A Spectroscopic and Microscopic Study on the Extraction Behavior of a Probe Dye, Neutral Red, by Aerosol OT in the Isooctane/Water System

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In the extraction system using Aerosol OT (AOT) in isooctane, the distribution of AOT between the isooctane and aqueous phases depended on the concentration of inorganic electrolytes, salts and/or acids. A colored dye, neutral red, was selected as a probe molecule, which exists in red cationic and yellow nonionic forms in acidic and alkaline regions, respectively. The extraction was investigated in the acidic region in detail. At lower NaCl concentrations, the cationic neutral red existed in the aqueous phase. At higher NaCl concentrations, the cationic neutral red was extracted into the isooctane phase. At intermediate NaCl concentrations, a small amount of third phase appeared in the interfacial region and almost all of the cationic neutral red was extracted into it. Observation by optical microscopy gave geometric information about the third bicontinuous microemulsion phase as well as microemulsions or emulsions formed in the isooctane and aqueous phases.

1. Introduction

Winsor-I, -II, and -III systems originally studied by Winsor are unique systems, which consist of an organic phase, an aqueous phase, and a surfactant that can form both water-in-oil (W/O) and oil-in-water (O/W) microemulsions [1]. The Winsor-I system consists of two phases, that is, an organic phase and an aqueous phase containing O/W microemulsions. The Winsor-II system also consists of two phases, that is, an organic phase containing W/O microemulsions and an aqueous phase. The Winsor-III system consists of three phases, that is, an organic phase, an aqueous phase, and a surfactant-rich phase. In the case of ionic surfactants, the reversible change between Winsor-I and Winsor-II is mainly controlled by inorganic salts added to the aqueous phase; lower and higher concentrations of inorganic salts lead to Winsor-I and Winsor-II, respectively [1]. The Winsor-II system has been used for the extraction of hydrophilic substances, such as water molecules [2] amino acids [3], and inorganic ions [2], and their extraction process
has been discussed in detail [2]. Recently, the extraction of water-soluble biological macromolecules, such as DNA and proteins, using Winsor-II systems has been investigated, where the macromolecules are incorporated into the W/O microemulsions in the organic phases [4,5].

Aerosol OT (sodium bis(2-ethylhexyl) sulfosuccinate, AOT; Figure 1) is one of the well-known surfactants that form W/O microemulsions in nonpolar organic solvents [6]. When the W/O microemulsions are formed artificially in an AOT organic solution by injecting a known amount of water, their size is simply determined by the concentrations of AOT and water [5]. There are a number of studies on W/O microemulsions in an organic phase; for example, the nature of the water molecules and chemical reactions in them [6]. On the other hand, the formation scheme of W/O microemulsions in two-phase systems is complicated, because the organic phase is saturated with water and AOT is distributed between the two phases [7]. Therefore, the size of the W/O microemulsions was determined not only by the concentrations of AOT and water but also by the concentration of inorganic electrolytes (salts or acids) in the aqueous phase [8]. Furthermore, the detailed features of the third surfactant-rich phase appearing in the Winsor-III system are still unknown.

The present study focuses on the effects of pH, salt concentrations, and types of salt on the extraction behavior of a hydrophilic probe molecule by AOT. It also focuses on the geometric and chemical features of the third AOT-rich phase. As the probe molecule, a water-soluble dye, neutral red (NR), was selected because it has a high light-absorptivity and exhibits acid dissociation. It is used as a pH indicator; it changes to its red cationic form (HR\textsuperscript{+}) in an acidic solution and it changes to its yellow nonionic form (R\textsuperscript{0}) in an alkaline solution, as shown in Figure 1. Therefore, the existence and form of the dye are easy to recognize by the naked eye, and quantitative studies can be carried out via absorption spectroscopy.

