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Probing the Relationship between Interfacial Concentrations and Lipase Activity in Cationic W/O Microemulsions: A Quantitative Study by Chemical Trapping

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Interfacial concentrations and/or space: which one is the predominant factor in regulating lipase activity at the water−oil interface? This work is an endeavor toward probing the relationship between lipase activity and interfacial concentrations in cationic water-in-oil (W/O) microemulsions through quantitative study by a chemical trapping method. The interfacial concentrations of water ($[H_2O_i]$), bromide ($[Br_i^-]$), and *n*-hexanol ($[HexOH_i]$) were estimated in the W/O microemulsions of six surfactants with varying headgroup architecture and hydrophilicity across a wide W_0 ($[H_2O]/[surfactant]$) range. The surfactants were prepared by the replacement of methyl groups of cetyltrimethylammonium bromide (**1**) by *n*-propyl (**2**−**4**), one hydroxyethyl (**5**), and one methoxyethyl (**6**) group. The estimated $[H_2O_i]$ was found not to change much (30.0−36.7 M) with the variation in headgroup hydrophilicity or size from **1**−**5**. However, $[Br_i^-]$ was found to increase with a decrease in the degree of dissociation (α), being higher for **1** and **5** (2.4−3.3 M) and relatively lower (0.9−1.9 M) for others depending on W_0. Interestingly, $[H_2O_i]$ was found to be little higher (41.5−42.2 M) in the case of **6**. The present study elucidates the importance of interfacial water and counterion concentrations in modulating the lipase activity in reverse micelles. In our previous report, the lipase activity was found to increase from **1**−**4** and in **6**, whereas that observed in **5** was comparable with **1**, being largely regulated by the surfactant head group size (Das, D.; Roy, S.; Mitra, R. N.; Dasgupta, A.; Das, P. K. *Chem.*−*Eur. J.* **2005**, *11*, 4881). The only other parameter that increased distinctly with lipase activity is the headgroup size, not $[H_2O_i]$. Thus, the role of $[H_2O_i]$ in comparison to the surfactant's headgroup size is not found to be that significant. Moreover, the lower $[Br_i^-]$ in **2**−**4** and **6** perhaps enhances the probability of enzyme and substrate localization at the interface, leading to higher lipase activity.

Introduction

Over the past decades, enzymology in self-organized aggregates such as water-in-oil (W/O) microemulsions of various surfactants with novel structures has been an area of increasing interest because of its potential biotechnological applications.[1−4] Despite the wide-ranging applications of W/O microemulsions, several microstructural parameters of reverse micellar aggregates are still incompletely understood. Beyond reports of the primary dependency of an enzyme's catalytic efficiency on the local concentrations of water and other ions present in vicinity of a biocatalyst,[5−10] the effects of different microstructural parameters on the enzyme activity has remained a somewhat neglected issue over the past few years.

Toward delineating the influence of interfacial compositions on surface-active enzyme−lipase in the initial stage, Das and Chaudhuri[7] showed that the activity of *Chromobacterium viscosum* (CV) lipase in cetyltrimethylammonium bromide (CTAB)/water/isooctane/*n*-hexanol W/O microemulsions across W_0 ($[water]/[surfactant]$) = 12−44 was unchanged, presumably as a result of the unaltered interfacial water concentration, $[H_2O_i]$ (28.1−31.8 M). The reported $[H_2O_i]$ is significantly lower than the bulk water concentration (55.5 M) and was thought to be responsible for the poor activity of lipase in CTAB microemulsions. At this point, Srilakshmi and Chaudhuri[9] found that although the activity of lipase was superior in AOT-based microemulsions, $[H_2O_i]$ = 27.9−32.0 M was similar to that observed in CTAB systems. In an attempt to improve the lipase activity, we found that on introduction of hydroxyethyl moieties at the polar heads of surfactants the catalytic activity of CV lipase increased up to 4−10-fold compared to that in widely used cationic (CTAB) reverse micelles.[8] The observed enhancement in activity was possibly thought to be due to the increase in $[H_2O_i]$ through hydrogen bonding with hydroxyl groups at the

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Scheme 1. Products of the Dediazoniation Reaction

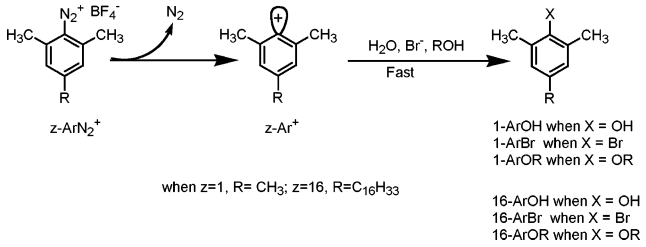
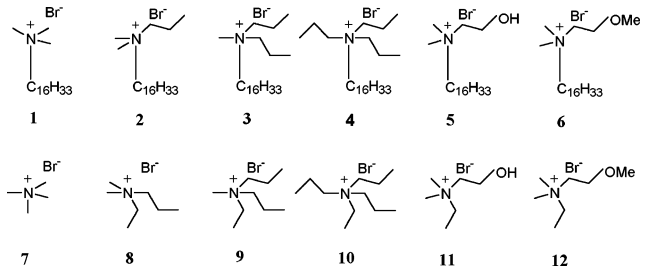


Chart 1. Surfactants with Varied Headgroups (1−6) and Their Short-Chain Analogues (7−12)



interface. However, the introduction of hydroxyethyl groups not only increased the hydrophilicity at the interface but also may have enhanced the head group size, owing to its bulky and flexible nature, and consequently the interfacial area. Thus, there is doubt as to which factor (hydrophilicity or interfacial area) the improvement in lipase activity with hydroxyethyl groups is due. Hence, to delineate the crucial role of head group size, the activity of CV lipase was estimated in cationic W/O microemulsions of three different series of surfactants comprising varying head group sizes and hydrophilicities.[11] The lipase activity was found to increase predominantly with headgroup size, underlining the importance of space at the interface.

