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Selina L. Günther[a]; David C. Stuckey[a]
[a] Department of Chemical Engineering, Imperial College London, London, UK

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Extraction of Human IgG4 Monoclonal Antibodies Using AOT- and HDEHP-Isooctane Reverse Micelles

Selina L. Günther and David C. Stuckey
Department of Chemical Engineering, Imperial College London, London, UK

The extraction of 0.05 and 1 mg mL^{-1} human IgG4 using reverse micelles (RMs) formed with anionic surfactants AOT or HDEHP in isooctane was evaluated. For both surfactants the use of 1 mg mL^{-1} IgG4 resulted in higher forward extraction (FE), and generally better backward extraction (BE) yields than 0.05 mg mL^{-1} IgG4, achieving optimum FE and BE yields at FE pHs of 5 at 3.13 mM AOT and 6 at 1.56 mM HDEHP. IgG4 precipitation at the interface was observed at the lower pHs during FE which appeared to cause low overall extraction yields. Water content analysis revealed AOT-RMs were much bigger than HDEHP-RMs.

Keywords AOT; backward extraction; forward extraction; HDEHP; isooctane; monoclonal antibodies; reverse micelles

INTRODUCTION

Due to the growing need for large-scale separation of proteins and other biopolymers from fermentation and cell culture broths, a readily scalable continuous liquid-liquid extraction technique for the separation of proteins with lower costs than chromatography would be highly desirable, and liquid-liquid extraction using reversed micelles (RMs) might serve this purpose. A RM is a nanometer-scale droplet of an aqueous solution, stabilized in an apolar environment by the presence of surfactant at the interface. It has been demonstrated that many proteins can be solubilized in RM solutions of apolar solvents, without denaturation or loss of function (FE) (1–3). It has also been observed that these proteins can be transferred from a micellar phase, back into an aqueous phase (BE). Therefore, the use of RMs to separate proteins has become of great interest in the last two decades.

The FE and BE has been shown to depend on several system parameters such as, pH, ionic strength, salt type and concentration, surfactant type and concentration, the type of solvent used, and the presence of cosurfactant; where the pH and the ionic strength appear to be the two parameters which dominate the process of micellar extraction. The system parameters can be adjusted and optimized, which will enable the degree of hydrophobic, electrostatic, and steric interactions between the proteins and the RMs to be successfully monitored, and result in protein purification; good results have been observed in terms of separation time, extraction yield, and protein activity recovery (4,5). As a result, fundamental studies of the factors determining selective separation of biomolecules are necessary to establish correlations between the physiochemical properties of the proteins and the RM system.

To date, RMs have shown great potential in the extraction of low molecular weight proteins, where the extraction of proteins such as ribonuclease A, cytochrome *c*, and lipase have been researched in great depth (6–12). Even though selective solubilization has been demonstrated and studied in great depth since the 1980s, many of the results published are contradictory. In addition, very few high molecular weight proteins such as antibodies and monoclonal antibodies (MAbs) have been examined. The group of Speiser were the first to show that RMs could be utilized as carriers for drugs (13). Gerhardt and Dungan (14,15) investigated the stability and structure as well as changes in the structure of protein molecules and water-in-oil (W/O) microemulsion aggregates using the large protein immunoglobulin G (IgG, molecular weight (MW) 155000 Da) and an equivolume oil/water mixture composed of brine, sulfosuccinic acid bis[2-ethylhexyl]ester (sodium salt) (AOT), and isooctane. Su and Chiang (16) investigated RM extraction to separate IgG from bovine colostral whey. Their objective was to obtain a purified product with immunological activity. Kuo (17) examined the effect of parameters such as pH, ionic strength, salt type, initial protein concentration, surfactant concentration, temperature, and hydrodynamics on the kinetics of antibody extraction. Lan (18) examined the feasibility of extracting MAbs from both a model buffer solution and fermentation broth using RMs; the influence of pH,

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Address correspondence to Selina L. Günther or David C. Stuckey, Chemical Engineering Department, Prince Consort Road, London, SW7 2AZ, United Kingdom. Tel.: +44 (0) 20 759 45591; Fax: +44 (0) 20 759 45629. E-mail: s.gunther05@imperial.ac.uk; d.stuckey@ic.ac.uk

temperature, surfactant concentration, and salt concentration on the yield of FE and BE was investigated.

Hence, until now, research carried out on the extraction of proteins using RMs has largely focused on the system parameters affecting FE; while some has also investigated the kinetics of extraction (19–22). Therefore, the objective of this work was to study the effects of system parameters such as pH, surfactant type (AOT and HDEHP) and concentration, and MAb concentration on both FE and BE in order to define the optimal extraction conditions and whether RM extraction using isooctane is feasible for the extraction of MAbs. In addition, we investigated whether surfactant type influences extraction yield and if the MAb concentration affects the results obtained. Finally, the effect of water content on the size of RMs was studied in isooctane.

MATERIALS AND METHODS

Bis(2-ethylhexyl)sulfosuccinate sodium salt (AOT), bis(2-ethylhexyl)phosphate (HDEHP), 2,2,4-trimethylpentane (isooctane: $\rho = 0.6920\,\mathrm{g\,mL^{-1}}$; Viscosity = 0.50 cP), sodium chloride, hydrogen chloride, sodium hydroxide, potassium chloride, acetic acid, sodium acetate, sodium bicarbonate, sodium carbonate, sodium phosphate dibasic, and sodium phosphate monobasic were obtained from Sigma-Aldrich Company Ltd. (Dorset, UK) and were of analytical grade. Potassium phosphate dibasic and potassium phosphate monobasic were Anal-R grade from Merck (Dorset, UK). Acetone was purchased from Fisher Scientific Ltd. (Leicestershire, UK) and was of analytical grade. The disinfectant Virkon was purchased from VWR International Ltd. (Leicestershire, UK). The monoclonal antibody used throughout this work was pure human IgG4 (pI = 7.2–7.7; MW = 146900 Da; $10\,\mathrm{mg\,mL^{-1}}$) in phosphate/NaCl buffer solution, which was kindly donated by Cambridge Antibody Technology (Cambridge, UK). The reagents for the Karl Fischer Automatic Titration to determine the water content of a RM phase sample were HYDRANAL® Coulomat A (anode reagent) and HYDRANAL® Coulomat CG (cathode reagent), and were purchased from Riedel-de Haën (Seelze, Germany) and were of analytical grade.

