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Synthesis, Micellization and Hemolysis Evaluation of Biodegradable quaternary Ammonium Compounds

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ABSTRACT

Surfactants have the special ability to interact with the lipid bilayer of cell membranes. The red blood cell is one of the most used cellular membrane models to study the mechanisms underlying surfactant – induced osmotic cell resistance.The aim of this study is the evaluation of the effect of the prepared surfactants on red blood cells (RBC) as a model for biological membranes. Also, in this study some of physicochemical and biodegradability properties were evaluated and studied. Series of cationic surfactants were prepared by the reaction of N,N\ dimethyl Tris(hydroxyl methyl) amino methane with fatty alkyl chlorides (C_{10}–C_{18}). We found a good correlation between the CMC and the concentrations resulting in maximum protection against hypotonic hemolysis for the cationic surfactants. The structures of the prepared compounds were confirmed by micro-analysis, IR and ^{1}HNMR spectra.

Key words: Cationic surfactant, biological membrane, hemolysis, biodegradability.

INTRODUCTION

Surfactants, due to their surface and interface properties are among the most versatile and frequently applied excipients in pharmaceutical, cosmetic applications, and technology-based industries. They are employed in large quantities every day open a worldwide scale as constituents of many different products[(1)].

The quaternary nitrogen atom of cationic surfactants is essential for many of their intrinsic properties such as adsorption onto negatively charged solids, antimicrobial activities, antielectrostatic properties and reactivity with anionic surfactant. Because of these properties, cationic surfactants find a number of applications as fabric softeners, disinfectants, wood preservatives, emulsifiers, wetting agents, and processing aids.

Cell lysis by surfactants is a process of great fundamental and practical importance[(2-3)]. Much research has been done in order to understand the mechanism underlying this process, mostly

using erythrocytes as a convenient model system. Because the human erythrocyte has no internal organelles and since it is the simplest cellular model obtainable, it is the most popular cell membrane system to study the surfactant membrane interaction[4]. Permeability enhancers are agents that decrease or remove extra cellular layer resistance reversibly and allow the drug to pass through and between epithelial cells toward blood and lymph. Recently, enhancing drugs permeability through cellular membrane becomes one of the main topics in pharmaceutical researches[5].

Adsorption enhancing ability of surfactants in formulation with low adsorption like peptides or proteins is used for drug delivery in non-injectable formulations. A broad spectrum of surfactants is used as enhancers including bile salts, anionic detergents, glycerides and lysophospholipids (lysolecithins); however, the efficacy of non-ionic surfactants with moderate polarity is better. On the other hand, it is reported that non-ionic polar surfactants do not have toxicity, while surfactants with moderate polarity show toxic effects [6-8]. In recent years permeability enhancing effects of some ionic and non-ionic surfactants were studied. Noudeh et al.[9] showed that some of non-ionic surfactants could increase mucosal adsorption of drugs with low absorption. One of the suggested mechanisms is inducing partial but reversible gap within cells membranes and consequently increasing the permeability by surfactants or other enhancers. Various models exist for evolution of membrane toxicity of surfactants including single cell models using erthrocytes, erythrocyte ghosts and liposomes. The erythrocyte model has been widely used as it presents a direct indication of toxicity of injectable formulations as well as general indication of membrane toxicity. Another advantage of erythrocytes model is that blood is readily available and that cells are easy to isolate from the blood; moreover, its membrane has similarities with other cell membranes[10] .

Effects of cationic surfactants derived from lysine on antimicrobial and hemolytic activities were evaluated[11].

Evaluating the permeability of enhancers using biological membranes plays an important role. Consequently, in the present study, the effects of cationic surfactants on biological membranes have been evaluated. Also, biodegradability of them has also been tested suggesting that they can be used without any environmental problems.