2. Experimental

2.1 Reagents

AOT (purity ≥ 99%, Fluka), isooctane (2,2,4-trimethylpentane; purity ≥ 99%, Kanto), and neutral red (Wako Pure Chemical) were used without further purification. Water was purified with a Milli-Q system (Milli-Q Sp. Toc., Millipore). AOT was dissolved in isooctane at a concentration of 1.0 × 10\textsuperscript{-3} M (1 M = 1 mol L\textsuperscript{-1}), and this solution was used as the organic phase. No water was added to the AOT solutions in advance. Salts, NaCl, CsCl, and NaSCN, were of analytical reagent grade and were used as received. These salts were individually dissolved in water. To maintain the pH, a phosphate buffer at 2 × 10\textsuperscript{-3} M was used in the pH range of 5 – 9. To adjust the pH, HCl and/or NaOH was added. The NR concentration was set at 1.0 × 10\textsuperscript{-5} M, which was 100-fold lower than the AOT concentration.

2.2 Extraction of neutral red by AOT

Commonly, solvent extraction is carried out by shaking extraction tubes vigorously. However, such
shaking resulted in the formation of much foam because AOT is a surfactant. Instead of shaking, each extraction tube, to which 5.0 mL of an AOT isoctane solution and 5.0 mL of an aqueous NR solution had been added, was stood quietly for a long interval (0.5 – 15 h) in a thermostated water-bath at 25 °C. After standing, the absorption spectra of the isoctane and aqueous phases were measured with a conventional spectrophotometer. The pH of the aqueous phases at equilibrium was measured with a glass electrode and a pH meter.

2.3 Optical microscope observation

For optical microscope observation, a two-phase thin-layer microcell was made as shown in Figure 2a [9]. After a small amount of the aqueous solution was put into the lower part, a small amount of the isoctane solution was gently added to the upper part. Then, the microcell was covered with a coverslip.

An inverted optical microscope (IX-51, Olympus) with an objective lens (UPlanFl, 10×, NA 0.30, Olympus) and a halogenated tungsten lamp (100 W, Olympus) was used for the observation of the interfacial phenomena. Microscope pictures with transmitted light were obtained with a monochromatic CCD camera (WAT-100N, Watec Co., Ltd.), and were transferred as digital pictures to a personal computer for analysis.

The existence of W/O emulsions in the isoctane phase was examined with another thinner microcell shown in Figure 2b. The same inverted microscope was used. For the detection of smaller W/O microemulsions in the isoctane phase, total internal reflection laser light scattering microscopy was applied, which has already been reported in detail [10,11]. Briefly, 532 nm cw-laser light (1.5 mW, Thrush Co., Ltd.), focused by a lens, was directed at about 75° (larger than the critical angle, 73°) onto the isoctane/water interface through a rectangular prism (1 × 1 × 1 cm) that was placed on the coverslip.

All of the microscope observations were carried out at 20 ± 2 °C.

3. Results and Discussion

3.1 Effect of standing time on the neutral red extraction

The standing time was varied in the range of 0.5 – 15 h in the presence of 0.1 M NaCl and 0.1 M HCl; under this condition, about 80% of NR was extracted into the isoctane phase. The extraction ratio was almost constant after a standing time of 10 – 15 h, and thus a standing time of 10 h was adopted.
3.2 Effect of the NaCl concentration on the neutral red extraction

AOT has a sulfonate group and thus it is an anion independent of pH. The distribution ratio of AOT between the isooctane and aqueous phases, $[\text{AOT}]_o/[\text{AOT}]_w$, increases with an increase in the inorganic electrolyte (salt or acid) concentrations in the aqueous phase, where the subscripts o and w denote the organic (isooctane) and aqueous phases, respectively [6,8]. The above-mentioned Winsor-II system corresponds to the extraction system, and thus a higher electrolyte concentration is necessary.

The acid-dissociation constant ($K_a$) for NR was reported to be $10^{-6.8}$ [12]. Therefore, NR changes to its cationic form (HR$^+$) under acidic conditions and it shows an attractive interaction with anionic AOT, whereas it changes to its nonionic form, which has a weak interaction with AOT, under alkaline conditions.

