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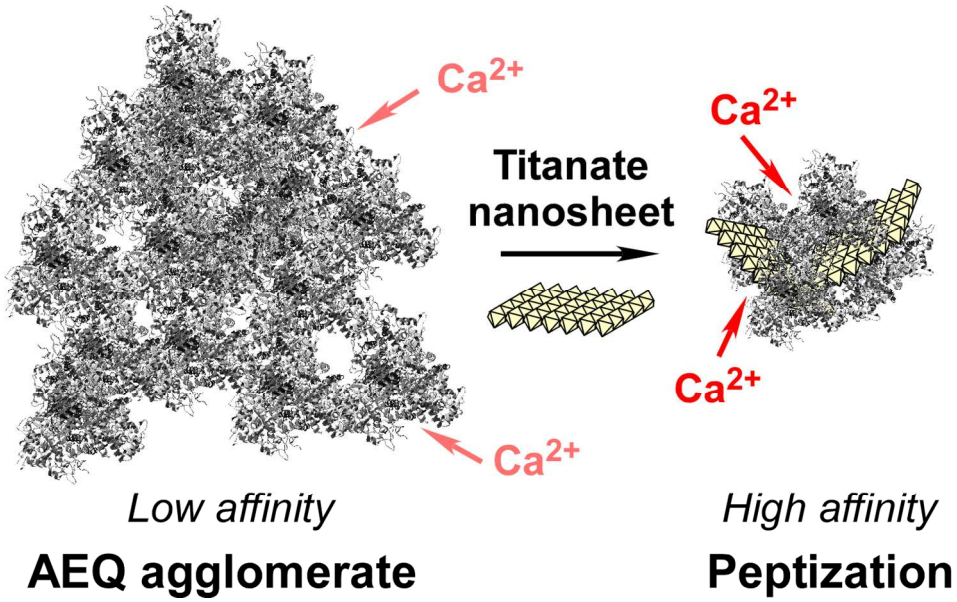

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ARTICLE

Intense Emission from Photoproteins Interacted with Titanate Nanosheets

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Bioluminescence of Ca^{2+}-binding photoprotein; aequorin (AEQ) is largely enhanced by co-presence of titanate nanosheets (TNS). Stable and transparent colloidal solution of TNS with a small hydrodynamic diameter less than 10 nm is synthesized through a simple hydrolysis reaction of titanium tetraisopropoxide followed by dialysis against pure water. Huge agglomerates of AEQ formed in an aqueous solution are peptized in the presence of TNS. The deflocculation of AEQ is considered to be derived from a weak electrostatic interaction with the TNS, which is indirectly demonstrated by an enhanced thermal stability of AEQ. As a result, the TNS addition causes an excellent affinity of Ca^{2+} for binding sites (so called EF hands) in AEQ and subsequent efficient bioluminescence. Similar phenomenon is also confirmed for chemiluminescence of luminol catalyzed by an oxidoreductase. These findings support that the TNS addition is useful to simultaneously solve faint emission and instability of photoproteins.

Introduction

Bioimaging is an essential technique to determine location (distribution) and concentration of target species in biotissues. Among several techniques, fluorescence imaging (FI) acquires through labeling of object with fluorogenic molecules. As an advanced technique, more than one target can be observed by means of multicolor fluorescence. It is well-known that there are several important points to take better images by FI. A suitable mechanism to eliminate scattering and reflection of excitation source from a fluorescence signal has to be equipped to a microscope system. Bleaching of fluorescence probe or functional/morphological damage of target is induced by exposure to excitation light for long duration. Moreover, effect of intrinsic fluorescence should be considered carefully. On the other hand, luminescence imaging (LI) utilizes bio- and molecular luminescence originated from photobiomolecules (photoproteins, photoenzymes) and light emitting molecules, respectively. Photobiomolecules bound to target emit visible light in the presence of specific substrate. Different from FI, no excitation source is needed, suggesting that the above-mentioned requirements for FI are not necessary to keep in mind. However, (bio-)chemically induced luminescence has serious drawbacks such as short lifetime (flash-type), weak emission, and susceptibility of photobiomolecules.

