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Visible-Light-Induced Activity Control of Peroxidase Bound to Fe-Doped Titanate Nanosheets with Nanometric Lateral Dimensions

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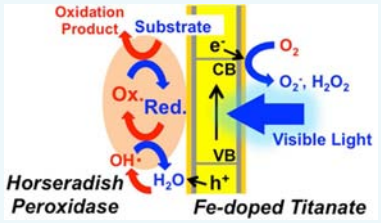
Supporting Information

ABSTRACT: Catalytic performance of horseradish peroxidase (HRP) electrostatically adsorbed on nanometric and semiconducting Fe-doped titanate (FT) nanosheets was successfully manipulated by visible light illumination. A colloidal solution of FT with a narrow band gap corresponding to a visible light region was fabricated through a hydrolysis reaction of metals sources. HRP could be easily bound to the FT at pH = 4 through an electrostatic interaction between them, and the formed HRP-FT was utilized for the visible-light-driven enzymatic reaction. Under exposure to visible light with enough energy for band gap excitation of the FT, catalytic activity of HRP-FT was dramatically enhanced as compared with free (unbound) HRP and was simply adjusted by light intensity. In addition, wavelength dependence of an enzymatic reaction rate was analogous to an optical absorption spectrum of the FT. These results substantiated an expected reaction mechanism in which the photoenzymatic reaction was initiated by band gap excitation of FT followed by transferring holes generated in the valence band of irradiated FT to HRP. The excited HRP oxidized substrates (amplex ultrared: AUR) accompanied by two-electron reduction to regenerate the resting state. In addition, the catalytic activity was clearly switched by turning on and off the light source.



INTRODUCTION

Recently, photochemical activity control of enzyme has attracted much attention because the activity can be easily adjusted by incident light intensity, wavelength, and so forth. To realize the photochemical control, enzymes are required to be attached to a support that can harvest photon energy. Then, the photon energy is transferred to enzymes in the form of thermal energy, aggressive radicals, or charge carriers to cause an enzymatic reaction. Photoswitching is also accomplished by turning on and off the light source. So far, gold nanoparticles,[1] quantum dots (calcogenides),[2] carbon nanodots,[3,4] or TiO_2−Au nanocomposites[5] have been used as host materials. As an analogous method, Kragl et al. have reported that pH-dependent enzymatic activity can be tuned by laser light irradiation that brings about a rapid pH change in solution including photosensitive carboxylates.[6] They have also claimed that spatially resolved enzymatic reaction is achieved, because light flux can be artificially focused on a specific position with desirable dimensions.[7]

Our group has also reported that peroxidase molecules intercalated into titanate layers are activated by ultraviolet (UV)-light irradiation and photoswitching of peroxidase activity takes place by turning on/off the UV light.[8] In the literature, the host titanate layers which were fabricated through a solid state reaction at high temperature had micrometric 2D dimensions, and hence tiny peroxidases deeply inserted into the layers would not participate in the photoenzymatic reaction. In addition, exposure to UV light for a long period deteriorated catalytic performance of peroxidase. Consequently, the advanced research targeted another semiconducting support (α-Fe_2O_3) with a narrow band gap that can be excited by visible light with moderate photon energy.[9] In this case, however, enzymes were adsorbed on the surface of the support, inducing a serious activity reduction due to desorption over a long reaction period. Furthermore, the α-Fe_2O_3 support was supplied as a thin film on a noble metal plate because of difficulty in fabricating a stable colloidal solution of α-Fe_2O_3. This would bring about a low photoenzymatic reaction rate due to a small effective surface area exposed to reactant solution.

