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Improving the Lipase Activity Profile in Cationic Water-in-Oil Microemulsions of Hydroxylated Surfactants

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The activity of *Chromobacterium viscosum* lipase (CV-lipase) in cationic water-in-oil (w/o) microemulsions is significantly lower compared to that in bis(2-ethylhexyl)sulfosuccinate sodium salt (AOT)-based (anionic) systems.[26,36,38,39] In the present study, we estimated the second-order rate constants k_2 in lipase-catalyzed hydrolysis of *p*-nitrophenylcaproate, in newly developed cationic w/o microemulsions of synthesized surfactants (**2**−**6**, Chart 1) containing hydroxyethyl moieties at the polar headgroup. The kinetic studies at pH = 6.0 (pH refers to the pH of the aqueous buffer solutions used in preparing the w/o microemulsions) show that the catalytic efficiency of CV-lipase was systematically increased with the sequential increment of hydroxyethyl groups at the polar heads of surfactants (**1**−**4**, Chart 1), possibly due to the increase in the interfacial concentration of water $[H_2O]_i$ in consequence of the added hydrogen bonding ability of hydroxyl groups. To this end, we found that in a 0.05 M **4**/water/32.3:1 (v/v) isooctane/*n*-hexanol w/o microemulsion, the activity of lipase is almost 4-fold higher than that in 0.05 M cetyltrimethylammonium bromide (CTAB)/water/9:1 (v/v) isooctane/*n*-hexanol[37] systems and is enhanced by an order of magnitude compared to that in the case of CTAB/water/heptane−chloroform (1:1, v/v) w/o microemulsions.[26] Moreover, to our knowledge, the observed k_2 is the highest-ever lipase activity, hitherto unattainable in any cationic w/o microemulsion. Simultaneously, for the first time, the estimated activity of lipase in the former cationic w/o microemulsion is akin to the best-ever activity of lipase found in a w/o microemulsion of AOT.[26] In addition, the pH profile and the stability of CV-lipase were investigated in newly developed w/o microemulsions.

Introduction

Water-in-oil (w/o) microemulsions are optically transparent nanometer scale aggregates of water and surfactants in an apolar bulk solvent.[1−8] Structurally, water forms a microdroplet surrounded by a monolayer of surfactant molecules organized with their polar heads toward the aqueous core, known as the water-pool, and the hydrophobic tails in contact with the bulk apolar solvent, thus forming an anisotropic interface separating the polar aqueous part from the nonpolar oily region.[9] Technological and biotechnological potentials of such thermodynamically stable w/o microemulsions are wide-ranging due to their increased interfacial area and improved ability to solubilize otherwise immiscible substrates.[10−12] A plethora of biomolecules including proteins, enzymes, and nucleic acids have been solubilized in the water-pool of the w/o microemulsions without the loss of their biological activities.[13−23] When dissolved in the small droplets of water, hydrophilic enzymes are afforded some protection from the denaturing effect of the oil.[2,13−19] On the other hand, interfacially active enzymes, such as lipase, horseradish peroxidase, and lactate dehydrogenase, when added to the w/o microemulsions, locate themselves at the anisotropic interfacial region of the aggregates.[24−27]

Lipases (surface-active enzymes) represent a group of enzymes that are most widely used for diversified transformation in microemulsions.[26−34] In most cases, the