Recently, the difficulties in these imaging techniques have been overcome through binding of fluorescence probe and photobiomolecule. For instance, So et al. have been reported a new imaging strategy without an excitation source by using photoenzyme (luciferase)-adsorbed fluorescent quantum dots (QDs).[1] Bioluminescence of luciferase excites fluorescence of QDs in darkness. Alternatively, Saito and coworkers have applied a nanocomposite composed of photoprotein and green fluorescent protein (GFP) to observation of a single cell or a small animal tumor.[2] The luminescence mechanism mimics green emission of jellyfish *Aequorea victoria* discovered by Simomura et al.[3]

The present study reports that bioluminescence of aequorin, which is one of photoproteins isolated from the *Aequorea victoria*, is largely enhanced in the presence of inorganic nanosheets. An aequorin molecule consists of apoprotein (apoaequorin) and a chromophore moiety (coelenterazine) bonded to a molecular oxygen.[4] When a trace amount of Ca^{2+} is present, the coelenterazine changes a conformation accompanied by a release of CO_2 and a blue emission.[5] Currently, aequorin is frequently employed as an indicator of free Ca^{2+} in cells or tissues, since the emission intensity depends on Ca^{2+} concentration over a wide range. However, the flash-type emission (rapid quenching) as denoted above is also one of disadvantages to be solved in the aequorin-based bioimaging system. The previous literature reported that existence of sugars, polyols, or electrolytes inhibits a gradual denaturation (improvement of thermal stability).[6] In contrast, within our knowledge, no method to accomplish enhancement of emission intensity without modifying molecular structure of apoaequorin or coelenterazine has been proposed so far.[7,8]

In a past decade, several groups including us have demonstrated that functional proteins can be immobilized in

interlayer space of inorganic nanosheets, and these hybrids possess a synergistic function of bio- and inorganic materials.[9-13] For example, hybrids consisting of oxidoreductase and semiconducting nanosheet achieved a photochemical enzymatic activity control based on a photoabsorption of nanosheet followed by an energy transfer to the bound enzyme.[14] The present study firstly reveals that hybridization with titanate nanosheets is effective to brighten the blue emission of aequorin. Such behavior is explained by an electrostatic interaction that influences dispersibility of aequorin molecules in an aqueous solution. Furthermore, it is also described that chemiluminescence catalyzed by an oxidoreductase is enhanced by co-presence of nanosheets. The observed phenomena would be employed to overcome the faint emission of photoproteins. On the other hand, it will develop a new aspect of inorganic nanosheets that has been applied for various fields including hosts of drug delivery or ion-exchange system,[15] active electrodes in batteries,[16] and fluorescent nanomaterials.[17]

Experimental

Titanate nanosheets (denoted below as TNS) dispersed in an aqueous solution was fabricated by means of hydrolysis of titanium tetraisopropoxide (TTIP) with tetrabutylammonium (TBA+) hydroxide solution followed by aging at 60 ^{o}C for 2 h.[18] An absolute ethanol solution of $EuCl_3$ (5 mol%) was added to the liquid TTIP in advance. The resultant transparent sol was dialyzed against pure water using a membrane filter several times to obtain the neutral aqueous sol of TNS. The Eu^{3+} incorporated could be used for quantitative analysis of TNS by fluorescence spectroscopy. The crystal structure of TNS was analyzed by X-ray diffraction (XRD) after drying the colloidal solution on a glass plate. The particle size of TNS was evaluated by dynamic light scattering (DLS) measurement on assumption that the crystal has a spherical morphology even though the nanosheets have a two-dimensional anisotropy in shape.