To overcome the aforementioned drawbacks, in the present study, we attempted to prepare Fe-doped layered titanate (FT) colloidal particles with nanosized lateral dimensions by means of one-step hydrolysis reaction of metal sources, then applied for a host material. The Fe doping transformed the layered titanate with a wide band gap into a visible-light-harvesting material. Moreover, the use of FT is beneficial as compared with the previously reported photoenzymatic reactions utilizing the nanoparticles of noble metals or calcogenides, because the FT composed of Fe and Ti is generally inexpensive and nontoxic. After hybridizing the FT with horseradish peroxidase (HRP) through electrostatic interaction, photochemical control of HRP activity in the hybrid colloids (HRP-FT) was achieved by irradiating visible light with different wavelengths or intensities. As a result of transfer of holes photogenerated in

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the valence band of FT, the HRP is oxidized to an activated state followed by enzymatic oxidation of substrates accompanied by regeneration of the resting state. The proposed reaction mechanism employed light irradiation as a trigger, and thus a clear photoswitching behavior was discernible by intermittent light irradiation. The present paper demonstrates the validity of an expected photoenzymatic reaction mechanism on the basis of experimental findings in detail.

RESULTS AND DISCUSSION

Colloidal solutions of Fe-doped layered titanate nanoparticles were synthesized through hydrolysis of Ti(IV) tetraisopropoxide (TTIP) mixed with an ethanol solution of $FeCl_2$ using an aqueous solution of tetrabutylammonium (TBA^+) hydroxide.[10,11] In the present paper, the Fe-doped titanate nanoparticle was described as "*x*FT", where *x* is the mol % of Fe for a total metal content ($x = 0-15$). A colorless and transparent colloidal solution that clearly scattered a laser beam was formed as a result of hydrolysis reaction of TTIP only (0FT), while the coexistence of $FeCl_2$ (*x*FT) changed the solution to brown and its strength was increased in proportion to *x*. The brown color is an indication of the presence of ferric ions (Fe^{3+}) by oxidizing Fe^{2+} in the raw material. Neutral colloidal solutions of *x*FT were obtained after dialysis against deionized water several times. As the filtrate during the dialysis to remove byproducts was colorless, most iron species are considered to remain in the colloidal solution. However, a neutral colloidal solution of titanate nanoparticles with a highest Fe doping amount (15FT) fabricated in the present study could not be obtained. In this case, the particles formed passed the membrane filter during the dialysis process probably due to their tiny dimensions. Since the MWCO (molecular weight cutoff) of the membrane filter used in the present study was 3000 Da, which corresponded to ca. 2 nm in pore diameter, the 15FT would be smaller than the diameter. Optical absorption properties of the colloidal solutions will be discussed later in detail. The color change by the Fe addition suggests that the *x*FT is possible to employ as a host for the visible-light-driven enzymatic reaction.

XRD patterns of 0FT and 10FT in a small angle region are displayed in Figure 1a and b, respectively. The XRD pattern of 0FT has peaks attributed to (*n*00) planes of tetratitanate ($Ti_4O_9^{2-}$) inserting TBA^+ counterions during the drying process.[11] The XRD patterns of 10FT were almost identical to that of 0FT without any peak shift, and no diffraction line indexed to chemical species including iron (oxide, hydroxide, and oxyhydroxide) was observed at a wide diffraction angle range. Hence, it can be judged that a ferric ion (Fe^{3+}) with a similar ionic radius (0.065 nm, 6-cordination, high spin) to Ti^{4+} (0.075 nm, 6-cordination) substituted for a Ti^{4+} site in the tetratitanate.[12] Figure 2a,b displays the particle size distribution

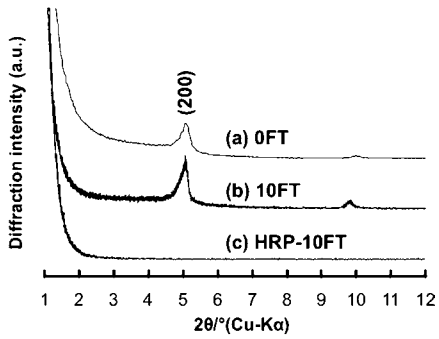

Figure 1. XRD patterns of (a) 0FT (undoped), (b) 10FT, and (c) HRP-10FT thin films prepared by drying their colloidal solutions on a glass plate.

