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Binding Behavior of Subphthalocyanine-Tagged Testosterone with Human Serum Albumin at the *n*-Hexane/Water Interface

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Subphthalocyaninatoboron(III) was applied for the first time as a novel marker-tag of testosterone (Subpc-test) for the binding analysis with human serum albumin (HSA) at a liquid/liquid interface. The binding interaction of Subpc-test with HSA at the *n*-hexane/water interface was studied by UV−visible absorption and circular dichroism (CD) spectroscopy combined with a centrifugal liquid membrane cell at different pHs of aqueous solutions. Complementary studies by a high-speed stirring experiment and an interfacial tension measurement were also performed to characterize the interfacial adsorptivity of Subpc-test and HSA molecules, respectively. The *n*-hexane solution of Subpc-test showed no optical chirality, but the contact with the aqueous solution of HSA induced its optical chirality, clearly suggesting the formation of Subpc-test/HSA complexes at the *n*-hexane/water interface. Furthermore, pH profiles of CD signals showed that the interaction between Subpc-test and HSA was very sensitive to the neutral-to-base transition (N−B transition). Interfacial formation of the Subpc-test/HSA complex was studied in the presence of the site-selective ligand, furosemide (site I) or cefaclor (site II). The experimental results suggested that Subpc-test is bound to site I of the HSA molecule at the *n*-hexane/water interface. This technique using subphthalocyanine as a tag molecule and the liquid/liquid interface as a two-dimensional nanoreaction field will be useful for the evaluation of the interaction between drugs or hormones and proteins.

The reversible protein binding ability of human serum albumin (HSA) in blood is an important factor in the transport and release of various drugs and hormones.[1] HSA, the most abundant protein in human blood plasma, has multiple hydrophobic binding sites and binds a diverse set of drugs and hormones, especially neutral and negatively charged hydrophobic compounds.[2,3] In short, interactions with HSA may control the efficacy and distribution of drugs and hormones. It is also known that the competition between hormones or drugs to interact on the same HSA site contributes to modify the activity and the toxicity of drugs.[4] Actually, there have been many reports on the independent, cooperative, and competitive interactions between many hormones or drugs and proteins.[5] For instance, the binding of testosterone, a male sex hormone, to HSA molecule is involved in an important phase in its general metabolism in vivo.[6−8] Despite much previous research, the protein binding phenomena at a liquid/liquid interface have hardly been explored so far.

The liquid/liquid interface between immiscible two liquids has been recognized as an important reaction and separation fields in biological systems, particularly in biomedical engineering, pharmacology, and food processing.[9] For instance, the hydrocarbon/water interface may provide an excellent model medium to cellular interfaces where many important biological functions and processes occur.[10] In biomedical applications, the behavior of drugs and hormones at biological membranes is an important factor in determining pharmacological activity. [11] In such cases, the oil/water interface can be used to characterize drug and hormone/protein interactions, since it can mimic cell membrane structure.

In recent years, there has been a growing interest in the use of phthalocyanine, porphyrin, and related compounds as therapeutic drugs.[12] They are applied in medicine as a cancer detection reagent and as photosensitizers in photodynamic therapy of cancer.[13,14] Potential applications of these compounds have recently appeared in the treatment of nonmalignant conditions such as

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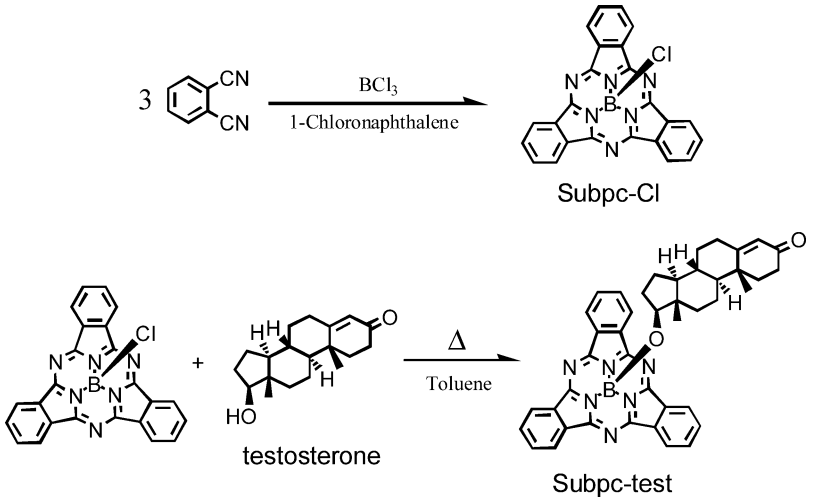
Scheme 1. Synthesis of Subphthalocyanine-Tagged Testosterone at the Axial Position



psoriasis, blocked arteries, and viral and bacterial infections, including human immunodeficiency virus (HIV).[15] Subphthalocyanine (Subpc) is the lowest homologue of phthalocyanine, and has a macrocycle structure formed by three coupled isoindole moieties having a delocalized 14-π-electron system.[16] Native Subpc has a high molar absorptivity (ϵ > 50 000) at around 560 nm and a halogen atom bound to the central boron atom, which is exchangeable with other nucleophiles. Thus, the Subpc molecule has potential as a high-sensitivity probe dye in the visible region.[17,18] In the present study, we have synthesized a novel subphthalocyanine compound with testosterone (test) tagged at the axial position (Subpc-test; see Scheme 1) as a binding marker for HSA molecule.