Stock solution of aequorin from jellyfish (denoted below as AEQ, 2 mg/ml) was prepared by dissolving commercial lyophilized powder (Sigma-Aldrich, A4140) with 10 mM ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) solution and was stored at -45^{o}C until use. The apparent molecular size of AEQ in the solution was measured by the DLS. Bioluminescence of AEQ in the presence or absence of TNS was evaluated as follows. The AEQ stock solution was diluted with 0.1 M tris-HCl buffered solution (pH 8) to 0.2 mg/ml. Equal volumes of the TNS colloidal solution (0 ~ 0.3 mg/ml) and the AEQ solution were mixed, and then the mixture was incubated at 25^{o}C for 30 min. An aliquot of solution (50 µl) was pipetted to a well of white microplate (96-well, round bottom). Luminescence of the solution through a bandpass filter of 460/40 nm (center wavelength / FWHM) was recorded for 10 s immediately after dispense of 100 mM $CaCl_2$ solution (15 µl) to the well. The identical measurement was repeated three times to secure reliable results.

Results and discussion

The hydrolysis of titanium tetraisopropoxide (TTIP) with the tetrabutylammonium (TBA^+) hydroxide solution and the subsequent dialysis against pure water resulted in formation of colorless and transparent colloidal solution (pH ~ 8). The colloidal solution could be preserved over several months without precipitation. Fig. 1a depicts the particle size distribution curve of the colloidal solution. Solid components in the solution had a relatively narrow size distribution with ca. 4 nm in mean hydrodynamic diameter. Our previous paper has demonstrated that the crystal structure of TNS produced by the hydrolysis of TTIP is akin to tetratitanate ($Ti_4O_9^{2-}$).[19] In fact, The diffraction line assigned to (200) plane of tetratitanate intercalated with TBA^+ counter ions appeared in the XRD pattern of solid after drying the colloidal solution (Fig. 1b). Hence, it was confirmed that stable and crystallized TNS were formed in the aqueous solution.

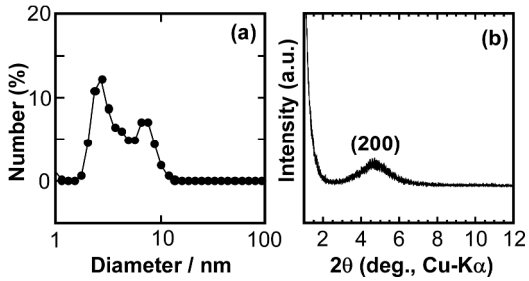

Fig. 1. Characterization of TNS prepared by hydrolysis of TTIP with TBAOH followed by dialysis against pure water. (a) Particle size distribution curve of colloidal solution of TNS (0.10 mg/ml). (b) X-ray diffraction pattern of solid thin film of TNS prepared by drying the colloidal solution.

AEQ releases a blue light with a wavelength peak at ca. 465 nm upon binding with Ca^{2+} (Fig. 1S). On the other hand, the emission intensity is quenched within less than 3 s (Fig. 2S). Fig. 2a shows the effect of TNS on relative luminescence unit (RLU) of AEQ. All test solutions contained an identical weight of AEQ (0.1 mg/ml) and the TNS amounts were expressed as weight ratios against the AEQ. The RLU in the presence of a small amount of TNS was more than twice as bright as that of AEQ only (TNS/AEQ = 0), and further addition had a little influence. In contrast, the co-presence of TNS did not affect the emission wavelength and lifetime (Fig. 1S and 2S). Consequently, it was demonstrated that the bio-luminescence of calcium-binding photoprotein AEQ is largely enhanced by the simple procedure, that is, the TNS addition.

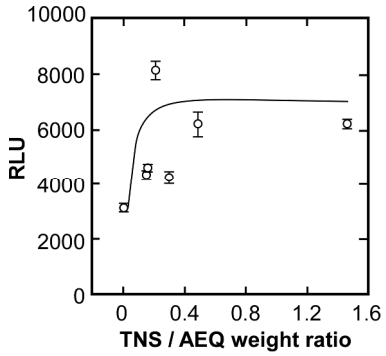

Fig. 2. Dependence of TNS/AEQ weight ratio on relative luminescence units (RLU) of AEQ. The AEQ content is fixed to 0.1 mg/ml.