Extraction Procedures

Two sets of conventional FE and BE procedures were carried out; FE was carried out for $0.05\,\mathrm{mg\,mL^{-1}}$ IgG4 between pHs 4–11. After which, $1\,\mathrm{mg\,mL^{-1}}$ IgG4 was forward extracted between pHs 5–7 for AOT and pHs 6–8 for HDEHP. Both sets of FE were then back extracted at pH 8. This was done using AOT and HDEHP, where all extraction procedures were conducted in duplicate at least. All coefficients of variation (COV) were determined based on at least three repetitions. The centrifugation speeds used for FE and BE were chosen based on previous research (11,17–19). The FE (E_f) and BE percentage (E_b) were calculated using Eqs. (1) and (2) respectively (23), while the OE percentage (E_o) was calculated using Eq. (3);

$$E_f[\%] = \frac{{C_{FE}}^{RM}}{{C_{Initial}}^{Aq}} 100 \quad (1)$$

$$E_b[\%] = \frac{{C_{BE}}^{Aq}}{{C_{FE}}^{RM}} 100 \quad (2)$$

$$E_o[\%] = \frac{{C_{BE}}^{Aq}}{{C_{Initial}}^{Aq}} 100 \quad (3)$$

where ${C_{FE}}^{RM}$ is the amount of forward extracted protein in the reversed micellar organic phase ($\mathrm{mg\,mL^{-1}}$), ${C_{Initial}}^{Aq}$ is the amount of protein in the initial aqueous phase ($\mathrm{mg\,mL^{-1}}$), and ${C_{BE}}^{Aq}$ is the amount of backward extracted protein in the aqueous phase ($\mathrm{mg\,mL^{-1}}$).

(a) Forward Extraction The RM phase consisted of an anionic surfactant, AOT or HDEHP, in isooctane, and the surfactants were used without further purification. Aqueous solutions for FE were prepared with 80% 0.1 M NaCl and 20% 0.1 M buffer solutions made up to the required pH (acetate, phosphate, and carbonate buffers, pHs 4–11). FE was performed by contacting the same volume (750 µL) of an aqueous phase containing $0.05\,\mathrm{mg\,mL^{-1}}$ IgG4 and (2 mL) of an aqueous phase containing $1.0\,\mathrm{mg\,mL^{-1}}$ IgG4, and an RM phase containing 1.56 to 50 mM AOT or HDEHP in isooctane, in a 1.5 mL (for $0.05\,\mathrm{mg\,mL^{-1}}$ IgG4) and a 12 mL (for $1.0\,\mathrm{mg\,mL^{-1}}$ IgG4) test tube. The test tube was then mixed by rotary inversion at 39 rpm for 40 min. Phase separation was achieved by centrifugation for 5 min at 3000 rpm. The COV for the entire FE process was ±1.2%.

(b) Backward Extraction BE was performed mixing the same volume (approximately 750 µL for $0.05\,\mathrm{mg\,mL^{-1}}$ IgG4 and approximately 2 mL for $1.0\,\mathrm{mg\,mL^{-1}}$ IgG4) of the protein RM phase with the same volume of 90% KCl solution and 10% 0.1 M buffer solution at the required concentration and pH by rotary inversion at 39 rpm for 2 hrs 50 min. The conditions used were 2 M KCl/potassium phosphate buffer pH 8 (the BE parameters were chosen based on previous research, showing that these are the ideal BE parameters when extracting IgG4 (17,18)). Phase separation was achieved by centrifugation for 5 min at 12000 rpm. The COV for the entire BE process was ±1.2%.

Protein Assay

Generally, protein concentration can be measured by absorption at 280 nm. However, past research showed that turbidity can be a problem at 280 nm due to colloidal matter (surfactant molecules), and more significantly, due to a

change in temperature (24). RM size decreases with decreasing temperature by expelling excess water into the organic phase, hence causing it to become turbid, and the use of a blank did not solve this problem since it was very difficult to generate identical conditions between the sample and the blank, and to achieve reproducible results (24). Goklen (25) suggested correcting protein absorption at 280 nm by the absorption at 310 nm, which provides a rough estimate of the turbidity. This procedure was used by several researchers (11,24) and found to be useful for both aqueous and RM solutions, and thus was adopted for this work. Hence, the concentration of human IgG4 in the aqueous phases was determined by using a Shimadzu UV 2101 Spectrophotometer at 280–310 nm. The RM phase was not analyzed using the spectrophotometer as these generated negative values. Antibody concentrations in the aqueous samples were determined using calibration curves, and were generated for each buffer used.

Measurement of pH

The pH of each solution was measured using a Hanna Instruments laboratory pH-123 Microprocessor pH/mV/°C Meter with a simple junction combined pH/reference electrode (VWR International UK). The accuracy of the pH and temperature measurements was ±0.01 pH and ±0.5°C, respectively.

Water Detection in Solvent Phase

The water content of the RM phase at the end of FE was determined by Karl Fischer titration using a Mettler DL37 KF Coulometer. A small (5 µL to 0.1 mL) sample was injected into the titrator using a microsyringe, and the COV for 1 mg water was <0.3%, where the minimum resolvable step was 0.1 µg water and the detection limit was 10 µg. The water concentration in the RM samples was calculated using Eq. (4) (24);

$$W_o = \frac{[Water]}{[Surfactant]} = \frac{(c_w - c_s)\rho_{RM}}{M_w[AOTorHDEHP]_{RM}} \qquad (4)$$

where c_w is the water concentration in the sample (ppm), c_s is the water concentration in the pure solvent (ppm), M_w is the molecular weight of water (18.02 g mol^{-1}), ρ_{RM} is the RM sample density (g cm^{-3}), and [surfactant]$_{RM}$ is the surfactant concentration in the RM sample (mM). The size of the RMs was calculated using Eq. (5) (24);