In this study, the binding characteristics of the Subpc-test compound to the HSA molecule at the *n*-hexane/water interface have been investigated by means of UV−visible absorption and circular dichroism (CD) measurements combined with a centrifugal liquid membrane (CLM) apparatus.[19,20] Subpc is an achiral molecule, while testosterone is a chiral molecule, but its own CD is very weak. Cotton effects of Subpc-test are, therefore, not observed in the visible region but were induced by the binding to the HSA molecule at the *n*-hexane/water interface. The present findings proposed a new way of characterization on drug/- or hormone/HSA interaction using Subpc as the probe dye and the liquid/liquid interface as the two-dimensional reaction field.

RESULTS AND DISCUSSION

Spectral Properties of Subpc-test Compound. According to the published procedure,[21,22] Subpc-Cl (shown in Scheme 1) was synthesized by the condensation reaction of phthalonitrile in the presence of boron trichloride. The axial chlorine atom in Subpc-Cl was replaced with testosterone, and Subpc-test was obtained. (Scheme 1) Subpc-test was highly soluble in dichloromethane, chloroform, toluene, dimethyl sulfoxide, tetrahydrofuran, *n*-hexane, acetone, and pyridine, and slightly soluble even in methanol and ethanol, but was insoluble in acetonitrile. Figure 1 shows the UV−visible absorption spectra of Subpc-test in *n*-hexane at room temperature, which has a Q-band and a B-band as in other isoindole aromatic macrocyclic compounds.[23] Similar to the original Subpc-Cl, the B-band of Subpc-test is seen at 298 nm, while the energetically higher lying Q-band appears at 555 nm. The two dominant absorption bands arise from the first two allowed $\pi-\pi^*$ transitions of the macrocyclic π-system consisting of C atoms and aza-N atoms. [16] As shown in the inset of Figure 1, the Lambert−Beer plot for the *n*-hexane solution of Subpc-test is linear over at least 3 orders of magnitude in concentration (from 1.0×10^{-6} to 1.0×10^{-4} M), ruling out any formation of aggregates in *n*-hexane.

Interfacial Adsorptivity of Subpc-test in the *n*-Hexane/Water System. In the high-speed stirring (HSS) experiments[24,25] of the *n*-hexane/water system, Subpc-test showed appreciable adsorption to the interface. The adsorption isotherm was obtained as shown in Figure 2 and analyzed by a Langmuir isotherm,[26]

$$[\text{Subpc-test}]_i = \frac{aK'_{\text{Subpc-test}}[\text{Subpc-test}]_o}{a + K'_{\text{Subpc-test}}[\text{Subpc-test}]_o} \qquad (1)$$

where $[\text{Subpc-test}]_i$ and $[\text{Subpc-test}]_o$ denote the concentrations of Subpc-test adsorbed at the liquid/liquid interface and in the bulk organic phase, respectively. The value a is the saturated interfacial concentration (mol cm^{-2}) and $K'_{\text{Subpc-test}}$ is the interfacial adsorption constant (cm) defined under the condition of $a \gg$

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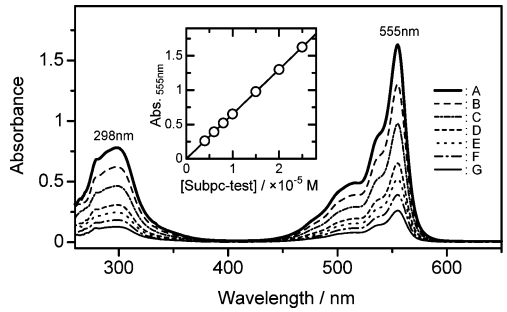

Figure 1. UV−visible absorption spectra of Subpc-test in *n*-hexane at different concentrations: (A) 2.5×10^{-5}, (B) 2.0×10^{-5}, (C) 1.5×10^{-5}, (D) 1.0×10^{-5}, (E) 8.0×10^{-6}, (F) 6.0×10^{-6}, and (G) 4.0×10^{-6} M. The insets show Lambert−Beer law plots for the absorbance at the Q-band of Subpc-test.