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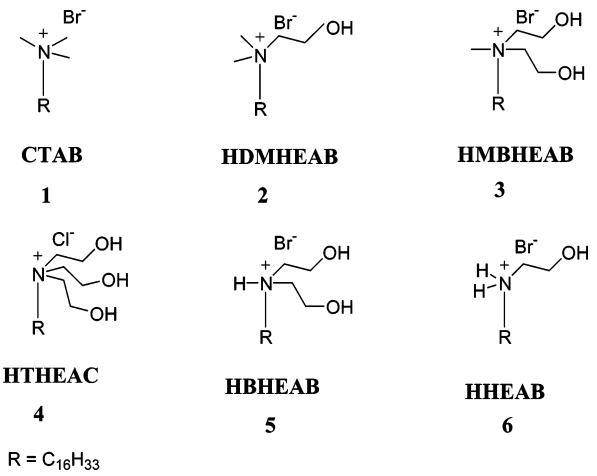
hydrolytic ability of lipase with triglyceride or nitrophenyl alkanoate esters as substrates is much higher in a bis-(2-ethylhexyl)sulfosuccinate sodium salt (AOT)-based system than in cationic or nonionic w/o microemulsions.[26,27,35−39] For instance, the activity of *Chromobacterium viscosum* lipase (CV-lipase) in AOT/water/heptane w/o microemulsions is more than an order of magnitude higher compared to that in cetyltrimethylammonium bromide (CTAB)/water/heptane−chloroform microemulsions and is equivalent with the intrinsic activity of lipase in bulk water.[26] The catalytic efficiencies of such encapsulated enzymes are likely to be influenced by the microstructural parameters (local molar concentration of water and other ions) present in the vicinity of the enzymes. A few investigations in the area of reversed micellar enzymology have implied the importance of local molar concentrations of water and other ions on the activities of biocatalysts.[40−42]

To this end, for the first time Das and Chaudhuri experimentally estimated[37] the local molar concentration of water, bromide ions, and *n*-hexanol in the interfacial region of CTAB/water/isooctane/*n*-hexanol (iso/hex) w/o microemulsions across a W_0 (mole ratio of water to surfactant) range of 12−44 using a phenyl cation trapping protocol developed by Romsted and co-workers.[43,44] A sensible correlation was observed between the catalytic efficiencies of lipase and the interfacial concentration of water $[H_2O]_i$ by deciphering the unchanged activities of lipase across the W_0 range as being due to the grossly unaltered $[H_2O]_i$ (28.1−31.8 M) in the same range of W_0.[37] To the same end, the role of the local concentration of water was also found to be crucial in modulating the activity of a predominantly hydrophilic enzyme (trypsin) solubilized inside the water-pool of CTAB/water/isooctane/*n*-hexanol w/o microemulsions.[45] The catalytic activities of trypsin have been observed to escalate and become comparable to that in bulk water with a concomitant increase in the molar concentration of water inside the water-pool from 43.8 to 55.4 M (the molarity of bulk water is 55.5 M) across the W_0 range of 12−44.[45]

The aforementioned observations clearly indicate that the local molar concentration of water has an important role in controlling the enzyme's activity. However, it is also distinct that in cationic w/o microemulsions, (i) the catalytic efficiencies of surface-active enzymes cannot be regulated simply by changing the W_0 due to unchanged $[H_2O]_i$ and (ii) the meagerness of $[H_2O]_i$ (almost half of the bulk water concentration) in addition to the remarkably high interfacial concentrations of bromide ions (3.4−2.5 M) and *n*-hexanol (8.1−6.4 M) in CTAB/water/isooctane/*n*-hexanol w/o microemulsions plays an important role in significantly reducing the lipase's activity. To increase the effective utilization of lipase-entrapped cationic w/o microemulsions in the midst of extensive potentials of micellar enzymology, enhancement of lipase activity is indispensable. To our knowledge, hitherto no such attempts were executed to modulate the catalytic efficiencies of lipase in cationic w/o microemulsions except changing W_0.

Chart 1



To this end, in the present investigation we have been able to dramatically enhance the activity of lipase in the newly developed cationic w/o microemulsions of hydroxylated surfactants (**2**−**4**, Chart 1). Introduction of hydroxyethyl groups, possessing significant hydrating ability due to the presence of hydrogen bond donor and acceptor atoms, at the polar headgroups of surfactants, possibly increases the $[H_2O]_i$. Consequently, lipase's activity boosts up to an extent hitherto unattainable in any cationic w/o microemulsions. Moreover, for the first time, the catalytic efficiencies of lipase in cationic w/o microemulsions attained a level comparable to that obtained to date only in AOT-based systems,[26,27,35−36] the best-ever w/o microemulsions used for the highest activity profile of lipase.

As rationalized in the preceding paragraphs, herein we describe the details of (a) the synthesis of five new hydroxylated surfactants (**2**−**6**, Chart 1) along with their critical micellar concentration (cmc) measurements and phase diagrams for pseudoternary systems, (b) the catalytic efficiencies of lipase estimated across the varying range of solution compositions using amphiphiles (**1**−**6**, Chart 1), and (c) the pH profile and stability of the enzyme in cationic w/o microemulsions of the synthesized surfactants.

