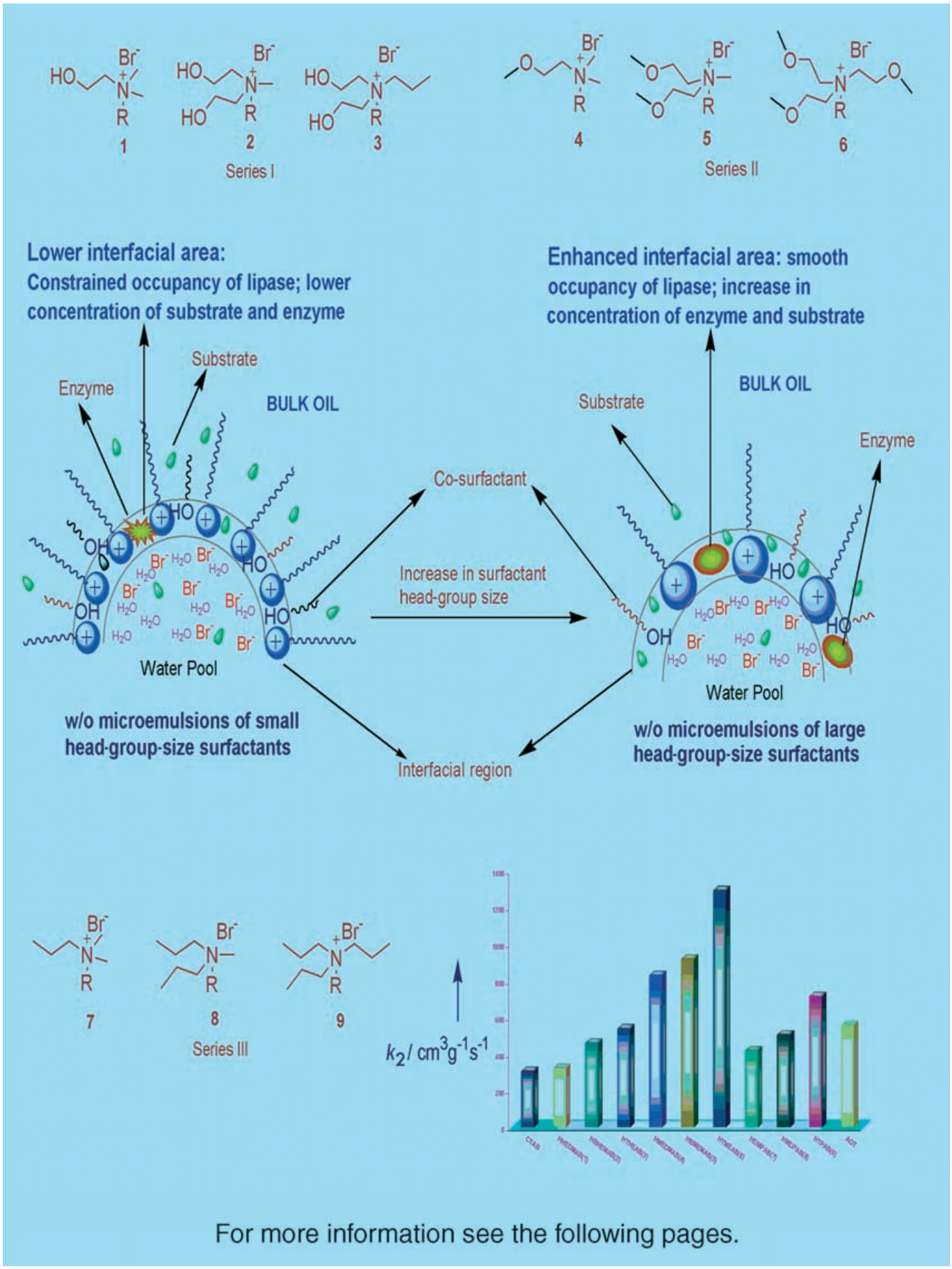

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Head-Group Size or Hydrophilicity of Surfactants: The Major Regulator of Lipase Activity in Cationic Water-in-Oil Microemulsions

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Abstract: To determine the crucial role of surfactant head-group size in micellar enzymology, the activity of *Chromobacterium Viscosum* (CV) lipase was estimated in cationic water-in-oil (w/o) microemulsions of three different series of surfactants with varied head-group size and hydrophilicity. The different series were prepared by subsequent replacement of three methyl groups of cetyltrimethylammonium bromide (CTAB) with hydroxyethyl (**1**–**3**, series I), methoxyethyl (**4**–**6**, series II), and *n*-propyl (**7**–**9**, series III) groups. The hydrophilicity at the polar head was gradually reduced from series I to series III. Interestingly, the lipase activity was found to be markedly higher for series II surfactants relative to their more hydrophilic analogues in series I. Moreover, the activity remained almost comparable for complementary analogues of both series I and III, though the hydrophilicity was drastically different. Noticeably, the head-group area per surfactant is almost similar for comparable surfactants of both series I and III, but distinctly higher in case of series II surfactants. Thus the lipase activity was largely regulated by the surfactant head-group size, which plays the dominant role over the hydrophilicity. The increase in head-group size presumably allows the enzyme to attain a flexible conformation as well as increase in the local concentration of enzyme and substrate, leading to the higher efficiency of lipase. The lipase showed its best activity in the microemulsion of **6** probably because of its highest head-group size. Furthermore, the observed activity in **6** is 2–3-fold and 8-fold higher than sodium bis(2-ethyl-1-hexyl)sulfosuccinate (AOT) and CTAB-based microemulsions, respectively, and in fact highest ever in any w/o microemulsions.

Keywords: enzymes · hydrophilicity · lipases · microemulsions · surfactants

Introduction

Enzymology in self-organized aggregates such as water-in-oil (w/o) microemulsions has been an area of interest for several decades, because of its potential biotechnological applications.[1–4] Lipases, a class of surface-active enzymes,[1] are widely exploited in diverse transformations in w/o microemulsions.[1,5–7] The catalytic efficiencies of these enzymes are believed to be dependent primarily on the local concentrations of water and other ions present in the vicinity of the enzyme.[8–11] To this end, Das and Chaudhuri 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 due to the unaltered interfacial water concentration, $[H_2O]_i$ (28.1–31.8 M).[10] The reported $[H_2O]_i$ is significantly lower than bulk water concentration (55.5 M) and held responsible for poor activity of lipase in CTAB microemulsions. At the same time, while determining the role of interfacial water with respect to the superior activity of lipase in AOT-based microemulsions (AOT = sodium bis(2-ethyl-1-hexyl)sulfosuccinate),[1,6] Srilakshmi and Chaudhuri found a value of $[H_2O]_i$ = 27.9–32 M,[12] similar to that observed in CTAB systems.[10] Thus, the correlation between the lipase activity and the $[H_2O]_i$ is far from straightforward and deserves further investigation to determine the role of several other parameters. However, the higher surface area occupied by the AOT-head group may have crucial role on the superior activity of lipase in addition to the influence of $[H_2O]_i$.