At this stage, it is crucial to determine whether varying the headgroup hydrophilicity and size has also altered [H_2O_i], which in turn may effectively regulate the lipase activity. Thus, an estimation of the interfacial compositions is of utmost importance, without which it would be difficult to single out the major/predominant factor responsible for modulating the lipase activity or establishing the large influence of headgroup size on lipase activity in W/O microemulsions. One of the most insightful methods of estimating the interfacial compositions of association colloids is based upon "chemical trapping" of weakly basic nucleophiles by an amphiphilic arenediazonium probe.[12−14] Given its successful application to conventional surfactant micelles, the arenediazonium probe trapping method (Scheme 1) has been equally useful for evaluating the interfacial compositions of cationic W/O microemulsions.[7,9,10]

In the present study, we have used chemical trapping protocol to estimate the interfacial concentrations of water, bromide ion, and *n*-hexanol in the W/O microemulsions of cationic surfactants (**1**−**6**, Chart 1) with varying head group sizes and hydrophilicities and put forward which microstructural property is mainly responsible for regulating the lipase activity.

Experimental Section

Materials. 2,4,6-Trimethylbenzenediazonium tetrafluoroborate (1-ArN_2^+), 2,4,6-trimethylphenyl-*n*-butyl ether (1-ArOBu), 4-*n*-hexadecyl-2,6-dimethylbenzenediazonium tetrafluoroborate (16-ArN_2^+), 4-*n*-hexadecyl-2,6-dimethylphenol (16-ArOH), 4-*n*-hexadecyl-2,6-dimethylbromobenzene (16-ArBr), and 4-*n*-hexadecyl-2,6-dimethylphenyl-*n*-hexyl ether (16-ArOHex) were synthesized according to the previously published synthetic procedure.[12] 1-ArOH and 1-ArBr were purchased from Aldrich (>99% pure) and were further purified by recrystallization and vaccum distillation, respectively. Analytical-grade CTAB was recrystallized from methanol and ether. HPLC-grade solvents were procured from Spectrochem (India) and SRL (India). Tetramethylammonium bromide (TMAB) was purchased from Lancaster, U.K. *N*,*N*-Dimethylethylamine, *N*-methyldipropylamine, and 1-bromo-2-methoxy-ethane were obtained from Aldrich. All other reagents used in the syntheses were obtained from SRL (India). Amberlyst A-26 bromide ion-exchange resin from Lancaster was used to convert the iodide salts to their corresponding bromide forms. ^{1}H NMR spectra were recorded on an Avance 300 MHz (Bruker) spectrometer. Mass spectrometric (MS) data were acquired by electrospray ionization (ESI) techniques on a Q-TOF micro-quadruple mass spectrometer (Micromass, U.K.). The syntheses of the five surfactants (**2**−**6**, Chart 1) were reported earlier,[11] and those of the short-chain analogues (**8**−**12**, Chart 1) are listed below.

Synthesis of *N*-Ethyl-*N*,*N*-dimethyl-*N*-propylammonium Bromide (8), *N*-Ethyl-*N*-methyl-*N*,*N*-dipropylammonium Bromide (9), and *N*-Ethyl-*N*,*N*,*N*-tripropylammonium Bromide (10). *N*,*N*-Dimethylethylamine (for **8**), *N*-methyldipropylamine (for **9**), and *N*,*N*,*N*-tripropylamine (for **10**) (1.0 equiv) were quaternized with propyl bromide (for **8**) and ethyl bromide (for **9** and **10**) (1.2 equiv for each) in methanol and allowed to stir overnight at room temperature. The reaction mixtures were evaporated on a rotary evaporator, and pure products were obtained by repeated crystallization of the compounds from methanol/ether. The yields were in the range of 70−75%.

^{1}H NMR (300 MHz, $CDCl_3$) of (**8**): δ 1.05 (t, 3H), 1.42 (t, 3H), 1.77−1.85 (m, 2H), 3.38 (s, 6H), 3.45−3.54 (m, 2H), 3.68−3.76 (m, 2H). Anal. Calcd for $C_7H_{18}BrN$: C, 42.87; H, 9.25; N, 7.14. Found: C, 42.56; H, 8.92; N, 6.96. MS (ESI) *m*/*z*: calcd for $C_7H_{18}N$ (100%), 116.22; found, 116.07 (M^+). ^{1}H NMR (300 MHz, $CDCl_3$) of (**9**): δ 1.06 (t, 6H), 1.4 (t, 3H), 1.73−1.86 (m, 4H), 3.31 (s, 3H), 3.40−3.46 (m, 4H), 3.62−3.69 (m, 2H). Anal. Calcd for $C_9H_{22}BrN$: C, 48.22; H, 9.89; N, 6.25. Found: C, 47.85; H, 9.50; N, 6.08. MS (ESI) *m*/*z*: calcd for $C_9H_{22}N$ (100%), 144.27; found, 144.04 (M^+). ^{1}H NMR (300 MHz, $CDCl_3$) of (**10**): δ 1.04 (t, 9H), 1.40 (t, 3H), 1.72−1.85 (m, 6H), 3.30−3.36 (m, 6H), 3.58−3.65 (m, 2H). Anal. Calcd for $C_{11}H_{26}BrN$: C, 52.38; H, 10.39; N, 5.55. Found: C, 51.94; H, 10.11; N, 5.31. MS (ESI) *m*/*z* calcd for $C_{11}H_{26}N$ (100%), 172.33; found, 172.05 (M^+).