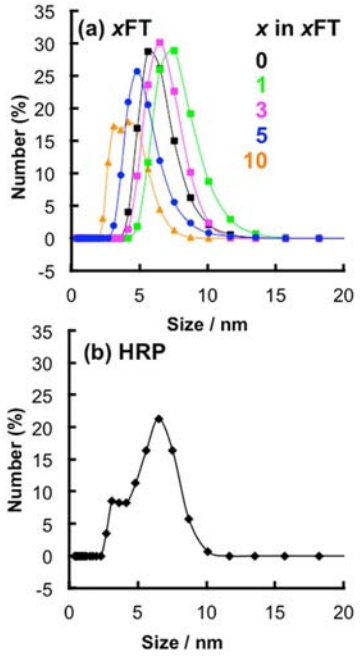

Figure 2. Particle size distribution curves of neutral colloidal solutions of (a) *x*FT with various *x* values and (b) HRP dissolved in 20 mM acetate buffer solution at pH = 4.

curves of *x*FT and HRP measured by the DLS technique. The curve of *x*FT consists of a single peak, indicating monodispersion of *x*FT nanoparticles. Fundamentally, the nanoparticle seems to exist as a small stack composed of several tetratitanate layers and TBA^+ ions rather than a single (exfoliated) layer.[13] The mean size of *x*FT tended to decrease with increasing *x* except for $x = 0$ (Figure 1S in Supporting Information). This tendency agrees with the fact that a neutral colloidal solution of highly doped FT was not obtained as stated above. It should be noted that the calculated mean size of 10FT was ca. 6 nm, comparable to the dimensions of an HRP molecule (Figure 2b). The size was significantly smaller than that the micrometric Fe-doped titanates fabricated via a solid-state reaction at high temperature.[14]

To confirm whether the *x*FT could be excited by visible light irradiation, optical absorption spectra of the colloidal solutions were measured and the results are displayed in Figure 3. The absorption edge of 0FT was located at ca. 340 nm, while those of *x*FT were considerably shifted to a longer wavelength side according to an increase in *x*. In our previous paper, FT layers prepared by a solid-state reaction had an absorption shoulder at ca. 400 nm even at a higher doping amount ($Fe_{0.8}Ti_{1.2}O_4^{0.8-}$),[15] requiring UV light for band gap excitation. By contrast, the *x*FT prepared here was capable of absorbing visible light even at a small doping amount of Fe. Hence, it was proven that *x*FT was useful as a host semiconductor. Judging from the maximum absorbance over a wide wavelength range, further studies focused on the 10FT.

In general, an electrostatic interaction between enzyme and oxide is frequently utilized to fabricate their conjugant because both surface charges can be simply controlled by adjusting a

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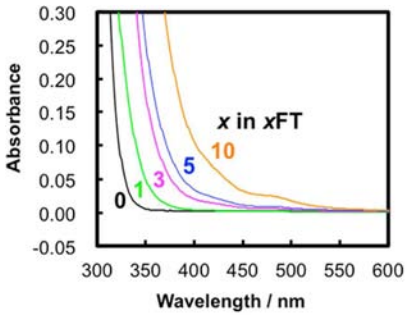

Figure 3. UV−vis absorption spectra of as-prepared *x*FT colloidal solutions after 100-fold dilution with deionized water. Each solution includes an identical total metal amount.

solution pH. Our previous paper has demonstrated that HRP charges positively in a weak acidic solution and 10FT would have a negative surface over a wide range of pH.[16] Hence, both components were mixed with an acetate buffer solution at pH = 4. Figure 1c shows an XRD pattern of HRP-10FT hybrid after collecting solid components in the mixed solution. The XRD pattern has no diffraction line attributed to the layered structure. When the FT layer with a micrometric lateral size was used as a support, broad diffraction peaks assigned to an expanded interlayer space containing HRP molecules appeared.[17,18] On the other hand, disappearance of the diffraction peaks assigned to the 10FT (Figure 1b) implies that the hybrids were composed of a random arrangement of HRP and 10FT. This is due to the nanometric lateral size of 10FT, as small as an HRP molecule, because of the difficulty piling up two kinds of components with similar dimensions periodically. Consequently, we succeeded in fabrication of the HRP-10FT hybrids through a simple electrostatic interaction. Our previous paper confirmed that catalytic activity of HRP was not altered even after hybridization with the nanometric *x*FT at pH = 4.[19] Thus, it is believed that HRP in the hybrids also possesses the original enzymatic activity.