In our previous study,[11] the hydrolytic ability of lipase was dramatically enhanced in cationic w/o microemulsions

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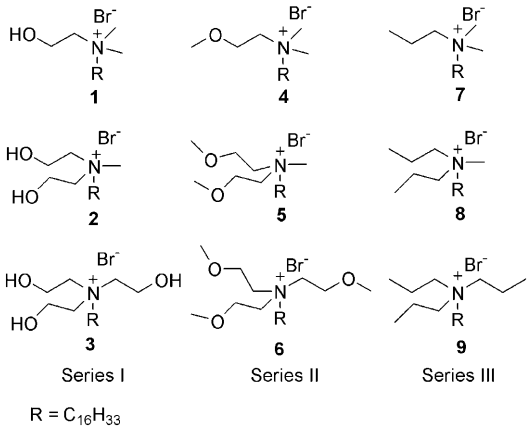
by the introduction of hydroxyethyl groups at the surfactant polar head. Hydroxyethyl groups were introduced with the aim of increasing the local concentration of water at the interface.[11] The hydroxyethyl groups not only improved the hydrophilicity, but at the same time also increased the head-group size and consequently the surface area per head group at the interface. The increase in the surface area with hydroxyethyl groups may provide enough space to attain flexible secondary conformation[13] of enzyme and also may allow a larger population of substrate and enzyme molecules at the interface,[14] leading to the enhancement in lipase activity.[15]

At this point, the issue remains unresolved whether the surfactant head-group size or the local concentration of water at the interface plays the dominant role in regulating the lipase's efficiency. A considerable amount of work has been reported toward investigating the effect of the head-group size and the hydrophilicity on numerous physical properties[16,17] and also different self-assembly-mediated chemical transformations.[18] The role of head-group size in modulating the enzyme activity is mostly a neglected parameter in micellar enzymology. To the best of our knowledge, no such attempt has been made to correlate the catalytic activity of the lipase with the head-group size and its hydrophilicity in reverse micelles.

In the present study, for the first time a possible correlation between the catalytic efficiency of lipase in cationic w/o microemulsions with the head-group size of surfactant is delineated. The catalytic activity of lipase was estimated in the w/o microemulsions of cationic surfactants (**1**–**9**) with varying head-group size and hydrophilicity. Interestingly, the efficiency of CV lipase was found to be similar in case of comparable head-group size, but with different hydrophilicity. The lipase activity distinctly increases with the head-group size regardless of its hydrophilic nature. Through the variation in the head-group size, we found that the use of surfactant **6** leads to the highest ever activity of CV-lipase in any w/o microemulsions. The observed activity in w/o microemulsion of **6** is almost 2–3-fold higher than that in AOT-based systems and eight times higher than that found in CTAB-based w/o microemulsions.



Results and Discussion

In deciphering the role of head-group size and hydrophilicity (the ability to increase the $[H_2O]_i$) on the efficiency of lipase, we have synthesized three series of surfactants. The hydrophilicity and size of the surfactant head groups in all the series have been methodically varied through the replacement of the methyl groups at the CTAB polar head by hydroxyethyl, methoxyethyl, and *n*-propyl groups (series I–III, respectively).

The aqueous critical micelle concentration (cmc) of the surfactants (**1**–**9**) has been measured tensiometrically using the ring method (Table 1). The cmc's were determined from plots of surface tension versus $\log c$ ($\log$[surfactant]). The cmc values obtained for each series were in good agreement with the fact that with increase in the head-group size the cmc of the surfactant decreases.[19] The minimum area per surfactant head group at the micellar interface, A_{min} in nm^2, was calculated from the following equations [Eqs. (1) and (2)], as obtained from literature.[16,19]

Table 1. The critical micellar concentration (cmc), area minimum (A_{min}) of surfactants **1**–**9**, isosbestic point, and the molar extinction coefficients (ε) of *p*-nitrophenol/*p*-nitrophenolate couple in the w/o microemulsions of surfactants **1**–**9**.

Surfactant	cmc [M][a]	A_{min} [nm^2]	Isosbestic point [nm]	ε [M^{-1}cm^{-1}]
1	2.02×10^{-4}	1.18 ± 0.02	340.0	4350
2	1.53×10^{-4}	1.42 ± 0.02	338.0	4250
3	0.36×10^{-4}	2.30 ± 0.03	339.8	4277
4	2.03×10^{-4}	2.12 ± 0.04	339.2	4529
5	1.22×10^{-4}	2.53 ± 0.03	337.0	4735
6	0.73×10^{-4}	2.90 ± 0.05	338.6	4420
7	6.12×10^{-4}	1.23 ± 0.01	338.0	4656
8	2.04×10^{-4}	1.40 ± 0.02	339.4	4509
9	1.38×10^{-4}	2.20 ± 0.04	338.2	4558

[a] Values were determined tensiometrically at 25 °C.

$$\Gamma_{max} = \frac{1}{4.606\,RT}\lim_{c\to c_{cmc}}\frac{d\pi}{d\log c} \quad (1)$$

$$A_{min} = \frac{10^{18}}{N\Gamma_{max}} \quad (2)$$

In these equations, π is the surface pressure calculated from the equation $\pi=\gamma_{water}-\gamma_{solution}$ (γ denotes the surface tension), Γ_{max} is the maximum surface excess concentration, and N is the Avogadro's number. The calculated A_{min} values show a proportional dependence on the head-group size. The surface area per head group for each series of surfactants increased with increasing head-group size and this trend is in accordance with the literature.[16]

The pseudoternary phase diagrams of the surfactants (**1**–**9**) with *n*-hexanol (1:2, w/w)/water/isooctane systems at 25 °C are presented in Figure 1. These three diagrams (Figure 1) have been the subject of a systematic phase behavior study to determine the water solubilization region