To elucidate origins of the favorable effect of TNS, the size distribution curve of AEQ in the solution including or excluding the TNS was measured by the DLS technique (Fig. 3). The curve of free AEQ had broad distribution with the mean molecular size of ca. 270 nm. According to the literature,[20] the hydrodynamic diameter of isolated AEQ molecule in an aqueous medium has been estimated to be 2.3 nm which is agreed with that estimated from the molecular weight (22 kDa). This implies that most AEQ molecules formed bulky aggregates. On the other hand, the existence of TNS dramatically reduced the apparent molecular size to ca. 12 nm. Taking account into the relatively small size of TNS (ca. 4 nm), the size reduction seems to be reflected by partial dissociation of the large AEQ aggregates. It has been noted that the proteins tend to associate at a high concentration[20] and gradually aggregate even in the presence of $(NH_4)_2SO_4$ which is recognized as a suitable stabilizer.[21] In this study, the TNS peptized the AEQ agglomerates, leading to an improved affinity of Ca^{2+} for the AEQ. As a result, the bioluminescence of AEQ was enlarged due to the TNS addition. The solid TNS may scatter or reflect the blue light from the AEQ and hence amplify apparent luminescence intensity. Since optical density (absorbance) of the concentrated TNS colloidal solution (35 mg/ml) was smaller than 0.002 at 460 nm, however, effect of light scattering/reflection on the variation in luminescence (Fig. 2) could be ignored.

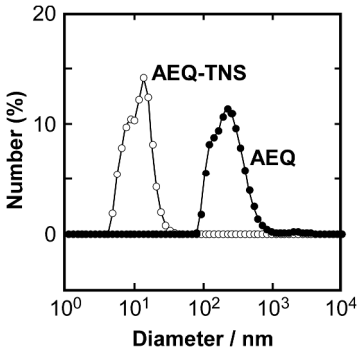

Fig. 3. Molecular size distribution curves of AEQ (0.033 mg/ml) and its mixture with TNS (TNS/AEQ = 3.2 (w/w)) in 5 mM tris-HCl buffer solution of pH 8.

In the past decade, several research groups including us have attempted to fabricate bio-inorganic nanohybrids composed of nanosheets and proteins.[9-14] The most facile approach for binding proteins to host nanosheets is to utilize an electrostatic attraction in an aqueous solution. One can select anionic or cationic nanosheets depending on surface potential of target protein.[15] When a solution's pH is adjusted between an isoelectric point (p*I*) of nanosheet and protein, they are spontaneously combined via an electrostatic interaction because both species have an opposite surface charge.[22] In general, metal oxide charges negatively in wide range of pH, for example, we have already determined that the p*I* of TNS is located at pH 2.[23] On the other hand, the zeta potential of AEQ was found to be negative in neutral solutions (Fig. 3S). Therefore, the anionic AEQ is likely to form electrostatic barrier against the TNS in the tris-HCl buffered solution used in this study (pH 8). However, the reduction of apparent molecular size of AEQ in the solution containing the TNS (Fig. 3) and the resultant enhancement of bioluminescence (Fig. 2) hint that some kind of interaction exists between them.

To reveal the existence of interaction, we evaluated thermal stability of AEQ in the presence or absence of TNS. If the AEQ is bound to the TNS, the thermal stability is improved in proportion to an equilibrium binding constant (K_b).[24] Fig. 4 displays effect of thermal treatment on the AEQ activity. The free AEQ or the TNS-AEQ solution was incubated at 35°C and the emission intensity was periodically recorded. When the incubation was carried out at a low temperature (4°C), the initial activity retained at least 20 h irrespective of the presence of TNS (data not shown). As shown in Fig. 4, the free AEQ was gradually denaturated at 35°C. The previous paper has noted that AEQ is abruptly deactivated over 40°C.[25] On the other hand, the AEQ-TNS solution preserved the initial activity more than 6 h, suggesting enhanced thermal stability of AEQ under the co-existence of TNS. If the AEQ was isolated from the TNS in the mixed solution, the thermal stability of AEQ should be independent of the TNS addition. Hence, these results indirectly exhibit that the TNS affects the dispersion of AEQ. As stated above, the Ca^{2+}-induced luminescence of AEQ were carried out at pH 8 giving a negative charge to both surfaces. Nevertheless, thanks to a local positive charge on N-terminal proline residues with a high pK_a, the AEQ might adsorb to the negative TNS surface. Alternatively, Na^+ ions derived from the EDTA-2Na (Ca^{2+} scavenger in the stock solution) might also mediate the binding of AEQ with the anionic TNS (ion-coupled protein binding).[26] As a result, these "weak" electrostatic attractions led to the improved thermal stability. Taking into account the variation in the apparent molecular size after the TNS addition (Fig. 3), it is envisaged that the TNS behaved as a surfactant and dissociated the AEQ agglomerates to smaller pieces which were dispersed well in the solution. Accordingly, the apparent emission intensity of AEQ was increased. The phenomenon is similar to the pervious knowledge that the dispersion stability of hydrophobic carbon nanotubes existed in an aqueous solution are improved by adsorbing of proteins.[27-29] On the other hand, the (200) peak of tetratitanate in XRD pattern (Fig.