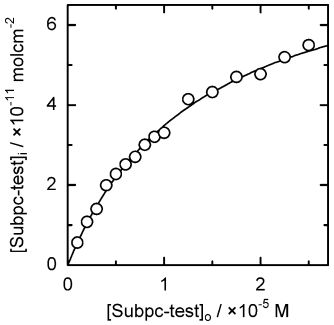

Figure 2. Adsorption isotherms of Subpc-test measured by the HSS method at the *n*-hexane/water interface at 25 °C. The solid line is the best fit of the data to eq 1. Concentrations: $[\text{Subpc-test}]_o = 1.0 \times 10^{-6}$−$2.5 \times 10^{-5}$ M and $[Na_2SO_4]_a = 3.3 \times 10^{-2}$ M, pH 7.3 (phosphate buffer).

$[\text{Subpc-test}]_i$,

$$K'_{\text{Subpc-test}} = [\text{Subpc-test}]_i/[\text{Subpc-test}]_o \qquad (2)$$

Herein, the difference between the absorbances of the bulk organic phase under high- and low-speed stirring, ΔA, and the absorbance of the bulk organic phase under high-speed stirring, A_o, can be described as follows,

$$\Delta A = \epsilon l[\text{Subpc-test}]_i(S_i/V_o) \qquad (3)$$

$$A_o = \epsilon l[\text{Subpc-test}]_o \qquad (4)$$

where ϵ, l, S_i, and V_o are the molar absorptivity of Subpc-test in *n*-hexane, the optical path length of the flow cell (1.0 cm), the total interfacial area under the stirring, and the volume of *n*-hexane phase, respectively. The total interfacial area, S_i, in the liquid/liquid system was already reported to be 2.0×10^4 cm^2.[27] From analysis with the Langmuir isotherm, the interfacial adsorption constants, $K'_{\text{Subpc-test}}$, and the saturated interfacial concentration, a, of Subpc-test are calculated to be 6.01×10^{-3} cm and 8.30×10^{-11} mol cm^{-2}, respectively. Using the obtained a value, the area per one molecule for Subpc-test was obtained to be ~200 Å^2. This value was comparable with the projection area of Subpc-test molecule of 155−230 Å^2 estimated by an optimized CPK model.

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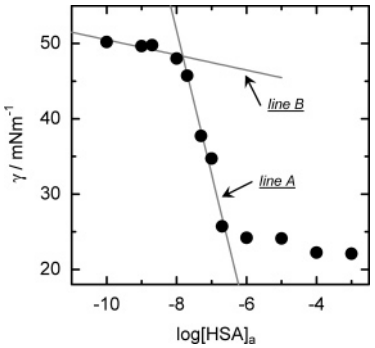

Figure 3. Interfacial tension lowering due to the adsorption of HSA in the *n*-hexane/water system at 25 °C. Lines A and B, respectively, are the best fits to eq 5 of the data at intermediate concentrations (1.0×10^{-8}−1.0×10^{-6} M) and low concentrations (<1.0×10^{-8} M) of HSA. Concentrations: $[\text{HSA}]_a = 1.0 \times 10^{-10}$−$1.0 \times 10^{-3}$ M and $[Na_2SO_4]_a = 3.3 \times 10^{-2}$ M, pH 7.3 (phosphate buffer).

Adsorption of HSA Molecules at the *n*-Hexane/Water Interface. Figure 3 illustrates the equilibrium interfacial tension in the *n*-hexane/water system as a function of the initial HSA concentration. The interfacial tension, γ, remains almost constant at ~50 mN m^{-1} for lower concentrations, i.e., from 1.0×10^{-10} to 1.0×10^{-8} M. With further increases in the bulk protein concentration, the interfacial tension declined steeply to ~25 mN m^{-1} at 10^{-6} M HSA. However, at concentrations above 1.0×10^{-6} M, γ remains again relatively constant at 25 mN m^{-1} (Figure 3). In view of this concentration dependence of the interfacial tension, the three regions are clearly identified. Some globular proteins have the ability to self-associate into a micellar arrangement under certain solution conditions.[28,29] Various reports revealed that albumins are more hydrophobic than other protein compounds.[30,31] As a result, we postulated that the HSA molecule would have a high tendency toward the formation of aggregates at *n*-hexane/water. That is, the adsorption of HSA molecules at <10^{-8} M forms a simple monolayer of isolated molecules. Above the interfacial saturation at ~10^{-8} M, these molecules started to form aggregates, and finally resulted in a plateau value for the interfacial tension, suggesting the micellar aggregation in the bulk aqueous phase. The micellar aggregates may still be present close to the interface.