The photoenzymatic reaction of HRP-10FT was evaluated using amplex ultrared (AUR) as a substrate to be oxidized. The expected reaction mechanism is illustrated in Figure 4. The

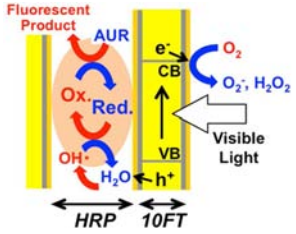

Figure 4. Schematic illustration of the mechanism of the visible light induced enzymatic reaction using HRP bound to 10FT.

10FT is excited under visible light illumination, resulting in the formation of electrons and holes in the conduction and valence band, respectively. Because of the high oxidizability, the holes produced in the valence band encourage oxidation of the resting HRP (Red., Fe^{III}) to Compound I (Ox.; production of oxy-ferryl (Fe^{IV}=O) and porphyrin radical cation) similar to a conventional peroxidase reaction using H_2O_2 as an initiator (electron acceptor).[20] The hydroxyl radicals, which are formed as a result of interaction between the hole and a water molecule, may be concerned with the oxidation of the resting HRP. Compound I oxidizes AUR and then is regenerated to the resting form accompanied by two-electron reduction. Dissolved O_2 molecules consume the electrons in the conduction band, inhibiting recombination of the photocarriers produced. Thus, photochemical control of the enzymatic reaction is realized on the basis of the photoirradiation to the 10FT adsorbing HRP molecules. Because the enzymatic reaction proceeds only under the band gap excitation of 10FT, the reaction rate appears to be tuned by the intensity of incident light that affects a hole concentration in the valence band.

Figure 5a shows variations in the relative fluorescence units (RFU) of AUR solutions containing HRP-10FT or free HRP

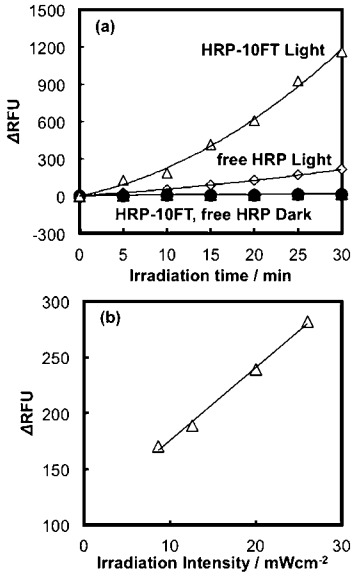

Figure 5. (a) Fluorescence variations (ΔRFU) of 25 μM AUR solution including free HRP or HRP-10FT in 5 mM citrate buffer solution at pH = 6. (b) Effect of irradiation intensity of blue light (430 nm) on ΔRFU after irradiation for 10 min.

under irradiation of blue light with a wavelength peak at 430 nm, which is shorter than the absorption edge of 10FT (Figure 3). To remove the influence of background fluorescence, all data were plotted after subtraction from initial values before irradiation, and thus the data was expressed as ΔRFU. The figure also describes ΔRFU changes in the dark. As a nonfluorescent AUR is transformed into a fluorogenic molecule, the ΔRFU is closely related to a change in a product concentration. As seen in Figure 5a, AUR was scarcely oxidized in the dark irrespective of the presence of 10FT. The ΔRFU of HRP-10FT under visible light irradiation was significantly increased with an increase in irradiation time. In the case of free HRP, only a minor increase in the ΔRFU caused by nonenzymatic photoxidation of AUR was observed. On the other hand, 10FT not adsorbing HRP had no photocatalytic oxidation ability for AUR (data not shown). These experimental results indicate that the enzymatic reaction of HRP adsorbed on 10FT was triggered by the visible light irradiation. In Figure 5a, the ΔRFU appears to elevate empirically obeying a quadratic function of time with a downward convex curve through the origin. That is