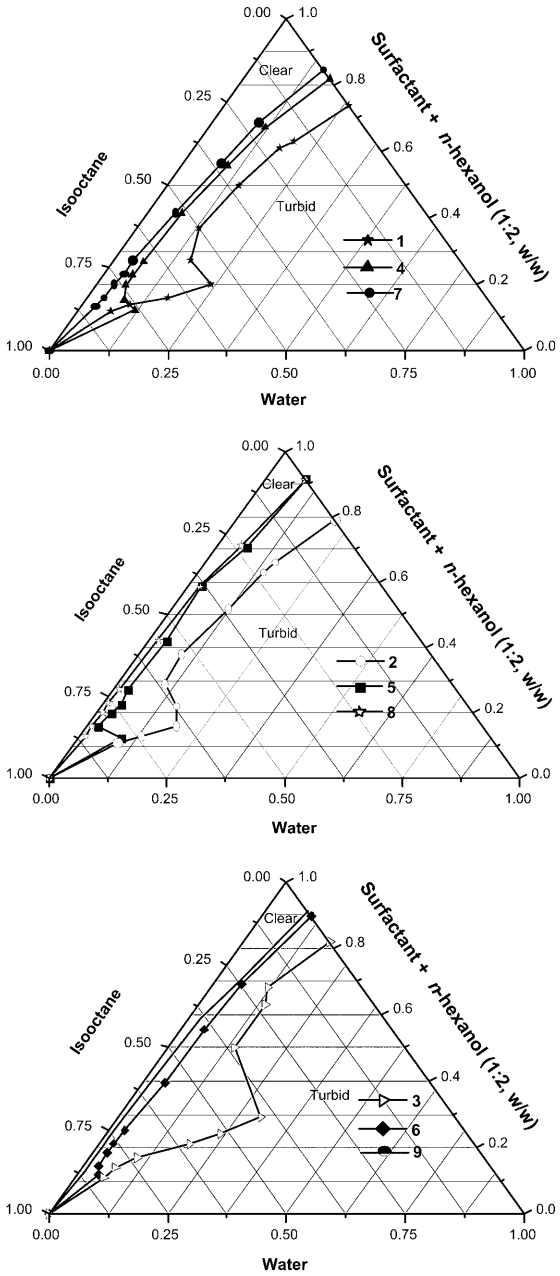


Figure 1. Pseudoternary phase diagrams of the quaternary systems of (**1**–**9**)/*n*-hexanol(1:2 w/w)/water/isooctane at 25 °C. Scale magnitudes are reduced by 1/100th of the plot.

with varying head-group size and hydrophilicity. In each phase diagram, complementary analogues of series I–III were merged together to observe the effect of variation in head-group hydrophilicity. The diagrams show that the presence of hydroxyethyl group(s) increases the hydrophilicity of the surfactant head, since the isotropic regions for the amphiphiles **1**–**3** are higher relative to the corresponding members of the other two series. The high isotropic area in the w/o microemulsion region for **1**–**3** is probably the result of high water uptake by the hydroxyethyl groups due to the presence of hydrogen-bond donor and acceptor atoms. The isotropic area is distinctly lower in the case of methoxyethyl-containing surfactants (**4**–**6**) than the corresponding hydroxyethyl-substituted analogues (**1**–**3**); possibly because of the replacement of hydrogen-bond acceptor atoms of latter analogues with methyl ether linkages. The isotropic area is further lowered (Figure 1) for *n*-propyl-substituted analogues (**7**–**9**), perhaps due to the complete absence of any hydroxyl moiety at the head group. Thus, the head-group hydrophilicity of the surfactant plays an important function in the molecular packing of organized aggregates.

Toward investigating the role of head-group hydrophilicity and size, the catalytic activity of CV lipase was examined in the w/o microemulsions of 0.05 M surfactant (**1**–**9**)/isooctane/*n*-hexanol/water at pH 6.0 (20 mM phosphate), z ([alcohol]/[surfactant]) = 4.8 and 25 °C across a varying range of W_0 (at which isotropic solutions are formed). The second-order rate constant, k_2 (Figure 2) for the lipase-catalyzed hydrolysis of *p*-nitrophenyl-*n*-hexanoate was found to be independent of W_0 for all the surfactants. In accordance with our previous report,[11] the activity of lipase increased (Figure 2) from **1**–**3** by ~65% (324 ± 13, 461 ± 10, and $540 \pm 8\ cm^3\,g^{-1}\,s^{-1}$ for **1**–**3**, respectively) with sequential increase in the number of hydroxyethyl moiety at the polar head group (series I). Under the similar experimental conditions, the k_2 was found to be $308 \pm 12\ cm^3\,g^{-1}\,s^{-1}$ in case of CTAB and $161 \pm 9\ cm^3\,g^{-1}\,s^{-1}$ when $z = 16$ (most commonly used solution compositions of CTAB w/o microemulsion).[11] Introduction of hydroxyethyl group(s), possessing significant hydrating ability due to the presence of hydrogen-bond donor and acceptor atoms at the polar head of surfactants (**1**–**3**), possibly increases the $[H_2O]_i$.[11,20] At the same time, the area per head group (A_{min}) of surfactants also increases (Table 1) from **1**–**3** (series I), due to the sequential substitution of hydroxyethyl groups. Thus, the influence of either of the parameters towards observed enhancement in lipase activity from **1**–**3** (Figure 2) is yet uncertain.