$$R_{wp} = (3.89 + 1.55W_o)10^{-10}m \qquad (5)$$

RESULTS AND DISCUSSION

Throughout this work, two anionic surfactants were used. The first surfactant used was sodium bis(2-ethylhexyl)sulfosuccinate (Aerosol-OT or AOT), a double-tailed anionic surfactant. The second was bis(2-ethylhexyl)phosphate (HDEHP) and was used to form a sodium bis(2-ethylhexyl)phosphate (NaDEHP) RM phase. NaDEHP was chosen as it has the same hydrocarbon tail as AOT, but a different polar head; and because the phase separation of NaDEHP is much faster than that of AOT. In addition, the surfactant can be readily recycled, and the RMs of its sodium salt, NaDEHP, have been used successfully for protein (cytochrome-c and α-chymotrypsin) extraction achieving high overall recoveries (10). Furthermore, NaDEHP RMs have been used in the hydrometallurgical industry because of their unique characteristics (short phase separation time, a very high recovery rate, easy recycle) (26). To date, hardly any work has been carried out on the extraction of IgG4 using AOT RMs, and, to our knowledge, no work has been published on the extraction of IgG4 using NaDEHP RM systems. All results presented here were from single experiments, with all analytical measurements being carried out in duplicate at least. Only "doubtful" data points were repeated several times, and an average value is presented. All experiments were carried out at 25°C.

Forward Extraction

The lack of usable data from the FE using various AOT concentrations (between 1.56 and 50 mM) at pHs 4–11 and various HDEHP concentrations (between 1.56 and 50 mM) at pHs 8, 9 and 11, of 0.05 mg mL^{-1} IgG4 (data not included) made it hard to come to any firm conclusions on optimal FE parameters, which was thought to be due to the small initial sample volumes used as well as the low IgG4 concentration. This is why 1 mg mL^{-1} IgG4 was

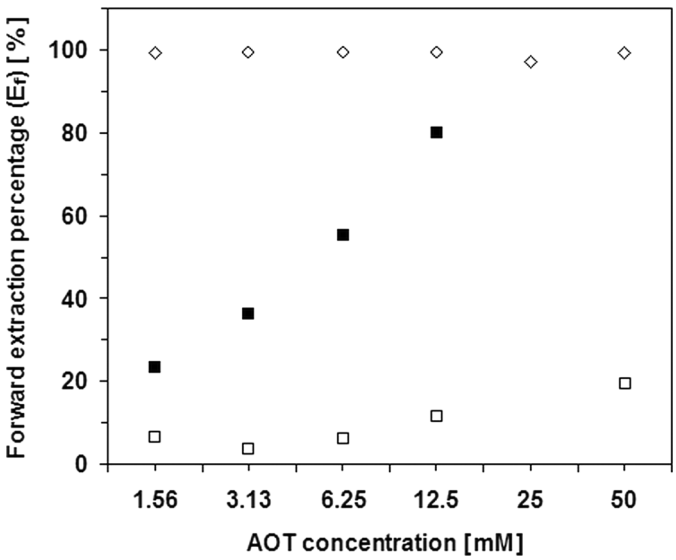

FIG. 1. Effect of AOT concentration on the FE of 1 mg mL^{-1} IgG4 at pH 5 (◇) acetate buffer, and at pHs 6 (■) and 7 (□) phosphate buffer with isooctane.

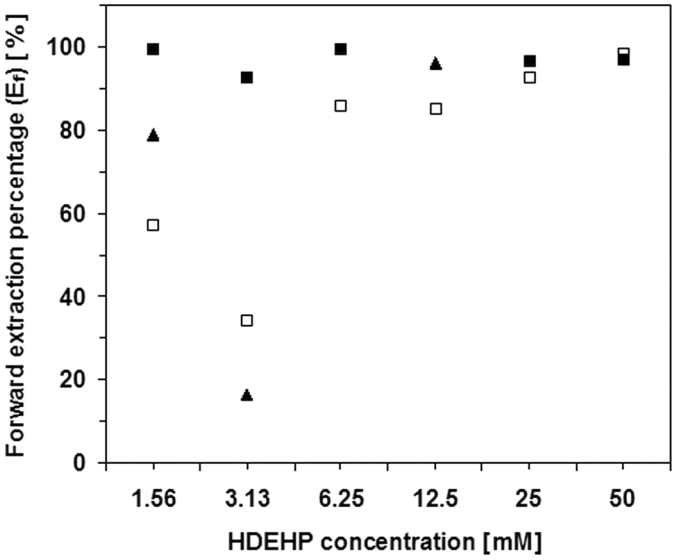

FIG. 2. Effect of HDEHP concentration on the FE of 1 mg mL^{-1} IgG4 at pHs 6 (■), 7 (□) and pH 8 (▲) phosphate buffer with isooctane.

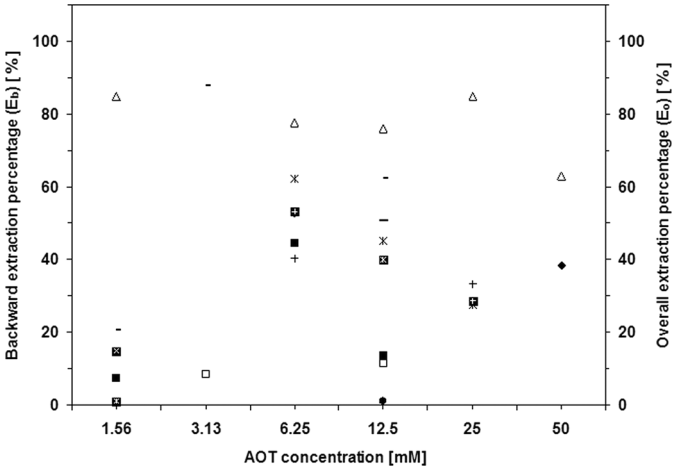

FIG. 3. Effect of AOT concentration on the BE using potassium phosphate buffer at pH 8 of 0.05 mg mL^{-1} IgG4, for a FE at pHs 4 (◆) and 5 (■) acetate buffer, at pHs 7 (●) and 8 (▩) phosphate buffer, and at pHs 9 (▩), 10 (✚) and 11 (–) carbonate buffer with isooctane and OE using potassium phosphate buffer at pH 8 of 0.05 mg mL^{-1} IgG4, for a FE at pHs 4 (◇) and 5 (□) acetate buffer, at pH 6 (△) phosphate buffer, and at pHs 9 (∗), 10 (+) and 11 (-) carbonate buffer with isooctane.