MATERIALS AND METHODS

2.1. Materials

Aliphatic alcohols with 10-18 carbon atoms, tris(hydroxyl methyl) amino methane, dimethyl sulfate and cetyl trimethyl ammonium chloride (CTAC) were supplied by Sigma. Aldrich Co. Sodium chloride, di-sodium hydrogen phosphate were purchased from Aldrich chemical Co., Germany. Drabkin's agent was supplied from Diamond diagnostics (DP, Cairo, Egypt).

Human red blood cells were obtained from healthy donors by venipuncture and collected in sodium-EDTA coated test tubes (Clinical pathology Department- National Liver Institute, Menoufiya University).
All other chemicals used in synthetic procedures were of reagent grade.

2.2. Methods

Preparation of N,N dimethyl tris(hydroxymethyl) amino methane was carried out by Badr, E.E. *et al.*[12].

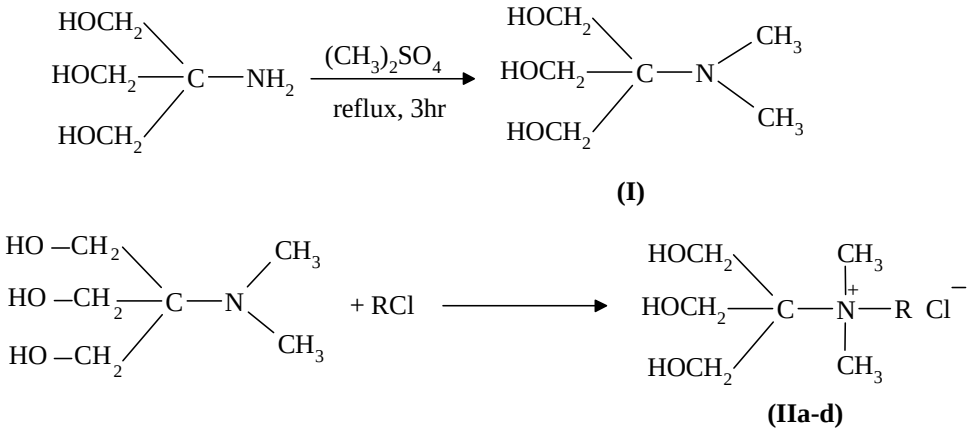
Fatty alkyl chloride (C_{10}–C_{18}) was synthesized by treatment of fatty alcohol with thionyl chloride according to the procedure described by Weil *et al.* [13].

General procedure for the preparation of cationic surfactants N-N-[Tris(hydroxymethyl) methyl]-N-alkyl-N,N-dimethyl ammonium chloride

A homologous series of cationic surfactants (II_{a-d}) was prepared by the reflux of (1 mol) of fatty alkyl chloride with (1 mol) of N,N\-dimethyl Tris(hydroxymethyl) amino methane in acetone at 70—85°C for 10-18 hrs (depending on the alkylchain length[14].

The products were purified by recrystallization three times in acetone. The chemical structure of the prepared compounds was confirmed by elemental analysis, IR and HNMR.

The cationic compounds were denoted as (II_{a-d}) and the chemical structures were represented in scheme 1.



Scheme 1 (R=C_{10-18})

2.3. Structural analysis

The elemental analysis for the synthesized surfactants was performed using a Perkin Elmer 2400 CHN apparatus - infrared (IR) spectra were obtained on 1600 FTIR perkin Elmer-spectrum in the 4,000-400 cm^{-1} range and Bruker model DRX-300. NMR, spectrometer with TMS as an internal standard for ^{1}HNMR using $CDCl_3$ as solvent.

2.4- Surface Tension Measurements :

Surface tension measurements were carried out on freshly prepared surfactants solutions in a concentration range of 10^{-1}–10^{-5} $molL^{-1}$was measured at 25°C using semi-automatic tensiometer apparatus (Küss K_6 Tensiometer) by Du Nouy platinum ring method.

