Modification of Near-infrared Cyanine Dyes by Serum Albumin Protein†

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Time-dependent nature of the complexes of IR806 dye and other similar near-infrared cyanine dyes in human serum albumin (HSA) have been studied by employing absorption, emission, and time-correlated single photon counting techniques. The complex formation of IR806 with HSA modifies the native structure of IR806 that results time dependent changes in the optical properties. The modification process of the dye and its complex formation with HSA is very slow and this takes about 90 min. The process of the formation of new complex is irreversible and totally controlled by the initial complex of IR806 and HSA. As far as we know, the new properties of cyanine dye have not been reported. These properties are very important for the near-infrared fluorescence imaging.

Near-infrared (NIR) cyanine dyes have been widely used for monitoring the biological function in living system owing to their absorption regions far beyond the absorption of most biological molecules. During the last decades remarkable efforts have been undertaken in the area of clinical uses of cyanine dyes, in particular famous indocyanine green, such as hepatic function tests and ophthalmic angiography.[1,2] The cyanine dyes have also shown the promising potential in the area of near-infrared (NIR) tissue imaging in small animal and human.[3–5] The absorption and scattering of the tissue in NIR region are much weaker than that of in ultraviolet-visible region, so the light can penetrate deeply into tissues.[6] Thus, cyanine dyes and its derivatives have been intensively investigated for the contrast agent *in vivo* application.[5,7,8] IR806 is one of the important classes of the cyanine dye which has absorption and emission maxima in the NIR region around 780–850 nm.

In the living biological systems, serum albumins are the most predominant protein in blood plasma and have known to act as a carrier to transport a variety of biologically active species including drugs. In HSA microenvironment, hydrophobic or amphiphilic ligands bind mostly to one of the two principal binding sites, located in the subdomain IIA (site 1) and IIIA (site 2) and these binding sites are often selective.[9,10] Elucidation of dye interaction to HSA is very important in thorough understanding of the potential uses *in vivo*, because binding affinity of a dye to HSA can affect the distribution, toxicity and excretion.

In this communication, we have reported the time dependent changes in the optical properties of the complex of IR806 (2-[2-[2-chloro-3-[2-[1,3-dihydro-3,3-dimethyl-1-(4-sulfobutyl)-2H-indol-2-ylidene]-ethylidene]-1-cyclopenten-1-y1]ethenyl]3,3dimethyl)-1(4-sulfobutyl)-3H-indolium hydroxide, inner sodium salt), and HSA (see Fig.1S in ESI). There are several reports on time dependent changes in electronic absorption spectrum of chromophores in protein by the replacement of central moiety or by the enzymatic reactions.[11–14] Here, our new finding is serum albumin induced spontaneous change in the chromophore of IR806, and this change formed a very stable modified dye complex.

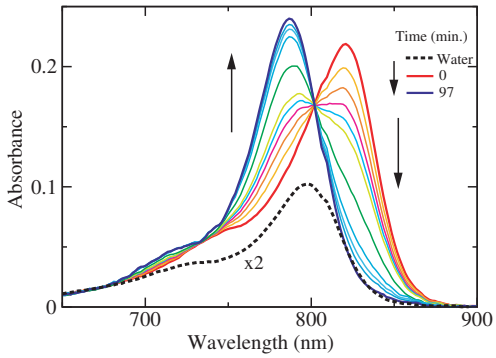

Fig. 1 Absorption of 1 μM IR806 in water (***dotted line***). Absorption of the complex of 1 μM IR806 and 50 μM HSA in the early stage of time is shown by red line. The arrow indicates the subsequent change in the spectrum as a function of time. The absorption of the complex after 97 min is shown by the blue line. The measurement times were 1, 3, 5, 7, 9, 11, 21, 37, 47, 57 and 97 min, respectively.

