

# Investigation of the physicochemical and biological properties of proline-based surfactants in single and mixed surfactant systems

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**Abstract** A series of surfactants derived from L-Proline, the free amine esters, the ester hydrochlorides and the quaternary ammonium compounds with varying chain lengths (C<sub>8</sub>–C<sub>14</sub>) were synthesised. The physicochemical and biological properties were determined in both single and sodium dodecyl sulphate (SDS) mixed systems with a view of enhancing the properties of the individual surfactants as potential ingredients in detergent formulations. The mode of action of the proline surfactants were investigated by their ability to form mixed micelles with the phospholipid 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC). The presence of a quaternary ammonium moiety and an increase in alkyl chain length were found to enhance the antibacterial activity of the proline QUAT derivatives. The SDS-C<sub>14</sub> QUAT mixed system displayed good antibacterial activity with optimum activity at mole fractions  $\alpha_{\text{QUAT}}$ : 0.4 and 0.6. The antibacterial activity of the mixed system was found to be governed by the monomers rather than the micelles. The SDS-C<sub>14</sub> QUAT mixed system also showed moderate irritancy which makes them potential candidates as detergents.

**Keywords** Proline · Quaternary ammonium compounds · Mixed micelles · Antibacterial · Foaming · Ocular irritation

## Introduction

Surfactants constitute an important class of chemicals widely used in almost every sector of modern industry, and are considered to be a key ingredient in detergents,

comprising 15 to 40 % of the total detergent formulations [1, 2]. With this view, the demand of the detergent industry for the development of formulations with improved detergency in the past few years has been the driving force for the synthesis of new surfactants as potential ingredients in cleaning agents. Alkylbenzenesulfonates, alkyl sulphates [1], alcohol ethoxylates [3], quaternary ammonium compounds [4] and betaine surfactants [5] are commonly used in detergent compositions, and their physicochemical behaviour such as good interfacial, surface activity and foaming abilities is well understood.

The increase in environmental awareness among consumers has led to the development of biodegradable and less toxic surfactants having good interfacial and biological properties for potential use in biomedical applications. In this perspective, amino acid surfactants as alternatives to conventional surfactants have attracted widespread attention over the last decade [6–10].

Amino acid surfactants, especially those of a cationic nature, are known to exhibit good antibacterial activity against a broad spectrum of microorganisms [11–13]. The mechanism of action of these surfactants on bacteria is known to involve electrostatic and hydrophobic interaction between the cationic agent and the phospholipid bilayer of the bacterial cell wall [14, 15]. These agents are also believed to form mixed micelles with the membrane phospholipid molecules [16]. These lead to the disruption of the membrane and permit release of electrolytes and nucleic materials, leading to cell death.

Proline-based surfactants have been found to possess interesting physicochemical as well as biological activities. Proline is a cyclic secondary amine which gives it an exceptional conformational rigidity compared to other amino acids. Optically active as well as racemic mixtures of *N*-acyl proline-based surfactants have been found to

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aggregate spontaneously in aqueous solutions [17]. Proline-based surfactants bearing an ester and amide linkage have been found to interact strongly with DNA [18, 19]. However, there have been limited studies on antibacterial properties of proline-based surfactants.

This study deals with the synthesis and physicochemical characteristics of a range of surfactants derived from proline esters. Their physicochemical and biological activities were determined as both single and mixed surfactant systems with SDS, in order to study their effectiveness as potential ingredients in detergents. The antibacterial activity against gram-positive and gram-negative bacteria were also investigated. In order to investigate the possible mechanism of the surfactant for their antimicrobial activity, mixed micelle-forming ability of selected proline surfactants with 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) were studied. The foaming characteristics of selected proline surfactants were investigated to evaluate their use as detergent formulations.

## Materials and Methods

L-Proline, octanol, decanol, dodecanol, and tetradecanol were obtained from Sigma-Aldrich (USA). *p*-Toluene sulfonic acid (PTSA) was obtained from Merck, Germany. Mueller–Hinton agar and broth were purchased from Oxoid Ltd., United Kingdom. The different bacterial strains were obtained from Microbiologics®, USA and Oxoid Ltd., United Kingdom. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was purchased from Avanti Polarlipids, Inc., USA. Cetyl trimethyl ammonium bromide (CTAB) and sodium dodecyl sulphate (SDS) were obtained from BDH Laboratory Supplies, England.

Conductivity measurements were carried out using a Jenway 4320 conductivity meter. Proton nuclear magnetic resonance ( $^1\text{H}$  NMR) and carbon-13 ( $^{13}\text{C}$ ) NMR spectra were recorded at 250 MHz and 62.9 MHz on a Bruker electro spin NMR spectrometer using  $\text{CDCl}_3$  as the solvent.

### Synthesis and Characterization of L-Proline Esters (1a–d) and Their Hydrochloride Derivatives (2a–d)

The L-proline esters were synthesised according to a modified procedure described by our group [14] by the reaction of L-proline (1.37 g, 12.1 mmol), PTSA (2.30 g, 12.1 mmol) and alcohol (14.5 mmol) in refluxing toluene (100 ml) for 48 h. The crude product was purified by column chromatography (Hexane/DCM 1:1) to give the esters (1a–d) as yellow oils. The proline esters were dissolved in EtOAc and then HCl gas was bubbled through the mixture and after evaporation of the solvent in vacuo, the proline ester hydrochloride derivatives (2a–d) were obtained as solids.

### Synthesis of Proline QUAT Derivatives (3a–d)

The L-proline ester (2.32 mmol) and methyl iodide (4 mL) were stirred in acetonitrile in the presence of  $\text{K}_2\text{CO}_3$  (1.20 g, 8.7 mmol) for 24 h at room temperature (25°C). The mixture was filtered and the filtrate was evaporated *in vacuo* to yield the product as white or yellow solid.

**Octyl pyrrolidine-2-carboxylate (1a)** Yield: 67%. IR,  $\nu_{\text{max}}$  ( $\text{cm}^{-1}$ ): 2956, 1741.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ),  $\delta$  (ppm): 0.86 (t,  $J$  7.0 Hz, 3H,  $\text{O}(\text{CH}_2)_7\text{CH}_3$ ), 1.25 (m, 10H,  $\text{OCH}_2\text{CH}_2(\text{CH}_2)_5\text{CH}_3$ ), 1.55 (m, 2H,  $\text{OCH}_2\text{CH}_2(\text{CH}_2)_5\text{CH}_3$ ), 1.72 (m, 2H,  $\text{CH}_2$ ), 1.87 (m, 2H,  $\text{CH}_2$ ), 2.88 (m, 1H,  $\text{CHH}$ ), 3.08 (m, 1H,  $\text{CHH}$ ), 3.62 (t,  $J$  7.0 Hz, 1H, CH), 4.10 (t,  $J$  7.0 Hz, 2H,  $\text{OCH}_2(\text{CH}_2)_6\text{CH}_3$ ).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ),  $\delta$  (ppm): 14.1 ( $\text{O}(\text{CH}_2)_7\text{CH}_3$ ), 22.7–30.3 ( $\text{OCH}_2(\text{CH}_2)_6\text{CH}_3$ ), 25.7 ( $\text{CH}_2$ ), 31.8 ( $\text{CH}_2$ ), 47.0 ( $\text{N}-\text{CH}_2$ ), 59.8 (CH), 63.1 ( $\text{OCH}_2(\text{CH}_2)_6\text{CH}_3$ ), 175.6 (C=O).

**Decyl pyrrolidine-2-carboxylate (1b)** Yield: 78%. IR,  $\nu_{\text{max}}$  ( $\text{cm}^{-1}$ ): 2954, 1743.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ),  $\delta$  (ppm): 0.86 (m, 3H,  $\text{O}(\text{CH}_2)_9\text{CH}_3$ ), 1.24 (m, 14H,  $\text{OCH}_2\text{CH}_2(\text{CH}_2)_7\text{CH}_3$ ), 1.54 (m, 2H,  $\text{OCH}_2\text{CH}_2(\text{CH}_2)_7\text{CH}_3$ ), 1.74 (m, 2H,  $\text{CH}_2$ ), 1.83 (m, 2H,  $\text{CH}_2$ ), 2.98 (m, 1H,  $\text{CHH}$ ), 3.15 (m, 1H,  $\text{CHH}$ ), 3.61 (t,  $J$  7.0 Hz, 1H, CH), 4.10 (t,  $J$  7.0 Hz, 2H,  $\text{OCH}_2(\text{CH}_2)_8\text{CH}_3$ ).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ),  $\delta$  (ppm): 14.1 ( $\text{O}(\text{CH}_2)_9\text{CH}_3$ ), 22.7–30.3 ( $\text{OCH}_2(\text{CH}_2)_8\text{CH}_3$ ), 25.7 ( $\text{CH}_2$ ), 31.9 ( $\text{CH}_2$ ), 47.0 ( $\text{N}-\text{CH}_2$ ), 59.8 (CH), 65.1 ( $\text{OCH}_2(\text{CH}_2)_8\text{CH}_3$ ), 175.6 (C=O).