tested, using greater sample volumes and for a smaller range of pHs, and this new data is shown in Figs. 1 and 2. Figure 1 shows the effect of AOT concentration on FE (E_f) of 1 mg mL^{-1} human IgG4 with isooctane at pHs 5–7, while Fig. 2 shows the effect of HDEHP concentration on FE (E_f) of 1 mg mL^{-1} human IgG4 with isooctane at pHs 6–8. In Fig. 1, the FE decreased with increasing pH; at pH 6 and pH 7 the E_f mainly increased with increasing AOT concentration as positively charged regions were taken-up by AOT, and the highest E_fs (97 to 99.3%) were at pH 5 at all AOT concentrations, reaching the highest E_f (99.3%) at pH 5 and 50 mM AOT. These findings are overall in accordance with those found by Lan (18) who studied the purification of whole IgG4 molecules using RMs, examining the relationship between the AOT concentration (between 5 and 50 mM) and the FE yield at different pHs (5, 6, and 7). In Fig. 2 the FE also decreased with increasing pH; at pHs 6–8, the E_f decreased with increasing HDEHP concentration between 1.56 and 3.13 mM, and at pHs 6 and 7 it increased with increasing HDEHP concentration between 12.5 and 50 mM as positively charged regions were taken-up by anionic HDEHP. The highest E_fs (92 to 99%) were at pH 6 at nearly all HDEHP concentrations between 50 and 1.56 mM, with the highest E_f (99%) at 1.56 and 6.25 mM. These findings suggest that the FE of human IgG4 is successful using both surfactants with isooctane, where pH 5 is optimal with AOT and pH 6 with HDEHP.

Backward and Overall Extraction

The effect of AOT concentration on the BE and overall extraction i.e., OE (E_b and E_o) of 0.05 mg mL^{-1} human IgG4 is shown at FE pHs 4–11 in Fig. 3; the effect of AOT concentration on the BE and OE (E_b and E_o) of 1 mg mL^{-1} human IgG4 with isooctane is shown at FE pHs 5–7 in Fig. 4; the effect of HDEHP concentration on the BE and OE (E_b and E_o) of 0.05 mg mL^{-1} human IgG4 with isooctane is shown at FE pHs 4–11 in Fig. 5; and the effect of HDEHP concentration on the BE and

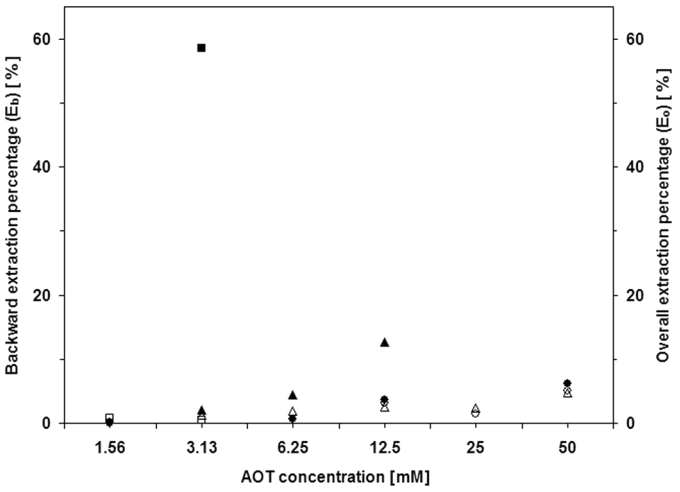

FIG. 4. Effect of AOT concentration on the BE using potassium phosphate buffer at pH 8 of 1 mg mL^{-1} IgG4, for a FE at pH 5 (■) acetate buffer, and at pHs 6 (▲) and 7 (●) phosphate buffer with isooctane and OE using potassium phosphate buffer at pH 8 of 1 mg mL^{-1} IgG4, for a FE at pH 5 (□) acetate buffer, and at pHs 6 (Δ) and 7 (○) phosphate buffer with isooctane.

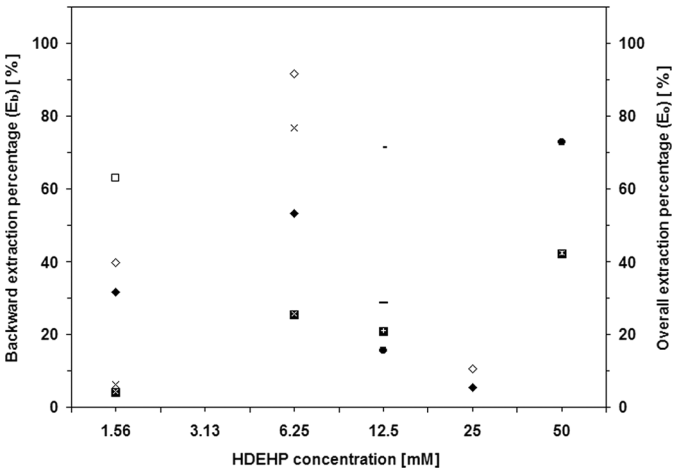

FIG. 5. Effect of HDEHP concentration on the BE of 0.05 mg mL^{-1} IgG4 using potassium phosphate buffer at pH 8, for a FE at pH 4 (◆) acetate buffer, at pHs 7 (●) and 8 (▪) phosphate buffer, and at pHs 9 (▪), 10 (▪) and 11 (–) carbonate buffer with isooctane and OE of 0.05 mg mL^{-1} IgG4 using potassium phosphate buffer at pH 8, for a FE at pHs 4 (◇) and 5 (□) acetate buffer, at pH 8 (×) phosphate buffer, and at pH 11 (-) carbonate buffer with isooctane.

OE (E_b and E_o) of 1 mg mL^{-1} human IgG4 with isooctane is shown at FE pHs 6–8 in Fig. 6.