The interfacial tension behavior is usually discussed using Gibbs' isotherm. This classical equation was based on the model of a nonpenetrable interface, where an adsorbate can substitute only one solvent molecule with similar size on the interface. [11] In the present case, amphipathic HSA molecules adsorbed at the liquid/liquid interface would substitute some molecules of both solvents; therefore, a classical equation such as Gibbs may be not applicable. [11,32] However, some researchers have demonstrated the close similarity at low protein concentration between the experimental and Gibbs' curves using β-casein and bovine serum

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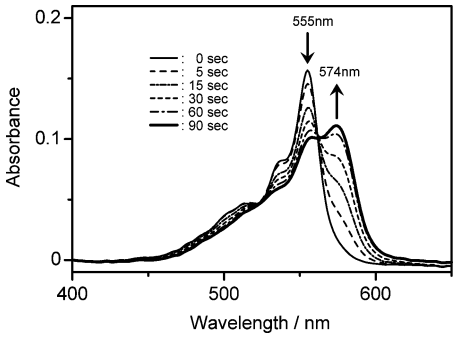

Figure 4. UV−visible absorption spectral changes due to the slow interfacial complexation of Subpc-test and HSA at the *n*-hexane/water interface measured by the CLM technique. The absorption spectra were recorded at 1-s intervals until the adsorption equilibrium was achieved. The arrows indicate the direction of the absorbance changes with time course. Concentrations: $[\text{Subpc-test}]_o = 1.0 \times 10^{-4}$ M, $[\text{HSA}]_a = 1.0 \times 10^{-3}$ M, and $[Na_2SO_4]_a = 3.3 \times 10^{-2}$ M, pH 7.3 (phosphate buffer).

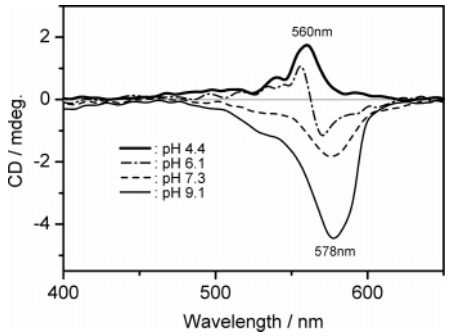

Figure 5. Change of CD spectra of Subpc-test/HSA complexes formed at the *n*-hexane/water interface as a function of various pH values in the aqueous phase. Concentrations: $[\text{Subpc-test}]_o = 1.0 \times 10^{-4}$ M, $[\text{HSA}]_a = 1.0 \times 10^{-3}$ M, and $[Na_2SO_4]_a = 3.3 \times 10^{-2}$ M, pH 4.4, 6.1, 7.3, and 9.1 (phosphate buffer).

albumin (BSA) as examples.[33,34] The Gibbs' adsorption equation is defined as eq 5.[35]

$$\Gamma_{\text{HSA}} = \frac{1}{2.303\,RT}\frac{d\gamma}{d(\log[\text{HSA}]_a)} \quad (5)$$

Herein, Γ_{HSA} is the interfacial excess (so-called the interfacial concentration), $[\text{HSA}]_a$ is the HSA concentration in the aqueous solution, R is the universal gas constant (8.31 N m^{-1} mol^{-1} K^{-1}), T is the absolute temperature, and γ is the interfacial tension. Line A in Figure 3 gave the interfacial concentration of HSA, Γ_{HSA}, of 6.09×10^{-10} mol cm^{-2}, which referred to the area per one molecule for HSA of ~27.4 Å^2. This value is 2 orders smaller than the molecular area at the saturated (close-packed) interfacial concentration of BSA (~2500 Å^2 for BSA as measured by radiotracer techniques),[34] although HSA and BSA have similar molecular weights and structures. We thought that this result suggested the formation of micellelike aggregates of HSA molecules at the *n*-hexane/water interface. In the region of low HSA concentrations (below 1.0×10^{-8} M), only four data points can be used to calculate the interfacial concentration. From line B in Figure 3, the interfacial concentration of Subpc-test was obtained as 8.41×10^{-12} mol cm^{-2}, which corresponded to the interfacial molecular area of ~1900 Å^2. This value is of the same order of magnitude as that of BSA.

Interfacial Formation of the Subpc-test/HSA Complex in the *n*-Hexane/Water System. The formation of Subpc-test/HSA complexes at the *n*-hexane/water interface was observed by UV−visible absorption and CD spectroscopy using a CLM technique.[19] Figure 4 shows the CLM absorption spectral change for the process of Subpc-test/HSA complex formation kinetics in pH 7.3 at the *n*-hexane/water interface. The preliminary experiment showed no extraction of the Subpc-test/HSA complex from the aqueous phase to the organic phase and no binding of Subpc molecule to HSA molecule in the two-phase system. In the CLM measurement (Figure 4), immediately after the initiation, the intense Q-band of Subpc-test ($\lambda_{max} = 555$ nm) decreased, and the lapse of time caused the growth of a new absorption band at $\lambda_{max} = 574$ nm with a red shift. The existence of the isosbestic point at 562 nm leads to the conclusion that the conversion between species is quantitative.[36] This result clearly indicated that the Subpc-test/HSA complex was formed at the *n*-hexane/water interface.