Experimental Section

Materials. CV-lipase (EC 3.1.1.3, Type XII) was purchased from Sigma and was used as received. Aerosol-OT (AOT) was procured from Aldrich Chemical Co. Analytical grade CTAB from Spectrochem (India) was recrystallized three times from methanol/ether, and the recrystallized CTAB was without minima in its surface tension plot. HPLC grade isooctane, *n*-hexanol, solvents, and all other reagents used in the syntheses were obtained from SRL (India) and were of the highest analytical grade. Mass spectrometric data were acquired by liquid secondary ion mass spectrometry (LSIMS) and electron impact (EI) techniques. The substrate *p*-nitrophenylcaproate was synthesized conventionally from an equimolar solution of caproic acid and *p*-nitrophenol in dichloromethane using an equivalent amount of *N*,*N*-dicyclo-

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Table 1. Critical Micellar Concentration Values for Hydroxylated (2−6) Surfactants

compound	method applied to determine cmc	cmc (M)[a]
HDMHEAB (**2**)	conductometry	8.3×10^{-4} [b]
HMBHEAB (**3**)	conductometry	7.6×10^{-4} [b]
HTHEAC (**4**)	conductometry	7.9×10^{-4}
HBHEAB (**5**)	tensiometry	3.5×10^{-5}
HHEAB (**6**)	tensiometry	1.7×10^{-5}

[a] Determined at 25 °C. [b] Values are taken from the literature (ref 46).

hexylcarbodiimide (DCC) and a catalytic amount of 4-*N*,*N*-(dimethylamino)pyridine (DMAP). The synthetic procedures of different surfactants (Chart 1) are given below (^{1}H NMR, elemental analysis, and mass spectrometric data are available in the Supporting Information).

Synthesis of *N*-Hexadecyl-*N*,*N*-dimethyl-*N*-(2-hydroxyethyl)ammonium Bromide (2) and *N*-Hexadecyl-*N*-methyl-*N*,*N*-bis(2-hydroxyethyl)ammonium Bromide (3). Both of the amphiphiles were prepared following the procedure mentioned in a recently published protocol.[46] Briefly, 1-bromohexadecane and the corresponding amines (*N*-methyldiethanolamine for **2** and *N*,*N*-dimethylethanolamine for **3**) were taken in the molar ratio 1.2:1 in 30% methanol/acetonitrile and refluxed. After 24 h of refluxing, the solvent was evaporated in 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 **2** and **3**, respectively.

Synthesis of *N*-Hexadecyl-*N*,*N*,*N*-tris(2-hydroxyethyl)-ammonium Chloride (4). An aqueous solution of NaOH (2.72 g, 0.068 mol, in 25 mL of doubly distilled water) was added dropwise to a mixture of 2-chloroethanol (6.5 g, 0.081 mol) and hexadecylamine (5 g, 0.021 mol) under refluxing conditions. 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 a 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) 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% (4.4 g).

Synthesis of *N*-Hexadecyl-*N*,*N*-bis(2-hydroxyethyl)ammonium Bromide (5). Compound **5** was synthesized following the same procedure for preparing **2** and **3**.

Synthesis of *N*-Hexadecyl-*N*-(2-hydroxyethyl)ammonium Bromide (6). This amphiphile was synthesized using a procedure similar to that applied to synthesize **2**, **3**, and **5**. In this case, the pure product ($R_f = 0.5$, 25% methanol in chloroform as the TLC developing solvent) was obtained by column chromatography of the reaction mixture on a 60−120 mesh silica gel column using 5% methanol in chloroform as the eluting solvent.

Critical Micellar Concentration. The cmc values for **2** and **3** determined by the conductometric method were obtained from the literature.[46] In the case of **5** and **6**, the surface tension method was used to determine the cmc, while that for amphiphile **4** was measured conductometrically. All of the cmc values are listed in Table 1.