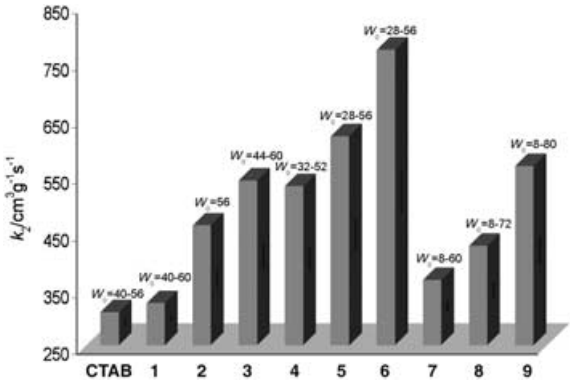


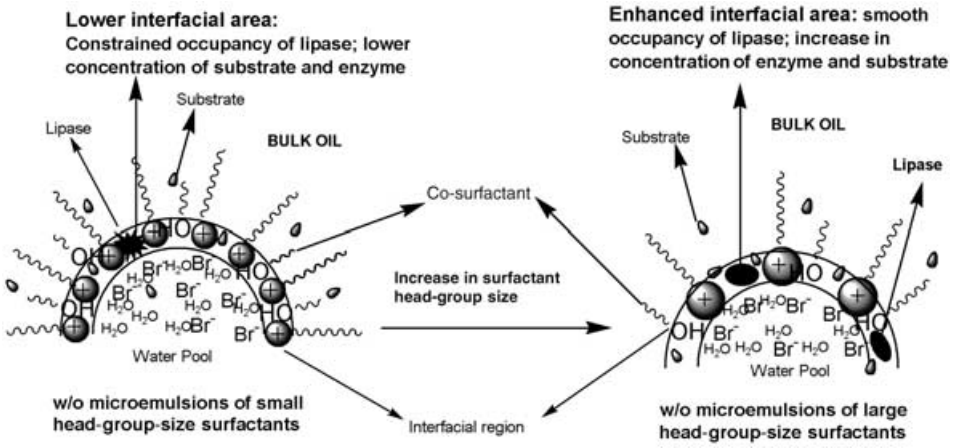
Figure 2. Variation of the second order rate constant (k_2) for the lipase-catalyzed hydrolysis of *p*-nitrophenyl-*n*-hexanoate in different cationic w/o microemulsions formed at $z = 4.8$, 25 °C, and pH 6.0 (20 mM phosphate). [surfactant] = 50 mM, [enzyme] = 1.02×10^{-6} gmL^{-1}, [substrate] = 3 mM. The experimental errors are given in the text.

To this end, we reduced the hydrophilicity of surfactant head groups (**1**–**3**) by protecting the −OH groups with a

methyl ether linkage to obtain the methoxyethyl analogues (**4–6**, series II) of the series I surfactants. Replacement of hydroxyethyl by methoxyethyl groups (series II) expectedly leads to a decline in the hydrophilicity at polar head, due to the removal of the hydrogen-bond acceptor atoms (H). The catalytic activity of lipase was measured in the w/o microemulsions of series II surfactants (**4–6**) keeping all other experimental conditions identical. The k_2 was found to be further increased by ~35–65% (530 ± 9, 618 ± 6, and $770\pm8\ cm^3\ g^{-1}\ s^{-1}$ for **4**, **5**, and **6**, respectively) compared to their hydroxyethyl analogues **1–3** (Figure 2). The hydrolytic efficiency of lipase was also found to improve with sequential increment in the number of methoxyethyl groups at the polar head of the surfactants **4–6** similar to that observed in series I (Figure 2). The decrease in the hydrophilicity at polar head of surfactant did not reduce the activity of lipase; instead it was increased (Figure 2) in spite of lowering the hydrophilicity. At this point, a distinct enhancement in the head-group size of series II surfactants compared to their corresponding surfactants of series I is evident from their A_{min} values (Table 1). Similarly, the head-group area is also increased with the sequential increase in the number of methoxyethyl groups at the polar head of surfactants from **4–6**. Thus, the increase in the head-group size of surfactants may be correlated with the enhanced efficiency of the lipase, while head-group hydrophilicity may not have significant influence on the catalytic activity of lipase in cationic w/o microemulsions.

In the preceding paragraph, lipase activity was measured in series II surfactants, whereby the head-group hydrophilicity was partially reduced with concurrent increase in the area per head group relative to series I surfactants. At this stage, to examine the straightforward correlation between the head-group size and lipase activity, series III surfactants (**7–9**) were synthesized in which the hydroxyethyl groups of series I were replaced by *n*-propyl groups. The hydrophilicity at polar head, due to the presence of hydroxyl moieties in series I, was completely removed in series III by substituting the −OH functionalities with methyl groups and keeping the head-group size similar (Table 1) for comparable surfactants of both series. The catalytic efficiency of lipase was measured in the w/o microemulsions of **7–9** under the same experimental conditions as mentioned above. As the number of propyl group increases at the polar head of the surfactants **7–9**, the k_2 again increased (365 ± 7, 425 ± 7, and $565\pm8\ cm^3\ g^{-1}\ s^{-1}$, respectively) with head-group size (Table 1) similar to the trend observed in series I and II (Figure 2). Interestingly, the lipase activity in **7–9** was found (Figure 2) to be similar to their complementary analogues of series I (**1–3**). The calculated A_{min} values (Table 1) indicate that the surface area per head group also does not vary for the corresponding surfactants of series I and III. Although the head-group hydrophilicity is distinctly different between series I and III surfactants, the almost unchanged lipase activity in comparable surfactants of both series is presumably attributed to their similar head-group size. Thus, a possible correlation can be established for the first time that the efficiency of lipase is steadily dependent on the head-group size of surfactant in cationic w/o microemulsions. Moreover, the dominating role of head-group size in modulating the efficiency of enzyme in organized solution is also delineated.

At this point an obvious question arises: how does the head-group size affect the catalytic efficiency of lipase in cationic w/o microemulsions? It is a well-known fact that the head-group structure of an amphiphile has an important role towards the molecular packing of organized self-assemblies.[16,17,21] An increase in the head-group size leads to increase in the interfacial area,[16,19] and accordingly the space between two head groups is also enhanced. The augmented space between two head groups presumably allows the lipase to solubilize itself smoothly at the interfacial region. Consequentially the enzyme may attain a more flexible secondary conformation (Scheme 1), leading to its higher efficiency. Furthermore, the possibility of increasing concentration of both enzyme and substrate is also improved at the enhanced interfacial region[14] (Scheme 1), probably because of alteration in partition coefficients.[6,9,22] As it was found that the lipase activity is larger in series II surfactants (**4–6**) relative to corresponding surfactants of series I and III, we can explain this from the present study exclusively on the basis of the head-group area (Table 1). Although the differences in A_{min} value did not appear to be significant in some cases, it is probably sufficient enough to affect the enzyme activity. At the same time, surfactants of different series with comparable head-group area (**3**, **4**, and **9**, Table 1)



Scheme 1. Pictorial representation of the interfacial region of w/o microemulsions prepared with varying head-group size. The increase in head-group size leads to smooth occupancy of lipase and increases in the local concentration of the enzyme and substrate.

showed similar activity (Figure 2) under equivalent solution compositions ($z = 4.8$). Therefore, the increase in the surfactant head-group size plays a crucial role in modulating the lipase efficiency, presumably by rendering a flexible conformation to the enzyme and increasing the local concentration of the reactants (Scheme 1) at the augmented interface.[13–16]