As shown in Figure 5, the interfacial formation of Subpc-test/HSA complexes at pH 7.3 resulted in an induced negative Cotton effect with a maximum around 570 nm in the Q-band region. Moreover, increasing pH values from 7.3 to 9.1 raised the negative CD intensity and shifted the maximum wavelength to 578 nm. In contrast, decreasing pH from 7.3 to 4.4 deformed completely the spectral shape: the negative CD spectrum around 570 nm disappeared, and a new, but small positive CD band appeared around 560 nm. Because unbound Subpc-test molecule is not optically active, and HSA does not produce any Cotton effects at these wavelengths, the observed Cotton effects are attributable to the interaction of the derivatized chromophore with an asymmetrical and chiral locus in the protein. Thus, extrinsic Cotton effects reflect the characteristics of specific chiral sites in the protein molecule and will provide information on the microenvironment of binding sites or ligand orientation in the sites. Increasing pH from 4.4 to 9.1, in which HSA is known to undergo the neutral-to-base transition (N−B transition),[4,37] caused changes in the CD spectrum of Subpc-test/HSA complex.

HSA and α1-acid glycoprotein (AGP) are known as the most important transport and depot proteins in the human body. AGP is considered to possess only one ligand-binding site, consisting of several overlapping regions for exogenous and endogenous substances.[38] However, HSA has at least three specific binding sites showing high affinity for specific drugs, namely, site I, site II, and site III, the warfarin-, benzodiazepine-, and digitoxin-binding sites, respectively, which are highly elongated hydrophobic pockets with charged lysine and arginine residues near the

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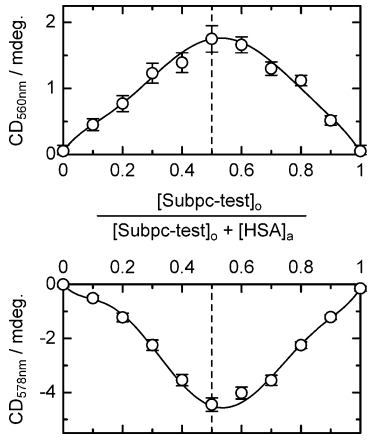

Figure 6. Job analyses of Subpc-test/HSA complexes formed at the *n*-hexane/water interface at (a) pH 4.4 and (b) pH 9.1. The sum of Subpc-test and HSA concentrations is 2.0×10^{-4} M.

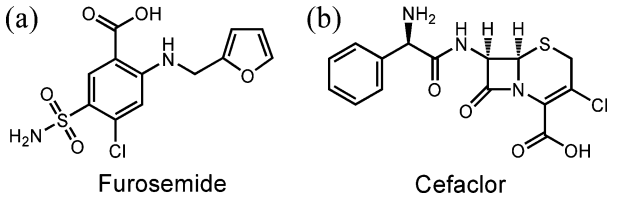

Figure 7. Chemical structures of (a) furosemide and (b) cefaclor.

surface.[4,39–41] Site I and site II of HSA undergo dramatic changes in tertiary structure as the N–B transition proceeds.[42] At pH 4.4, HSA essentially exists as the N-conformation, whereas at pH 9.1, almost all of the HSA are in the B-conformation.[4] Therefore, pH-dependent CD spectra in this system are related to the structural transition of HSA's N–B transition.

The stoichiometric composition of Subpc-test/HSA complexes at the *n*-hexane/water interface was determined by the continuous-variation plots (Job plots)[43] for the CD spectral changes at pH 4.4 and 9.1. As shown in Figure 6, the continuous variation plots gave maximums at 0.5, indicating the formation of 1:1 Subpc-test/HSA complexes at both pH values.

Interaction Mechanism between Subpc-test and Specific Site Ligands in the *n*-Hexane/Water System. When an auxiliary ligand that can bound specifically to site I or site II of HSA is added, the binding ability of Subpc-test to the independent site will become clear. Therefore, the binding ability of Subpc-test to HSA in the presence of furosemide or cefaclor in the aqueous phase was studied at several concentrations. It is well known that furosemide and cefaclor (Figure 7) interact specifically with site I and site II of HSA, respectively.[44–46] The effect of the auxiliary ligands on CD intensity at 675 nm of Subpc-test/HSA

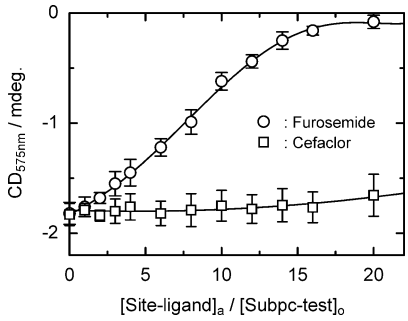

Figure 8. CD intensity (575 nm) of Subpc-test/HSA complexes formed at the *n*-hexane/water interface dependence upon the site ligand concentration in the aqueous phase at pH 7.3. The lines are a guide for the eyes. Concentrations: $[\text{Subpc-test}]_o = 1.0 \times 10^{-4}$ M, $[\text{HSA}]_a = 1.0 \times 10^{-3}$ M, $[\text{site ligand}]_a = 1.0 \times 10^{-5}$–$2.0 \times 10^{-3}$ M, and $[Na_2SO_4]_a = 3.3 \times 10^{-2}$ M, pH 7.3 (phosphate buffer).