At first, the extraction of NR was investigated by changing the NaCl concentration range from 0 to 0.2 M in the presence of 0.1 M HCl. At this HCl concentration, NR changes to its cationic form. Figure 3 shows the resultant photographs. At lower NaCl concentrations ($\leq 0.03$ M), NR exists in the aqueous phase near the interface. At higher NaCl concentrations ($\geq 0.07$ M), NR was extracted into the isooctane phase, as was expected. At 0.05 M, a third phase appeared at the interface and NR was found to exist only in this phase. When the AOT concentration increased, the thickness of the phase increased. This interesting interfacial phenomenon will be discussed in section 3.6.

3.3 Absorption spectra of neutral red in isooctane in the visible region

Absorption spectra in the visible region can be used for quantitative analysis as well as for qualitative discussion. The extraction of NR by $1.0 \times 10^{-3}$ M AOT was carried out at pH 0.34 and 13.21 with 0.2 M NaCl, and the absorption spectra of the isooctane phases after extraction are shown in Figure 4. It also includes the absorption spectra of the original aqueous solutions.

Under acidic conditions (Figure 4a), the absorption maximum for the isooctane phase (529 nm) is close to that for the aqueous solution (533 nm), and the shapes of the absorption spectra are similar. These facts suggest that the solvation of HR$^+$ is similar in both phases. Therefore, HR$^+$ is considered to be solvated by water molecules incorporated in the AOT W/O microemulsions in the isooctane phase. The hydrodynamic radius ($r_{em}$) of the AOT W/O microemulsion droplets depends on the NaCl concentration in the heptane/water system, and its values are estimated to be 7.7 and 4.8 nm for 0.1 and 0.2 M NaCl, respectively [6]. These values would be approximately valid in isooctane.

AOT W/O microemulsions can be artificially prepared by injecting water into AOT organic solutions. In heptane, the $r_{em}$ of...
AOT W/O microemulsion droplets is empirically related to the ratio of the water and AOT concentrations, \( w_0 = \frac{[H_2O]}{[AOT]} \), as [3]:

\[
    r_{em} = 0.16w_0 + 1.2
\]  

(1)

This equation qualitatively means that the size of the W/O microemulsion droplets is approximately controlled by a geometric requirement, that is, the relationship between the number of water molecules in each droplet and the number of AOT molecules surrounding the droplet [13]. Therefore, Eq. (1) should be applicable to isooctane solutions. When \( w_0 = 10, 20, \) and \( 30, \) \( r_{em} \) was calculated to be 2.8, 4.4, and 6.0 nm, respectively, using Eq. (1), which correspond to the above-mentioned values in the two-phase system (4.8 and 7.7 nm).

To discover the nature of the incorporation of HR\(^+\), AOT W/O microemulsions were artificially prepared by injecting aqueous NR solutions containing 0.1 M NaCl and 0.1 M HCl into 1.0 \( \times \) \( 10^{-3} \) M AOT isooctane solutions. Figure 5 displays the resultant absorption spectra, which are shown as molar absorption coefficients (\( \varepsilon_{HR} \)). Even when \( w_0 \) was changed to 10, 20, and 30, the absorption peaks (531 – 532 nm) and maximum \( \varepsilon \) values were barely affected. Also, the shapes of the absorption spectra are similar to those of Figure 4a, implying that HR\(^+\) is incorporated into the AOT W/O microemulsions in isooctane.

On the other hand, under alkaline conditions (Figure 4b), the absorption maximum for the isooctane phase (425 nm) is far from that for the aqueous solution (450 nm). This fact suggests that R\(^0\) solvation in each phase is different. Therefore, R\(^0\) is considered to be solvated by isooctane molecules, that is, it simply dissolves in the isooctane phase. In fact, NR can be dissolved in isooctane without AOT and the dissolved NR shows an absorption spectrum similar to that of the isooctane phase in Figure 4b. In this alkaline region, the absorption spectra of the aqueous phases before and after the extraction and the absorption spectrum of the isooctane phase after the extraction were measured. The molar absorption coefficients (\( \varepsilon_R \)) of R\(^0\) in the isooctane phase were calculated from these three spectra using the principle of mass balance.