Preparation of Microemulsions (Phase Behavior). Microemulsions were prepared by titrating the mixtures of surfactants, *n*-hexanol, and water with isooctane. 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. These solutions were then titrated with isooctane from a microburet at 25 °C until just turbid or phase separation. The pseudoternary phase diagrams of the different surfactants (**1**−**6**) are presented in Figure 1. To compare the effect of hydroxyethyl substitutions in the headgroup region of the amphiphiles, the phase diagrams of **1**−**6** are merged together. The optical isotropy of the solutions was checked by the naked eye, which means that the measured phase boundaries are of fairly poor accuracy and should be taken as rough estimates only.

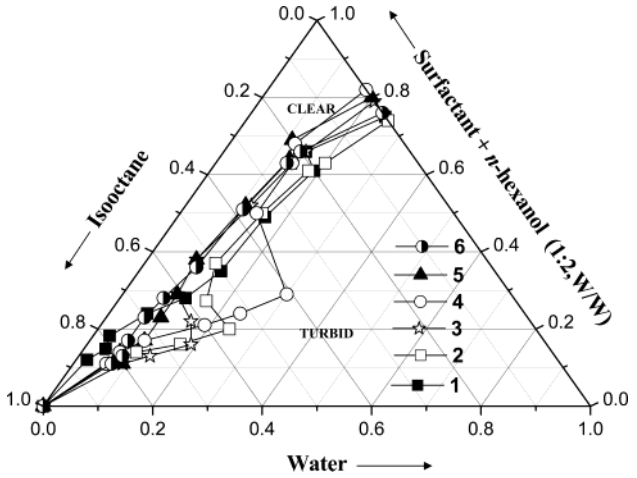

Figure 1. Pseudoternary phase diagrams of the quaternary system of (**1**−**6**)/*n*-hexanol (1:2, w/w)/water/isooctane at 25 °C. The scale magnitudes are reduced by 1/100th of the plot.

Activities of Interfacially Solubilized Lipase. The second-order rate constants (k_2) in lipase-catalyzed hydrolysis of *p*-nitrophenylcaproate in cationic w/o microemulsions were determined spectrophotometrically (on a Hitachi U-2000 spectrophotometer) at the isosbestic points as described previously.[26,37,47,48] In a typical experiment, 9 μL of the aqueous enzyme stock solution (0.34 mg/mL) and the substrate (10 μL, from a 0.45 M stock solution in isooctane) were added to the 1.5 mL w/o microemulsion (previously prepared with the desired surfactant concentration and pH (pH refers to the pH of the aqueous buffer solutions used in preparing the w/o microemulsions)) in a cuvette to attain the desired W_0 and reactant concentration. Gentle shaking produced clarification of the microemulsion within 1 min. The initial linear rate of increase of the 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*-nitrophenylcaproate are 2.04×10^{-6} g cm^{-3} and 3×10^{-3} M, respectively. Although lipase is essentially confined to the dispersed water droplets, for simplicity, the concentrations of reactants were referred to the overall concentration[26,37,41,47,48] to avoid the complexity of the volume fraction of water droplets in the w/o microemulsions and the partitioning coefficient of the substrate. 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**−**6**)/water/(iso/hex) were determined spectrophotometrically. The λ_{iso} and ϵ (in parentheses) were (339 nm, 4370 M^{-1} cm^{-1}), (340 nm, 4350 M^{-1} cm^{-1}), (338 nm, 4250 M^{-1} cm^{-1}), and (341 nm, 4250 M^{-1} cm^{-1}) for **1**, **2**, **3**, and **4**, respectively. It was not possible to determine the isosbestic points for compounds **5** and **6** since no point of intersection was found in the wavelength scan because of the turbidity formed after the addition of the NaOH in w/o microemulsions of **5** and **6** containing *p*-nitrophenol. Thus, for simplicity, ϵ was measured to be 4640 and 5640 M^{-1} cm^{-1} at 340 nm in w/o microemulsions of **5** and **6**, respectively.