complexes formed at the *n*-hexane/water interface is shown in Figure 8. Incidentally, furosemide, cefaclor, and their HSA complexes showed no measurable CD spectrum around the Q-band region of Subpc-test. As seen in Figure 8, the CD intensity decreased with the gradual addition of furosemide, whereas that was hardly affected with increasing cefaclor concentration in the aqueous phase. When the $[\text{furosemide}]_a/[\text{Subpc-test}]_o$ ratio was increased to 20, the CD spectra of Subpc-test/HSA complexes almost vanished. Thus, the formation of Subpc-test/HSA complexes at the *n*-hexane/water interface is more seriouly brocked by furosemide, site I ligand.

From the experimental results, probable schemes for the binding mechanisms between Subpc-test and HSA at the *n*-hexane/water interface and the effect of furosemide and cefaclor are given in Scheme 2. Since it is known that the testosterone molecule is a ligand having affinity to site I,[5] at pH 4 (N-conformation), we inferred that Subpc-test binds to site I of the HSA molecule. This was supported by the result of a positive Cotton effect in the Q-band region. Increasing pH from 4 to 9 modifies the conformation of the binding site, i.e., the N–B transition. Therefore, the observed large negative Cotton effect at pH 9.1 is ascribable to the binding of Subpc-test to HSA in the B-conformation. Moreover, furosemide molecule inhibited the binding of Subpc-test to the site I of HSA, although the presence of cefaclor did not affect the formation of Subpc-test/HSA complexes at the *n*-hexane/water interface. These results indicated that Subpc-test bonds selectively to site I at the *n*-hexane/water interface.

CONCLUSION

We have determined experimentally the adsorptivity of Subpc-test and HSA and the binding mechanism of Subpc-test to HSA at the *n*-hexane/water interface, which must be biochemically and pharmacologically important. Especially, since free Subpc-test molecules show no optical activity at all, the appearance of the induced CD band becomes strong experimental evidence for the existence of specific binding interaction between Subpc-test and HSA at the *n*-hexane/water interface. The CD band behavior of Subpc-test/HSA complexes upon changing pH values in the aqueous phase was indicative of the stereochemical nature (e.g., size, shape) of the binding site. Moreover, cooperative and competitive interactions between Subpc-test and site ligands

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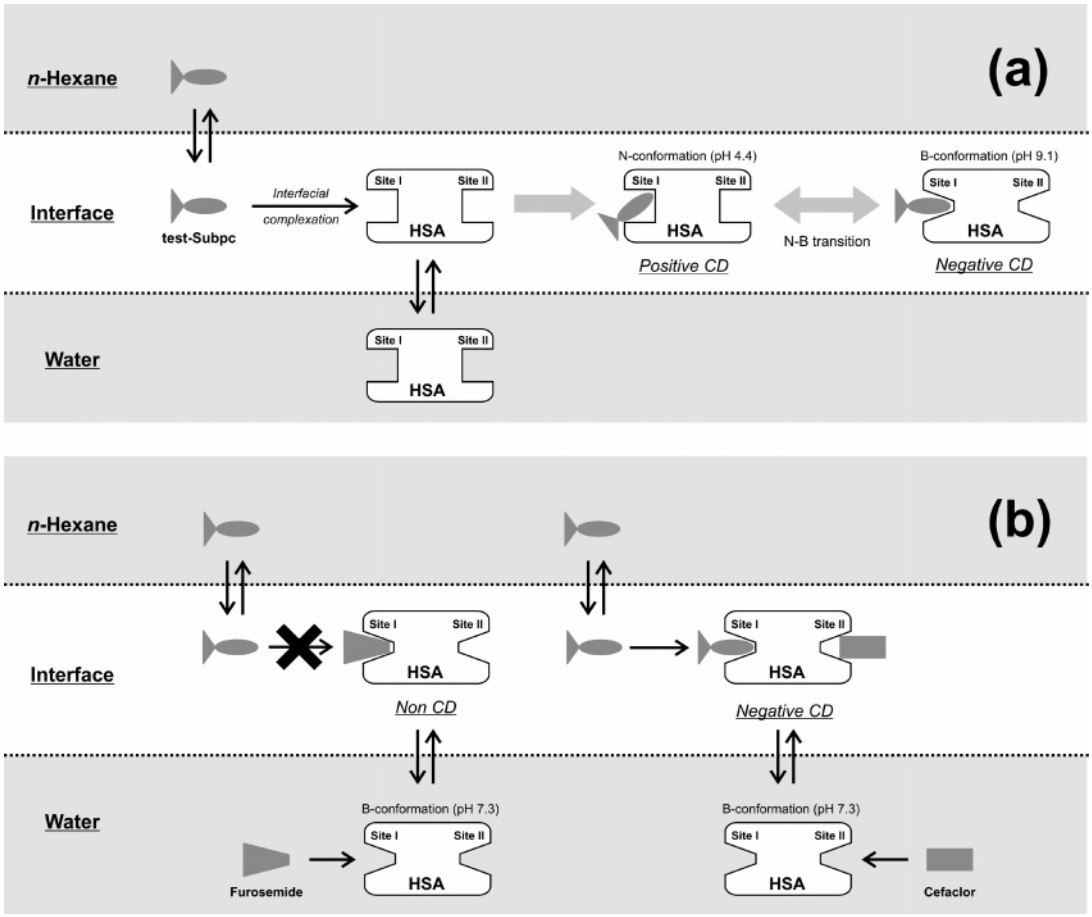
Scheme 2. Schematic Model for (a) Formation of Different Subpc-test/HSA Complexes at pH 4.4 and 9.1 and (b) Inhibition of Subpc-test/HSA Complexation by a Site I-Selective Ligand (Furosemide) and No Effect of Site II-Selective Ligand (Cefaclor) on Complexation at the *n*-Hexane/Water Interface