**Dodecyl pyrrolidine-2-carboxylate (1c)** Yield: 56%. IR,  $\nu_{\text{max}}$  ( $\text{cm}^{-1}$ ): 3377, 2957, 1737.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ),  $\delta$  (ppm): 0.88 (m, 3H,  $\text{O}(\text{CH}_2)_{11}\text{CH}_3$ ), 1.26 (m, 18H,  $\text{OCH}_2\text{CH}_2(\text{CH}_2)_9\text{CH}_3$ ), 1.61 (m, 2H,  $\text{OCH}_2\text{CH}_2(\text{CH}_2)_9\text{CH}_3$ ), 1.74 (m, 2H,  $\text{CH}_2$ ), 1.80 (m, 2H,  $\text{CH}_2$ ), 2.88 (m, 1H,  $\text{CHH}$ ), 3.06 (m, 1H,  $\text{CHH}$ ), 3.75 (m, 1H, CH), 4.11 (t,  $J$  7.0 Hz, 2H,  $\text{OCH}_2(\text{CH}_2)_{10}\text{CH}_3$ ).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ),  $\delta$  (ppm): 14.1 ( $\text{O}(\text{CH}_2)_9\text{CH}_3$ ), 22.7–30.3 ( $\text{OCH}_2(\text{CH}_2)_8\text{CH}_3$ ), 25.9 ( $\text{CH}_2$ ), 31.9 ( $\text{CH}_2$ ), 47.0 ( $\text{N}-\text{CH}_2$ ), 59.8 (CH), 65.1 ( $\text{OCH}_2(\text{CH}_2)_8\text{CH}_3$ ), 175.6 (C=O).

**Tetradecyl pyrrolidine-2-carboxylate (1d)** Yield: 75%. IR,  $\nu_{\text{max}}$  ( $\text{cm}^{-1}$ ): 3420, 2921, 1739.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ),  $\delta$  (ppm): 0.88 (m, 3H,  $\text{O}(\text{CH}_2)_{13}\text{CH}_3$ ), 1.26 (m, 22H,  $\text{OCH}_2\text{CH}_2(\text{CH}_2)_{11}\text{CH}_3$ ), 1.52 (m, 2H,  $\text{OCH}_2\text{CH}_2(\text{CH}_2)_{11}\text{CH}_3$ ), 1.78 (m, 2H,  $\text{CH}_2$ ), 1.86 (m, 2H,  $\text{CH}_2$ ), 3.03 (m, 2H,  $\text{CH}_2$ ), 3.64 (t,  $J$  7.0 Hz, 1H, CH), 4.13 (t,  $J$  7.0 Hz, 2H,  $\text{OCH}_2(\text{CH}_2)_{12}\text{CH}_3$ ).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ),  $\delta$  (ppm): 14.1 ( $\text{O}(\text{CH}_2)_9\text{CH}_3$ ), 22.7–30.3 ( $\text{OCH}_2(\text{CH}_2)_8\text{CH}_3$ ), 25.7 ( $\text{CH}_2$ ), 31.9 ( $\text{CH}_2$ ), 46.6 ( $\text{N}-\text{CH}_2$ ), 59.5 (CH), 65.8 ( $\text{OCH}_2(\text{CH}_2)_8\text{CH}_3$ ), 173.1 (C=O).

**Octyl pyrrolidine-2-carboxylate hydrochloride (2a)** Yield: 49%. Elem. Anal. Found: C, 58.93; H, 9.66; N, 5.00. Calcd. for  $\text{C}_{13}\text{H}_{26}\text{NO}_2\text{Cl}$ : C, 59.19; H, 9.93; N, 5.31.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ),  $\delta$  (ppm): 0.88 (m, 3H,  $\text{O}(\text{CH}_2)_7\text{CH}_3$ ), 1.27 (m, 10H,  $\text{OCH}_2\text{CH}_2(\text{CH}_2)_5\text{CH}_3$ ), 1.62 (m, 2H,

OCH<sub>2</sub>CH<sub>2</sub>(CH<sub>2</sub>)<sub>5</sub>CH<sub>3</sub>), 2.04 (m, 2H, CH<sub>2</sub>), 3.54 (m, 2H, CH<sub>2</sub>), 4.10 (m, 2H, OCH<sub>2</sub>(CH<sub>2</sub>)<sub>6</sub>CH<sub>3</sub>), 4.50 (m, 1H, CH).

**Decyl pyrrolidine-2-carboxylate hydrochloride (2b)** Yield: 52%. Elem. Anal. Found: C, 62.14; H, 10.32; N, 4.53; Calcd. for C<sub>15</sub>H<sub>30</sub>NO<sub>2</sub>Cl: C, 61.73; H, 10.36; N, 4.80. <sup>1</sup>H NMR (CDCl<sub>3</sub>), δ (ppm): 0.86 (m, 3H, O(CH<sub>2</sub>)<sub>9</sub>CH<sub>3</sub>), 1.27 (m, 14H, OCH<sub>2</sub>CH<sub>2</sub>(CH<sub>2</sub>)<sub>7</sub>CH<sub>3</sub>), 1.64 (m, 2H, OCH<sub>2</sub>CH<sub>2</sub>(CH<sub>2</sub>)<sub>7</sub>CH<sub>3</sub>), 2.03 (m, 2H, CH<sub>2</sub>), 3.53 (m, 2H, CH<sub>2</sub>), 4.12 (m, 2H, OCH<sub>2</sub>(CH<sub>2</sub>)<sub>8</sub>CH<sub>3</sub>), 4.48 (m, 1H, CH).

**Dodecyl pyrrolidine-2-carboxylate hydrochloride (2c)** Yield: 63%. Elem. Anal. Found: C, 64.17; H, 11.14; N, 4.75; Calcd. for C<sub>17</sub>H<sub>34</sub>NO<sub>2</sub>Cl: C, 63.82; H, 10.71; N, 4.38. <sup>1</sup>H NMR (CDCl<sub>3</sub>), δ (ppm): 0.87 (m, 3H, O(CH<sub>2</sub>)<sub>11</sub>CH<sub>3</sub>), 1.26 (m, 18H, OCH<sub>2</sub>CH<sub>2</sub>(CH<sub>2</sub>)<sub>9</sub>CH<sub>3</sub>), 1.66 (m, 2H, OCH<sub>2</sub>CH<sub>2</sub>(CH<sub>2</sub>)<sub>9</sub>CH<sub>3</sub>), 2.04 (m, 2H, CH<sub>2</sub>), 3.53 (m, 2H, CH<sub>2</sub>), 4.12 (m, 2H, OCH<sub>2</sub>(CH<sub>2</sub>)<sub>10</sub>CH<sub>3</sub>), 4.48 (m, 1H, CH).

**Tetradecyl pyrrolidine-2-carboxylate hydrochloride (2d)** Yield: 61%. Elem. Anal. Found: C, 65.83; H, 11.27; N, 4.09; Calcd. for C<sub>19</sub>H<sub>38</sub>NO<sub>2</sub>Cl: C, 65.58; H, 11.01; N, 4.03. <sup>1</sup>H NMR (CDCl<sub>3</sub>), δ (ppm): 0.87 (m, 3H, O(CH<sub>2</sub>)<sub>13</sub>CH<sub>3</sub>), 1.25 (m, 22H, OCH<sub>2</sub>CH<sub>2</sub>(CH<sub>2</sub>)<sub>11</sub>CH<sub>3</sub>), 1.61 (m, 2H, OCH<sub>2</sub>CH<sub>2</sub>(CH<sub>2</sub>)<sub>11</sub>CH<sub>3</sub>), 2.04 (m, 2H, CH<sub>2</sub>), 3.55 (m, 2H, CH<sub>2</sub>), 4.19 (m, 2H, OCH<sub>2</sub>(CH<sub>2</sub>)<sub>12</sub>CH<sub>3</sub>), 4.51 (m, 1H, CH).