Synthesis of *N*,*N*-Dimethyl-*N*-ethyl-*N*-(2-hydroxyethyl)ammonium Bromide (11). This was reported earlier.[10] Briefly, *N*,*N*-dimethylaminoethanol (1 equiv) was quaternized with ethyl bromide (in excess) in methanol at room temperature for 4 h. The reaction mixtures were evaporated on a rotary evaporator, and pure products were obtained by crystallization of the reaction mixtures from methanol/ethyl acetate. The yields were in the range of 65−70%. ^{1}H NMR (300 MHz, $CDCl_3$) of (**11**): δ 1.27 (t, 3H), 3.02 (s, 6H), 3.33−3.41 (m, 4H), 3.95 (br, 2H). Anal. Calcd for $C_6H_{16}BrNO$: C, 36.36; H, 8.08; N, 7.07. Found: C, 36.13; H, 8.12; N, 6.92. MS (EI) *m*/*z*: calcd for $C_6H_{16}BrNO$ (100%), 197.04; found, 197 (M^+) and 249 [(M + 2A) − (CH_2CH_3)]$^+$.

Synthesis of *N*,*N*-Dimethylammonium-*N*-ethyl-*N*-(2-methoxyethyl) Bromide (12). A mixture of 1-bromo-2-methoxy-ethane (1.0 equiv) and *N*,*N*-dimethylethylamine (1.1 equiv) was taken in a sealed tube and heated at 100−110 °C for 12 h. The reaction mixture was then washed with ether and crystallized from ethanol/acetone. The yields were in the range of 80−90%. ^{1}H NMR (300 MHz, D_2O) of (**12**): δ 1.24 (t, 3H), 2.99 (s, 6H), 3.29 (s, 3H), 3.31−3.36 (m, 2H), 3.42−3.45 (m, 2H), 3.77−3.79 (m, 2H). Anal.

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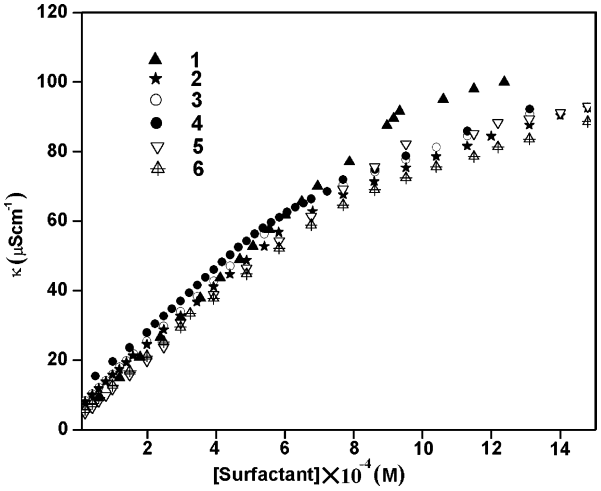

Figure 1. Plots of specific conductance (κ) vs concentration of surfactants **1**−**6** at 25 °C.

Table 1. Critical Micelle Concentration (cmc) and Degree of Dissociation (α) of Surfactants 1−6

surfactant	conductometric cmc (M)	degree of dissociation (α)
1	9.5×10^{-4}	0.32
2	7.26×10^{-4}	0.37
3	6.33×10^{-4}	0.40
4	6.29×10^{-4}	0.47
5	8.95×10^{-4}	0.25
6	7.64×10^{-4}	0.45

Calcd for $C_7H_{18}BrNO$: C, 39.63; H, 8.55; N, 6.60. Found: C, 39.23; H, 8.28; N, 6.44. MS (ESI) *m/z*: calcd for $C_7H_{18}NO$, the 4° ammonium ion (100%), 132.22; found, 132.01 (M^+).

Preparation of Reverse Micelles. To a measured amount (required to prepare 5 mL of a 50 mM solution) of surfactants **1**−**6**, isooctane/*n*-hexanol (*z* ([alcohol]/[surfactant]) = 4.8) was added and allowed to disperse by stirring with a vortex mixer. Then a measured amount of a 20 mM phosphate buffer (pH 6) was added to prepare a particular W_0 (molar concentration of water to surfactant), and the volume was made up to the mark by isooctane/*n*-hexanol; the mixture was stirred with a vortex mixer to obtain a macroscopically homogeneous solution.

Conductometry. In the conductance method, a concentrated solution of the surfactant was added in installments with a micropipette in 20 mL of water (doubly distilled having a specific conductivity of 2−4 μS cm^{-1} at 25 °C) and placed in a wide-mouthed test tube fitted with a dip-type conductivity cell with a cell constant of 1 cm^{-1}. After each addition, the specific conductance (κ) of the solution was measured with a conductometer (Toshniwal Instruments Mfg. Pvt. Ltd, India) at 25 ± 0.1 °C. The specific conductance values were reproducible within the limit of ±3−5%. The cmc values (Table 1, Figure 1) were obtained from the break point in the plot of specific conductance (κ) versus concentration of surfactant. The degree of dissociation (α) is the ratio of the slopes of the lines above and below the cmc.

Activity of Interfacially Solubilized Lipase. The second-order rate constant (k_2) in the lipase-catalyzed hydrolysis of *p*-nitrophenyl-*n*-hexanoate in cationic W/O microemulsions was determined spectrophotometrically as reported in our earlier work.[11] Briefly, in a typical experiment, 4.5 μL of the aqueous enzyme stock solution (0.34 mg/mL) and the substrate (10 μL, from 0.45 M stock solution in isooctane) was added to 1.5 mL of the W/O microemulsion previously prepared with the desired surfactant concentration and pH in a cuvette to attain the particular W_0 and reactant concentrations. Gentle shaking produced a clarification of the microemulsion within 1 min. The initial linear rate of increase in absorbance (i.e., the absorbance of the liberated *p*-nitrophenol) was then recorded at the isosbestic points (λ_{iso}). The overall concentrations of lipase and *p*-nitrophenyl-*n*-hexanoate are 1.02×10^{-6} g cm^{-3} and 3×10^{-3} M, respectively. k_2 was found to be independent of W_0 for all of the surfactants.