(furosemide and cefaclor) on the HSA molecule were observed. CD monitored experiments performed with these defined site ligands for HSA provided critical information on the binding location of the Subpc-test molecule in this system. The present study demonstrated for the first time that, by selecting subphthalocyanine as a probe dye, HSA binding of a hormone molecule at the liquid/liquid interface can be measured from the change of the induced CD in the absorption band region of subphthalocyanine. We believe that, by induced CD spectral measurement of dye-tagged drug or hormone/protein complexes formed at the liquid/liquid interface, significant information on drug- or hormone-binding properties of proteins will be obtained without crystallization soon.

EXPERIMENTAL SECTION

Unless specified in particular, the ionic strength of aqueous solutions was fixed at 0.1 M (1 M = 1 mol L^{-1}) with sodium sulfate and all measurements were carried out in a thermostated room at 25 ± 2 °C.

Chemicals. Fatty acid-free HSA, furosemide, and cefaclor were purchased as pure substances from Sigma Chemicals (St. Louis, MO). The molecular mass of HSA was assumed to be 66 500 Da. Testosterone and phthalonitrile were obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Other chemicals (reagent grade) were obtained from Nacalai Tesque Inc. (Kyoto, Japan). *n*-Hexane was purified according to the reported method[47] and then saturated with distilled water prior to interfacial tension, HSS, and CLM measurements. The water used was first distilled and then passed through a Milli-Q system (Millipore), resulting in the specific resistivity of 18.2 MΩ cm. Phosphate buffer (1.0×10^{-3} M) was used as a standard buffer, and it was made from sodium phosphate dibasic and sodium phosphate monobasic salts.

Synthesis of Subphthalocyaninatoboron(III)-Tagged Testosterone. (a) Chloro[subphthalocyaninato]boron(III) (Subpc-Cl). Following the procedure of Meller and Ossko,[16,20] boron trichloride (10 mL, 1 M solution in dichloromethane) was added through a capillary to 10 mL of 1-chloronaphthalene containing phthalonitrile (2.0 g, 0.016 mol) under argon atmosphere. The reaction mixture was stirred under heating at ~120 °C for 6 h. The resulting purple mixture was then washed with methanol and was purified by column chromatography on silica gel using chloroform as eluent, giving 0.46 g (yield 20% on the basis of phthalonitrile) of Subpc-Cl as a deep purple solid: mp > 250 °C; ^{1}H NMR (300 MHz, $CDCl_3$, 298 K) δ 7.88 (dd, 6H, Ar), 8.82 (dd, 6H, Ar).

(b) Androst-4-ne-3-one-17β-oxo[subphthalocyaninato]boron(III) (Subpc-test). Testosterone (1.00 g, 0.003 46 mol) and Subpc-Cl (0.50 g, 0.0012 mol) were refluxed in 25 mL of toluene

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for 12 h. The reaction mixture was cooled to room temperature, and then the solvent was evaporated. The resulting residue was purified by column chromatography on silica gel using a mixture acetone/chloroform (3:7 v/v) as eluent, giving a magenta solid Subpc-test: 0.55 g (yield 67% on the basis of Subpc-Cl); mp >250 °C; ^{1}H NMR (300 MHz, $CDCl_3$, 298 K) δ 0.57−2.42 (m, 25H, test), 3.47 (t, 1H, 17α-C), 5.60 (s, 1H, 4-C), 7.88(dd, 6H, Ar), 8.82(dd, 6H, Ar); MALDI-TOF/MS m/z 682 [M^+], 395 [$(M - test)^+$] (matrix, α-cyano-4-hydroxycinnamic acid); UV−visible (*n*-hexane) λ_{max}/nm (logϵ/M^{-1} cm^{-1}) 555 (4.81), 513 (sh), 298 (4.49). Anal. Calcd for $C_{43}H_{39}BN_6O_2$: C, 75.66; H, 5.76; N, 12.31. Found: C, 76.13; H, 6.04; N, 12.57.