**2-Octyloxycarbonyl-1,1-dimethyl-pyrrolidinium iodide (3a)** Yield: 57%. Elem. Anal. Found: C, 47.27; H, 8.03; N, 3.21; Calcd. for C<sub>15</sub>H<sub>30</sub>NO<sub>2</sub>I: C, 47.00; H, 7.89; N, 3.65. <sup>1</sup>H NMR (CDCl<sub>3</sub>), δ (ppm): 0.87 (m, 3H, O(CH<sub>2</sub>)<sub>7</sub>-CH<sub>3</sub>), 1.28 (m, 10H, OCH<sub>2</sub>CH<sub>2</sub>(CH<sub>2</sub>)<sub>5</sub>CH<sub>3</sub>), 1.52 (m, 2H, OCH<sub>2</sub>CH<sub>2</sub>(CH<sub>2</sub>)<sub>5</sub>CH<sub>3</sub>), 2.11 (m, 2H, CH<sub>2</sub>), 2.50 (m, 1H, CHH), 2.82 (m, 1H, CHH), 3.22 (s, 3H, CH<sub>3</sub>), 3.63 (s, 3H, CH<sub>3</sub>), 4.04 (m, 1H, CHH), 4.20 (m, 2H, OCH<sub>2</sub>(CH<sub>2</sub>)<sub>6</sub>CH<sub>3</sub>), 4.58 (m, 1H, CHH), 5.31 (m, 1H, CH). <sup>13</sup>C NMR (CDCl<sub>3</sub>), δ(ppm): 14.1 (O(CH<sub>2</sub>)<sub>7</sub>CH<sub>3</sub>), 22.7–30.3 (OCH<sub>2</sub>(CH<sub>2</sub>)<sub>8</sub>-CH<sub>3</sub>), 25.7 (CH<sub>2</sub>), 31.9 (CH<sub>2</sub>), 46.6 (N-CH<sub>2</sub>), 47.0 (CH<sub>3</sub>), 51.7 (CH<sub>3</sub>), 59.5 (CH), 63.2 (OCH<sub>2</sub>(CH<sub>2</sub>)<sub>8</sub>CH<sub>3</sub>), 165.8 (C=O).

**2-Decyloxycarbonyl-1,1-dimethyl-pyrrolidinium iodide (3b)** Yield: 43%. Elem. Anal. Found: C, 49.17; H, 8.56; N, 3.03. Calcd. for C<sub>17</sub>H<sub>34</sub>NO<sub>2</sub>I: C, 49.64; H, 8.33; N, 3.40. <sup>1</sup>H NMR (CDCl<sub>3</sub>), δ (ppm): <sup>1</sup>H NMR (CDCl<sub>3</sub>), δ (ppm): 0.86 (m, 3H, O(CH<sub>2</sub>)<sub>9</sub>CH<sub>3</sub>), 1.25 (m, 14H, OCH<sub>2</sub>-CH<sub>2</sub>(CH<sub>2</sub>)<sub>7</sub>CH<sub>3</sub>), 1.52 (m, 2H, OCH<sub>2</sub>CH<sub>2</sub>(CH<sub>2</sub>)<sub>7</sub>CH<sub>3</sub>), 2.14 (m, 2H, CH<sub>2</sub>), 2.48 (m, 1H, CHH), 2.76 (m, 1H, CHH), 3.21 (s, 3H, CH<sub>3</sub>), 3.73 (s, 3H, CH<sub>3</sub>), 4.04 (m, 1H, CHH), 4.18 (m, 2H, OCH<sub>2</sub>(CH<sub>2</sub>)<sub>8</sub>CH<sub>3</sub>), 4.58 (m, 1H, CHH), 5.42 (m, 1H, CH). <sup>13</sup>C NMR (CDCl<sub>3</sub>), δ (ppm): 14.1 (O(CH<sub>2</sub>)<sub>13</sub>CH<sub>3</sub>), 22.7–29.5 (OCH<sub>2</sub>(CH<sub>2</sub>)<sub>10</sub>CH<sub>3</sub>), 25.9 (CH<sub>2</sub>), 31.9 (CH<sub>2</sub>), 47.2 (CH<sub>3</sub>), 51.9 (CH<sub>3</sub>), 63.4 (OCH<sub>2</sub>(-CH<sub>2</sub>)<sub>10</sub>CH<sub>3</sub>), 68.4 (N-CH<sub>2</sub>), 73.6 (CH), 166.0 (C = O).

**2-Dodecyloxycarbonyl-1,1-dimethyl-pyrrolidinium iodide (3c)** Yield: 37%. Elem. Anal. Found: C, 52.12; H, 9.06; N, 3.24; Calcd. for C<sub>19</sub>H<sub>38</sub>NO<sub>2</sub>I: C, 51.93; H, 8.72; N, 3.19. <sup>1</sup>H NMR (CDCl<sub>3</sub>), δ (ppm): 0.88 (m, 3H, O(CH<sub>2</sub>)<sub>11</sub>CH<sub>3</sub>), 1.26 (m, 18H, OCH<sub>2</sub>CH<sub>2</sub>(CH<sub>2</sub>)<sub>9</sub>CH<sub>3</sub>), 1.51 (m, 2H, OCH<sub>2</sub>CH<sub>2</sub>(CH<sub>2</sub>)<sub>9</sub>CH<sub>3</sub>), 2.25 (m, 2H, CH<sub>2</sub>), 2.51 (m, 1H, CHH), 2.83 (m, 1H, CHH), 3.24 (s, 3H, CH<sub>3</sub>), 3.65 (s, 3H, CH<sub>3</sub>), 4.04 (m, 1H, CHH), 4.22 (m, 2H, OCH<sub>2</sub>(-CH<sub>2</sub>)<sub>10</sub>CH<sub>3</sub>), 4.62 (m, 1H, CHH), 5.31 (m, 1H, CH). <sup>13</sup>C NMR (CDCl<sub>3</sub>), δ (ppm): 14.1 (O(CH<sub>2</sub>)<sub>11</sub>CH<sub>3</sub>), 19.2 (CH<sub>2</sub>), 22.7–31.9 (OCH<sub>2</sub>(CH<sub>2</sub>)<sub>11</sub>CH<sub>3</sub>), 25.9 (CH<sub>2</sub>), 47.3 (CH<sub>3</sub>), 52.3 (CH<sub>3</sub>), 63.1 (OCH<sub>2</sub>(CH<sub>2</sub>)<sub>11</sub>CH<sub>3</sub>), 68.0 (N-CH<sub>2</sub>), 73.1 (CH), 165.8 (C=O).

**2-Tetradecyloxycarbonyl-1,1-dimethyl-pyrrolidinium iodide (3d)** Yield: 78%. Melting point: 121 °C. Elem. Anal. Found: C, 53.62; H, 9.26; N, 3.09; Calcd. for C<sub>21</sub>H<sub>42</sub>NO<sub>2</sub>I: C, 53.96; H, 9.06; N, 3.00. TOF MS, m/z: 340.32 (without the iodide ion). <sup>1</sup>H NMR (CDCl<sub>3</sub>), δ (ppm): 0.88 (m, 3H, O(CH<sub>2</sub>)<sub>13</sub>CH<sub>3</sub>), 1.25 (m, 22H, OCH<sub>2</sub>CH<sub>2</sub>(CH<sub>2</sub>)<sub>11</sub>CH<sub>3</sub>), 1.53 (m, 2H, OCH<sub>2</sub>CH<sub>2</sub>(CH<sub>2</sub>)<sub>11</sub>-CH<sub>3</sub>), 2.22 (m, 2H, CH<sub>2</sub>), 2.49 (m, 1H, CHH), 2.84 (m, 1H, CHH), 3.22 (s, 3H, CH<sub>3</sub>), 3.75 (s, 3H, CH<sub>3</sub>), 4.12 (m, 1H, CHH), 4.22 (m, 2H, OCH<sub>2</sub>(CH<sub>2</sub>)<sub>12</sub>CH<sub>3</sub>), 4.52 (m, 1H, CHH), 4.30 (m, 1H, CH). <sup>13</sup>C NMR (CDCl<sub>3</sub>), δ(ppm): 14.1 (O(CH<sub>2</sub>)<sub>13</sub>CH<sub>3</sub>), 19.1 (CH<sub>2</sub>), 22.7–31.9 (OCH<sub>2</sub>(CH<sub>2</sub>)<sub>12</sub>-CH<sub>3</sub>), 25.7 (CH<sub>2</sub>), 47.3 (CH<sub>3</sub>), 52.2 (CH<sub>3</sub>), 63.1 (OCH<sub>2</sub>(-CH<sub>2</sub>)<sub>12</sub>CH<sub>3</sub>), 67.9 (N-CH<sub>2</sub>), 73.5 (CH), 165.0 (C = O).