$$\Delta\mathrm{RFU} = k_1 t^2 + k_2 t \quad (1)$$

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where k_i and t are constants and irradiation time, respectively. Because a reaction rate at a certain time could be defined as a slope, i.e., a first derivative ($d(\Delta RFU)/dt = 2k_1t + k_2$), the equation implies that a reaction rate tended to increase gradually with t. At the moment, we cannot find any reason to explain the phenomenon. One of the possibilities is temperature rising of a solution exposed to visible light.[21] As a result of curve fitting of the data set to eq 1, the reaction rate of free HRP and HRP-10FT at t = 30 min was estimated to be 9.7 and 64.7 ΔRFU/min, respectively, with high squared correlation coefficients (R^2) over 0.995. The large difference again validated that the enzymatic activity of HRP was incited by visible light irradiation to the HRP-10FT. The influence of light intensity on the photoenzymatic reaction of HRP-10FT was also investigated at λ = 430 nm. Figure 5b plots ΔRFU immediately after 10 min irradiation against the light intensity. As a result, the ΔRFU was linearly elevated in proportion to the intensity, suggesting that the reaction rate could be easily manipulated by the light intensity.

To study wavelength dependence, the photoenzymatic reaction was performed using three kinds of violet or blue light sources with peak wavelengths at 410, 430, or 474 nm. These experiments maintained irradiation intensity of 27 mW cm^{-2} in order to pay attention to the wavelength dependence only. Figure 6 summarizes the influence of irradiation

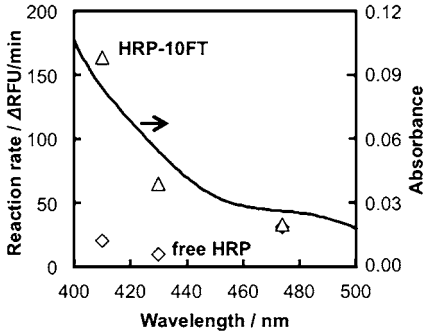

Figure 6. Wavelength dependence of reaction rate (ΔRFU/min) of 25 μM AUR solution including free HRP or HRP-10FT at pH = 6. The reaction rates were calculated from the slopes of ΔRFU against irradiation time at 30 min after nonlinear fitting to eq 1. The solid line indicates a magnified absorption spectrum of 10FT shown in Figure 3.

wavelength on the photoenzymatic reaction rate at t = 30 min along with the magnified absorption spectrum of 10FT in Figure 3 (solid line). The irradiation at 474 nm longer than the absorption edge of 10FT (ca. 450 nm) had no effect on the enzymatic activity. In contrast, the irradiations with shorter wavelengths led to the photoenzymatic reaction, and the wavelength dependence was quite akin to the absorption spectrum of 10FT. This result demonstrated that the oxidation of AUR advanced through the photoenzymatic reaction mechanism proposed in Figure 4, and that the reaction rate can be adjusted by wavelength as well as intensity of incident light.

In the previous paper describing photoinduced reaction on the HRP-adsorbed quantum dots (QDs), reactive oxygen species (superoxide anions or hydroxyl radicals) generated by UV-irradiation to the QDs had activated the catalytic performance of HRP.[3] Because of the long lifetime of superoxide anions in neutral−basic solution, however, the enzymatic reaction continued even after turning off the light. The phenomenon seems to be inappropriate to attain a precise time and/or spatial resolution of the enzyme reaction. On the other hand, Kang and co-workers have tuned laccase activity on carbon nanodots with visible light irradiation.[4] In this case, photon energy can be beneficial to acceleration of an enzymatic reaction, whereas photoswitching attributed to distinct activity fluctuation by turning on/off an incident light was impossible. Therefore, the photoswitching ability of peroxidase activity was investigated using the present reaction system. Figure 7