One of the aims of our research is to improve the lipase's efficiency in cationic w/o microemulsions.[11] To this end, besides the molecular architecture of surfactant, solution compositions also play a role in regulating the enzyme activity. Till now we have prepared the w/o microemulsions using the solution composition surfactant, (0.05 M)/water/isooctane/*n*-hexanol at $z = 4.8$. At this stage the competitive inhibition by alcohols on the lipase activity has also been iterated several times in the literature.[11,23] Hence, to enhance the lipase's efficiency in cationic w/o microemulsions it is essential to prepare the microemulsions without or with a minimum amount of alcohol (co-surfactant). To reduce the inhibitory action of *n*-hexanol, we prepared the cationic w/o microemulsions of 0.05 M **1**–**9**/water (pH 6.0)/isooctane with the minimum amount of *n*-hexanol needed to form isotropic solutions (z values for each surfactant are given on top of the activity bar in Figure 3). Noticeably, the *n*-hexanol content could be reduced in case of series II (**4**–**6**) and III (**7**–**9**) surfactants, which are distinctly less hydrophilic at the interface relative to series I analogues (**1**–**3**). As expected, the activity of lipase was found to increase (Figure 3) by ~15–70% (820 ± 15, 920 ± 13, 1290 ± 12, 420 ± 8, 500 ± 10, and $715 \pm 10\ cm^3 g^{-1} s^{-1}$ for **4**–**9**, respectively) with decreasing alcohol content in the microemulsions of **4**–**9**. Here also the observed k_2 values show similar trend of head-group-size dependence, as the activity of lipase enhanced with the increasing head-group size of series II and III surfactants. The observed k_2 values for **4**–**6** (Figure 3) is 2–2.5-fold higher than the corresponding more hydrophilic analogues **1**–**3** in series I and also compared to AOT/isooctane/water microemulsions.[1,6] Moreover, this activity is 4- and 8-fold higher than that found in CTAB w/o microemulsions at $z = 4.8$ and 16, respectively. Thus, overall we can conclude that the head-group size of surfactant is the major regulator of lipase activity profile in cationic w/o microemulsions.

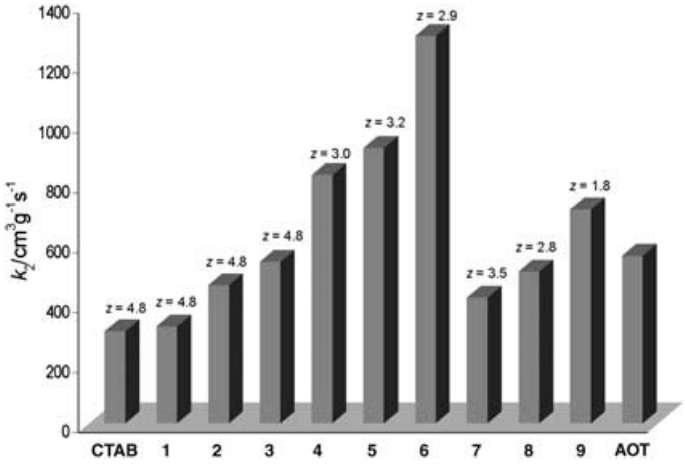


Figure 3. Variation of the second-order rate constant (k_2) for the lipase-catalyzed hydrolysis of *p*-nitrophenyl-*n*-hexanoate in different cationic w/o microemulsions of surfactants using least amount of *n*-hexanol required at 25 °C and pH 6.0 (20 mM phosphate). [surfactant] = 50 mM. [enzyme] = 1.02×10^{-6} gmL^{-1}. [substrate] = 3 mM. The experimental errors are given in the text. The W_0 ranges where activity was measured are 40–56, 40–60, 56, 44–60, 32–44, 36–52, 32–44, 12–16, 8–20, 28–32, and 12 for CTAB, **1**–**9**, and AOT respectively.

Since co-surfactant plays a significant role in the packing of the microemulsions, it is necessary to verify whether this head-group size effect on lipase activity profile is dependent on the co-surfactant architecture. Accordingly, the activity of enzyme was studied (Figure 4) in the microemulsions of

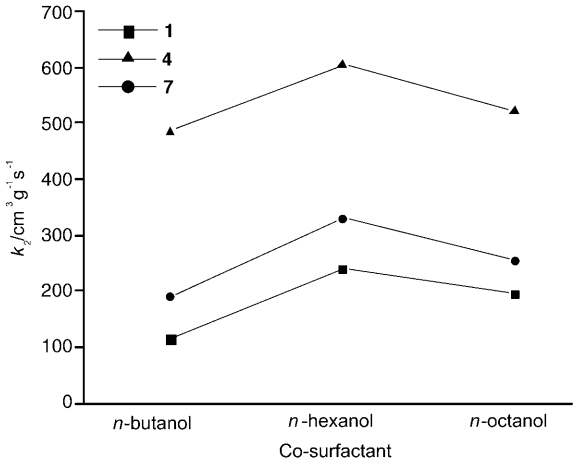


Figure 4. Effect of varying co-surfactant on the second-order rate constant for lipase-catalyzed hydrolysis of *p*-nitrophenyl-*n*-hexanoate in different cationic w/o microemulsions at $z = 9.6$, 25 C, and pH 6.0 (20 mM phosphate). [surfactant] = 50 mM. [enzyme] = 1.02×10^{-6} gmL^{-1}. [substrate] = 3 mM.

1, **4**, and **7** (comparable analogues of each series) with *n*-butanol, *n*-hexanol, and *n*-octanol as the co-surfactant at $z = 9.6$ (solution compositions at which each surfactant can form w/o microemulsions at same z value with any co-surfactant). Irrespective of the co-surfactants, the efficiency of lipase (Figure 4) increased with head-group size from **1** to **4**. Although **1** and **7** are of comparable head-group size (Table 1), the k_2 was found to be lower in case of **1**, possibly because of the different inhibiting effect of alcohols at such high $z = 9.6$. However, for each of the surfactants the lipase activity increased with increasing alcohol chain length, and was highest in the case of *n*-octanol.