### 3.4 Effect of pH on the neutral red extraction

In the present extraction system, extractability is affected not only by the AOT concentration but also by the nature of the AOT W/O microemulsion. The size of the W/O microemulsion droplets is an important factor, and thus salt solutions of a fixed concentration (0.2 M NaCl) was used to keep the size of the AOT W/O microemulsion droplets constant.

As mentioned above, the \( K_a \) value for NR is \( 10^{-6.8} \) and thus the extractability (\( %E \)) should depend on the pH. Thus, the extraction was carried out over a pH range of 0.3 – 13.2. The aqueous phase sometimes became slightly cloudy after extraction, and thus the \( %E \) values were calculated with the absorption spectra of the isooctane phases after the extraction. Both HR\(^+\) and R\(^0\) were extracted into the isooctane phase, and thus the \( \varepsilon_{HR} \) and \( \varepsilon_R \) values in the isooctane phase at 529 and 425 nm (peaks of HR\(^+\) and R\(^0\), respectively) were used for the calculation of HR\(^+\) and R\(^0\) concentrations in the isooctane phase.

Figure 6 shows the dependence of \( %E \) on the pH. In the acidic region, \( %E \) values are relatively high...
and almost constant, indicating that the nature of the AOT W/O microemulsion is barely affected by the pH. Then, they rapidly fall around pH 7 and they are almost constant again in the alkaline region.

Under the conditions of Figure 6, the third phase did not appear and thus the pH dependence of $%E$ was analyzed with a distribution model for a two-phase system. The distribution ratio of $HR^{+}$ in the acidic region should be constant because of the constant concentrations of AOT and the salt (NaCl), and thus this is defined as an apparent distribution constant, $K_{D}'$. The distribution constant of $R_0^0$ is defined as $K_{D}$. Then, the overall distribution ratio, $D$, of NR can be expressed as:

$$D = \frac{[HR^{+}]_0 + [R_0^0]_0}{[HR^{+}]_w + [R_0^0]_w} = \frac{K_{D} + K_{D}'[H^{+}]_w}{1 + K_{D}'[H^{+}]_w} \quad (2).$$

$%E$ is expressed together with the volumes of isoctane and aqueous phases, $V_o$ and $V_w$, respectively, as:

$$%E = \frac{100D}{D + V_w/V_o} \quad (3).$$

The pH dependence of $%E$ in Figure 6 is fitted to Eqs. (2) and (3) by the least-squares method with $K_a = 10^{-6.8}$ and the obtained fitting curve is shown as the line in Figure 6. The experimental data were somewhat scattered but the line agrees well with the data, implying that the above-mentioned assumption is valid. The $K_{D}'$ and $K_{D}$ values were determined to be 1.7 and 0.41, respectively.

### 3.5 Effect of salts on the neutral red extraction

As shown in Figure 3, the NaCl concentration affects the extraction of NR considerably. The type of inorganic salt is known to affect the nature of W/O microemulsions and thus the effects of some inorganic salts were investigated.

Other salts, CsCl and NaSCN, were used independently. Figure 7 shows the results. The extractability in the presence of NaSCN is almost the same as that for NaCl, meaning that the anions only slightly affect the nature of the AOT W/O microemulsions. On the other hand, the extractability with CsCl is much higher than that with NaCl in the range of 0.01 – 0.07 M, suggesting that the cations significantly affect the formation of the AOT W/O microemulsions.