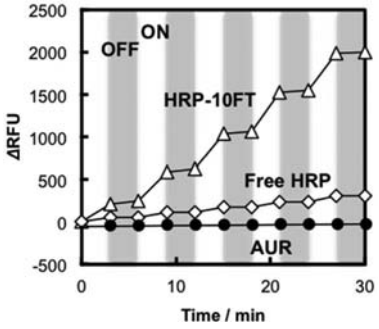

Figure 7. Photoswitching behaviors of catalytic oxidation of 25 μM AUR by HRP-10FT or free HRP under intermittent blue light irradiation (430 nm, 27 mW cm^{-2}).

describes changes in ΔRFU of AUR solutions including free HRP or HRP-10FT under intermittent visible light irradiation (λ = 430 nm, 27 mW cm^{-2}) every 3 min at 298 K. Needless to say, the irradiation to the solution including only AUR did not elevate RFU (closed circles). In the free HRP solution, only a slight increase in the ΔRFU assigned to the nonenzymatic oxidation of AUR was observed when exposed to the light. On the other hand, the ΔRFU of HRP-10FT solution was largely increased under the illumination (ON) in contrast to little change in the dark (OFF). Judging from these facts, it was concluded that the peroxidase activity of HRP attached to the 10FT can be switched by turning on or off the visible light with sufficient energy to excite the 10FT. Thus, the photoswitching ability is one of important features of the photoenzymatic reaction using the semiconducting FT.

Our interest was directed at another enzyme besides HRP in order to extend the feasibility of the photoenzymatic reaction. Taking into account the reaction mechanism depicted in Figure 4, various biocatalysts stimulating a redox reaction should be judged to be applicable. Therefore, a functional protein, myoglobin (Mb), which is not enzyme but exhibits peroxidase-mimetic activity,[18] was employed for the purpose. Mb was bound to the 10FT at pH = 4.0, and then the photoenzymatic reactivity was evaluated in an AUR solution. The measured ΔRFU data were given as Figure 2S in the Supporting Information. As a matter of course, no dark reaction arose. When exposed to visible light, the ΔRFU was increased in a similar manner to the HRP-10FT (Figure 5a). The reaction rate of Mb-10FT (13.3 ΔRFU/min at t = 30 min) was much greater than that of free Mb (4.1 ΔRFU/min). Even though the reaction rate under illumination was smaller than the HRP-10FT, it was verified that the visible light irradiation also triggers the peroxidase-mimetic activity of Mb adsorbed on the 10FT as a light harvesting material.

In the present study, photoinduced activity control of peroxidase (HRP) adsorbed on a visible light harvesting support was carried out. First of all, x mol % Fe-doped layered

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titanate (*x*FT; $x = 1-15$) nanoparticles were fabricated through a one-step hydrolysis reaction of liquid titanium tetraisopropoxide mixed with $FeCl_2$. The resultant FT had nanometric 2D sizes less than 10 nm. According to UV−vis photoabsorption spectroscopy, an absorption edge of *x*FT was shifted to longer wavelength with an increase in *x*. Due to the widest absorption wavelength window, 10FT was selected as the solid support for HRP. We fabricated HRP-10FT hybrid materials via an electrostatic interaction between positively charged HRP and the FT with an opposite surface charge at pH = 4. The XRD measurement revealed that the hybrids were composed of an irregular arrangement of them. The photoenzymatic reaction of HRP-10FT was undertaken in an aqueous solution of amplex ultrared (AUR) as a substrate under visible light irradiation with different wavelengths and intensities. The relative fluorescence unit (ΔRFU) correlated with the concentration of the oxidation product of AUR was quadratically increased with an increase in irradiation time. The reaction rate under exposure to visible light was much greater than that of free HRP. Moreover, the dependence of incident wavelength on the reaction rate was almost in agreement with the optical absorption spectrum of 10FT. This meant that the photoenzymatic reaction mechanism proposed here was accomplished, that is, holes generated in the valence band of irradiated FT excited HRP followed by the oxidation of AUR by the excited HRP. The reaction rate could be simply adjusted by light intensity or wavelength, and the photoswitching behavior was observed by turning on and off the light source. The activity control was also realized for peroxidase-like activity of myoglobin. Taking into account the present photoenzymatic reaction mechanism, the activity of other oxidases will be manipulated by visible light. Furthermore, reductases may be excited by electrons in the conduction band of FT when a redox potential of reductase is more positive than the conduction band edge. Therefore, a feasibility study of the photoenzymatic reaction for various enzymes is in progress.