2.5- Biodegradability

Die-away tests in river water were performed employing the surface tension method[15]. Samples taken daily were filtered through No. 1 Whatman filter paper before measuring the surface tension. Biodegradation was calculated by the following equation:.

$$D = \gamma_t - \gamma_o / \gamma_{bt} - \gamma_0$$

(γ_t : surface tension at time t ; γ_0 : surface tension at time zero (initial surface tension; γ_{bt}: surface tension of the control sample at time t).

2.6- Buffer and reagents preparation

Mcllvaine's buffer was prepared as follows: solution 1, containing 21 g of citric acid (100 mM) and 8.775 g of sodium chloride (150 mM) made up to 1000 ml with deionized water, was mixed with solution 2, containing 28.4 g of di-sodium hydrogen phosphate (200 mM) and 8.775 g of sodium chloride (150 mM) made up to 1000 ml with deionized water, to produce the required pH of 7.0, Solution pH

2.7 Preparation of red blood cells suspension

Human blood was collected from a healthy individual with 46.7% hematocrit and added to four heparinized tubes. After centrifuging at 3000 rpm for 10 min (Hermle 230 ZA, Germany), plasma and buffy coat were removed and the erythrocytes were washed three times in at least five times of their volume with Mcllvaine's buffer, pH = 7.0. Afterward, by adding Mcllvaine's buffer, an erythrocyte suspension with 12% hematocrit was prepared and kept in 4°C for experiments[6].

2.8 Hemolytic method

A suspension of erythrocyte (200 µl) within a micro-tube was incubated for the required times with an equal volume of the test sample of surfactants mixture, including II_{a-d} prepared in Mcllvaine's buffer, at 25 and 37°C. After incubation, the mixture was spun in a microcentrifuge at 3000 rpm for 35 s (Spectrafuge 161M, England) and 200 µl of the resulting supernatants was added to 3 ml of Drabkin's reagent. To assay for the amount of hemoglobin released, the absorbance of samples was assessed in 540 nm wavelength using spectrophotorneter (Shimadzu, 3100, Japan). Positive controls consisted of 200 µl of uncentrifuged mixtures of erythrocyte suspensions and 200 µl of buffer, which was added to 3 ml Drabkin's reagent to obtain a value for 100% haemolysis. A negative control, included to measure the level of spontaneous haemolysis, comprised 200 µl buffer mixed with 200 µl erythrocytes, and after centrifugation for 35 s, a 200 µl sample of supernatant was added to 3 ml of Drabkin's reagent. Haemolysis percentage for each sample was calculated by dividing sample's absorbance on positive control absorbance complete haemolysis) multiplied by 100[6].

RESULTS AND DISCUSSION

3.1. Preparation

In this study the results of elemental analysis of the final product of the cationic surfactants (IIa-d) were collected in Table 1 .Atypical IR spectrum of the derivatives prepared in the present study displayed bands at 3365cm^{-1} (OH), 2851-2940cm^{-1} (C–H), 1108cm^{-1} (C–Nstr.), 720cm^{-1} due to CH rocking from long alkyl chain $(CH_2)_x$ The structure of the compound was further supported by the ^{1}HNMR spectrum, shows signals at δ 0.8-1.0 ppm (t, 3H, CH_3), 1.2-1.4 ppm (m, 20H, CH_2), 1.44 - 1.51 (m, 2H, NCH_2), 2.1 (s,3H, OH) , 3.21 (s, 6H, N $(CH_3)_2$), 4.2-4.4 (s, 6H, 3CH2OH) .