### Critical Micelle Concentration and Phase Behaviour

The critical micelle concentrations (CMCs) of the proline surfactants were determined by a conductivity method [14]. Mixed SDS-C<sub>14</sub> QUAT **3d** systems were prepared by mixing precalculated volumes of the stock solutions of both surfactants in water and the solutions were stirred for 1 h. In order to study the counterion effect, the mixed micelle was also studied in 5 mM NaI. The composition of each solution was expressed as a mole fraction of the QUAT:

$$\alpha_{\text{QUAT}} = \frac{[\text{QUAT}]}{[\text{QUAT}] + [\text{SDS}]}$$

where [QUAT] and [SDS] are the concentrations of QUAT **3d** and SDS in the mixed solutions, respectively.

Mixed systems with varying mole fractions of QUATS ( $\alpha_{\text{QUAT}} = 0, 0.2, 0.4, 0.6, 0.8$ ) were prepared and the CMCs of the different mixed systems were determined by adding successive amounts of the stock solutions to deionised water/5 mM NaI in the form of a titration.

Mixed systems of SDS with varying mole fractions of QUAT **3d** ( $\alpha_{\text{QUAT}} = 0$  to 1) were prepared by mixing the required volume of equimolar (20 mM) QUAT **3d** and

SDS in 5 mM NaI. The solutions were stirred for 24 h and the phase behaviour of the different mixed systems was investigated by visual inspection and by conductivity measurements.

Mixed surfactant-phospholipid (DMPC) systems were prepared according to the method previously described by Faustino et al. (2011) [16]. Equimolar stock solutions (500 mM) of proline surfactants (**2d** and **3d**) and DMPC were prepared in deionized water. Binary mixtures of DMPC with varying mole fractions (0, 0.2, 0.4, 0.6, 0.8, 1) of the surfactants were prepared by mixing calculated amounts of the stock solutions of both components in aqueous phase. All the solutions were stirred for about 1 h to ensure complete mixing of the phospholipid and the surfactant. The CMC of the mixed surfactant-phospholipid system was determined as mentioned above.

The mole fractions ( $\alpha_L$ ) of the phospholipid in the mixtures were calculated as per the equation below:

$$\alpha_L = \frac{[\text{Lipid}]}{[\text{Lipid}] + [\text{surfactant}]}$$

where [lipid] and [surfactant] are the concentrations (M) of the phospholipid and surfactant in the solution, respectively.

The phase behaviour of the binary mixture of QUAT **3d** with varying mole fraction of DMPC ( $\alpha_L = 0$  to 1) was determined by mixing the required volume of equimolar (0.01 M) QUAT **3d** and DMPC. After mixing for 24 h, the phase behaviour was noted by visual observation and conductivity measurements as a function of  $\alpha_L$ .

### Antibacterial Activity

The antibacterial activities were evaluated against *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Bacillus cereus* (ATCC 11778, ATCC 10876), *Klebsiella pneumoniae* (ATCC 13883), *Escherichia coli* (ATCC 22922) and *Salmonella typhimurium* (ATCC 14028). The antibacterial activity was expressed as the minimum inhibitory concentrations (MICs) which was defined as the lowest concentration that inhibits the growth of bacteria. CTAB was used as a positive control. The antibacterial activities of the different SDS-QUAT **3d** mixed micelle systems ( $\alpha_{\text{QUAT}} = 0, 0.2, 0.4, 0.6, 0.8$ ) were determined starting from solutions with a total surfactant concentration ( $C_T$ ) of 0.01 M [(QUAT) + (SDS) = 0.01 M].

The antimicrobial susceptibility of the proline surfactants was determined by the Kirby–Bauer disc diffusion method [20]. Solutions of esters of free amine L-proline (**1a–d**) and their hydrochlorides (**2a–d**) in dimethyl sulfoxide (DMSO; 10  $\mu\text{L}$ , 10 mg/mL) were pipetted onto discs on Mueller–Hinton Agar plates containing bacteria an

allowed to incubate at 37 °C for 24 h. For each compound, the experiment was done in triplicate. The antibacterial activity was determined by measuring the average zone of inhibition in mm. The MIC was determined by the microdilution assay in a 96-well ELISA plate [21]. All wells were inoculated with 50  $\mu\text{L}$  of a bacterial suspension adjusted to 0.5 McFarland in physiological solution. Microplates were covered and incubated for 24 h at 37 °C. The minimum inhibitory concentration (MIC) of the surfactants was detected following addition of 20  $\mu\text{L}$  iodinitrotetrazolium chloride (0.4 mg/mL) and incubation at 37 °C for 30 min. MIC is defined as the lowest surfactant concentration that prevented the colour change from yellow to pink, due to inhibition of bacterial growth.

### Foamability and Foam Stability Measurements

The surfactant solution (20 mL, 0.1 wt%) was shaken manually in a 100-mL glass cylinder for 10 s at 25 °C. The height of the foam (cm) was measured at different time intervals (5 min, 30 min, 1 h and 24 h). A 10-mL glass cylinder was used for the measurement of foamability of the different mixed micelle SDS-QUAT systems. 5-mL portions of 500 mM mixed micelle solution ( $\alpha_{\text{QUAT}} = 0, 0.2, 0.4, 0.6, 0.8, 1$ ) were shaken vigorously for 10 s, and the initial heights of the foam were recorded. All the measurements were performed at 25 °C in triplicate and the results are reported as the mean value  $\pm$  standard deviation.

### Hemolytic Activity and Eye Irritation Potential of an SDS-QUAT Mixed Micelle System

Hemolytic activity of the SDS-QUAT mixed micelles was determined using the previously reported procedure [15]. The results were expressed by the concentration of the mixed micelle that induced 50 % hemolysis ( $\text{HC}_{50}$ ).

The eye irritation potential of the mixed micelle system was determined by the ratio of hemolysis to the protein denaturation index [23]. Protein denaturation was determined from the ratio  $R_x$  of absorbance at 575 and 540 nm in a UV–Vis spectrophotometer.  $R_1$  was the ratio obtained with the distilled water lysis and  $R_2$  was the ratio obtained with the denaturant SDS. The ratio obtained with each test material concentration was  $R_i$ . The ratios were used to calculate the haemoglobin denaturation index (DI), where  $\text{DI} = R_1 - R_i / R_1 - R_2$ . The relation between the hemolytic activity ( $\text{HC}_{50}$ ) and the denaturation index (DI) called the  $L/D$  ratio was used to classify the irritancy of the different surfactant mixtures as: non-irritant  $>100$ , weak irritant  $>10$ , moderate irritant  $>1$ , irritant  $>0.1$  and very irritant  $<0.1$ .

## Results and Discussions

Esters of L-proline-free amine (chain lengths C<sub>8</sub>–C<sub>14</sub>) **1a–d** were synthesized by the reaction of proline with the corresponding alcohols. The cationic surfactants were obtained either by protonation of the amino group or by reacting the esters with excess MeI yielding the hydrochlorides **2a–d** and the quaternary ammonium derivatives (QUATS) of proline **3a–d** (Fig. 1). Quaternisation was confirmed by the presence of two singlets corresponding to (2 × 3H) at δ 3.21–3.74 ppm. Compared to the L-proline ester hydrochloride series **2a–d**, the L-proline QUATS have a permanent positive charge on the head group, irrespective of the pH.

The purity of the proline surfactants were confirmed by spectral and analytical data. The <sup>1</sup>H and <sup>13</sup>C NMR of the different proline derivatives are concordant with the expected structures.

### Critical Micelle Concentration

The CMCs of the cationic surfactant derivatives of proline, namely the ester hydrochlorides (**2a–d**) and the QUATS (**3a–d**), are summarised in Table 1.

The CMCs of the ester hydrochlorides (**2a–d**) were found to be higher compared to the QUAT derivatives (**3a–d**), implying that the QUATS have a stronger tendency to form micelles than the ester hydrochlorides.