Measurement of Interfacial Adsorption Behavior of Supbc-test Compound. The interfacial adsorption of Subpc-test at the *n*-hexane/water interface was observed by using the HSS apparatus.[25] The *n*-hexane phase (50.0 cm^3) containing Subpc-test compound and an aqueous phase of the same volume were placed into the stirring cell thermostated at 25 ± 0.1 °C. The extent of dispersion of the liquid/liquid system was controlled by changing the rotation rate of the stirrer with the motor speed controller (Nikko Keisoku, SC-5), from 200 to 5000 rpm. The absorption spectrum from 300 to 700 nm of the organic phase, which was continuously separated from the dispersed liquid/liquid system by means of a PTFE phase separator and circulated through the flow cell, was measured at 1.0-s intervals by a photodiode array UV−visible detector (Shimadzu SPD-M6A). The flow cell was a cylindrical quartz cell with an optical path length of 1.0 cm. We could determine the amount of interfacial Subpc-test from the difference between the absorbances of the bulk organic phase under high-speed (5000 rpm) and low-speed stirring (200 rpm).

Interfacial Tension Measurement of HSA Adsorbed at the *n*-Hexane/Water Interface. The interfacial tension, γ, of the *n*-hexane/water interface adsorbed by HSA was measured by the Wilhelmy balance technique with a 10.0 × 10.0 mm filter paper plate (Hybrid Instruments Co.). A glass sample tube (i.d. = 30 mm) was treated with dichlorodimethylsilane for hydrophobic coating of the inner wall to make a flat *n*-hexane/water interface and thermostated with a water jacket at 25 ± 0.1 °C. Into the tube, water (25 mL) and then *n*-hexane (25 mL) were introduced. A measured small volume of an aqueous solution of HSA was added into the aqueous phase with a microsyringe. This procedure was repeated to increase stepwise the concentration of HSA. It took at least 1 h until the interfacial tension reached an equilibrium. The interfacial tension was measured with a precision of ±0.05 mN/m.

UV−Visible Absorption and Circular Dichroism Spectra of Subpc-test/HSA Complexes Formed at the *n*-Hexane/Water Interface. The formation of Subpc-test/HSA complexes at the *n*-hexane/water interface was directly measured spectrophotometrically by the centrifugal liquid membrane-absorption and circular dichroism spectroscopy (CLM-Abs and CLM-CD).[19,20] The principle of the CLM method was described elsewhere.[48] The apparatus for the CLM-Abs and CLM-CD measurements was essentially the same as the one reported previously.[49−51] The CLM cell is a cylindrical cell, which is 3.3 cm in length and 2.1 cm in outer diameter, and has a 1.0-mm-i.d. hole as a sample inlet at the bottom. It was placed horizontally in a sample chamber of a diode array UV−visible spectrophotometer (8453, Agilent) or spectropolarimeter (J-810E, Jasco) and rotated at 10 000 rpm by a speed-controlled motor (NE-22E, Nakanishi Inc.). The *n*-hexane solution of Subpc-test and the aqueous solution of HSA (each 0.500 mL) were introduced into the cylindrical cell using a microsyringe. The sum of UV−visible absorption and CD spectra of the bulk phases and the interface was measured in the range of 300−700 nm.

Effect of Site Ligand on the Formation of Subpc-test/HSA Complexes at the *n*-Hexane/Water Interface. To determine the effect of increasing the site ligand concentration in the aqueous phase on the CD signal of Subpc-test/HSA complexes, the site ligand, furosemide (site I) or cefaclor (site II), was dissolved in water/acetone (95:5 v/v) and added to the aqueous solution with HSA (pH 7.4). The final concentration of acetone in the HSA aqueous solution was less than 1% (v/v). Using the CLM−CD technique, the variation in CD intensity of Subpc-test/HSA complexes formed at the *n*-hexane/water interface at 575 nm with increasing concentration of furosemide or cefaclor was determined.

Other Apparatus. UV−visible and CD spectra in *n*-hexane solution were obtained on a V-570 spectrometer (Jasco) and J-810 spectropolarimeter (Jasco), respectively. ^{1}H NMR spectra were recorded on a Unity 300 spectrometer (Varian). Chemical shifts of ^{1}H NMR spectra were values relative to an internal standard of TMS. Elemental analyses were performed using a CHNS/O analyzer 2400 (Perkin-Elmer). Chromatographic separations were performed on a column with silica gel (Wako Gel C-200). pH values of the aqueous phase were conducted using a F-14 pH meter (Horiba) equipped with a 6366-10D glass electrode. Melting points were determinated with a MP-500D melting point apparatus (Yanako).

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