BE was carried out at a pH>pI of IgG4 (pI = 7.2–7.7) using potassium phosphate buffer (pH 8) with isooctane. The BE parameters were the optimal BE parameters based on previous research (17,18). Even though the E_os shown in Fig. 4 were extremely low compared to those in Fig. 3, the same general conclusions apply; they all showed that the results obtained for a FE at pH 6 seemed to be the best

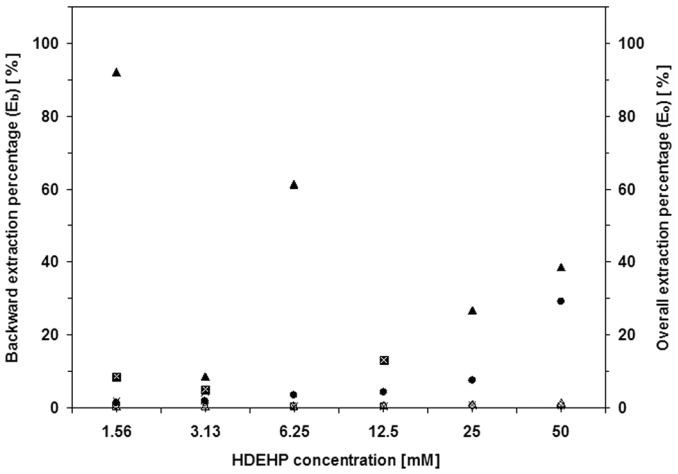

FIG. 6. Effect of HDEHP concentration on the BE of 1 mg mL^{-1} IgG4 using potassium phosphate buffer at pH 8, for a FE at pHs 6 (▲), 7 (●) and 8 (▪) phosphate buffer with isooctane and OE using potassium phosphate buffer at pH 8 of 1 mg mL^{-1} IgG4, for a FE at pHs 6 (Δ), 7 (○) and 8 (×) phosphate buffer with isooctane.

overall, achieving the highest E_os (76–85%) in Fig. 3. On the other hand, the E_bs shown in Fig. 4 were similar overall to those in Fig. 3; the E_b results obtained for a FE at pH 5 in both Figs. 3 and 4 seemed generally the best, achieving an E_b ranging between 8 and 45% when 0.05 mg mL^{-1} IgG4 was used (Fig. 3), reaching 45% at 6.25 mM AOT and an E_b of 58% (single data point) at 1 mg mL^{-1} (Fig. 4) at an AOT concentration of 3.13 mM. These results for both the BE and OE yields with AOT agree with the results obtained by Gerhardt and Dungan (14,15) of an "optimum" FE pH of 5.55 and Lan (18) of approximately 6. The recovery yield was then found to be lower overall for all other FE pHs compared to the recovery generated at the optimum FE pH of 6, which was also observed by Lan (18). Data in Fig. 3 shows that at a FE pH of 9, a decrease in OE with increasing AOT concentrations from 6.25 to 25 mM is observed; similar results were obtained by Ichikawa and Furusaki (23), Lan (18), and Naoe et al. (27). Figure 4 also shows that BE and OE yields obtained for all FE pHs tend to increase with increasing AOT concentrations.

On the other hand, Fig. 5 shows that in order to achieve an E_b of 16 to 73%, FE pHs of 4, 7, 8, 9, 10, and 11 should be used with HDEHP concentrations of 1.56, 6.25, 12.5, and 50 mM depending on the pH chosen. In contrast, Fig. 6 shows generally better E_bs compared to when a smaller IgG4 concentration (i.e., 0.05 mg mL^{-1} in Fig. 5) was used, and in order to achieve high E_bs (between 9 to 92%), a FE pH of 6 should be used with all HDEHP concentrations except 12.5 mM, where the highest E_b of 92% was found when a FE pH of 6 was used at an HDEHP concentration of 1.56 mM. Figure 5 also showed that in order to achieve E_os of 63 to 92%, FE pHs of 4, 5, 8, and 11 should be used with HDEHP concentrations of 1.56, 6.25, and 12.5 mM depending on the pH chosen. On the other hand, Fig. 6 showed much lower E_os compared to when a smaller IgG4 concentration (i.e., 0.05 mg mL^{-1} in Fig. 5) was used. In order to achieve high E_os (between 1 to 4.5%), FE pHs of 6–8 should be used with all HDEHP concentrations except 6.25 mM depending on the pH chosen, where the highest E_o of 4.5% was found when a FE pH of 8 was used at an HDEHP concentration of 3.13 mM. In the literature, there is no data on the use of the surfactant NaDEHP for the extraction of MAbs. Hu and Gulari (10) studied protein extraction using a NaDEHP RM system, and achieved overall recoveries of 98% for cytochrome-c and 67% for α-chymotrypsin, which accords with the results (Fig. 5), where OE yields up to 92% were achieved. From the results obtained, even though E_b and E_o were lower than E_f, reasonable results were obtained, suggesting that the BE of 1 mg mL^{-1} human IgG4 can yield an E_b of up to 58% for AOT and 92% for HDEHP depending on the surfactant concentration and the pH chosen.

Since all BEs were carried out using the same stripping solution, this implies that the variations observed in the E_bs in both figures were due to the surfactant concentration, buffer type, and pH used during FE, as the IgG4 transferred into the organic phase during FE are solubilized in a water pool. For Fig. 4, the differences in E_bs for a FE pH of 5 (acetate buffer) compared to those at pHs 6 and 7 (phosphate buffer) and that in Fig. 6 at pH 6 (phosphate buffer) compared to FE at pHs 7 and 8 (phosphate buffer), were not due to the ionic strength of the buffers used. This was because the buffer strengths were calculated based on their required pH and ionic strength, and the ionic strength of all buffers used was set at 0.1 M buffer solution. This was decided because the ionic strength of the aqueous solution in contact with a RM phase affects protein partitioning, first by altering electrostatic interactions between the protein surface and the surfactant headgroups by modification of the electrical double layers next to both the charged inner micelle wall and the protein surface. Hence, an increase in ionic strength causes a reduction in attraction between surfactant headgroups and the protein. Such an electrostatic screening effect is also accountable for decreasing the surfactant headgroup repulsion resulting in the formation of smaller RMs which can induce a reduction in protein solubilization by means of the size exclusion effect. Secondly, another effect of ionic strength is to ''salt out'' the protein from the RM phase due to the increased tendency of the ionic species to migrate to the RM water pool, reduce the size of the RMs, and move the protein (24). This can be explained by the lyotropic/Hofmeister series, where the ions of the added electrolyte dehydrate the hydrophilic RM by competing for its water of hydration, hence the ''salting-out'' efficiency of an electrolyte relies upon the predisposition of its ions to hydrate. This suggests that the difference in performance with different buffers was due to pH and surfactant type and concentration.