3.2. Surface activity

Surface tension of cationic surfactants (II_{a-d}) has been measured using Küss K6 tensiometry. The surface tension decrease with increasing concentrations and then reach clear break-points, which are taken as (CMC) as indicated in Fig. 1. The values of critical micelle concentration (CMC), apparent surface excess (Γ_{CMC}), minimal area/molecule at the interface (A_{min}), efficiency of surface tension reduction (PC_{20}: negative log of the surfactant molar concentration required to reduce the surface tension of the solvent (water) by 20mNm^{-1}) and the CMC/C_{20} ratio (which estimates the tendency of the surfactant to form micelles relative to the tendency to adsorb at the air/water interface) were indicated in Table 2 and calculated according to Gibbs adsorption

isotherm equation[16]. Indeed, the area per molecule at air/water interface of head group in the surfactants of the cationic series was found to be within a very close range of 93-109A^2, showing a somewhat smaller area per molecule with increasing tail length. This, would be attributable to the intermolecular Van der Waals forces at increasing chain lengths[17] .

The value of CMC can be used to obtain the Gibbs free energy of micellization ($\Delta G°_{mic}$.) and the standard free energy of adsorption $\Delta G°_{ads}$. It is clear that increasing the carbon chain length increases the negativity of $\Delta G°_{mic}$. which indicates that the micelle formation is thermodynamically favored for the prepared surfactants and the micellization process proceeds spontaneously.

The values of $\Delta G°_{mic}$. are smaller than the values of $\Delta G°_{ads}$ in all the series, indicating that the adsorption is promoted more than the micellization. In addition, the values of $\Delta G°_{mic.}$ increase with increasing hydrocarbon chain length of compounds, suggesting that a driving force of micellization is derived from the hydrophobic moieties due to the interaction between hydrocarbon tails.

3.3. Hemolytic activity

The surfactant may be absorbed and penetrate to the cell membrane, where it makes osmotic phenomenon by altering the permeability of membrane, which in turn causes the cellular lysis[18]. The results showed that hemolytic effects of all tested compounds increase with increasing surfactant concentration Fig. 2-3. Hence, the ability to increase membrane permeability and after it osmotic cellular lysis are related to concentration of surfactant[7].

In this study, the hemolytic effects of surfactants increased as temperature increased (Table 3-4). Liquid characteristics and fluidity of bilayer liquid is one of its special features. Some parts of the membrane can easily move throughout the surface and this characteristic is due to membrane phospholipids which converts to jelly in temperatures lower than physiologic temperature. This conversion of phospholipids helps in more stabilized and regular membrane and increases its resistance.In Table 3, the amount of haemolysis at 37°C is greater than, at 25°C; the reason is that with increase in temperature, the membrane fluidity and accordingly its permeability increase.

The fact that, in solutions with higher concentration of surfactants hemolysis amount was greater can be described by Fick's law that, the diffusion flux from a membrane is proportional to concentration difference on both sides[19-20] .

The hemolytic behaviour of the prepared compounds depends on the number of carbon atoms in the hydrophobic chains. Thus, Surfactant with 10 carbon atoms (II_a) in the hydrophobic chain resulted in the highest hemolysis .

Accordingly, it can be concluded that higher content of hydrophobic part may lead to reduction in permeability and hemolytic effects Tables 3-4.

According to the results, at 0.005mM concentration almost all surfactants caused about 50% haemolysis of erythrocytes. (Table 3-4). Further haemolysis was observed by increasing the temperature, thus at 37°C , 0.1 mM of compounds II_{b-d} caused 72-75% of erythrocytes destruction compared with II_a and CTAC which effected 91 and 93% of destruction, respectively.

The erythrocyte haemolysis showed that compound II_d had the lowest destruction level and less toxicity on cellular membrane. The maximum hemolytic effect of synthesized surfactants was observed at concentration above critical micelle concentration.

In addition, the potential uses of surfactants as drug delivery systems make of great importance the evalution of haemolysis.

3.4. Biodegradability

After use, all surfactants, which used in laundry detergents, cleaning agents and dyeing auxiliaries, are passed quantitatively into wastewater. Due to this fact, the constant input of surfactants into the environment requires a particular ecological characterization of this class of compounds. In this study, the biodegradability of these surfactants was evaluated by surface tension measurements in 7 days. The results are shown in Fig. 4. Apparently, these are considerably more highly, the biodegradable because they would be the natural foods of bacteria present in sewage or raw river water[21-22]. Compound II_c, II_d had the best results (97-98%).