To ascertain whether or not the observed head-group size effect is substrate specific, *p*-nitrophenylalkanoates of various chain length (C_4 to C_{16}) were hydrolyzed (Figure 5) by CV lipase in the cationic w/o microemulsions of **2**, **5**, and **8** (corresponding surfactants of each series) keeping all other experimental conditions identical. As it can be seen in Figure 5, surfactants **2** and **8**, with comparable head-group size (Table 1), had very similar activity profiles across the range of substrates. Whereas regardless of the substrate

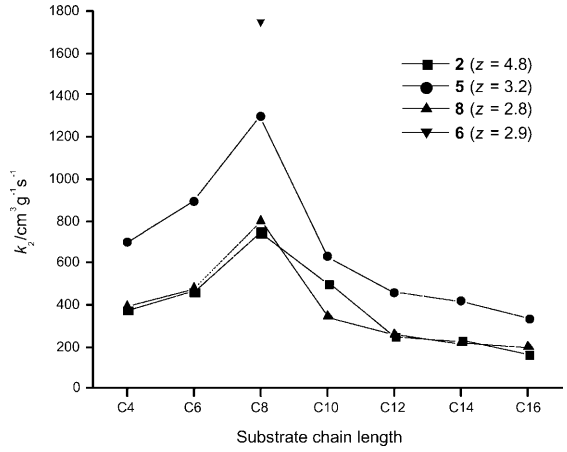


Figure 5. Effect of the substrate chain length on the second-order rate constant (k_2) for the lipase-catalyzed hydrolysis of *p*-nitrophenylalkanoates in different cationic w/o microemulsion systems at 25 C and pH 6.0 (20 mM phosphate). [surfactant] = 50 mM. [enzyme] = 1.02×10^{-6} gmL^{-1}. [substrate] = 3 mM. The W_0 ranges where activity was measured are 56, 36–52, 32–44, and 8–20 for **2**, **5**, **6**, and **8**, respectively.

chain length, surfactant **5** exhibited markedly higher activity (Figure 5), presumably due to its larger head-group size (Table 1). Furthermore, irrespective of the surfactants, the highest activity was found for *p*-nitrophenyloctanoate (C_8), and bell-shaped activity profiles were obtained in all cases. Variations in the number of methylene groups in the hydrophobic domain of the substrates alter the hydrophilic–lipophilic balance (HLB) of the molecules. The HLB probably reaches an optimal value at C_8, at which the interfacial localization of the molecules becomes highest for isooctane/*n*-hexanol bulk oil composition. Accordingly, the local concentration of C_8 substrate possibly increases at the interface and consequently the lipase activity increases. Throughout the investigation the best lipase activity was observed either in the microemulsions of **6** or by using C_8 substrate in respective series of surfactants and substrates. In continuation of our efforts to improve the lipase activity in w/o microemulsions, we measured the enzyme's efficiency in **6**/water/isooctane-*n*-hexanol ($z = 2.9$, $W_0 = 32$–44) using *p*-nitrophenyloctanoate as the substrate. The observed k_2 value (1748 ± 24 cm^3 g^{-1} s^{-1}) is more than three times higher than that found in the widely used AOT-based system and in fact the highest ever activity of lipase found in any w/o microemulsions.

Conclusion

The regulatory role of the surfactant head-group size on the lipase activity is distinct throughout the investigation. The activity was found to increase markedly for series II surfactants relative to their more hydrophilic analogues in series I. At the same time the activity in case of series III surfactants remained almost comparable to that with series I, in spite of large differences in their hydrophilicity. Notably, the head-group area per surfactant is similar for comparable surfactants of both series I and III, but distinctly higher in case of series II surfactants. Thus, the hydrophilicity of the surfactant head group does not have much influence over the enzyme efficiency, while the head-group size was found to largely regulate the activity. The increase in the head-group size presumably provides greater space for the enzyme to attain a flexible conformation and also increases the local concentration of enzyme and substrate molecules at the interface, leading to the higher efficiency of lipase. The overall results showed for the first time that the hydrolytic activity of lipase steadily increased with the head-group size of surfactant irrespective of its hydrophilicity. The observed k_2 value in microemulsions of **6** is 2–3-fold higher than that found in AOT-based systems and 8-fold higher compared to the CTAB/water/isooctane-*n*-hexanol ($z = 16$) system; it is also the highest ever in any w/o microemulsions.

Experimental Section

Materials: *Chromobacterium viscosum* Lipase (EC 3.1.1.3, Type XII) was purchased from Sigma and was used as received. Analytical grade CTAB from Spectrochem (India) was recrystallized three times from methanol/diethyl ether; the recrystallized CTAB was without minima in its surface tension plot. HPLC grade isooctane, *n*-butanol, *n*-hexanol, *n*-octanol, solvents, and all other reagents used in the syntheses were obtained from SRL (India) and were of the highest analytical grade. 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. Chemical shifts are reported in ppm, by using TMS for ^{1}H NMR as internal standard. Mass spectrometric (MS) data were acquired by electrospray ionization (ESI) techniques on a Q-TOF Micro-Quadruple mass spectrometer, Micromass, UK. The *p*-nitrophenylalkanoate substrates were synthesized conventionally from an equimolar solution of the corresponding acid and *p*-nitrophenol in dichloromethane with an equivalent amount of *N,N*-dicyclohexylcarbodiimide (DCC) and catalytic amount of 4-*N,N*-dimethylaminopyridine (DMAP). The syntheses of different surfactants are listed below.

Synthesis of *N*-hexadecyl-*N*-(2-hydroxyethyl)-*N,N*-dimethylammonium bromide (1) and *N*-hexadecyl-*N,N*-bis(2-hydroxyethyl)-*N*-methylammonium bromide (2): Both the amphiphiles were prepared following the procedure mentioned in a recently published protocol.[11,17a] Briefly, 1-bromohexadecane and the corresponding amines (*N,N*-dimethylethanolamine for **1** and *N*-methyldiethanolamine for **2**) were taken in the molar ratio 1.2:1 in 30% methanol/acetonitrile and refluxed. After 24 h, the solvent was evaporated on a rotary evaporator and pure products were obtained by crystallization of the reaction mixture from methanol/ethyl acetate. The yields were 87% and 80% for **1** and **2**, respectively.

Data for 1: ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 4.14$ (br, 2H), 3.69 (br, 2H), 3.48–3.45 (br, 2H), 3.41 (s, 6H), 1.77 (br, 2H), 1.33–1.18 (m, 26H), 0.86 ppm (t, $J = 6.9$ Hz, 3H); elemental analysis calcd (%) for $C_{20}H_{44}BrNO$: C 60.89, H 11.24, N 3.55; found: C 60.85, H 11.16, N 3.39; MS (ESI): *m/z* calcd for $C_{20}H_{44}NO$ (the 4° ammonium ion, 100%): 314.34; found: 314.2605 [M^+].