Mixed surfactant systems are known to have physical properties different from that of the individual components and these systems are encountered in several applications [24, 25]. The electrostatic interactions between cationic and anionic surfactants are known to enhance their surface

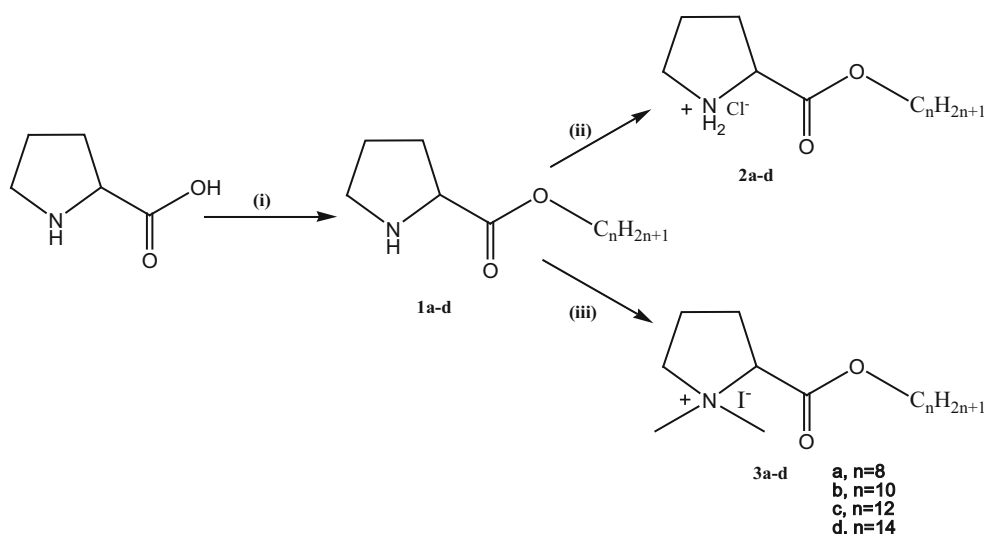
**Table 1** CMC of cationic surfactants derived from L-proline

Chain length	Ester hydrochlorides		QUATS	
	Compound	CMC (μM)	Compound	CMC (μM)
8	<b>2a</b>	1530	<b>3a</b>	1380
10	<b>2b</b>	909	<b>3b</b>	745
12	<b>2c</b>	741	<b>3c</b>	385
14	<b>2d</b>	566	<b>3d</b>	194

activities [26]. In this study, the QUAT C<sub>14</sub> derivative of proline **3d** displayed the highest micelle-forming ability. In view of providing superior properties, the use of the QUAT **3d** in mixed micelle solutions with the anionic surfactant SDS have been investigated. To study the effect of the counterions on the mixed micelle solutions, the CMCs of the mixed systems were also determined in a swamping excess of the counterions; i.e., 5 mM NaI solution. The CMC values of the different binary combinations of SDS and QUAT **3d** are presented in Table 2.

The CMC values of the SDS-QUAT C<sub>14</sub> surfactant was lower compared to the CMC of the pure SDS solution, suggesting that micellar formation is more favoured in the mixed micellar system. This is mainly due to the electrostatic attraction between the anionic SDS molecules and the cationic QUAT molecules, which result in a better packing in the mixed micelle system. The lower CMCs of the different SDS-QUAT C<sub>14</sub> mixed systems in NaI compared to those observed in deionised water indicate that the presence of NaI facilitates micellar formation of the different mixed systems.

The ideal CMC of the binary mixture can be predicted by the Clint equation [27] (Eq. 1):



**Fig. 1** Synthesis of proline surfactants. (i) C<sub>n</sub>H<sub>2n+1</sub>OH/ PTSA, Refluxing Toluene, 48 h (ii) HCl, room temperature (iii) MeI, room temperature, 24 h

**Table 2** CMC of mixtures of SDS with QUAT 3d

Mole fraction		CMC in water ( $\mu\text{M}$ )	CMC in 5 mM NaI ( $\mu\text{M}$ )
QUAT	SDS		
0	1	8300	7500
0.2	0.8	909	833
0.4	0.6	536	455
0.6	0.4	370	227
0.8	0.2	283	192
1	0	194	142

$$\frac{1}{\text{cmc}^*} = \frac{\alpha_{\text{QUAT}}}{\text{cmc}_{\text{QUAT}}} + \frac{(1 - \alpha_{\text{QUAT}})}{\text{cmc}_{\text{SDS}}} \quad (1)$$

where  $\alpha_{\text{QUAT}}$  is the mole fraction of the QUAT in the mixture,  $\text{cmc}_{\text{QUAT}}$  and  $\text{cmc}_{\text{SDS}}$  correspond to the CMCs of pure components of QUAT and SDS, respectively, and  $\text{cmc}^*$  is the value under ideal mixing.

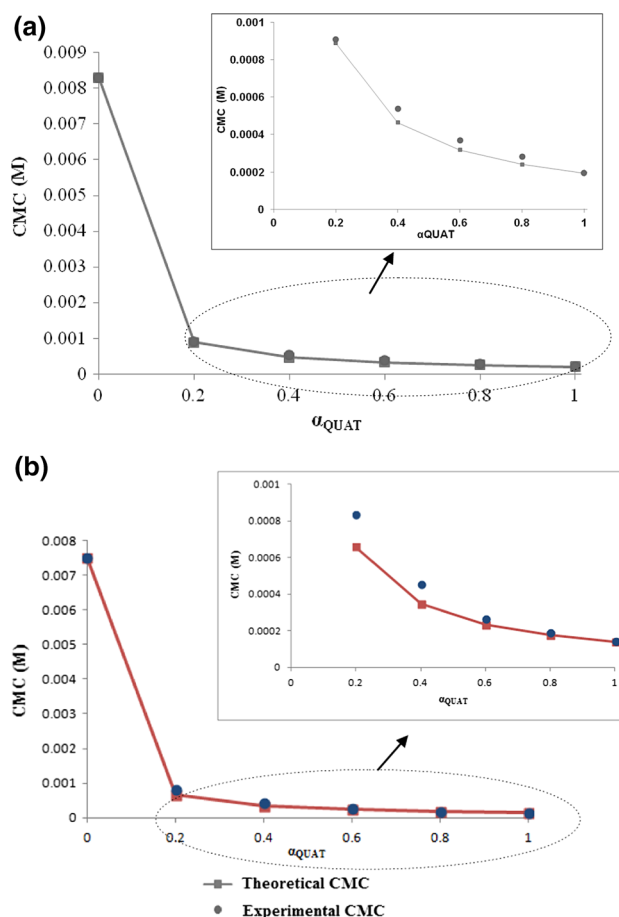
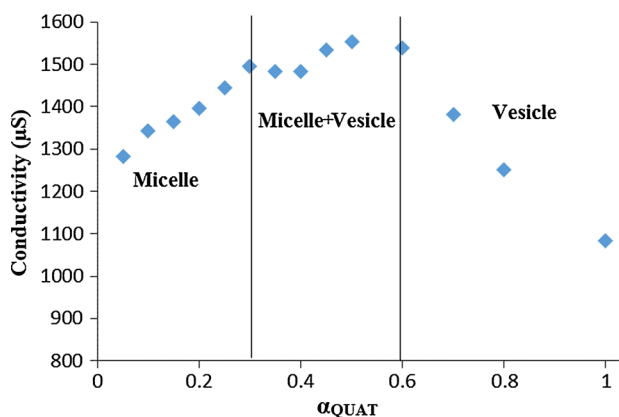
The micellar molar fraction of the QUAT in the ideal state was evaluated according to Eq. 2, assuming binary ideal mixture.

$$\chi_{\text{QUAT}}^{\text{id}} = \frac{\alpha_{\text{QUAT}} \text{cmc}_{\text{SDS}}}{\alpha_{\text{QUAT}} \text{cmc}_{\text{SDS}} + (1 - \alpha_{\text{QUAT}}) \text{cmc}_{\text{QUAT}}} \quad (2)$$

The experimental value and theoretical value predicted by the Clint equation for the CMC of each of the surfactant binary mixture is shown in Fig. 2.