Dediazoniation Reactions. HPLC product distribution analyses were carried out with a Waters 2487 UV detector and a Waters 1525 pump using a Waters (Nova Pak) reverse-phase C-18 column (4.6 × 150 mm^2) to separate the dediazoniation products. The sample loop size used for determining the nonmicellar product distribution of 1-ArN_2^+ in the presence of *n*-BuOH was 200 μL, and a loop size of 20 μL was used for determining the product distributions in all other dediazoniation reactions. The wavelength of the UV detector was set at 230 nm for detecting 1-ArOH and 1-ArBr, at 205 nm for 1-ArOBu, and at 219 nm for the dediazoniation products from 16-ArN_2^+. The products of nonmicellar dediazoniation of 1-ArN_2^+ in the presence of *n*-BuOH were separated with the initial mobile phase of CH_3CN/H_2O (60/40 v/v) which was changed to CH_3CN/H_2O (80/20 v/v) at 3.5 min and maintained up to 20 min. The initial flow rate (0.9 mL/min) was gradually decreased to 0.4 mL/min at 3.5 min and again increased to 0.9 mL/min at 5.0 min and maintained up to 20 min. Typical retention times were 3.9 and 12.0 min for 1-ArOH and 1-ArOBu, respectively. The nonmicellar thermal dediazoniation (Scheme 1) was performed by adding 15 μL of a freshly prepared ice-cold stock solution of 1-ArN_2^+ (∼0.1 M in CH_3CN) to aqueous (pH 6; 20 mM phosphate buffer) solutions (1.5 mL) of 0−3.0 M **7**−**12** (Chart 1) to give a $\sim 1 \times 10^{-3}$ M reaction concentration of 1-ArN_2^+. The concentration of water (Table 2) in each case was determined by the following equation: [(total solution weight of any salt at a particular concentration − weight of only the salt) × 1000]/(18 × 1.5). Cyclohexane (50 μL) was layered on top of each solution to prevent product loss by evaporation. Reactions were diluted to 5 mL with MeOH/CH_3CN (70/30 v/v) before injection into the HPLC. The separation of the products (1-ArOH and 1-ArBr) obtained from the dediazoniation of 1-ArN_2^+ was carried out with the mobile phase CH_3CN/H_2O (70/30 v/v) at 0.6 mL/min. Typical column pressures were 500−600 psi, and typical retention times were, respectively, 1-ArOH at 4.1 min and 1-ArBr at 16.6 min. Similarly, interface-associated thermal dediazoniation of 16-ArN_2^+ was initiated by adding ∼2.5 mg (the concentration of diazonium salt calculated separately in each case) of solid 16-ArN_2^+ to each of the 5 mL reverse micellar solutions of **1**−**6** (0.05 M)/water/isooctane/*n*-hexanol systems at $z = 4.8$, $W_0 = 40$, and 25 °C. All of the dediazoniation reactions were carried out for at least 10 half-lives (24 h at room temperature), and the reaction mixtures were diluted 10-fold with MeOH/iPrOH (75/25 v/v) before injection into the HPLC. Half-lives were in the range of 1 to 2 h and were determined by a procedure published earlier.[12] The mobile phase used for reverse micellar dediazoniation was MeOH/*i*-PrOH (72/28 v/v) at 0.4 mL/min. Typical column pressures were 950−1000 psi, and typical retention times were, respectively, 16-ArOH at 8.2 min, 16-ArBr at 22.0 min, and 16-ArOHex at 24.0 min.

Estimation of the Interfacial Concentrations. The local interfacial concentrations of bromide counterions ([Br_i^-]), *n*-hexanol ([$HexOH_i$]), and water ([H_2O_i]) in cationic reverse micelles (Scheme 1) were estimated by applying the chemical trapping method.[12−14a] Briefly, assuming the nonmicellar dediazoniation reactions of 1-ArN_2^+ as a good model for the aggregate-bound dediazoniation of 16-ArN_2^+ (Scheme 1). [Br_i^-] values across the W_0 range (Table 3) in each case were estimated by substituting the percent 16-ArBr yields of the interface-associated dediazoniation of 16-ArN_2^+ in the correlations between percent 1-ArBr yields and corresponding values (short-chain analogues) observed for the nonmicellar dediazoniation of 1-ArN_2^+ in the presence of varying amounts (0.1−3.0 M) of short-chain analogues (Table 2, Figure 2). Similarly, [$HexOH_i$] values were estimated by substituting the percent 16-ArOHex yields observed in the interface-associated dediazoniation of 16-ArN_2^+ into the correlation between percent 1-ArOBu yields and the concentrations of 1-butanol observed in the aqueous nonmicellar dediazoniation of 1-ArN_2^+ in the presence of varying concentrations (0.4−0.9 M) of added 1-butanol (Supporting Information).

The interfacial concentrations of water, [H_2O_i] (Table 3), were estimated by substituting values for [Br_i^-], [$HexOH_i$], and the percent

Table 2. Normalized Product Yields and Water Concentrations for Room-Temperature Dediazoniation of 1-ArN$_2^+$ in Aqueous Solutions of 7−12 at pH 6.0