EXPERIMENTAL PROCEDURES

Fe-doped layered titanate (*x*FT) nanoparticles were fabricated through a protocol reported by the other group with minor modifications.[11] In the case of undoped titanate, titanium(IV) tetraisopropoxide (TTIP) was mixed with an aqueous tetrabutylammonium (TBA^+) hydroxide solution, where the molar ratio of Ti for TBA^+ was set to 1.0. To fabricate the FT nanoparticles, $FeCl_2$ dissolved in absolute ethanol ($x = 1$, 3, 5, 10, and 15 mol % for a total metal amount) was substituted for a portion of TTIP. The mixture was maintained at 333 K for 2 h with viscous shaking. The resultant basic solution was dialyzed using a membrane filter (MWCO: 3000) under centrifugal force (22 000*g*) to remove byproducts such as isopropanol. After adding deionized water to the concentrated solution, the dialysis was conducted again. The procedure was repeated several times until a nearly neutral colloidal solution (pH ∼ 8) was obtained. Characterization of the colloidal solutions produced was performed by X-ray diffraction (XRD), dynamic light scattering (DLS), and UV−vis photoabsorption spectroscopy. To prepare a thin film sample for the XRD measurement, the colloidal solution was deposited on a glass plate followed by drying under vacuum at room temperature.

To hybridize HRP molecules with *x*FT, the colloidal solution of *x*FT was added to a 20 mM acetate buffer solution (pH = 4) dissolving HRP, then the mixture was gently shaken at 298 K for 60 min. The concentration of HRP and *x*FT in the mixture was fixed to 0.12 mg/mL and 0.76 mM, respectively. In this condition, all HRP molecules were adsorbed on *x*FT nanoparticles (HRP-*x*FT). The fact was verified by means of optical absorption measurement of supernatant after removal of solid components by centrifugation, that is, no Soret absorption band of HRP at 403 nm was detected in the supernatant.[19]

The visible-light-induced enzymatic reaction was conducted according to the following procedure. An amplex ultrared (AUR, Invitrogen) was a substrate to be oxidized by HRP. A dimethyl sulfoxide (DMSO) solution of AUR was diluted with a 0.1 M citrate buffer solution (pH = 6) to prepare 0.050 mM AUR. The AUR solution (0.1 mL) was mixed with an identical volume of the HRP-*x*FT colloidal solution in a well of 96-well black microplate with round bottoms; therefore, final concentrations of AUR, HRP, and *x*FT were 0.025 mM, 0.06 mg/mL, and 0.38 mM, respectively. After the microplate was set in a chamber thermostated at 298 K, photoenzymatic reaction was initiated by irradiation of monochromatic violet−blue LED lights with three different wavelengths (410, 430, and 474 nm) to the well. Light intensity was measured and adjusted by using a multichannel spectroscopic analyzer. According to the protocol released by the manufacturer of AUR, nonfluorescent AUR is transformed into a fluorescent product as a result of oxidation with HRP. Therefore, concentration of the enzymatic oxidation product after visible light irradiation was estimated by measuring fluorescence of the solution using a microplate reader. The band-pass filters (peak wavelength/fwhm; excitation: 528/20 nm and emission: 590/35 nm) were employed for the fluorescence measurement. As a reference, an AUR solution including the same amount of free HRP was also tested with the identical technique.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00464.

Figures describing mean particle sizes of *x*FT and enzyme-mimetic activity of myoglobin bound to 10FT under visible light irradiation (PDF)

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Notes

The authors declare no competing financial interest.

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