According to hemolytic data and biodegradability effect, II_d had the least toxicity and the best properties for biodegradable. Due to its low toxicity to the membranes, it would be considered a suitable surfactant in drug formulations and biocide .

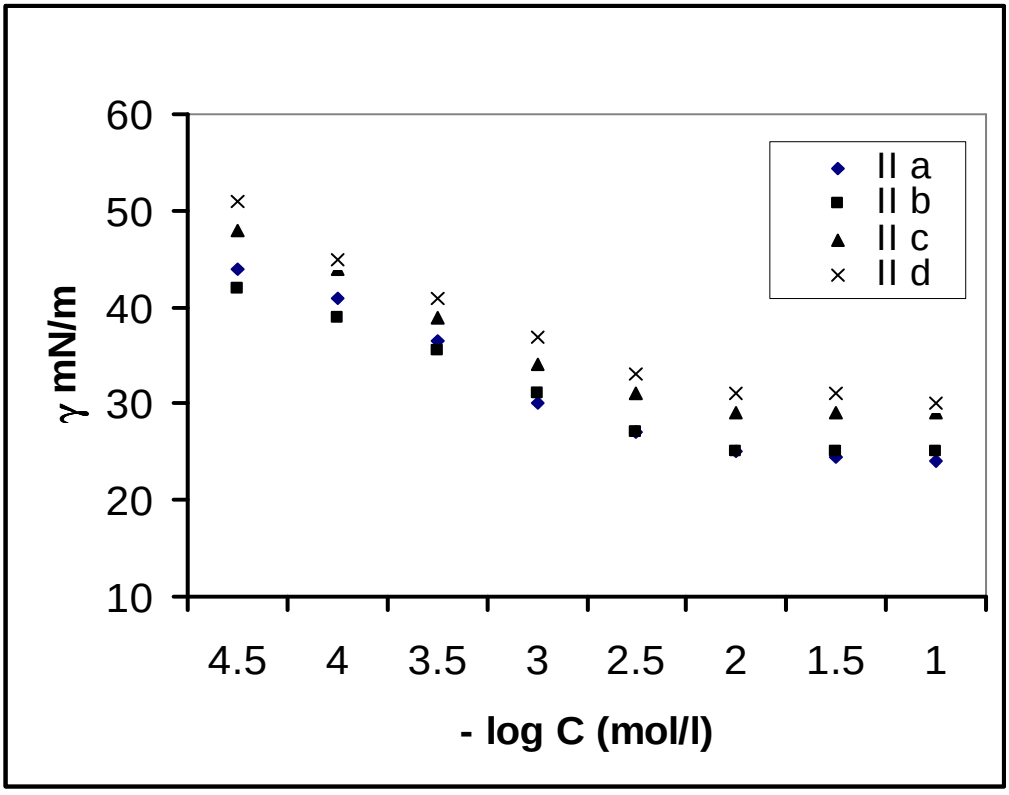


Fig. 1. Surface tension versus logarithm of concentration C, for surfactants II_{a-d} at 25°C.