	7			8			9			10			11			12		
[salt] (M)	1-ArOH (%)	1-ArBr (%)	[H$_2$O] (M)	1-ArOH (%)	1-ArBr (%)	[H$_2$O] (M	1-ArOH (%)	1-ArBr (%)	[H$_2$O] (M)	1-ArOH (%)	1-ArBr (%)	[H$_2$O] (M)	1-ArOH (%)	1-ArBr (%)	[H$_2$O] (M)	1-ArOH (%)	1-ArBr (%)	[H$_2$O] (M)
0.1	96.1	3.9	55.2	93.4	6.6	55.5	94.8	5.2	55.3	93.5	6.5	54.9	96.6	3.4	54.5	92.6	7.4	54.8
0.5	87.7	12.3	52.7	79.7	20.3	53.5	82.1	17.9	51.8	79.7	20.3	51.6	89.3	10.7	51.0	77.1	22.9	52.4
1.0	82.9	19.1	49.6	68.5	31.5	48.7	65.0	35.0	45.7	65.9	34.1	44.2	80.9	19.1	47.1	63.0	37.0	47.2
1.5	74.4	25.6	46.6	60.8	39.2	43.4	59.2	40.8	40.3	57.7	42.3	37.4	75.2	24.8	43.5	54.0	46.0	42.8
2.0	69.3	30.7	43.3	48.4	51.6	40.1	46.9	53.1	35.9	46.4	53.6	31.9	69.8	30.2	39.7	46.3	53.7	38.6
2.5	64.3	35.7	40.2	39.8	60.2	36.7	38.2	61.8	31.5	38.2	61.8	26.7	62.6	37.4	36.2	39.1	60.9	34.6
3.0	59.6	40.4	37.1	34.0	66.0	32.1	29.7	70.3	26.5	29.1	70.9	22.5	56.7	43.3	31.2	32.4	67.6	30.2

Table 3. Estimated Interfacial Concentrations of Water, Bromide, and *n*-Hexanol in Different Cationic W/O Microemulsions of 1−6/Water/Isooctane/*n*-Hexanol Systems at *z* ([Alcohol]/[Surfactant]) = 4.8, across the W_0 Range, in the Dediazoniation of 16-ArN$_2^+$ at Room Temperature

	[H$_2$O$_i$] (M)						[Br$_i^-$] (M)						[HexOH$_i$] (M)					
W_0	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**
8		31.8	31.2	30.0				1.9	1.7	1.6				4.7	4.3	4.4		
16		35.0	34.1	33.4				1.5	1.4	1.2				3.8	4.2	3.6		
24		35.8	35.6	34.2				1.3	1.3	1.1				3.9	3.7	3.5		
32		36.3	35.9	34.6		41.5		1.2	1.2	1.0		0.95		3.7	3.8	3.3		3.54
40	32.6	35.6	36.4	34.7	33.1	42.1	3.28	1.1	1.1	1.0	2.5	0.92	3.4	3.3	3.4	3.2	3.9	3.45
48	33.4	36.2	36.5	34.7	33.0	42.2	2.58	1.1	1.1	0.97	2.6	0.9	4.0	4.2	3.3	3.3	3.9	3.3
56	33.5	34.6	36.7	34.6	33.4		2.56	1.0	1.1	0.96	2.4	-	3.9	3.7	3.5	3.4	3.9	
64			36.4	34.4					1.1	0.98					3.8	3.4		
72			36.6	34.7					1.1	0.9					3.7	3.4		
80				34.4						0.9						3.7		

16-ArOH yields observed in reverse micellar dediazoniation of 16-ArN$_2^+$ into eq 1

$$[H_2O_i] = \{(\%\ 16\text{-ArOH})(S_W^{Br}[Br_i^-] + S_W^{A}[HexOH_i])\}\frac{1}{100 - \%\ 16\text{-ArOH}} \quad (1)$$

$$\frac{\%\ 1\text{-ArBr}}{\%\ 1\text{-ArOH}} = \frac{S_W^{Br}[salt]}{[H_2O]} \quad (2)$$

$$\frac{\%\ 1\text{-ArOBu}}{\%\ 1\text{-ArOH}} = \frac{S_W^{A}[n\text{-BuOH}]}{[H_2O]} \quad (3)$$

where S_W^{Br} and S_W^{A} are the selectivity values for the dediazoniation reaction with bromide ions and 1-butanol with respect to water. These selectivity parameters were determined from the product distribution results in nonmicellar aqueous dediazoniation reactions of 1-ArN$_2^+$ using eqs 2 and 3; S_W^{Br} = 8.4, 18.6, 18.5, 18.3, 7.9, and 24.3, respectively, for **7**−**12**, and S_W^{A} = 0.54. The selectivity values for bromide ions were found to increase with the headgroup size of short-chain analogues as the percent yields of 1-ArBr were found to rise (Supporting Information). To determine [H$_2$O$_i$] for each of surfactant, the S_W^{Br} value is taken at the short-chain analogue concentration, which is similar to the observed [Br$_i^-$]. The details of the nonmicellar- and reverse micellar-associated dediazoniation reactions, HPLC product distribution results, HPLC conditions, and the observed correlations between % 1-ArBr and [**7**−**12**] and those between % 1-ArOBu and [1-BuOH] used in estimating [Br$_i^-$] and [HexOH$_i$] are provided in the Supporting Information.

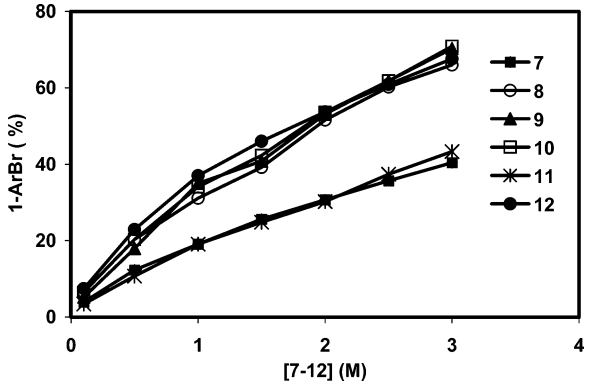

Figure 2. Correlation curves obtained through curve fitting the nonmicellar dediazoniation product results of **7**−**12**. Correlation equations for 1-ArBr: % 1-ArBr = 19.2 [Br$^-$]$^{0.685}$, % 1-ArBr = 31.52[Br$^-$]$^{0.677}$, % 1-ArBr = 31.1 [Br$^-$]$^{0.77}$, % 1-ArBr = 32.8 [Br$^-$]$^{0.70}$, % 1-ArBr = 18.6[Br$^-$]$^{0.75}$, and % 1-ArBr = 34.57[Br$^-$]$^{0.65}$ for **7**−**12**, respectively.