Results and Discussion

The role of the local molar concentration of water in the vicinity of the enzymes is the prime key factor to control the activity of the enzyme. As illustrated in the Introduction, both the catalytic efficiency of lipase and $[H_2O]_i$ are grossly unchanged across a varying range of W_0 in CTAB-based cationic w/o microemulsions.[26,37] The most optimistic

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Table 2. Second-Order Rate Constant, k_2, for Lipase-Catalyzed Hydrolysis of *p*-Nitrophenylcaproate in Different Cationic w/o Microemulsions with Varying W_0 Range at pH = 6.0 (20 mM Phosphate) and 25 °C

		9:1 iso/hex (v/v)		4:1 iso/hex (v/v)[a]		15.7:1 iso/hex (v/v)[a]		32.3:1 iso/hex (v/v)[b]	
surfactant	concn (M)	W_0 range	k_2 ($cm^3\ g^{-1}\ s^{-1}$)	W_0 range	k_2 ($cm^3\ g^{-1}\ s^{-1}$)	W_0 range	k_2 ($cm^3\ g^{-1}\ s^{-1}$)	W_0 range	k_2 ($cm^3\ g^{-1}\ s^{-1}$)
1	0.05	8−36	161 ± 9					40−56	308 ± 1 2
	0.10	8−44	146 ± 8	6−20	94 ± 5	24−68	236 ± 13		
2	0.05	8−40	165 ± 7					48−60	324 ± 13
	0.10	8−76	143 ± 9	6−24	85 ± 6	36−72	240 ± 14		
3	0.05	8−40	161 ± 10					56	461 ± 10
	0.10	8−80	144 ± 9	6−24	85 ± 4	40−76	260 ± 10		
4	0.05	8−48	172 ± 11					52−56	582 ± 9
	0.10	8−84	161 ± 10	6−30	109 ± 7	40−76	310 ± 11		
5	0.05	8−28	95 ± 6					36−52	227 ± 12
	0.10	8−36	103 ± 7	8−16	61 ± 6	16−40	185 ± 10		
6	0.05	8	87 ± 5						
	0.10	12	99 ± 6	12	46 ± 3				

[a] [Surfactant] = 0.10 M. [b] [Surfactant] = 0.05 M.

pathway by which the $[H_2O]_i$ can be modulated is the variation in the molecular architecture of the basic building block of micellar aggregates, the surfactant. Thus, to increase the extent of hydration at the interface of cationic w/o microemulsions and consequently to enhance the activity of interfacially solubilized lipase, we have synthesized a series of surfactants (**2**−**6**, Chart 1) with the sequential increment of hydroxyethyl groups at the polar head.

The pseudoternary phase diagrams of surfactants (**1**−**6**) with *n*-hexanol (1:2, w/w)/water/isooctane systems at 25 °C are represented in Figure 1. The diagram (Figure 1) shows the first indicative evidence that the insertion of the hydroxyethyl moiety in place of the methyl group at the polar head of the surfactant increases the isotropic region for the amphiphiles **2**, **3**, and **4** (Figure 1) compared to CTAB (**1**) as a result of enhanced water content in the microemulsion. Presumably, this enhanced water content is due to the added hydrogen bonding ability of the hydroxyl groups, which possess both hydrogen bond donor and acceptor atoms. In this regard, recently Wettig[49] et al. emphasized that hydroxyl substitution at the headgroup (interfacial region) makes its hydration cosphere more hydrophilic than the nonsubstituted one. Furthermore, in accordance with our expectation and intention, increased water content in the isotropic area (Figure 1) is the region of w/o microemulsion for **2**−**4**. On the contrary, as hydrogen atoms replace the methyl groups in **5** and **6**, the area ratio of the clear and anisotropic regions (Figure 1) decreased compared to that for the corresponding methyl analogues (**2** and **3**) and CTAB. Such reduced isotropic area and the absence of any appreciable improvement of water content albeit in the presence of hydroxyl groups specify that the headgroup size has an important function to play in the molecular packing of organized aggregates.[50] The decrease in the surfactant headgroup area might have reduced the interfacial area of the aggregates and favored tighter binding of the counterion;[51] thus the occupancy of more water molecules plummeted at the interfacial region.