In both deionised and NaI solution, the CMC values obtained for the SDS-QUAT binary system were slightly higher than those predicted by the Clint model. The larger CMC values than those predicted by the Clint model are generally due to unfavourable interactions between the two surfactants, which is highly unusual in the SDS-QUAT system due to the oppositely charged surfactants. Similar behaviour of cationic and anionic surfactant binary mixtures has also been reported by Bakshi et al. [28]. Based on this, it was assumed that the higher CMC values of the mixed SDS-QUAT compared to the ideal values obtained from the Clint equation was due to the precipitation of the neutral catanionic complex formed between equimolar anionic SDS and cationic QUAT. For the different ratios, the excess charges cause destabilisation of the precipitate, leading to vesicle formation [29]. This causes the concentration of the SDS and QUAT to decrease and, hence, fewer monomers would be available for the mixed micelle formation process. Thus, the CMC is reached later and becomes greater than the predicted CMC.

SDS exists mainly in the form of micelles at a concentration of 20 mM which is well above its CMC (8.3 mM). The phase transitions of the SDS-QUAT binary system were investigated. Addition of QUAT 3d ( $\alpha_{\text{QUAT}} = 0\text{--}0.3$ ) to an aqueous solution of SDS caused an increase in the

**Fig. 2** Variation of CMC OF SDS with QUAT 3d in (a) deionised water and b 5 mM NaI**Fig. 3** Variation of conductivity of an SDS-QUAT 3d binary system at different mole ratios of QUAT 3d

electrical conductivity values due to the increase in number of ions (Fig. 3). A further increase in mole fraction of QUAT 3d above 0.3 caused the solution to change from clear to turbid, leading to the formation of a white precipitate at  $\alpha_{\text{QUAT}} = 0.5\text{--}0.6$  (Fig. 4). At  $\alpha_{\text{QUAT}} = 0.3\text{--}0.6$ , the

conductivity values were found to vary slightly due the formation of vesicles in the solution. An increase in  $\alpha_{\text{QUAT}}$  to 0.8 caused the solution to become clear and highly viscous, together with a drastic decrease in conductivity values. This is attributed to the adsorption of the counterions in the vesicles that are being formed, hence, decreasing the number of conducting ions. Similar observations were made by Ghosh et al. with the binary system of cetyltrimethyl ammonium bromide and the anionic surfactant 1-butyl-3-methylimidazolium octyl sulfate [30]. Based on these observations, it can be inferred that the SDS-QUAT mixtures above  $\alpha_{\text{QUAT}} = 0.6$  exist predominantly as vesicles.

The extent of non-ideality of surfactant interactions is usually evaluated using the regular solution theory (RST) which includes an interaction parameter,  $\beta_{12}$ , to characterise the interactions between the two components within the mixed micelles. This parameter is related to the activity coefficients,  $f_i$ , of the surfactants within the micelle, according to:

$$f_1 = \exp \beta_{12}(1 - \chi_{\text{QUAT}})^2 \quad (3)$$

$$f_2 = \exp \beta_{12}\chi_{\text{QUAT}}^2 \quad (4)$$

where  $\chi_{\text{QUAT}}$ , the molar fraction of QUAT **3d** in the mixed micelle, can be obtained solving the following equation iteratively:

$$\frac{\chi_{\text{QUAT}}^2 \ln(\alpha_{\text{QUAT}}\text{cmc}/\chi_{\text{QUAT}}\text{cmc}_{\text{QUAT}})}{(1 - \chi_{\text{QUAT}})^2 \ln[(1 - \alpha_{\text{QUAT}})\text{cmc}/(1 - \chi_{\text{QUAT}})\text{cmc}_{\text{SDS}}]} = 1 \quad (5)$$

The interaction parameter  $\beta_{12}$  can then be evaluated from

$$\beta_{12} = \frac{\ln(\alpha_{\text{QUAT}}\text{cmc}/\chi_{\text{QUAT}}\text{cmc}_{\text{QUAT}})}{(1 - \chi_{\text{QUAT}})^2} \quad (6)$$

A negative  $\beta_{12}$  value accounts for synergism while a positive value indicates antagonism behaviour for the mixed micelle formation. In the SDS-QUAT system, positive  $\beta_{12}$  (2.5–40.9) values were obtained for the different mole ratios studied. Positive  $\beta_{12}$  values were also observed in the presence of 5 mM NaI. Similar results were observed by Bakshi et al. [28] and based on this, we assume that the

positive  $\beta$  values are not a result of antagonistic behaviour, but due to the higher value of the CMC of the mixed micelle compared to the those predicted by the Clint model which arises from the dimerisation of the oppositely charged SDS and QUAT monomers.

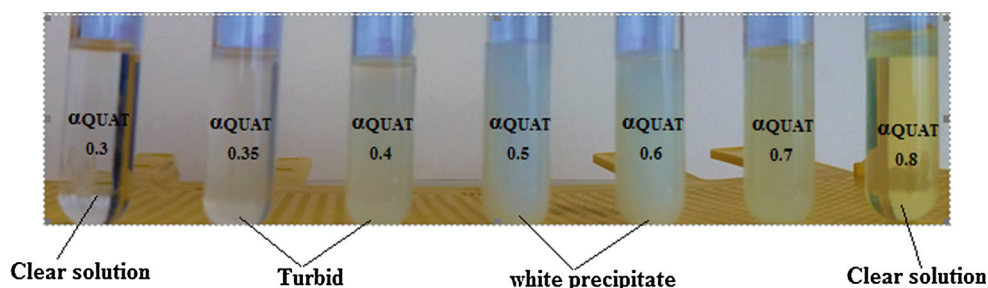
### Antibacterial Activity of Proline Surfactants in Single and Mixed Surfactant Systems

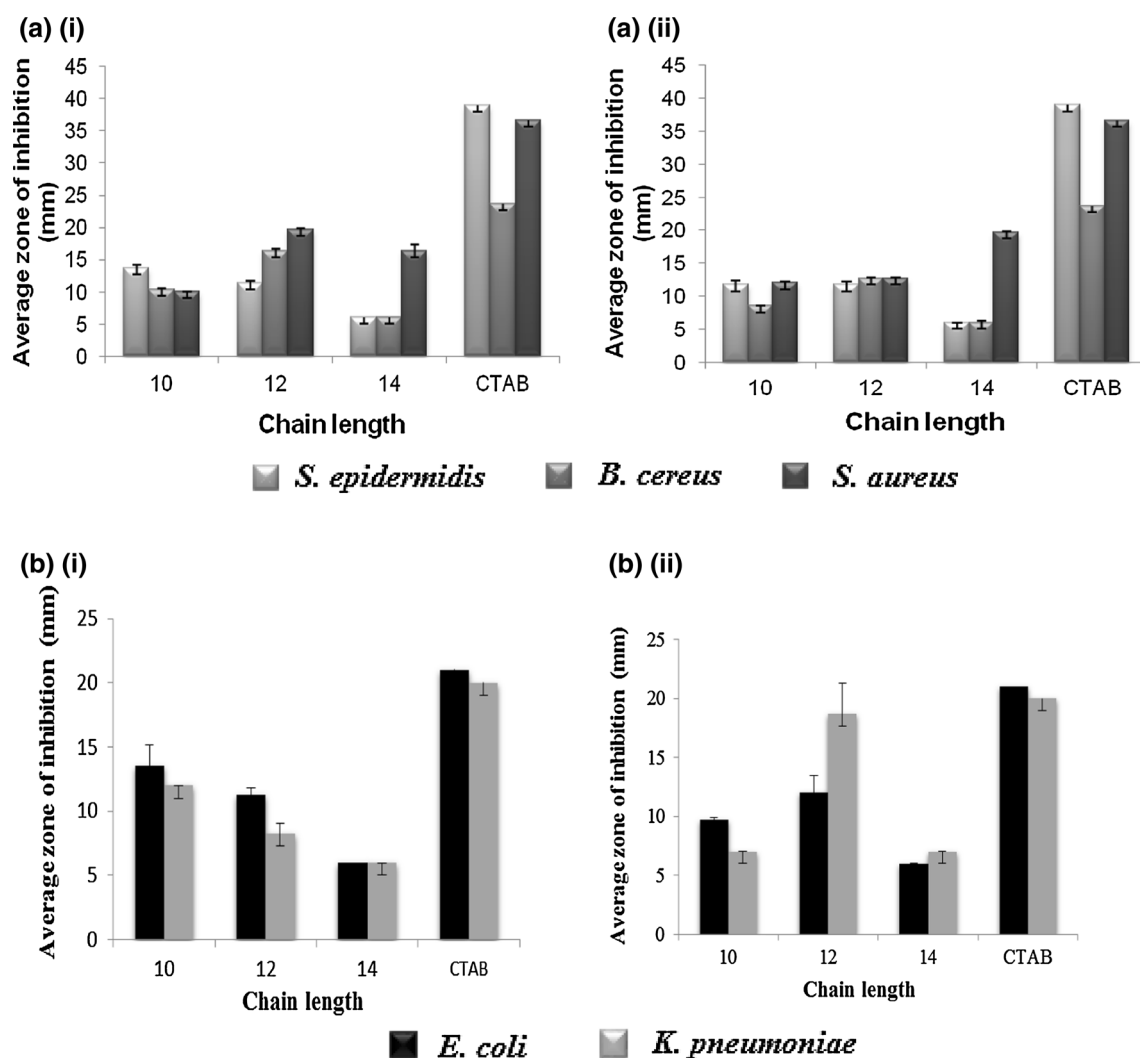
The esters of free amine L-proline (**1a–d**), their hydrochlorides (**2a–d**) as well as the QUATS (**3a–d**) were screened for their antibacterial activity. They were found to exhibit considerable activity towards gram-positive bacteria and were less active with respect to gram-negative strains (Fig. 5). In the case of the free amine ester series (**1a–d**), the highest activity was observed at  $C_{12}$  for gram-positive bacteria. For the ester hydrochloride series (**2a–d**), the highest activity was observed at  $C_{12}$  with respect to *S.aureus* and *B.cereus*, while in the case of *S.epidermidis*, the activity was found to increase up to  $C_{14}$ . For gram-negative bacteria, the activity was found to decrease from  $C_{10}$  to  $C_{14}$  for the free amine esters, while in the case of the ester hydrochlorides, the cut-off point was observed at  $C_{12}$ .