Results and Discussion

In deciphering the role of local molar concentrations of water and ions present at the interfacial region in modulating the activity of interfacially encapsulated lipase, we have determined the interfacial compositions of the W/O microemulsions of representative cationic surfactants (**1**−**6**, Chart 1). The surfactants were chosen by keeping in mind the variation in their headgroup size and hydrophilicity by a methodical, sequential replacement of methyl groups of CTAB with three *n*-propyl groups (**2**−**4**), one hydroxyethyl (**5**) group, and one methoxyethyl (**6**) group. Physicochemical characterizations such as the critical micelle concentration (cmc) and degree of dissociation (α) of the surfactants were measured in order to promote a better understanding of the chemical trapping results.

The cmc for each surfactant was determined with the help of electrical conductivity measurements at 25 °C by plotting the specific conductivity (κ) of the surfactant solutions as a function of concentration. For each surfactant, a reproducible break was observed in the κ versus [surfactant] plot, indicating the onset of aggregation (Figure 1). The degree of dissociation (α) at the micellar interface is taken as the ratio of the slopes of the lines above and below the cmc[15] (Table 1). The results show that with the increase in headgroup area the α value increases.

To ascertain whether the observed differences in α due to the alteration in head group structure has any correlation with the interfacial compositions or the reported lipase activity, it was essential to determine the local molar concentrations of water

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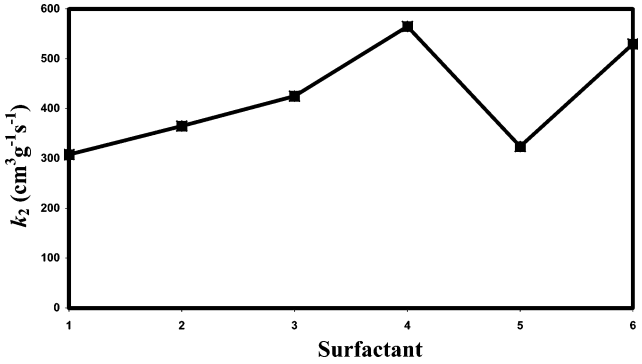

Figure 3. Variation of the second-order rate constant (k_2) for the lipase-catalyzed hydrolysis of *p*-nitrophenyl-*n*-hexanoate with surfactant headgroup size in cationic W/O microemulsions of **1**−**6** (across the W_0 ranges of 40−56 for **1**, 8−60 for **2**, 8−72 for **3**, 8−80 for **4**, 40−60 for **5**, and 32−52 for **6**) formed at $z = 4.8$, 25 °C, and pH 6.0 (20 mM phosphate) as reported earlier.[11] [Surfactant] = 50 mM, [enzyme] = 1.02×10^{-6} gmL^{-1}, and [substrate] = 3 mM. The experimental errors in the determination of k_2 were within the range of ±1−5%.

([H_2O_i]), bromide counter ions ([Br_i^-]), and *n*-hexanol ([$HexOH_i$]) at the interface of W/O microemulsions. Interfacial concentrations in **1**−**6** (0.05 M)/isooctane/*n*-hexanol/water at *z* ([alcohol]/[surfactant]) = 4.8 were estimated by the chemical trapping method using 16-ArN_2^+ as a probe on the basis of the pseudophase model of aqueous/W/O microemulsions and the extraordinary insensitivity of the dediazoniation reaction.[12−14a,16,17] The product distribution was carried out at $z = 4.8$ because it is the common solution composition at which all of the surfactants form W/O microemulsions and exhibit distinct differences in lipase activity (Figure 3).

The nonmicellar dediazoniation reactions were carried out using headgroup-mimicking short-chain salts (**7**−**12**, Chart 1), synthesized by replacing the long chain of surfactants with an ethyl group (except **7**) because the short-chain methyl salts are extremely hygroscopic in nature and some of them are also liquids, even below room temperature. It was very difficult to purify such ionic liquids; therefore, we prepared one-homologue-higher salts with an ethyl group, which are also water-soluble non-micelle-forming salts.[10] Moreover, they are solid in nature at room temperature and could be purified easily by crystallization.

The product distribution results were obtained by nonmicellar aqueous thermal dediazoniation (determined by quantitative HPLC analysis) of 1-ArN_2^+ with varying amounts (0−3.0 M) of added short-chain structural analogues **7**−**12** (Chart 1) of surfactants **1**−**6**, respectively, at pH 6.0 (20 mM phosphate buffer) and room temperature (Table 2, Figure 2). Correlations between the normalized percent yields (Table 2) of products (1-ArBr, 2, 4, 6-trimethylbromobenzene) and the molar concentration of [Br^-] in the nonmicellar dediazoniation were obtained through curve fitting in each of the cases. (Details of HPLC product distribution data and curve-fitting equations provided in Supporting Information.) The obtained correlation equations (0.990−1.00 correlation coefficients for all of the curve-fitting equations) are % 1-ArBr = $19.2[Br^-]^{0.685}$; % 1-ArBr = $31.52[Br^-]^{0.677}$; % 1-ArBr = $31.1[Br^-]^{0.77}$; % 1-ArBr = $32.8[Br^-]^{0.70}$; % 1-ArBr = $18.6[Br^-]^{0.75}$; and % 1-ArBr = $34.57[Br^-]^{0.65}$ for **7**−**12**, respectively. In the nonmicellar dediazoniation reactions (Table 2), at the same salt concentrations, [H_2O] decreases with an increase in the size of the quaternary ammonium group, yielding increased %1-ArBr and plummeting %1-ArOH values, as reported earlier.[18] In a similar concentration range (0.1−3.0 M) of salt, %1-ArBr increases from **7** (3.9−40.4%) to **10** (6.5−70.9%). This trend of increasing %1-ArBr is also evident on moving from **11** to **12** (43.3 to 67.6%) at 3.0 M.