The pH of a solution affects the solubilization of a protein primarily by modifying charge distribution on the protein surface. During FE the important system parameters are pH and salt concentration in the aqueous phase, and larger polypeptides such as IgG4 require a larger number of charged residues on their surface in order to be transferred into the RMs, so the larger the protein, the greater the pH difference of maximal transfer is from the pI (IgG4 pI = 7.2–7.7). This in turn affects the interaction between the antibody and the surfactant headgroups (AOT and HDEHP), and thus the transfer of IgG4 into the RMs. On the other hand, during BE, in order to recover IgG4 from RMs, the pH of the stripping solution needs to be altered towards the pI, resulting in a reduction of the IgG4 interaction with oppositely charged headgroups, and high salt concentrations that form small RMs favor BE; this is why all BEs were carried out at pH 8 (potassium phosphate buffer). Hence, in Figs. 4 and 6, the lower the FE pH was (i.e., pH 5 for AOT and pH 6 for HDEHP), the larger the number of positively charged residues on the IgG4 surface. When the IgG4 molecule was taken-up by the RMs and transferred into the organic phase, it maintained these residues on its surface and a strong interaction with the negatively charged anionic surfactant headgroups. However, the larger ions (K^+) used in the aqueous stripping solution during BE compared to the smaller ions (Na^+) used in all FE buffers, cause a salting-out effect. This effect during BE was stronger at low FE pHs resulting in the higher E_bs observed (Figs. 4 and 6). As soon as the K^+ ions start to hydrate, they exposed the positively charged IgG4 surface to the aqueous stripping solution, and since at lower pHs the number of charged residues on the surface of IgG4 is greater, the attraction and tendency for these charged residues to become negative will be greater resulting in an increase in BE yield.

Surfactant concentration and type also affect extraction: this was observed in FE with increasing surfactant concentration resulting in an increase in the number of micelles, which in turn enhances the capacity of the RM phase to solubilize proteins (20). This is observed in Fig. 4 as the BE yields obtained for all FE pHs (except pH 5 as only one data point is plotted) increase with increasing AOT concentration, and in Fig. 6 as the BE yields obtained for FE pH 6 (except for 1.56 and 6.25 mM HDEHP), pH 7 and pH 8 (except for 1.56 mM) increase with increasing HDEHP concentration. Furthermore, the interaction between the surfactant type and IgG4 solubilized will also play a part. AOT and HDEHP are both anionic surfactants, but with different polar heads. The fact that at pH 6 in Fig. 6 (HDEHP), higher E_bs were obtained than at pHs 7 and 8 using the same buffer could be because NaDEHP forms more rod-like RMs compared to the spherical RMs formed by AOT, and that at pHs further from the pI of IgG4, the RMs are less tightly packed, enabling the larger K^+ ions in the stripping solution to penetrate the rod-like NaDEHP RMs more easily thus increasing the BE yields. This analysis implies that the type of ion used in the buffer will also influence the radius of curvature as these ions interpolate themselves between the surfactant headgroups. In addition, the fact that the polar head of NaDEHP is structurally similar to that of the phosphate buffer could be ''tricking'' the IgG4 molecule, and increasing the E_bs at the lower FE pH of 6. The lower the FE pH is from the pI of IgG4, the larger the amount of positively charged residues on the IgG4 surface. This could explain why this is not observed in Fig. 4 for AOT at pH 6 when using phosphate buffer, as the polar head of AOT contains a sulfur instead of phosphorus.

Hence, the above discussion has shown that the buffer type, pH, surfactant type, and surfactant concentration,

all played a role in the different E_bs observed in Figs. 4 and 6, showing that the sensitivity of certain parameters such as pH have an effect on the BE yields obtained, and slight variations can dramatically effect the E_bs obtained.

Precipitate Formation

Extraction of human IgG4 using AOT resulted in the formation of a precipitate at the interface which was observed at low FE pHs (pHs 4 and 5), and at low protein transfers below the proteins pI (pI = 7.2–7.7 for human IgG4). No precipitation was observed when the FE pH was higher than the pI of IgG4, suggesting that precipitate formation may be due to a strong interaction between the protein and AOT headgroups. Lan (18) also found that precipitation decreased with increasing pH, and it is possible that the rate of precipitate formation at low pH is actually faster than at higher pHs. However, since no apparent precipitate was observed at the interface during FE for pHs 6 to 11 ($0.05\,mg\,mL^{-1}$) and for pH 6 and pH 7 ($1\,mg\,mL^{-1}$), it was assumed that the IgG4 removed from the aqueous phase during FE was transferred into the RM phase. In contrast, when extracting human IgG4, precipitate formation was observed at low FE pHs (at pHs 4–8 for $0.05\,mg\,mL^{-1}$), indicating that some of the IgG4 removed from the aqueous phase was precipitated at the interface forming an IgG4-HDEHP complex. However, since no apparent precipitate was observed at the interface during FE for pH 9 and pH 11 (at $0.05\,mg\,mL^{-1}$), and for pHs 6 to 8 (at $1\,mg\,mL^{-1}$), it was assumed that the IgG4 removed was transferred into the RM phase.

Nonetheless, since all IgG4 in the RM phase (when using both AOT and HDEHP) was back extracted using the same stripping solution, and no significant precipitation was observed at the end of BE, regardless of the pH used during FE, this would suggest that any variations in the OE yields were likely to be caused by the RM phase containing slightly different IgG4 concentrations. This suggests that the OE yield depends on the BE mechanism, which again is in accordance with Lan (18). Similar findings were also observed by Mat (24) during his research on protein extraction.

Research carried out by Gerhardt and Dungan (14,15) on the changes in microemulsion and protein structure in IgG-AOT-Brine-Isooctane systems, and on IgG's time-dependent solubilization into these microemulsions have shown that the presence of the large protein IgG within a W/O microemulsion phase leads to changes in phase structures and behavior, signaled by the emergence of a third, middle phase (i.e., a precipitate). Their results suggested that IgG precipitates are partially coated by surfactant shells, and that this precipitation is most likely a result of either denaturation in an unfavorable organic environment during the breakdown of the complex, or the unfolding of IgG in the microemulsion over time because of binding of surfactant to the protein surface. Kuo (17) showed that AOT RMs worked well for the extraction of IgG4 when FE and BE were carried out on the same day; however, Lan (18) showed that if several days were left between FE and BE, denaturation of IgG4 at low surfactant concentration occurred, and a precipitate formed. This is why for this work, both FE and BE were carried out on the same day, thus reducing the amount of precipitate formation. Nonetheless, the very low OE yields obtained for both surfactants with IgG4 at $1\,mg\,mL^{-1}$ could also be due to: when calculating the percentage removal of IgG4 in the RM phase, the precipitate was included in the calculations, whether it was recovered or not. Therefore, the "true percentage removal" is in fact, the "calculated percentage removal" minus the "precipitate".