1b) disappeared after the hybridization with the AEQ (data not shown), indicating that the TNS and the AEQ were randomly stacked. FTIR spectra were collected to follow a conformational change of AEQ during the hybridization (Fig. 4S). There is no change in the amide I (1633 cm^{-1}) band after the hybridization, suggesting that the process took place without any deformation of the secondary structure of AEQ. The schematic peptization model is illustrated in Fig. 5. It is predictable that the TNS attract not only the AEQ but also the Ca^{2+} substrate, inducing Ca^{2+}-rich environment at the AEQ-TNS interface, because inorganic polyvalent cations including Ca^{2+} have a high affinity with titanate nanosheets.[30] In fact, an addition of Ca^{2+} to the TNS colloidal solution caused spontaneous formation of large aggregates consisting of negatively charged TNS interstratifying the Ca^{2+}. This may be one of reasons for the enhanced bioluminescence.

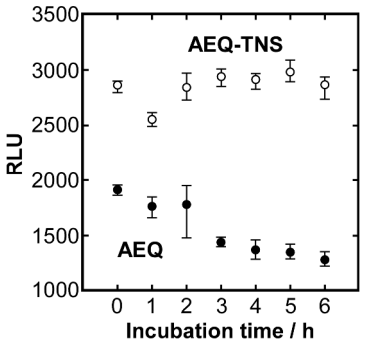

Fig. 4. Influence of thermal treatment at 35°C on AEQ activity (0.05 mg/ml) in the absence or presence of TNS (0.18 mg/ml) at pH 8.

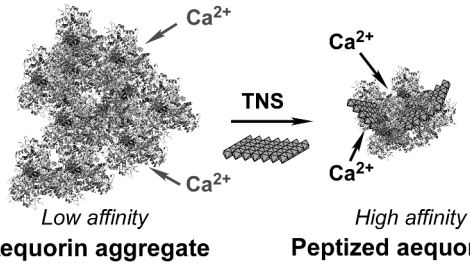

Fig. 5. Schematic illustration of deflocculation of AEQ agglomerates by addition of TNS.

To study whether the TNS-assisted bright luminescence is applicable for other protein-mediated reaction system is of great interest. Therefore, we examined an influence of TNS on chemiluminescence caused by luminol oxidation in the presence of peroxide (H_2O_2) and oxidoreductase (horseradish peroxidase, HRP).[31] In this case, the HRP (p*I* ~ 5,[23] 0.01 mg/ml, 200 µl) and the TNS (p*I* ~ 2, 0 ~ 0.037 mg/ml, 200 µl) were mixed for 30 min in the 20 mM potassium acetate buffer solution of pH 4. Judging from the p*I*s, the positively charged HRP would adsorb on the negative TNS surface. The mixed solution was diluted two times with a concentrated tris-HCl buffer solution (0.1 M, pH 7.6), then 50 µl of the aliquot was injected to a well of white microplate. After dispensing 15 µl of 0.1 mM H_2O_2 containing 0.01 mM luminol to the well, the chemiluminescence attributed to luminol oxidation was measured through a bandpass filter (460/40 nm) for 20 s. Prior to the measurements, it was confirmed that the HRP immobilized to the TNS surface at pH 4 was not detached by the dilution with the buffer solution of pH 7.6 reversing the surface charge of HRP.