To examine the proposition outlined in the Introduction, first we studied the activity of CV-lipase in the w/o microemulsions formed by both 0.1 and 0.05 M **1**−**6** in the 9:1 (v/v) (iso/hex) and water system at pH 6.0 (20 mM phosphate; pH within the water-pool of w/o microemulsions does not vary significantly, <1 unit[26,52]) and 25 °C (unless mentioned, the temperature will be same throughout the investigation) across a varying range of W_0 (Table 2). To our surprise, the second-order rate constants k_2 (as the initial rates were found to be first order with respect to substrate concentration in the case of lipase-catalyzed hydrolysis in w/o microemulsions reported by Fletcher et al.[26]) were virtually unchanged (143 ± 9 to 172 ± 10 $cm^3\ g^{-1}\ s^{-1}$, Table 2) for the w/o microemulsions of **1**−**4** at any W_0. The observed unaltered catalytic activities of lipase are similar to the results obtained by Das et al. and Fletcher et al. in CTAB/water/(iso/hex) and CTAB/water/heptane−chloroform (1:1, v/v) w/o microemulsions, respectively.[26,37] Moreover, under the same experimental conditions, in the case of w/o microemulsions prepared with **5** and **6**, k_2 was distinctly lower (87 ± 5 to 103 ± 7 $cm^3\ g^{-1}\ s^{-1}$, Table 2) than that obtained in the case of **1**−**4**.

In contrast to the expected enhancement (in accordance with the increase of water content in phase behavior (Figure 1)) of lipase activity in the w/o microemulsions of **2**−**4**, the foregoing observations point toward the role of other compositional parameters of the self-assembled aggregates. As described earlier, in deciphering the correlation between the interfacial concentrations and the activities of lipase in CTAB/water/(iso/hex) (9:1, v/v) w/o microemulsions, Das and Chaudhuri found an extraordinarily high (otherwise unattainable) interfacial concentration of *n*-hexanol (8.1−6.4 M) across the W_0 range of 12−44,[37] while the inhibitory action of alcohols over the catalytic activity of lipase through the competitive inhibition mechanism has been reported several times in the literature.[53−58] In this context, we decided to check the inhibiting ability of *n*-hexanol on the hydrolytic activity of lipase in 4:1 (v/v) (iso/hex) (increasing the alcohol content) w/o microemulsions of **1**−**6**, keeping all other experimental conditions identical. As was our expectation, the k_2 decreased by ∼40−50% (Table 2) for all the microemulsions irrespective of surfactant, probably due to the rise in the *n*-hexanol concentration at the interface of aggregates. The interfacial concentration of *n*-hexanol

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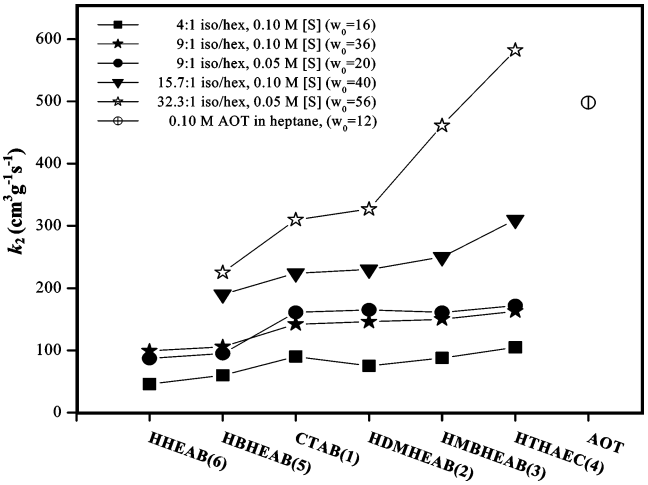

Figure 2. Variation of the second-order rate constant (k_2) for the lipase-catalyzed hydrolysis of *p*-nitrophenylcaproate in different cationic w/o microemulsion systems at 25 °C and pH = 6.0 (20 mM phosphate). In 9:1 iso/hex, for 0.05 and 0.10 M **6** the W_0 values are 8 and 12, respectively. Similarly, in 4:1 iso/hex, in the case of 0.10 M **6** W_0 is 12 and that for 0.05 M **5** in 32.3:1 iso/hex is 52. The ratio of isooctane to *n*-hexanol is in v/v. [S] is the concentration of surfactant. The concentrations of lipase and *p*-nitrophenylcaproate are 2.04×10^{-6} g cm^{-3} and 3×10^{-3} M, respectively.

has been found to be 10.4−7.3 M (data not shown) in the CTAB (0.1 M)/water/(iso/hex) (4:1, v/v) systems in the W_0 range of 6−20, determined using the chemical trapping method developed by Romsted and co-workers.[43,44]

On the basis of the aforementioned observations, primarily it is essential to decrease the interfacial concentration of *n*-hexanol for enhancing the catalytic efficiencies of lipase at the interface of w/o microemulsions. In addition, it could be possible that the extent of improving the hydrating ability of hydroxyethyl groups at the interfacial region is being suppressed by the presence of high molar concentration of alcohol.