According to the basic assumptions of phenyl cation trapping protocol,[19−21] we assumed that the correlation between %1-ArBr yields and [Br^-] (Figure 2) observed in the nonmicellar aqueous dediazoniation also holds true in the case of thermal dediazoniation of the aggregate-bound long-chain 16-ArN_2^+ at the interface of reverse micelles. In general, if

$$\%\ \text{1-ArBr} = x[Br^-]^y \tag{4}$$

then

$$\%\ \text{16-ArBr} = x[Br_i^-]^y \tag{5}$$

where *x* and *y* are the respective constant values as given in the correlation equations for **7**−**12**. Thus, the correlation equations observed in the nonmicellar dediazoniations of 1-ArN_2^+ with varying amounts of **7**−**12** are used to estimate [Br_i^-] by substituting % 16-ArBr yield in eq 5 for W/O microemulsions of **1**−**6**, respectively. As mentioned earlier,[18] in brief, "when the yields are the same, the concentrations are the same". Similarly, the correlation between % 16-ArOHex and [$HexOH_i$] in the interface-bound dediazoniation of 16-ArN_2^+ is assumed to be the same as that between % 1-ArOBu and [*n*-BuOH] in the aqueous nonmicellar dediazoniation of 1-ArN_2^+ in the presence of varying amounts of added *n*-butanol. The correlation equation for short-chain alcohols was developed using *n*-butanol because the solubility of *n*-hexanol is extremely low in water (∼0.1 M). The correlation equation for % 1-ArOBu and [*n*-BuOH] is

$$\%\ \text{1-ArOBu} = 1.214[n\text{-BuOH}] - 0.1 \tag{6}$$

so

$$\%\ \text{16-ArOHex} = 1.214[HexOH_i] - 0.1 \tag{7}$$

Therefore, in eq 7, by substituting % 16-ArOHex yields obtained from the interface-associated dediazoniation of 16-ArN_2^+, [$HexOH_i$] was calculated for W/O microemulsions of **1**−**6**. Finally, the interfacial concentrations of water, [H_2O_i] (Table 3), were estimated by substituting values of [Br_i^-], [$HexOH_i$], and the % 16-ArOH yields observed in the reverse micellar dediazoniation of 16-ArN_2^+ in eq 1, given in the Experimental Section.

Table 3 shows the product distribution results for the interface-bound dediazoniation of 16-ArN_2^+ in **1**−**6** (50 mM)/isooctane/*n*-hexanol/water microemulsions at $z = 4.8$ across the wide W_0 range. In all, dediazoniations yields were normalized because the observed yields (the actual yields in duplicate experiments varied within ± 1%) were almost quantitative (89−106%, the variation in the observed yields is mainly due to experimental errors such as weighing the diazonium salt, the addition of diazonium salt solution, etc.) with no significant extraneous peaks in the HPLC chromatograms (Supporting Information). The results of the interface-bound dediazoniation reaction shows that [Br_i^-] decreases with the increase in the headgroup size of

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surfactants as the smaller number of counterions being attached to the interface. On moving from surfactants **1**−**4** with an increase in the headgroup area, (the surface area per headgroup, A_{min}, was reported in our previous work[11]), α (Table 1) rises, leading to a decrease in [Br_i^-] from 3.28 to ∼0.9 M (Table 3). It is very difficult to measure the α in the W/O microemulsions. Thus, the observed α in aqueous solution may provide an explanation of the decrease in [Br_i^-] with increasing headgroup size, considering a similar trend in their behavior as in aqueous micelles. Following the same trend, [Br_i^-] is also a bit lower (∼2.5 M) in **5** compared to that in **1**. The interfacial [Br_i^-] in **1** (3.28−2.56 M, at $z = 4.8$) was found to be in agreement with that reported earlier (3.7−2.5 M at $z = 16$) in **1** W/O microemulsions.[7] However, it is markedly higher than that found in **4** owing to decrease in both the headgroup size and α. Further increases in headgroup size in **6** due to the methylation of the hydroxyl group of **5** led to an increase in α and a decrease in [Br_i^-] of ∼0.9 M.

Now it was important to determine how the concentration of water at the interface influences lipase activity. It was observed that [H_2O_i] remained almost unchanged (32.6−33.5 M in **1**, 31.8−34.6 M in **2**, 31.2−36.6 M in **3**, and 30.0−34.4 M in **4**) in W/O microemulsions of **1**−**4** across the W_0 range (Table 3). Strikingly, irrespective of the headgroup size from **1** to **4**, where the methyl groups of **1** were subsequently replaced by *n*-propyl groups (a hydrophobic moiety), [H_2O_i] did not change at all. In our earlier work,[11] it was found that the catalytic activity of lipase significantly increases in the W/O microemulsions of **1**−**4** (Figure 3, second-order rate constants, k_2, of 308 ± 12, 365 ± 7, 425 ± 7, and 565 ± 8 $cm^3\ g^{-1}\ s^{-1}$ for **1**−**4**, respectively), having similar solution compositions that were used for a product distribution study. The only other parameter that also increases with lipase activity is the surface area per head group with sequential *n*-propyl substitution from **2** to **4** ($A_{min} \approx 1.20$ to 2.20 nm^2).[11] Thus, with increasing headgroup size and hydrophobicity, lipase activity increases while [H_2O_i] remains unaltered.