Based on the data obtained on the average zone of inhibitions with respect to the different bacteria tested, the  $C_{12}$  and  $C_{14}$  derivatives of the free amine esters and ester hydrochlorides showed promising antibacterial activity. Therefore, the MIC values of only the  $C_{12}$  and  $C_{14}$  derivatives of the free amine ester (**1c** and **1d**) and ester hydrochlorides (**2c** and **2d**) were evaluated. The MIC of the QUATS **3a–d** were also evaluated since the presence of a quaternary ammonium moiety has been known to enhance antibacterial activity [15]. The results are summarised in Table 3.

In general, the compounds showed moderate to good antibacterial activities with respect to the different bacterial strains tested. Lower MIC values were observed in the case of gram-positive bacteria, showing that the proline derivatives exhibited better activity with respect to these bacterial strains. The QUAT **3d**, with a chain length of  $C_{14}$ , was found to be the most active among the series, displaying the lowest MIC values with respect to all the bacteria tested. The QUAT **3d** even showed better antibacterial effect compared to the positive control CTAB.

**Fig. 4** Phase behaviour of the binary SDS-QUAT **3d** mixtures





**Fig. 5** Antibacterial activities with respect to (a) gram-positive bacteria (b) gram-negative bacteria (i) esters and (ii) ester hydrochlorides in terms of zone of inhibition at 10 mg/mL

**Table 3** Minimum inhibitory concentrations of selected proline surfactants

Microorganisms	MIC ( $\mu\text{M}$ )							
	1c	1d	2c	2d	3a	3c	3d	CTAB
Gram-positive								
<i>S. aureus</i>	34.5	125	90.7	112.2	203.98	16.67	2.61	13.39
<i>S. epidermidis</i>	68.9	125	61.0	336.7	203.98	5.56	2.61	3.35
<i>B. cereus</i>	68.9	125	61.0	224.8	407.96	16.67	1.31	3.35
Gram-negative								
<i>K. pneumoniae</i>	68.9	4019.3	244.5	3597	2447.8	177.96	208.99	53.5
<i>S. typhimurium</i>	4416.96	4019.3	978.0	3597	3263.7	711.85	2676.65	53.5
<i>E. coli</i>	137.81	4019.3	61.0	3597	3263.7	177.96	1338.3	53.5

The antibacterial activities of the proline esters are attributed to the surfactant structure of these compounds which allows them to interact with the bacterial membrane, leading to cell lysis and eventually cell death [31].

Quaternisation of the amino group was found to enhance the antibacterial activity of the QUATS. This might be due to their relatively larger head group that causes greater membrane disruption, leading to a larger free volume in the



bacterial membrane, hence facilitating the destruction of the microorganisms [15].

Subsequently, the antibacterial activity of the binary mixture of the surfactant, **3d**, with the conventional surfactant SDS was evaluated against 2 g of gram-positive and 1 g of gram-negative bacteria. The results are shown in Table 4.

SDS exhibits some antimicrobial properties against the bacteria tested. An increase in activity was observed upon increasing the amount of the QUAT **3d**. The binary mixture  $\alpha_{\text{QUAT}} = 0.4$  and  $0.6$  exhibited better antimicrobial activity. From Fig. 6, it can be seen that the antimicrobial activity of the binary mixture as a function of the  $\alpha_{\text{QUAT}}$  do not show a linear trend. Comparing the MIC values with the CMC obtained for the different binary mixtures of SDS-**3d**, it was found that the antibacterial activities of these surfactants are observed below their CMC, showing that the mixed monomers influence their activity.

Pure SDS showed moderate antibacterial activity which might be due to the absence of a positive charge, which plays an essential role in electrostatic interaction with the negatively charged bacterial membrane. Addition of the QUAT to the SDS enhances the antibacterial effect due to the involvement of the positively charged head group of the QUAT in the electrostatic interaction with the negatively charged bacterial membrane, followed by hydrophobic interaction with the bacterial membrane giving rise to cell lysis and cell death.

### Surfactant-DMPC Mixed Micelle Formation

The biological membrane is crucial for bacterial survival and serves as a permeability barrier for transport of molecules in and out of the cell. Phospholipid bilayers in membranes play a key role in the regulation of *in vivo* barriers. Phospholipids having long hydrocarbon chains are major components of cell membranes. In aqueous solutions, these phospholipids form closed spherical liposomes, responsible for cellular partitioning which is crucial for biological activity.

Phosphatidyl choline is the most abundant membrane phospholipid in cells and can be found in significant amounts in bacterial membranes. DMPC has been used as

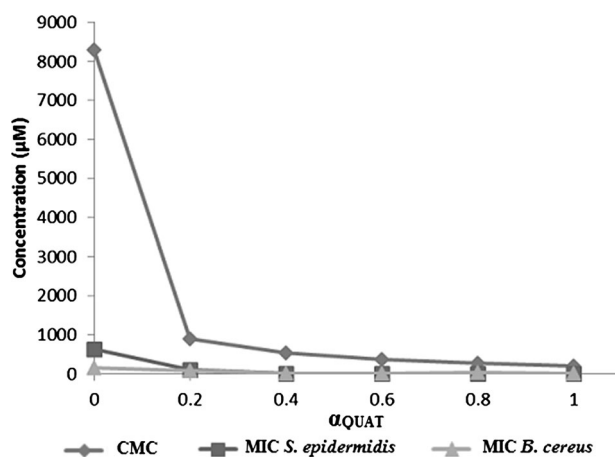


Fig. 6 Relationship between CMC and MIC of the different SDS-**3d** mixed systems

membrane models to study the antibacterial properties of diacyl glycerol arginine-based surfactants [32]. DMPC has also been used to study the mixed micelle formation with the anionic Gemini surfactants derived from cysteine, leading to the formation of lipid-surfactant systems [16].

In this study, the mixed micelle formation between the cationic proline surfactants, namely the ester hydrochloride and the QUAT derivatives with the zwitterionic phospholipid DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine) were studied, in order to investigate the possible mode of action of these compounds based on their effectiveness in solubilising bacterial membranes. The effect of the head group structures on the formation of the mixed micellar aggregates was also compared.

The mixed micelle formation process of the proline ester hydrochloride **2d** with a varying mole fraction of DMPC is shown in Fig. 7.

The CMC values obtained for the different binary mixtures of the surfactant and DMPC were lower than the CMC of the pure surfactant, a consequence of attractive interaction between the surfactant and the phospholipid. These two factors lead to a net decrease in the hydrophilicity and, therefore, leading to lower CMC values.