Figure 8 shows an illustration of the AOT W/O microemulsion. There is an electrostatic repulsion force among anionic AOT molecules of the W/O microemulsion, and cations can weaken the repulsion force. The crystal ionic radius of Na$^+$ is smaller than that of Cs$^+$, but the radius of hydrated Na$^+$ (0.18 nm) is larger than that of hydrated Cs$^+$ (0.12 nm) [14]. The alkali metal ions are highly hydrated and few water molecules of hydration are lost by the ionic interaction. Therefore, a smaller hydrated Cs$^+$ shows a larger attractive interaction with anionic AOT, and thus the AOT W/O microemulsions are formed at lower
concentrations, as shown in Figure 7.

As mentioned in section 3.3, the $r_{em}$ value decreases with an increase in the salt concentration in the Winsor-II system. This phenomenon is explained as follows. When the cation concentration increases, the electrostatic repulsion force among AOT molecules decreases. This causes a reduction in the volume of the hydrophilic part of the AOT molecules, leading to smaller W/O microemulsions by a geometric effect [6]. When the salt concentration increases at a constant concentration of AOT, the total volume of water in the W/O microemulsions decreases. This would cause a decrease in the total amount of HR$^{+}$ incorporated in the AOT W/O microemulsions. This phenomenon typically appears in the CsCl case as shown in Figure 7.

There is another possibility that the cationic HR$^{+}$ was extracted with the anionic AOT by an ion-association extraction. Such an ion-association means that HR$^{+}$ is directly associated with anionic AOT, and that the nonionic associates so formed are extracted into the organic phase. The associates are not incorporated into the W/O microemulsion but are solvated by isooctane molecules. If this ion-association extraction occurs, the effect of the salt would be a salting-in or salting-out effect. Anions have a larger salting-out effect than cations, and the order of the salting-out effect is known to be the Hoffmeister series; $\text{Cl}^{-} > \text{SCN}^{-}$ [15,16]. This order means that $\text{Cl}^{-}$ has a higher salting-out effect than $\text{SCN}^{-}$, but the results in Figure 7 do not show this, meaning that the extraction is not due to ion-association.

3.6 Microscope observation at the isooctane/water interface

As shown in Figure 3, HR$^{+}$ exists near the interface in the aqueous phase in the lower NaCl concentration range ($0 - 0.03$ M). This fact suggests that AOT O/W microemulsions are formed in the aqueous phase (Winsor-I) and that HR$^{+}$ was incorporated into them. Their rise to near the interface implies that larger O/W microemulsions are formed and they undergo a buoyancy force caused by the density difference. The interface in the absence of NaCl was observed by optical microscopy with transmitted light. At first, there are no substances at the interface, but many bubbles appeared and grew gradually. An interfacial picture at $15$ h after the sample preparation is shown in Figure 9a. Their size is in a range of $1 - 5$ $\mu$m, and they moved at the interface horizontally by Brownian motion. In the isooctane and aqueous phases, no substances were observed. These bubbles are considered to correspond to AOT O/W emulsion droplets formed in the aqueous phase near the interface.

Figure 3 shows that HR$^{+}$ exists only at the interface at an NaCl concentration of $0.05$ M (Winsor-III). A microscope picture of this interface is shown in Figure 9b. Larger amoeba-like moving...
substances were observed at the interface from an early period (5 min). They gathered together and finally some larger substances existed at the interface; their shape was always changing. These moving substances correspond to the third AOT-rich phase. The physical structure of this phase is thought to be bicontinuous, as shown in Figure 10 [17]. It consists of AOT, isooctane, and water molecules, and contains a much broader interface. To satisfy electroneutrality, the counterion Na\(^+\) must be incorporated into this phase. The anionic AOT is one of the essential components of this phase, and therefore other cations can be incorporated into this phase by exchanging with Na\(^+\). This phase can have both hydrophobic and hydrophilic natures. It is known that the randomized bicontinuous microemulsion phase does not show birefringence, whereas partially-ordered liquid crystal phases show birefringence. To confirm this, two polarizing plates were placed in crossed Nichol configuration (crossed-polarized light); one was placed between the light source and the microcell, and the other was placed between the objective lens and the tube lens. The third phase did not show any bright images, suggesting a bicontinuous microemulsion phase.