Water Content Measurements and their Effects on the Size of Reverse Micelles

The water content (W_o) and radius of the water pool (R_{wp}) after FE for $1\,mg\,mL^{-1}$ are shown in Table 1 at different pHs and AOT concentrations, and in Table 2 at different pHs and HDEHP concentrations. The R_{wp} and W_o in the RM phase after FE of HDEHP in isooctane against

TABLE 1
W_o and R_{wp} in the isooctane RM phase after FE of $1\,mg\,mL^{-1}$ IgG4 at different pHs and AOT concentrations

		AOT concentration [mM]					
pH		50	25	12.5	6.25	3.13	1.56
5	W_o	15.8	18.0	9.9	1.2	1.4	13.5
	R_{wp} [Å]	28.35	31.42	19.24	5.68	6.09	24.81
6	W_o	29.8	25.1	15.3	16.6	2.7	4.3
	R_{wp} [Å]	50.06	42.84	27.63	29.63	8.02	10.50
7	W_o	40.0	33.4	25.0	32.1	9.1	16.3
	R_{wp} [Å]	66.20	55.60	42.56	53.57	17.93	29.21

TABLE 2
W_o and R_{wp} in the isooctane RM phase after FE of 1 mg mL^{-1} human IgG4 at different pHs and HDEHP concentrations, where the values marked as "–" were disregarded as the Karl Fischer Titrator was unable to read those samples

		HDEHP concentration [mM]					
pH		50	25	12.5	6.25	3.13	1.56
6	W_o	0.01	–	–	–	–	–
	R_{wp} [Å]	3.89	–	–	–	–	–
7	W_o	–	0.04	0.05	0.05	4.6	2.0
	R_{wp} [Å]	–	3.95	3.97	3.96	11.05	6.98
8	W_o	0.1	0.1	0.1	–	0.4	0.4
	R_{wp} [Å]	4.04	4.03	4.06	–	4.50	4.53

deionized water for different sample volumes and HDEHP concentrations is shown in Table 3.

Past work has shown that for an AOT/isooctane/H_2O system, the maximum W_o is around 60, and above this the transparent RM solution becomes a turbid emulsion, and phase separation may occur (20). However, on the basis of geometric considerations, a W_o value of 28 corresponds to an effective hydrodynamic radius of approximately 55 Å, which is the size of an IgG molecule (15,28). Table 1 shows W_o values ranging from 1.2 to 40.0. All W_o values greater than 28 suggest that the extracted IgG4 in these RM phases was encapsulated within a single RM. This was observed at pH 6 (at an AOT concentration of 50 mM) and at pH 7 (at AOT concentrations of 6.25, 25, and 50 mM). In contrast, all W_o values below 28 suggest that the extracted IgG4 was likely to be encapsulated via the aggregation of multiple RMs, and this is observed at pH 5 (at all AOT concentrations), at pH 6 (at all AOT concentrations below 50 mM), and at pH 7 (at AOT concentrations of 1.56, 3.13, and 12.5 mM). An increase in W_o with increasing AOT concentration at pH 5 between 6.25 and 25 mM AOT, and at pHs 6 and 7 between 12.5 and 50 mM AOT was also observed in Table 1; this suggested that at AOT concentrations above 12.5 mM, and at pHs closer to the pI of IgG4 (7.2–7.7), the IgG4 molecule was encapsulated by one RM. The reason that higher W_os/R_{wp}s were obtained at higher pHs could be because no precipitate formed at pHs 6 and 7 during FE, and the decrease in water transfer could thus be due to the formation of a precipitate at the interface which was most likely an IgG4-AOT complex. As a result, less AOT remained in the organic phase compared to when no or less precipitate formed as the W_o increased. The W_os (R_{wp}s) obtained were mainly higher at higher AOT concentrations (at 50 and 25 mM for pHs 5, 6, and 7) due to an increase in the number of RMs formed, enabling a larger quantity of water to be transferred to the organic phase during FE. The observation that in Table 1 at 1.56 mM AOT the W_os obtained are bigger than at the higher AOT concentration of 3.13 mM, could be explained by the small sample volumes analyzed (the differences in these W_o values are statistically different (95%) (29)). If the samples were not uniformly mixed, this could result in

TABLE 3
W_o and R_{wp} in the RM phase after FE of HDEHP in isooctane against DI water (i.e., no antibody) for different sample volumes and HDEHP concentrations, where the values marked as "–" were disregarded as the Karl Fischer Titrator was unable to read those samples

		HDEHP concentration [mM]					
Sample [mL]		50	25	12.5	6.25	3.13	1.56
0.005	W_o	0.01	0.01	0.01	0.01	0.01	0.01
	R_{wp} [Å]	3.89	3.89	3.89	3.89	3.89	3.89
0.0985	W_o	0.01	0.01	0.01	–	0.05	–
	R_{wp} [Å]	3.91	3.91	3.90	–	3.96	–
0.1	W_o	0.01	0.03	0.02	0.06	0.12	0.24
	R_{wp} [Å]	3.91	3.93	3.92	3.99	4.07	4.27
1.0	W_o	0.01	0.01	0.02	0.01	0.07	0.15
	R_{wp} [Å]	3.90	3.89	3.92	3.90	3.99	4.12

the variation in W_os and consequently R_{wp}s obtained. Furthermore, these variations can also depend on the amount of IgG4 present in the organic phase, as at lower AOT concentrations less surfactant is present and the amount of antibody molecules transferred to the organic phase is expected to take place via the aggregation of multiple small RMs. Each small RM would have its own water pool that would be in direct contact with the antibody molecule solubilized by the multiple RMs. However, the transfer of IgG4 to the organic phase can also be achieved via ion-pair interactions between the charged headgroups of the surfactant and ionized side chains of the antibody molecule, and this would result in less water being transported to the organic phase, which in turn could also explain the variation in W_os obtained.