Data for 2: ^{1}H NMR (300 MHz, $CDCl_3$) : $\delta = 4.13$ (br, 4H), 3.71 (br, 4H), 3.49 (br, 2H), 3.31 (s, 3H), 1.75 (br, 2H), 1.36–1.18 (m, 26H), 0.88 ppm (t, $J = 6.9$ Hz, 3H); elemental analysis calcd (%) for $C_{21}H_{46}BrNO_2$: C 59.42, H 10.92, N 3.30; found: C 59.45, H 10.86, N 3.08; MS (ESI): *m/z* calcd for $C_{21}H_{46}NO_2$ (the 4° ammonium ion, 100%): 344.60; found: 344.4506 [M^+].

Synthesis of *N*-hexadecyl-*N,N,N*-tris(2-hydroxyethyl)ammonium bromide (3): Aqueous solution of NaOH (2.72 g, 0.068 mol, in 25 mL doubly dis-

tilled water) was added dropwise to a mixture of 2-bromoethanol (6.5 g, 0.081 mol) and hexadecylamine (5 g, 0.027 mol) under reflux. After 24 h of refluxing, the reaction mixture was extracted with chloroform (3× 50 mL). Chloroform was removed on a rotary evaporator followed by drying under vacuum. The residue was then crystallized from methanol/ethyl acetate and filtered. The resulting mixture showed three spots (with $R_f = 0.55$, 0.4, and 0) on thin-layer chromatography (TLC) by using 25:75 (v/v) methanol/chloroform as the TLC developing solvents. The dried product (with $R_f = 0.55$) was purified from the white solid obtained from crystallization by column chromatography in a 230–400 mesh silica gel column with 7% methanol/chloroform. The yield was 40%. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 4.08$ (br, 6H), 3.49–3.35 (br, 6H), 3.23–3.18 (br, 2H), 1.83 (br, 2H), 1.34–1.25 (m; 26H), 0.88 ppm (t, $J = 6.9$ Hz, 3H); elemental analysis calcd (%) for $C_{22}H_{48}BrNO_3$: C 58.15, H 10.57, N 3.08; found: C 58.13, H 10.64, N, 3.08; MS (ESI): m/z calcd for $C_{22}H_{48}NO_3$ (the 4° ammonium ion, 100%): 374.62; found: 374.2458.

Synthesis of *N*-hexadecyl-*N*-(2-methoxyethyl)-*N*,*N*-dimethylammonium bromide (4), *N*-hexadecyl-*N*,*N*-bis(2-methoxyethyl)-*N*-methylammonium bromide (5), and *N*-hexadecyl-*N*,*N*,*N*-tris(2-methoxyethyl)ammonium bromide (6): Compounds **4**–**6** were prepared from compounds **1**–**3**, respectively. The corresponding starting materials were treated with excess methyl iodide in dry DMF in presence of sodium hydride (2, 4, and 6 equiv for **4**–**6** respectively) under a nitrogen atmosphere for 48 h. After completion of the reaction, water was added to the reaction mixtures, which were then extracted with chloroform. The organic layer was washed with aqueous sodium thiosulfate solution followed by brine, concentrated on a rotary evaporator, and finally under reduced pressure. The dried material was washed with dry diethyl ether and then passed through Amberlyst A-26 bromide ion exchange column to get the bromide form of the compound. The white solids were then washed with dry diethyl ether three times to get the pure forms of compounds **4**, **5**, and **6**.

Data for 4: ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 3.94$–3.9 (br, 2H), 3.87 (br, 2H), 3.56–3.53 (br, 2H), 3.45 (s, 6H), 3.33 (s, 3H), 1.79–1.74 (br, 2H), 1.25 (br, 26H), 0.88–0.84 ppm (t, $J = 6.48$ Hz, 3H); elemental analysis calcd (%) for $C_{21}H_{46}BrNO$: C 61.74, H 11.35, N 3.43; found: C 61.40, H 11.50, N 3.33; MS (ESI): m/z calcd for $C_{21}H_{46}NO$ (the 4° ammonium ion, 100%): 328.36; found: 328.4758 [M^+].

Data for 5: ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 3.94$–3.84 (br, 4H), 3.80 (br, 4H), 3.55–3.46 (br, 2H), 3.42 (s, 3H), 3.33 (s, 6H), 1.69 (br, 2H), 1.26 (br, 26H), 0.83–0.78 ppm (t, $J = 6.96$ Hz, 3H); elemental analysis calcd (%) for $C_{23}H_{50}BrNO_2$: C 61.04, H 11.14, N 3.10; found: C 60.88, H 11.09, N 3.20; MS (ESI): m/z calcd for $C_{23}H_{50}NO_2$ (the 4° ammonium ion, 100%): 372.38; found: 372.4155 [M^+].

Data for 6: ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 3.95$–3.9 (br, 6H), 3.87 (br, 6H), 3.54–3.52 (br, 2H), 3.38 (s, 9H), 1.7 (br, 2H), 1.25 (br, 26H), 0.88–0.86 ppm (t, $J = 6.92$ Hz, 3H); elemental analysis calcd (%) for $C_{25}H_{54}BrNO_3$: C 60.46, H 10.96, N 2.82; found: C 60.30, H 10.88, N 2.96; MS (ESI): m/z calcd for $C_{25}H_{54}NO_3$ (the 4° ammonium ion, 100%): 416.41; found: 416.5907 [M^+].