The effect of TNS on the chemiluminescence of luminol is shown in Fig. 6. As expected, the mixing with the TNS was effective to strengthen an emission based on the HRP-catalyzed oxidation of luminol. Especially, small amounts of TNS dramatically increased the chemiluminescence more than 10 times. The concentration dependence of apparent hydrodynamic diameter of free HRP reveals that the HRP molecules aggregate in the present condition (0.0025 mg/ml, Fig. 5S). Consequently, the TNS played a role as a dispersant for the HRP molecules, and therefore the bright luminescence was detected in the identical manner to the bioluminescence of AEQ. On the contrary, the further addition of TNS gave rise to lowering of chemiluminescence. While the diluted TNS solution did not cause any sedimentation, large particles observed in naked eyes were precipitated in the solutions containing much amount of TNS due to a firm electrostatic interaction at pH 4. As a result, the dispersion stability of HRP related to the luminescence intensity was degraded. Thus, it was proved that the chemiluminescence of luminol induced by the HRP is largely increased especially at the small TNS / HRP ratio.

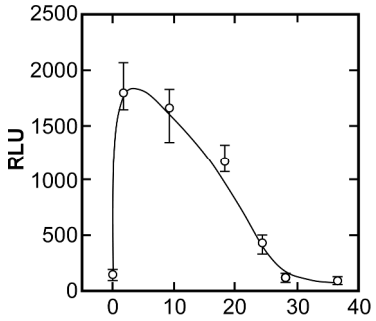

Fig. 6. Relative luminescence units of luminol oxidation induced by HRP and H_2O_2 in solution with various TNS / HRP weigh ratio.

Conclusions

The present study reported that the presence of titanate nanosheets (TNS) in the solution enhances the luminescence intensity of Ca^{2+}-binding photoprotein aequorin (AEQ). Such favorable behavior could be explained by the increase in dispersion stability of AEQ molecules. Concretely, the adsorption of AEQ to the TNS brought about the peptization of the AEQ aggregates. Hence the affinity of Ca^{2+} substrate for the AEQ was improved and then the intense emission was observed in the presence of TNS. That is, the TNS practically acted as a surfactant for the AEQ. The chemiluminescence catalyzed by the oxidoreductase (HRP) was also enhanced via the identical mechanism. This preferred effect of TNS should be an important technique to overcome faint illumination as one of

drawbacks recognized for photoproteins. In addition, the interaction with the TNS resulted in a reinforcement of AEQ against thermal denaturation. The protecting effect of TNS may be also effective for proteolysis degradation of AEQ. Since the TNS used in the present study is extremely small and physicochemically stable, they could not disturb bio-recognition or detection in practical usages of AEQ. There-fore, the hybridization with TNS would effectively elevate functions of photoproteins.

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Notes and references

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Electronic Supplementary Information (ESI) available: Additional figures showing emission spectra and transient emission curves of AEQ and TNS-AEQ, pH dependence of zeta potential of AEQ, and concentration dependence of apparent molecular size of HRP. See DOI: 10.1039/b000000x/

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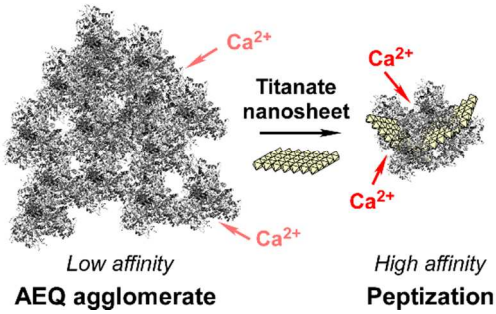

Bioluminescence of Ca^{2+}-binding photoprotein (aequorin, AEQ) is largely enhanced by co-presence of titanate nanosheets that peptize AEQ agglomerates via a weak electrostatic interaction.