Figure 1 shows the absorption of IR806 in water and in HSA solution. Absorption spectrum of IR806 in water shows

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a band maximum at 798 nm. Formation of the complex with HSA was observed by the new absorption band at 822 nm with 24 nm red shift from the native absorption band, and the optical density is also accelerated by the complex formation. The red-shifted absorption was observed within the time required for the manual preparation of the sample of IR806 and HSA complex, just after the measurement in water. Absorption of this complex surprisingly diminishes with the passes of time, and a new peak appears at 786 nm with 36 nm blue shift. The intensity of this new peak also increases with the passage of time. Finally, within 90 min the absorption band at 822 nm totally disappears and a new absorption band with a peak maximum at 786 nm was observed. The isosbestic points were clearly observed at 801 nm and 740 nm. The presence of isosbestic point indicates the conversion of one absorptive species to another.

The red-shifted peak of the complex indicates hydrophobic environment around dye due to the binding in HSA microenvironment. Hydrophobic interaction of dye with HSA can be confirmed by the solvent dependent absorption of IR806. The solvent dependent measurement clearly shows the red shift of absorption band with decreasing the solvent polarity, which is commonly observed in cyanine dyes (see Fig.2S in ESI).[15,16] On the other hand, the blue-shifted absorption cannot be merely attributed to the hydrophobic interaction of IR806 with HSA; the electronic state of dye is strongly modified by HSA.

The denaturation and the ultrafiltration studies were conducted to characterize the blue-shifted complex. The denaturation process with 4 M guanidine hydrochloride only shows spectral broadening of the blue-shifted absorption spectrum not peak shift as shown in Fig.2. The spectral broadening is most probably due to the fluctuation or inhomogeneity of the dye environment by the relaxation of protein conformation in the presence of denaturant. Further, the blue-shifted complex with and without 4 M of guanidine hydrochloride was filtered by a centrifugal filter device. In both cases, all dye remained in the retentate (protein side solution) and no separated dye from HSA was detected in the ultrafiltrate (low molecular weight side). These results indicate that the blue-shifted complex is very stable even the HSA structure is almost unfolded with guanidine hydrochloride[17] and the dye molecule does not dissociate from the complex. It suggests that the blue-shifted complex is not simple physical binding.

In contrast, the primary complex is easily dissociated by the denaturant and after dissociation it never formed the secondary complex. Therefore, it is suggested that the blue-shifted complex is totally controlled by the red-shifted complex formation.

Similar time dependent changes were observed for IR806 and bovine serum albumin (BSA) complex. The dyes, ICG, IR783, IR820 and IR786, which are structurally similar to

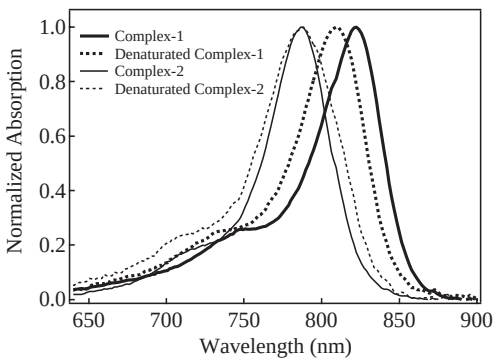

Fig. 2 Complex of the 1 μM IR806 and 50 μM HSA in the early stage of time (complex-1) shown by thick solid line, and the spectrum after the denaturation of HSA (***thick dotted***). Complex of the modified IR806 and HSA (complex-2) shown by thin solid line, and the spectrum after the denaturation of HSA (***thin dotted***).

IR806, were also tested (see Fig.1S in ESI). These phenomena were not observed in ICG, IR820 and IR786 perchloride and HSA or BSA complexes. On the other hand, IR783 shows a very similar change in HSA microenvironment. The time dependent changes in the optical properties are peculiar to the complexes of IR806 and IR783 in HSA or BSA microenvironment.

