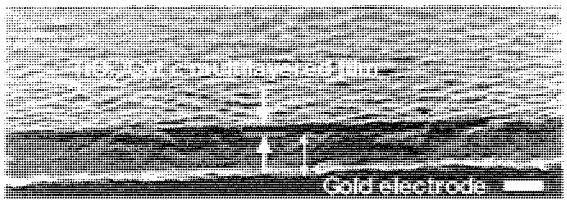

Figure 3. Scanning electron micrograph of a TiO_2/cyt *c* film on a Au-coated resonator. Total frequency shift : 3821 Hz. Scale bar represents 200 nm.

The stepwise procedure is indispensable for construction of the cyt *c*/TiO_2 multilayers without denaturation. When cyt *c* was added to an aqueous solution containing $(NH_4)_2TiF_6$ (0.1 M, 3 ml) and boric acid (0.25 M, 3ml) at a concentration of 1 x 10^{-4} M (pH 4), it was co-deposited on a quartz plate together with TiO_2 (absorption edge of TiO_2 ; 340 nm, Soret absorption peak of cyt *c* ; 403 nm). The Soret peak in the co-deposited film is considerably altered from that in aqueous $(NH_4)_2TiF_6$ (0.1 M) and in borate buffer (λ_{max}, 409 nm), and thus cyt *c* encapsulated in the TiO_2 matrix may undergo denaturation. The extensive exposure of cyt *c* to deposited TiO_2 surfaces is avoided in the alternative assembly, and it might relieved cyt *c* of their deteriorating influences.

In conclusion, spatially controlled manipulation of cyt *c*/ TiO_2 nano-assembly is achieved by repeating the stepwise deposition/adsorption procedure. This technique will be widely extended to the preparation of varied protein/metal oxide multilayers that are not accessible from conventional sol-gel approaches.[2-10] We envisage that this strategy would facilitate creation of photo-functional protein/inorganic superlattices.

References and Notes

1 G. Krampitz and G. Graser, *Angew. Chem., Int. Ed. Engl.*, **27**, 1145 (1988).
2 M. T. Reez, *Adv. Mater.*, **9**, 943 (1997) and references therein.
3 V. Glezer and O. Lev, *J. Am. Chem. Soc.*, **115**, 2533 (1993).
4 I. Gill and A. Ballesteros, *J. Am. Chem. Soc.*, **120**, 8587 (1998).
5 Y. Tang and B. C. Dave, *Adv. Mater.*, **10**, 1536 (1998).
6 N. Shibayama and S. Saigo, *J. Am. Chem. Soc.*, **121**, 444 (1999).
7 I. Ichinose, H. Senzu, and T. Kunitake, *Chem. Lett.*, **1996**, 831.
8 I. Ichinose, H. Senzu, and T. Kunitake, *Chem. Mater.*, **9**, 1296 (1997).
9 I. Ichinose, T. Kawakami, and T. Kunitake, *Adv. Mater.*, **10**, 535 (1998).
10 S.-W. Lee, I. Ichinose, and T. Kunitake, *Chem. Lett.*, **1998**, 119.
11 F. S. Mathews, *Prog. Biophys. Mol. Biol.* **45**, 1 (1985).
12 S. Deki, Y. Aoi, O. Hiroi, and A. Kajinami, *Chem. Lett.*, **1996**, 433.
13 S. Deki, Y. Aoi, Y. Asaoka, A. Kajinami, and M. Mizuhata, *J. Mater. Chem.*, 7, 733 (1997).
14 Y. Lvov, K. Ariga, I. Ichinose, and T. Kunitake, *J. Am. Chem. Soc.*, **117**, 6117 (1995).
15 M. Wahlgren and T. Arnebrant, *J. Colloid Interface Sci.*, **142**, 503 (1993).
16 This value was estimated for closely packed monolayers of cyt *c* formed on both sides of a quartz plate, by using molecular extinction coefficient of 96 $mM^{-1}cm^{-1}$ and a coverage of 2.2 x 10^{-11} mol cm^{-2}.[17]
17 L. L. Wood, S-S. Cheng, P. L. Edmiston, and S. S. Saavedra, *J. Am. Chem. Soc.*, **119**, 571 (1997).
18 S. Tunesi and M Anderson, *J. Phys. Chem.* **95**, 3399 (1991).