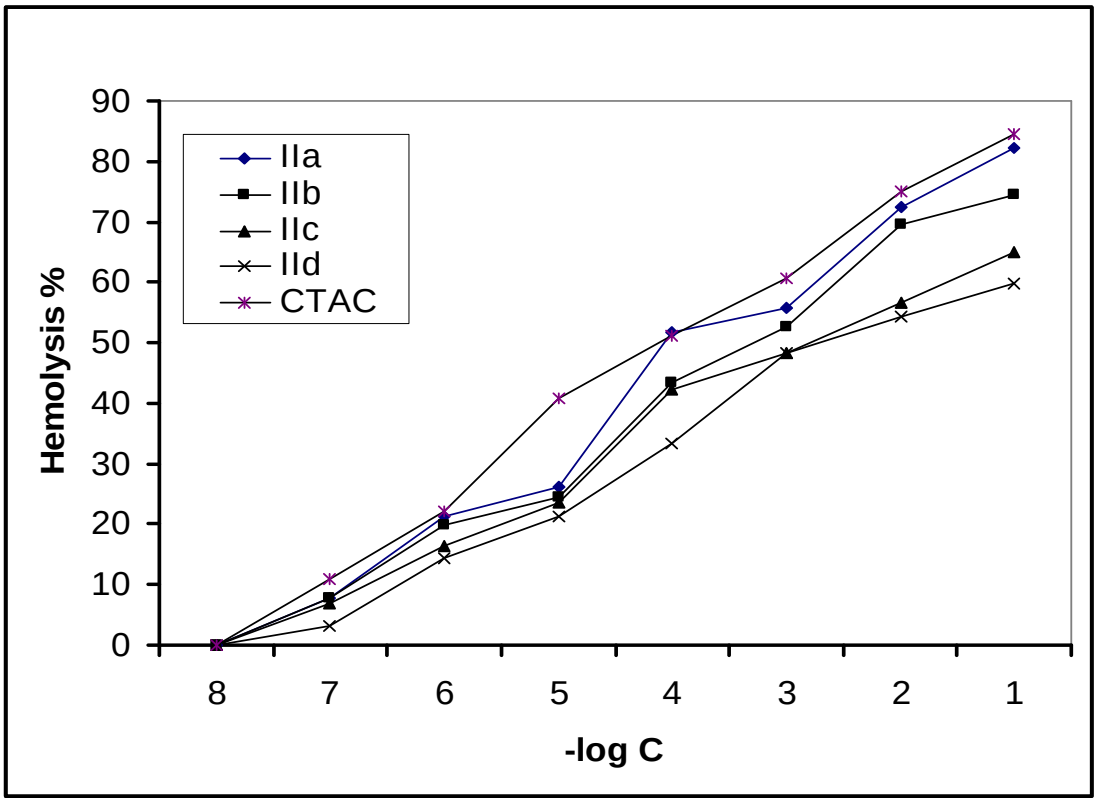


Fig. (2) Hemolysis versus logarithm of concentration C, for surfactants II_{a-d} and cetyl trimethyl ammonium chloride (CTAB) at 25°C.