The AOT O/W emulsions and the AOT-rich phase at the interface shown in Figure 9a and b were also observed without NR, indicating that the formations of these substances are intrinsic phenomena in the systems consisting of AOT, isooctane, and the aqueous phases.

3.7 AOT W/O microemulsions in the isooctane phase

Generally, microscope observation with transmitted light can detect substances larger than about 100 nm, but laser light scattering microscopy can detect substances larger than about 10 nm. The detection limit of the former and latter methods is restricted by the diffraction limit of light and the efficiency of light scattering \((\sigma_s)\), respectively. \(\sigma_s\) of scattering particles can be expressed as:

\[
\sigma_s \propto \frac{NV_p^2}{\lambda^4} \left( \frac{n_p^2 - n_m^2}{n_p^2 + 2n_m^2} \right)^2
\]  \hspace{1cm} (4),

where \(N\) is the number density of the particles, \(V_p\) is the volume of the particle, \(\lambda\) is the wavelength, \(n\) is the refractive index, and subscripts p and m denote particle and medium, respectively [18]. The isooctane phase and the isooctane/water interface were observed by total internal reflection laser light scattering microscopy.

In the Winsor-I system, AOT O/W microemulsions will be formed in the aqueous phase, but the transient formation of larger W/O emulsions in the organic phase has been observed [7]. In the present study, some larger W/O emulsions were also detected in the isooctane phase near the interface with 0.1 M HCl (no NaCl), which was consistent with the previous results [7].
In the Winsor-II system, smaller W/O microemulsions were expected. For example, their hydrodynamic radii \( r_{em} \) are 7.7 and 4.8 nm for 0.1 and 0.2 M NaCl, respectively, as reported in section 3.3. Laser light scattering microscopy was applied to the isooctane phase near the interface, and the resultant picture is shown in Figure 11. In this figure, the white line corresponds to the path of the focused laser light. The brightness of the laser path in Figure 11 is much stronger than that in the isooctane phase near the interface in the absence of AOT. This fact means that many smaller scattering substances, that is, W/O microemulsion droplets, exist and they move vigorously. If the position of a single microemulsion droplet can be measured as a function of time, the analysis of its Brownian motion enables one to determine its hydrodynamic radius [7]. However, the present W/O microemulsions are too small and move too fast to detect any individual positions. A similar result to Figure 11 was obtained in the presence of 0.2 M NaCl instead of 0.1 M NaCl. No substances were observed by optical microscopy with transmitted light, suggesting that the size of the observed W/O microemulsion droplets is in the 10–100 nm range.

**Conclusion**

The present study investigated the extraction behavior of a colored dye, neutral red, by AOT in the isooctane/water system using spectrophotometry, which gave the chemical forms, the solvation, and the concentration of neutral red. The pH dependence of \( %E \) was quantitatively analyzed and the effects of the concentration and the types of inorganic salts added to the aqueous phase were interpreted qualitatively. Furthermore, observation by optical microscopy near the isooctane/water interface clarified the geometrical natures of the AOT O/W emulsions in the aqueous phase, the AOT W/O microemulsions in the isooctane phase, and the AOT-rich bicontinuous microemulsion phase at the interface.

The basic knowledge obtained in the present study will be useful for the extraction of hydrophilic substances, such as biological compounds, with W/O microemulsions (Winsor-II). Furthermore, the surfactant-rich bicontinuous microemulsion phase appearing in the Winsor-III system has both hydrophobic and hydrophilic natures and includes a much broader interface. Therefore, this phase would show specific functions in the extraction system, such as a high dissolving ability, a high concentrating ability, and an effective reaction field. More detailed investigation should be carried out in the future.

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**References and footnotes**