For such changes, some reactive residues in HSA, such as lysines and free cystein (Cys34), might have played important role.[12,18] The nucleophilic groups, such as free amino groups of lysine in HSA, might replace the chloro moiety in the central polymethine chain or sulfonate groups.[18,19] It is also known that Cys34, which is the only cystein with free SH group, locates on the surface of HSA and is reactive with exogenous compounds.[12] To check the interaction to lysine groups, the absorption spectrum of IR806 in a lysine and tryptophan random copolymer solution was studied. This solution shows only the red-shifted peak, which is same as the early stage of the IR806-HSA system (see Fig.3S in ESI) and does not induce the blue-shift of the spectrum.

Therefore, the significant blue shift of the spectrum seems to be more reasonable by the modification of the chloro moiety in the central polymethine chain. Actually, ICG, which does not possess a chlorine atom on the conjugated bridge, does not show the blue shift. On the other hand, IR783 and IR820, which have the same structure around the chloro moiety, show opposite properties; IR783 shows the similar blue-shifted property as IR806 but IR820 does not. This suggests that the aromatic rings near by the sulfonate groups at both edges are affecting the reaction.

The changes in the ground state also reflect the similar changes in the excited state as shown in Fig.3. The emission spectrum of IR806 in water shows the peak maximum at 827 nm. The complex of IR806 and HSA shows 14 nm

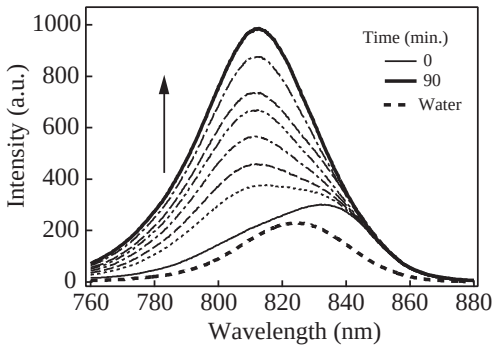

Fig. 3 Time-resolved emission spectra of the complex of 1 μM IR806 and 50 μM HSA. The emission of the complex in the early stage of time is shown by red line. The arrow indicates the subsequent change in the spectrum as a function of time. Emission spectrum of IR806 in water is also shown with black dotted line. The excitation wavelength was an isosbestic point at 740 nm. The measurement times were 0, 5, 7, 12, 20, 30, 40 and 90 min, respectively.

red-shifted emission peak (∼841 nm) in the early stage of time. With the passage of time, a shifted band arises in the shorter wavelength region, grows and reaches maximum at 813 nm, which corresponds to the different species than the early stage of time. The intensity ratio of the red-shifted and the blue-shifted complexes in the fluorescence is quite different from the intensity ratio in the absorption, indicating the smaller quantum efficiency of the red-shifted complex.

The emission decay of IR806 is determined by a single lifetime (0.25 ns) in the aqueous solution (see Fig.4S in ESI). The emission decay of the complex of IR806 and HSA in the early stage of time (complex-1) requires at least two exponentials; observed lifetimes are 0.44 ns (44%) and 0.80 ns (56%). The emission of the later complex (complex-2) also shows bi-exponential decay with lifetimes of 0.35 ns (4%) and 1.2 ns (96%). In contrast to the single exponential decay in aqueous solution, the emission decays in HSA solution show a bi-exponential decay. The bi-exponential decay of the complex-1 is probably due to the partial isolation of IR806 from the water environment but somewhat it might be fluctuated condition or intermediate condition before the conversion. On the other hand, complex-2 shows almost single exponential decay with longer lifetime. This suggests that the dye is either buried deep into the protein and/or the chemical structure is modified by HSA. The lifetime measurements are consistent with the steady state spectrum. The optical properties are summarized (see Table 1 in ESI).

The spontaneous modification of cyanine dye with the protein has not been known and this type of properties of dye-protein complex might be applicable to the protein labeling. The modified complex is highly fluorescent than the ICG-HSA complex and stable. Therefore the IR806 complex is expected to lead the significant biomedical imaging applications.

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