Toward this issue, we reduced the volume ratio of *n*-hexanol to isooctane in the bulk system up to an extent (1:15.7, v/v) beyond which isotropic w/o microemulsions cannot be prepared using 0.1 M **1**−**5**; except for **6**, the system is anisotropic under similar compositions. Lipase's activity was investigated in **1**−**5** (0.1 M)/water/(iso/hex) (15.7:1, v/v) w/o microemulsions in the varying range of W_0 (Table 2, Figure 2) at pH 6.0 (20 mM phosphate). In the case of CTAB (**1**)-based w/o microemulsions, for the first time k_2 (236 ± 13 cm^3 g^{-1} s^{-1}) attained such a high level which is increased by almost 60% (Table 2) compared to that obtained in the 9:1 (v/v) (iso/hex) bulk system otherwise under identical conditions. Also, in **2**−**4**-based w/o microemulsions prepared using a 15.7:1 (iso/hex) bulk composition, the k_2 (Table 2, Figure 2) improved by 60−100%. More importantly, in accordance with our strategy explained earlier, the catalytic efficiencies of lipase systematically improved up to 30% with the chronological increment of hydroxyethyl groups at the polar head of **1**−**4** (Table 2, Figure 2). This is the first investigative verification that hydroxyethyl groups at the polar head of surfactants can bring more water molecules to the interfacial region, that is, increasing the $[H_2O]_i$, as the lipase's activity is escalating in the order of **1**−**4**. However, in the case of **5**, lowering the alcohol/isooctane ratio also has an encouraging effect as k_2 improved by almost 100% as compared to that in the 9:1 (v/v) (iso/hex) bulk system. Noticeably, for any surfactant, under particular bulk compositions, the activity of lipase is virtually the same across the entire range of W_0.

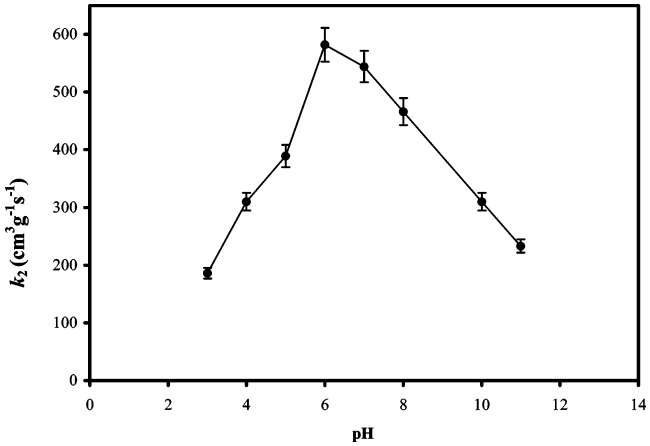

Figure 3. Variation of the second-order rate constant (k_2) for the lipase-catalyzed hydrolysis of *p*-nitrophenylcaproate with pH (20 mM buffer) in 0.05 M **4**/water/32.3:1 (v/v) iso/hex (W_0 = 56) at 25 °C. The buffers used for different pH solutions were hydrochloric acid/KCl (pH 3), citric acid/trisodium citrate (pH 4 and 5), phosphates (pH 6 and 7), Tris (pH 8), and bicarbonate/carbonate (pH 10 and 11).