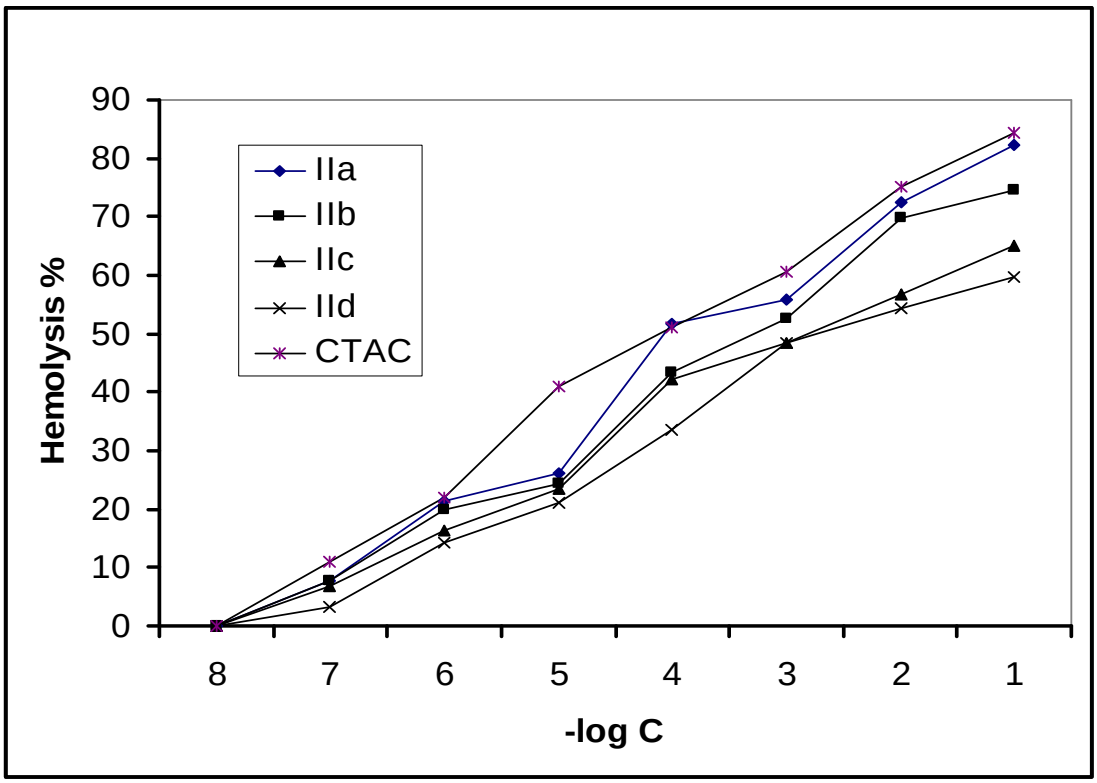


Fig. 3. Hemolysis versus logarithm of concentration C, for surfactants II_{a-d} cetyl trimethyl ammonium chloride (CTAB) at 37°C.

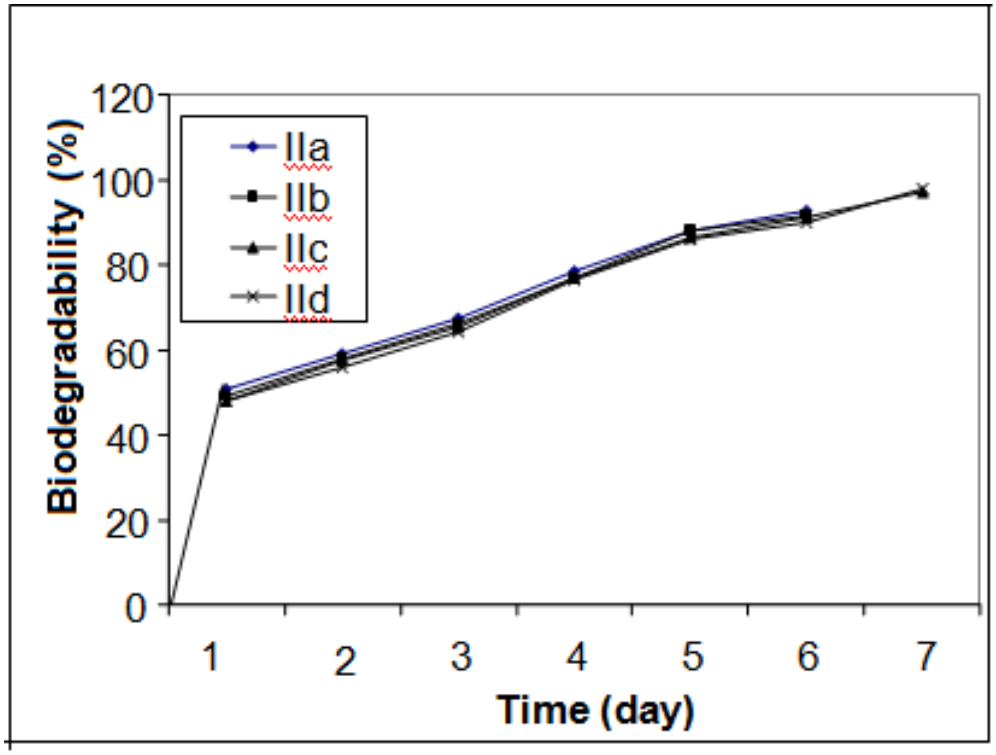


Fig. .4. Biodegradability of cationic surfactants (IIa-IId)