Table 2 shows W_o values ranging from 0.01 to 4.6, suggesting that the extracted IgG4 was most likely encapsulated via the aggregation of multiple RMs. As in Table 1, the observation that at lower HDEHP concentrations (3.13 and 1.56 mM) higher W_os were obtained (4.6 and 2.0 respectively at pH 7 and 0.4 at pH 8) compared to between 6.25 and 50 mM HDEHP, where lower W_os were obtained (0.01 at pH 6 and 50 mM HDEHP, 0.04 at 25 mM HDEHP and 0.05 at 12.5 and 6.25 mM HDEHP, and 0.1 at 50, 12.5, and 6.25 mM HDEHP) could be due to the small sample volumes analyzed. The lack of values obtained in Table 2 are most probably due to the small size of the samples analyzed since the RM samples were mostly isooctane which is 0.00024% w/w (i.e., 2.4 mg/L of water in solvent). Hence these values were disregarded, and only the samples in which water was detected were used to calculate W_o values and the corresponding water pool radius (R_{wp}). Nevertheless, in order to check the W_o and R_{wp} values of the HDEHP micelles, a FE without IgG4 was carried out using deionized water as the aqueous phase and HDEHP at varying concentrations (between 1.56 to 50 mM) in isooctane as the RM phase. This is shown in Table 3, where samples of various volumes (between 0.005 and 1.0 mL) were analyzed (triplicates). Various volumes were tested since the data in Table 2 was obtained from samples of different volumes (0.005 mL for pH 6 at 6.25, 3.13, and 1.56 mM HDEHP, 0.0985 mL for pH 6 at 50, 25, and 12.5 mM HDEHP, and 0.1 mL for pHs 7 and 8 at all HDEHP concentrations). Table 3 shows that even though no antibody was present, the W_o values obtained (ranging from 0.01 to 0.24) and the corresponding R_{wp} were in the same range as those in Table 2. This confirmed the assumption that the extracted IgG4 in the RM phase was most probably encapsulated via the aggregation of multiple RMs. By comparing Tables 2 and 3 in the presence and absence of IgG4, it can be seen that the volume of the samples in the absence of antibody molecules has a minimal effect on the actual W_os obtained since these are all between 0.01 and 0.24. Thus, these W_os could be deemed as negligible taking into account the COV, especially at the lower sample volumes (0.005 and 0.0985 mL). Where these variations are slight they could be due to moisture absorbance which occurs during Karl Fischer titration and increases with the number of samples analyzed, resulting in regular reagent replacement, and not to the formation of RMs in the organic phase. Nonetheless, it could be concluded as for Table 2, that at 3.13 and 1.56 mM HDEHP and 0.1 and 1.0 mL, higher W_os were obtained (0.12 and 0.24 respectively at 0.1 mL, and 0.07 and 0.15 respectively at 1.0 mL) compared to between 6.25 and 50 mM HDEHP (W_os between 0.01 and 0.06) and this could also be due to whether the samples analyzed were uniformly mixed.

These findings are in accordance with past research carried out by Mat (24), where he studied the influence of protein and RM size on the extent of protein transfer, and found that in order for the protein to solubilize in the RMs, they need to be roughly the same size as the protein. Since protein transfer also occurs when the RM sizes are nominally smaller than the protein, this process might involve RM re-aggregation to form larger RMs that are capable of solubilizing the protein; thus concluding that the extent of protein transfer depended on the type of protein, salt concentration as well as pH. Pérez de Ortiz and Stuckey (20) came to similar conclusions when examining the water solubilization capacity, they found that micelle size depends on the salt type and concentration, solvent, surfactant type and concentration, and also temperature. The findings in this study are also in accordance with work carried out by Gerhardt and Dungan (14,15) on the changes in microemulsion and protein structure in IgG-AOT-Brine-Isooctane systems and on the role of cluster formation in such systems. From their research, they found that cluster formation occurs more readily with higher surfactant or salt concentration, when the protein and droplet are comparable in size (15). They also found that since IgG partitions into the microemulsion droplets despite its large size, this may indicate the presence of hydrophobic interactions between protein and surfactant, which together with electrostatic forces drives the protein to reside in the microemulsion phase. Under conditions in which the protein cannot be solubilized within a single droplet and at higher pH values, the protein-containing microemulsion formed was more stable (14).

CONCLUSIONS

Comparison of FE and BE of 0.05 and 1 mg mL^{-1} human IgG4 under the same experimental conditions concluded that for both surfactants (AOT and HDEHP), 1 mg mL^{-1} generated a more stable and reliable, wider range of results; overall achieving high FE, good BE, and reasonable OE yields. The OE yield was found to decrease significantly with increasing initial IgG4 concentration,

which would suggest that lower IgG4 concentrations enhance BE. The precipitate layer observed at the lower pHs during FE explained the low OE yields, causing the protein to remain trapped in the precipitate at the interface during extraction. Water content analyses for $1\,mg\,mL^{-1}$ human IgG4 revealed that the RMs formed when using HDEHP were much smaller than those using AOT. Thus, the initial results for the extraction of $1\,mg\,mL^{-1}$ human IgG4 in isooctane using AOT and HDEHP RMs appear equally promising, suggesting that this novel extraction technique may have some potential. However, more work is needed to obtain commercially viable recoveries.

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NOMENCLATURE

AOT	Bis(2-ethylhexyl)sulfosuccinate sodium salt
BE	Backward extraction
$C_{BE}{}^{Aq}$	Amount of backward extracted protein in the aqueous phase ($mg\,mL^{-1}$)
$C_{FE}{}^{RM}$	Amount of forward extracted protein in the reversed micellar organic phase ($mg\,mL^{-1}$)
$C_{Initial}{}^{Aq}$	Amount of protein in the initial aqueous phase ($mg\,mL^{-1}$)
c_w	Water concentration in the sample (ppm)
c_s	Water concentration in the pure solvent (ppm)
E_b	Backward extraction percentage (%)
E_f	Forward extraction percentage (%)
E_o	Overall extraction percentage (%)
FE	Forward extraction
HDEHP	Bis(2-ethylhexyl)phosphate
MW	Molecular weight (Da)
NaDEHP	Sodium bis(2-ethylhexyl)phosphate
OE	Overall extraction
RMs	Reverse micelles
R_{wp}	Water pool radius
$[surfactant]_{RM}$	Surfactant concentration in the reverse micelle sample (mM)
W/O	Water-in-oil
W_o	Molar ratio of water to surfactant

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