To decipher the role of hydrophilic groups in the alteration of [H_2O_i] and thereby its effect on lipase activity, the interfacial composition was determined in W/O microemulsions of **5**, where one methyl of **1** was replaced by a hydroxyethyl group serving as a representative of the hydrophilic headgroup surfactants.[11] The introduction of a hydroxyethyl group at the polar head of a surfactant was done with a view that it possess significant hydrating ability due to the presence of hydrogen bond donor and acceptor atoms, which presumably increase [H_2O_i].[8,22] In contrast, the product distribution results revealed that there was no increase in [H_2O_i] (33.0−33.4 M, Table 3) in W/O microemulsions of **5** compared to that in **1**−**4**. The unchanged [H_2O_i] might be due to the scarcity of free water because most of it is bound to the hydroxyl group owing to its hydrogen bonding ability. The catalytic activity of lipase in **5** was found to be slightly higher ($k_2 = 324 \pm 13$ $cm^3\ g^{-1}\ s^{-1}$, Figure 3) than that of **1** but lower than that of **2**, with A_{min} remaining between values for **2** (1.23 nm^2) and **5** (1.18 nm^2). As a whole, the product distribution results indicate that irrespective of the nature of the polar headgroup [H_2O_i] remains almost the same in W/O microemulsions of **1**−**5** while the lipase activity was found to vary linearly with alterations in headgroup size.

Notably, in W/O microemulsions of methoxyethylated surfactant headgroups, lipase showed its highest activity ranges,[11] which were ∼35−65% higher than their hydroxyethyl analogues. The replacement of hydroxyethyl by methoxyethyl expectedly leads to a decline in the hydrophilicity at polar headgroup due to the removal of the hydrogen bond acceptor atoms (H). Therefore, an obvious question arises of how much the hydrophilicity of a surfactant headgroup is responsible for the alteration of [H_2O_i] and, consequently, enzyme activity. Does the interfacial water concentration change with the reduction of hydrophilicity of the surfactant head group by means of protecting the −OH groups with a methyl ether linkage? To answer this question, the interfacial composition was measured in a W/O microemulsion of **6**, a representative of methoxyethylated headgroup surfactants. Interestingly, [H_2O_i] was found to increase to 41.5−42.2 M. This rise in [H_2O_i] might be due to more free water because the hydrophilicity was reduced by having only a hydrogen bond donor atom. In comparison to CTAB (**1**) and the corresponding hydrophobic (**2**) and hydrophilic (**5**) headgroup analogues, lipase activity (Figure 3) in **6** ($k_2 = 530 \pm 9$ $cm^3\ g^{-1}\ s^{-1}$) was enhanced[11] with increases in both [H_2O_i] (Table 3) and headgroup size ($A_{min} = 2.12$ nm^2) but was found to be in a comparable range with **4** ($k_2 = 565 \pm 8$ $cm^3\ g^{-1}\ s^{-1}$). Importantly, the A_{min} values of **4** and **6** (2.20 and 2.12 nm^2) are in a comparable range although their [H_2O_i] values differ by ∼20% with similar W_0 values. Despite the differences in [H_2O_i], similar lipase activity in comparable headgroup areas of **4** and **6** once again denotes the predominant role of headgroup size and probably the negligible influence of the interfacial water concentration in regulating lipase activity. Besides [H_2O_i] and [Br_i^-], another interfacial component whose presence has been left unmentioned is [$HexOH_i$]. The concentration of the cosurfactant remains in the comparable range in W/O microemulsions of all of the surfactants at similar W_0 values, leading to analogous influences in the regulation of lipase activity.

An increase in the headgroup size leads to an increase in the space between two headgroups and consequently the interfacial area. The augmented interface presumably allows for the smooth solubilization of lipase and also increases the possibility of increasing the concentrations of both enzyme and substrate, leading to a higher activity of lipase.[1b,6,23] Although [H_2O_i] remains almost unchanged, the concentration of another interfacial component (i.e., [Br_i^-]), the essential component of the headgroup, was found to change considerably. Also, we have attempted to correlate lipase activity in the cationic W/O microemulsions of **1**−**6** with the [H_2O_i]/[Br_i] ratio. The lipase activity was found to increase with [H_2O_i]/[Br_i] on moving from a surfactant of smaller to larger headgroup size, but no linear or curve-fitted correlation was found between them (Figure S1, Supporting Information).

[Br_i^-] was found to be greater in **1** and **5** than in **2**−**4** and **6** (Table 3). Therefore, with increasing headgroup size, the α value increases (Table 1), leading to the lowering of [Br_i^-], which in turn also enhances the space available at the interface for the localization of the enzyme and substrate.[1b,6,23] As a result, the lipase activity increases, possibly as a result of the improved local concentration of the enzyme and the substrate at the interface. Thus, the headgroup size and counterion are the two parameters that play complementary roles in controlling the water−oil interfaces and consequently the behavior of the surface-active enzyme.

Conclusions

To delineate the independent/predominant roles of headgroup size/hydrophilicity and interfacial composition in controlling the catalytic activity of lipase, the concentration of interfacial

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components in the cationic reverse micelles of varying surfactants (**1**−**6**) was quantitatively determined by a chemical trapping protocol. The presumed crucial parameter, $[H_2O_i]$, was found to remain almost unaltered (30.0−36.7 M) from **1**−**5** but increased a little up to 41.5−42.2 M in the case of W/O microemulsions of **6**. However, despite the difference in $[H_2O_i]$, the lipase activity was found to increase primarily with surfactant headgroup size and decrease in counterion concentration, which is inversely proportional to the degree of dissociation. Importantly, irrespective of the differences in $[H_2O_i]$, lipase had similar activity in W/O microemulsions of **4** and **6** in agreement with comparable α values, headgroup size, and $[Br_i]$. Thus, it may be safely concluded that it is the headgroup size that primarily regulates lipase activity in micellar enzymology.

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Supporting Information Available: Details of the HPLC product distribution data: HPLC peak areas, observed and normalized product yields in reverse micellar and nonmicellar aqueous dediazoniation reactions, all of the calibration equations used, and local molar concentrations of interfacial components in reverse micellar dediazoniation reactions. This material is available free of charge via the Internet at http://pubs.acs.org.

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