Synthesis of *N*-hexadecyl-*N*,*N*-dimethyl-*N*-propylammonium bromide (7) and *N*-hexadecyl-*N*-methyl-*N*,*N*-dipropylammonium bromide (8): Hexadecylamine(1eq) and *n*-propyl bromide (1.2 equiv) were refluxed in 30% MeOH/MeCN solvent mixture for 36 h, and then the white solid was crystallized from MeOH/ethyl acetate three times. The desired compound with $R_f = 0.5$ (in 10% MeOH/$CHCl_3$) was isolated by column chromatography on a 60–120 mesh silica gel column using MeOH/$CHCl_3$ as the mobile phase. The ammonium salt thus obtained was basified with ammonia and then extracted with diethyl ether; the ether layer was washed with brine to neutrality and concentrated. The secondary amine (*N*-propyl-*N*-hexadecylamine) thus obtained was divided in two parts and the first part was quarternized with excess methyl iodide in presence of K_2CO_3 (2.2 equiv) and a pinch of [18]crown-6 ether in dry DMF for 2 h. The reaction mixture was extracted with ethyl acetate and washed with 5% aqueous sodium thiosulfate solution and brine. It was then concentrated to get the iodide form of surfactant **7**. Another part of the secondary amine was again refluxed with *n*-propyl bromide (1.2 equiv). The corresponding tertiary amine was isolated following the similar procedure mentioned above. The tertiary amine thus obtained was then quaternized with methyl iodide in methanol for 2 h and then methanol was evaporated on a rotary evaporator and the material was taken in ethyl acetate and washed with sodium thiosulfate solution followed by brine wash. The organic part was concentrated to get the iodide form of surfactant **8**. The iodides were then passed through Amberlyst A-26 bromide ion exchange column to get the corresponding bromides **7** and **8**. These salts were crystallized from MeOH/diethyl ether three times to get the pure compounds.

Data for 7: ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 3.48$–3.40 (br, 4H), 3.32 (s, 6H), 1.76–1.56 (br, 4H), 1.18–1.11 (br, 26H), 1.03–0.97 (t, $J = 7.2$ Hz, 3H), 0.83–0.79 ppm (t, $J = 6.82$ Hz, 3H); elemental analysis calcd (%) for $C_{21}H_{46}BrN$: C 64.26, H 11.81, N 3.57; found: C 64.35, H 11.60, N 3.49; MS (ESI): m/z calcd for $C_{21}H_{46}N$ (the 4° ammonium ion, 100%): 312.36; found: 312.4034 [M^+].

Data for 8: ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 3.47$–3.41 (br, 6H), 3.34 (s, 3H), 1.81–1.69 (br, 6H), 1.25 (br, 26H), 1.09–1.04 (t, $J = 7.23$ Hz, 6H), 0.90–0.85 ppm (t, $J = 6.81$ Hz, 3H; elemental analysis calcd (%) for $C_{23}H_{50}BrN$: C 65.69; H 11.98; N 3.33; found: C 65.85, H 11.80, N 3.42; MS (ESI): m/z calcd for $C_{23}H_{50}N$ (the 4° ammonium ion, 100%): 340.9; found: 340.4234 [M^+].

Synthesis of *N*-hexadecyl-*N*,*N*,*N*-tripropylammonium bromide (9): Amphiphile **9** was synthesized by refluxing *n*-hexadecyl bromide (1.2 equiv) and tripropylamine (1 equiv) in 30% methanol/acetonitrile. After 48 h the solvents were removed on a rotary evaporator and the mixture was then crystallized from methanol/diethyl ether three times to get the pure ammonium salt **9**. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 3.31$–3.29 (br, 8H), 1.74–1.60 (br, 8H), 1.17 (br, 26H), 1.01–0.96 (t, $J = 7.11$ Hz, 9H), 0.79–0.77 (t, $J = 6.6$ Hz, 3H); elemental analysis calcd (%) for $C_{25}H_{54}BrN$: C 66.93, H 12.13, N 3.12; found: C 66.59, H 11.93, N 3.03; MS (ESI): m/z calcd for $C_{25}H_{54}N$ (the 4° ammonium ion, 100%): 368.43; found: 368.4215 [M^+].

Critical micelle concentration and minimum surface area: Unless otherwise mentioned, the aqueous critical micelle concentration (cmc) values of the surfactants were determined by measuring surface tension with a temperature-controlled Krüss tensiometer by means of the ring method at 25 ± 0.1 °C. The cmc values are listed in Table 1. The accuracy of measurements in duplicate experiments was within ± 2%.

Preparation of microemulsions (phase behavior): The mixture of surfactants, *n*-hexanol, and water were titrated with isooctane to prepare the microemulsions. A constant mass ratio (1:2) of the surfactant and *n*-hexanol was dissolved in water, forming solutions of different concentrations taken in different screw-topped test tubes and stirred until the solutions became clear. Isooctane was then added to these solutions in measured quantities at 25 °C until just turbid or phase separation. The pseudoternary phase diagrams of the different surfactants (**1**–**9**) are presented in Figure 1. The isotropy/turbidity of the solutions were checked by the naked eye, which means the measured phase boundaries are of fair accuracy.

Activity of interfacially solubilized lipase: The second-order rate constant (k_2) in lipase-catalyzed hydrolysis of *p*-nitrophenyl-*n*-hexanoate in cationic w/o microemulsions was determined spectrophotometrically (on a Shimadzu 1700 spectrophotometer) at the isosbestic points as described previously.[6,10,11,22] In a typical experiment, the aqueous enzyme stock solution (4.5 μL, 0.34 mg mL^{-1}) and the substrate (10 μL, from 0.45 M stock solution in isooctane) were added to the w/o microemulsion (1.5 mL) previously prepared with the desired surfactant concentration and pH (pH refers to the pH of the aqueous buffer solutions used for preparing the w/o microemulsions; pH within the water pool of w/o microemulsions did not vary significantly, < 1 unit),[6,24] in a cuvette to attain the particular W_0 and reactant concentrations. Gentle shaking produced clarification of the microemulsion within 1 min. The initial linear rate of increase in absorbance, that is, 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 were 1.02×10^{-6} g cm^{-3} and 3×10^{-3} M, respectively. Although the lipase was essentially confined to the dispersed water droplets (at the oil/water interface), for simplicity, the concentration of reactants were referred to the overall concentration to avoid the complexity of the volume fraction of water droplet in the w/o

microemulsions and the partitioning coefficient of the substrate.[6,9,22] Moreover, for the sake of consistency with the previous observations, we measured the second-order rate constant (k_2) instead of first-order Michaelis–Menten catalytic constant (K_{cat}), since the initial rate of lipase-catalyzed hydrolysis of *p*-nitrophenyl alkanoate were observed to be first order with respect to the substrate concentration.[6,10,11,22] Similar observations of second-order rate constant have also been found in micelle-mediated organic transformations in chemical systems.[18c–e] The isosbestic points (λ_{iso}) and the molar extinction coefficients (ε) at λ_{iso} of the *p*-nitrophenol/*p*-nitrophenolate couple in w/o microemulsions of different surfactants (**1**–**9**)/water/isooctane/*n*-hexanol were determined spectrophotometrically (Table 1).

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