To enhance the lipase activity further and to ascertain our rationale more firmly, we decided to decrease the concentration of **1**−**6** from 0.1 to 0.05 M since it is well established that the activity of surface-active enzymes (lipase) increases with decreasing concentration of amphiphile.[16,25,59,60] In view of the results in the preceding paragraph, lowering the surfactant concentration to 0.05 M will additionally help to enhance the efficiency of lipase in self-assembled aggregates as the amount of *n*-hexanol required to form a w/o microemulsion reduced by 2-fold (iso/hex (v/v), 32.3:1) as compared to that needed for 0.1 M surfactant. Kinetic studies for the hydrolytic activity of lipase in the w/o microemulsions formed using 32.3:1 (v/v) (iso/hex) bulk solvent with **1**−**5** (here also an isotropic w/o microemulsion cannot be prepared with **6**) exemplified (Table 2, Figure 2) that (i) the k_2 was amplified by 200−300% depending on the amphiphiles **1**−**4** in comparison with the 9:1(iso/hex) (v/v) bulk system otherwise under the same conditions and (ii) the activity of lipase, in compliance with our underlying principle, was methodically enhanced by almost 100% with the sequential increment of hydroxyethyl groups in **1**−**4**. In the case of **4**, where three hydroxyethyl moieties are present at the headgroup of the surfactant, k_2 was 582 ± 9 cm^3 g^{-1} s^{-1} (Table 2, Figure 2), which is almost 4-fold higher than that in 0.05 M CTAB/water/9:1 (v/v) (iso/hex)[37] systems and an order of magnitude higher compared to that in the case of CTAB/water/heptane−chloroform (1:1, v/v) w/o microemulsions.[26] To our knowledge, the obtained catalytic efficiency of lipase in the cationic w/o microemulsion of **4** using the 32.3:1 (v/v) isooctane/*n*-hexanol bulk system is the highest ever shown by CV-lipase in any cationic w/o microemulsion. More significantly, for the first time, the activity of lipase in a cationic w/o microemulsion is comparable with the catalytic efficiency of the same lipase obtained in an AOT-based w/o microemulsion (AOT/water/heptane)[26] (Figure 2), the best-ever w/o microemulsions used for the highest activity profile of lipase.

Interestingly, in the case of **5**, the k_2 rose to 100% due to lowering the ratio of *n*-hexanol/isooctane to 1:32.3, but it is lowered by 2-fold (Table 2) with respect to its methyl

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analogue (**3**)-based w/o microemulsion. However, regardless of any bulk composition, the activity of lipase is always less in the **5**- and **6**-based w/o microemulsions (Table 2, Figure 2) than in those of **1**−**4**. As described above in the phase behavior section, due to the decrease in headgroup size for **5** and **6**, probably the interfacial area is reduced, resulting in tight counterion binding.[51] Thus, both the $[H_2O]_i$ and the activity of lipase diminished concurrently.

Next, we investigated the pH dependence of lipase-catalyzed hydrolysis of *p*-nitrophenylcaproate in **4**-based w/o microemulsions under the conditions illustrated in Figure 3. It was found that the k_2 varied across pH 3−11 and showed a maximum at pH 6.0, while in CTAB-based systems the activity remains unaltered[26] for the pH range of 5−11. Once again, for the first time, the pH profile (Figure 3) in a cationic w/o microemulsion is similar to that found in an AOT/water/heptane system.[26]

Experiments were then conducted to determine the stability of the enzyme in the **4** (0.05 M)/water/(iso/hex) (32.3:1, v/v) w/o microemulsion (the figure is provided in the Supporting Information). It was observed that there is no significant change in the activity up to 4 days, but after that a sharp decrease in the activity was noticed. Thus it can be said that the enzyme is at least stable up to 4 days in the newly developed **4**-based w/o microemulsion.

Conclusion

In closing, we have established that the introduction of hydroxyethyl groups at the polar heads of the surfactants dramatically enhances the catalytic efficiencies of lipase by means of increasing the interfacial concentration of water. Our results show that the hydrolytic activity of lipase in the newly developed cationic w/o microemulsions of **2**−**4** increases up to 4-fold and 10 times compared to that in widely used cationic w/o microemulsions, the CTAB/water/(iso/hex) (9:1, v/v) and CTAB/water/heptane−chloroform (1:1, v/v) systems, respectively. Furthermore, for the first time, the catalytic efficiencies of lipase in cationic w/o microemulsions attained a level comparable to that obtained till now only in AOT-based systems, the best-ever w/o microemulsions used for the highest activity profile of lipase.

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Supporting Information Available: ^{1}H NMR, elemental analysis, and mass spectrometric data of the newly synthesized surfactants **2**−**6**; the stability profile of the CV-lipase in 0.05 M **4**/water/32.3:1 (iso/hex). This material is available free of charge via the Internet at http://pubs.acs.org.

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