Table 1. Elemental analysis of cationic surfactants (II_{a-d})

Compared Molecular formula MW	m.p. (°C)	Yield %	Elemental analysis calculated (%) found		
			C	H	N
IIa $C_{16}H_{36}O_3NCl$ 325.92	Oily	73	58.91 58.85	11.04 10.97	4.29 4.25
IIb $C_{18}H_{40}O_3NCl$ 353.98	Oily	78	61.02 60.96	11.30 11.26	3.95 3.89
IIc $C_{22}H_{48}O_3NCl$ 410.08	44	82	64.37 64.31	11.70 11.66	3.41 3.35
IId $C_{24}H_{52}O_3NCl$ 438.14	55	84	65.73 65.68	11.86 11.82	3.19 3.17

Table 2. Surface Active properties of cationic surfactants (II_{a-d}) at 25°C

Comp. No.	γ_{CMC} mN/m	CMC mol/L)	Γ_{max} (mol/m^2 x 10^6)	A (m^{2}x10^{20})	C_{20} (mol/L)	PC_{20}	CMC/C_{20}	$\Delta G°_{mic.}$	$\Delta G°_{ads}$	π_{cmc}
II_a	24	6.30 x10^{-3}	1.85	93.25	4.35 x10^{-3}	2.36	1.44	-12.34	-35.58	43
II_b	25	5.62 x10^{-3}	1.73	95.80	2.49 x10^{-3}	2.60	2.48	-12.62	-39.78	47
II_c	29	3.98 x10^{-3}	1.71	96.86	2.48 x10^{-3}	2.60	1.60	-13.46	-38.54	43
II_d	32	3.16x10^{-3}	1.51	109.93	2.22 x10^{-3}	2.64	1.42	-14.02	-40.51	40

γ_{CMC}, surface tension at the CMC, CMC critical micelle concentration; Γ_{max} = the surface excess at the air/water interface; A, area occupied by the surfactant molecule at the interface; PC_{20}, (-log) to reduce the surface tension of water 20mN/m; $\Delta G°_{mic}$, standard free energies of micellization;' $\Delta G°_{ads}$, standard free energies of adsorption.

Table 3. Hemolysis induced by cationic surfactants (IIa-d) after 15 min 25°C.

Compound Conc.(mM)	Hemolysis (%)				
	IIa	IIb	IIc	IId	CTAC*
0	0.00	0.00	0.00	0.00	0.00
0.00005	7.81	7.74	6.91	3.22	10.90
0.0001	21.42	19.88	16.30	14.32	22.09
0.0005	26.28	24.38	23.48	21.15	40.96
0.001	51.75	43.39	42.30	33.47	51.10
0.005	55.92	52.70	48.40	48.42	60.61
0.01	72.33	69.70	56.76	54.26	75.02
0.1	82.16	74.45	64.99	59.70	84.48

** CTAC : cetyl trimethyl ammonium chloride as a reference*

Table 4. Hemolysis induced by cationic surfactants (IIa-d) after 15 min 37°C.

Compound Conc.(mM)	Hemolysis (%)				
	IIa	IIb	IIc	IId	CTAC*
0	0.00	0.00	0.00	0.00	0.00
0.00005	8.22	11.57	10.70	7.68	18.81
0.0001	17.65	21.55	17.12	16.57	36.00
0.0005	33.82	29.70	29.02	27.62	46.91
0.001	53.50	51.09	49.95	48.65	59.05
0.005	64.15	63.38	60.77	59.08	69.56
0.01	79.11	73.46	71.21	65.17	77.23
0.1	91.77	75.16	74.32	72.50	93.68

** CTAC : cetyl trimethyl ammonium chloride as a reference*

CONCLUSION

All the surfactants prepared in this work have good surface activity, absorption enhancer properties and biodegradability of them has also been tested suggesting that they can be used without any environmental problems.

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