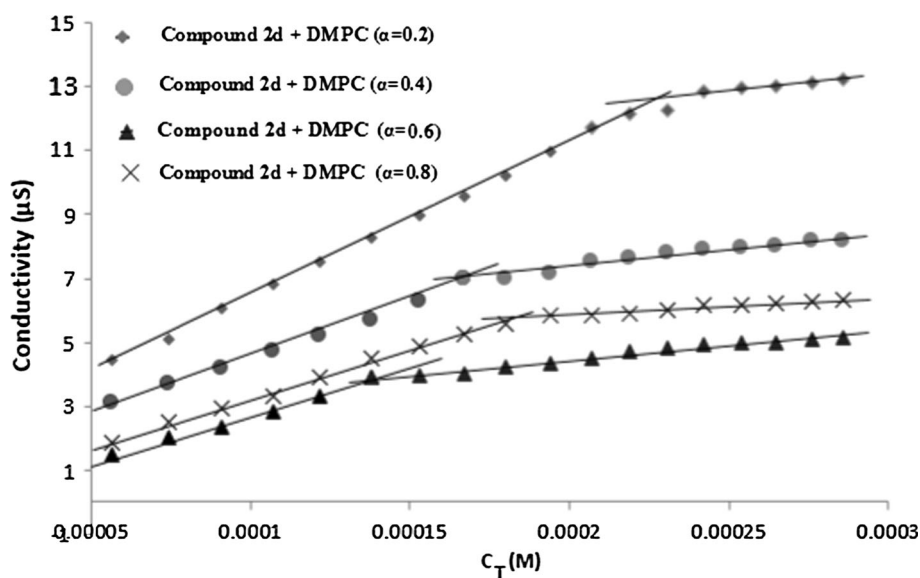
Normalized CMCs ( $\text{CMC}/\text{CMC}_0$ , where  $\text{CMC}_0$  is the CMC of pure surfactant) for the DMPC-surfactant mixtures studied, are shown in Fig. 8. An increase in the mole

Table 4 Antibacterial activity of SDS-**3d** mixed system

Microorganisms	MIC ( $\mu\text{M}$ )					
	Mole fraction SDS	1	0.8	0.6	0.4	0.2
	Mole fraction <b>3d</b>	0	0.2	0.4	0.6	0.8
<i>S. Epidermidis</i> (ATCC 12228)		625	117	2.44	4.89	14.6
<i>B. Cereus</i> (ATCC 11778)		156	78.1	4.89	4.89	39.0
<i>K. Pneumoniae</i> (ATCC 13883)		–	–	313	313	313

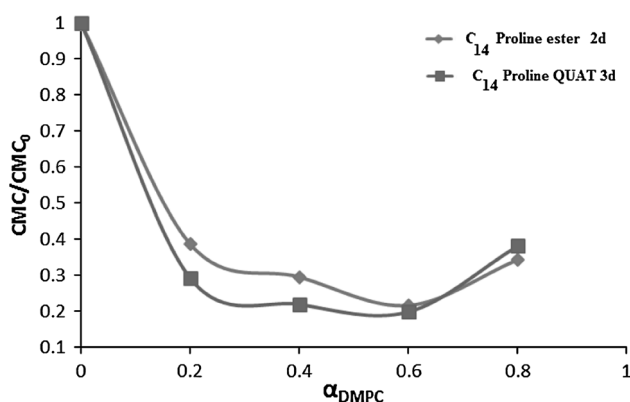
– Indicates no activity

**Fig. 7** Variation of solution conductivity with total surfactant concentration ( $C_T$ )



fraction of DMPC leads to a substantial decrease of the CMC, and further increase above  $\alpha_{\text{DMPC}}$  of 0.6. From Fig. 8, it was observed that the decrease in  $\text{CMC}/\text{CMC}_0$  was more pronounced in the case of the QUAT derivative than the ester hydrochloride, showing that the QUATS-DMPC mixed micelle formation is more favoured compared to the ester hydrochloride-DMPC mixed micelle. This might be the reason for the relatively enhanced antibacterial activity of the QUATS over the proline ester hydrochloride derivatives.

The addition of phospholipid to the micellar solution of proline surfactants leads to a decrease in CMC values. Similar observation has been observed by Faustino et al. [16]. Based on this, the decrease in CMC might be assumed to be due to the incorporation of DMPC molecules into the proline micelles, causing less ionic head group repulsions between the similar proline surfactants and, hence, causing the DMPC-proline surfactant mixed micelles to be more

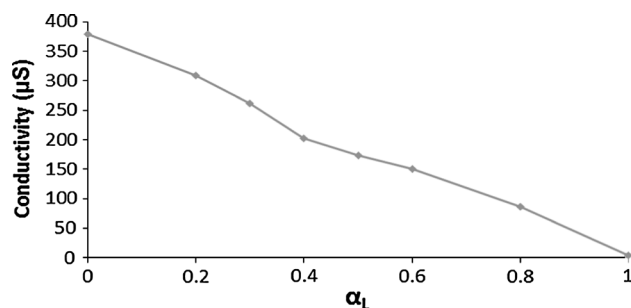


**Fig. 8** Variation of normalised CMC with DMPC molar fraction in the bulk for the  $C_{14}$  derivative of proline ester (2d) and QUAT derivative (3d)

stable. However, an increase in the  $\text{CMC}/\text{CMC}_0$  is observed at higher concentrations of DMPC. DMPC normally forms vesicles rather than micelles in aqueous solutions [16]. Therefore, a higher concentration of DMPC in the mixed-system can cause a transition from mixed micelle to the formation of vesicles and, hence, causing an increase in the CMC values. The phase behaviour of the mixture of QUAT 3d with varying mole ratio of DMPC was studied. An increase in  $\alpha_{\text{DMPC}}$  was found to increase the turbidity of the solutions together with a decrease in conductivity (Fig. 9). The decrease in conductivity confirmed the formation of vesicles, which absorbs the ions present in the mixtures, hence making the solution less conducting.

### Foamability and Foam Stability of the Proline Surfactants

Foaming is a property inherent to all surfactant solutions and this is widely used in detergents and cosmetics. Foam is an important aspect in detergency and, hence, the design of a product is often focused upon foaming abilities,



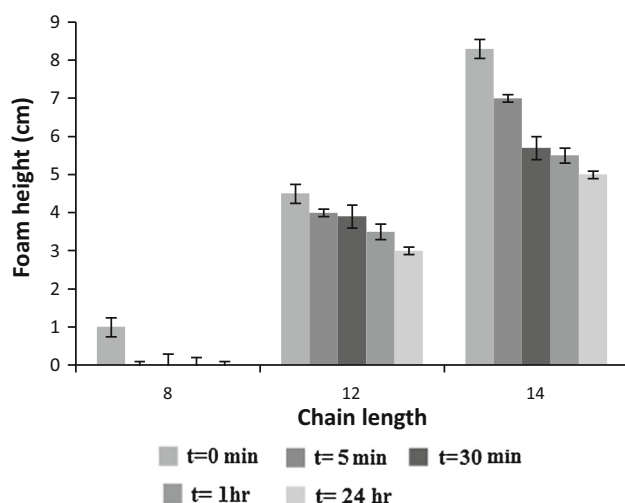
**Fig. 9** Variation of conductivity with DMPC molar fraction in a binary DMPC-QUAT 3d mixture

rendering investigation of foaming an active field of research. Due to their high level of interfacial free energy, foams are thermodynamically unstable, and, therefore, the control of foam stability is important in many applications.

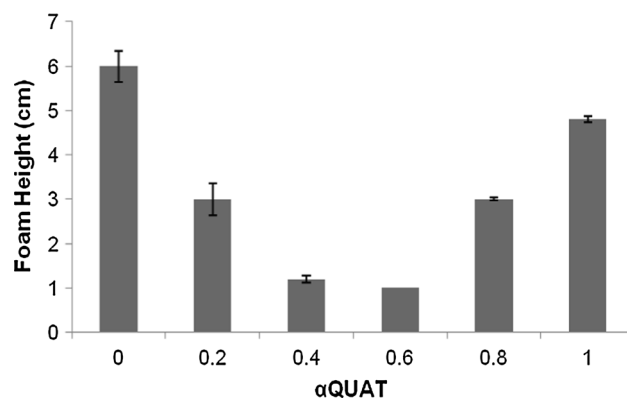
The foaming property of the proline QUAT derivatives, which exhibited good antibacterial activity, was studied in view of investigating the use of these surfactants as potential ingredients in detergent-like formulations. The foam stability of the QUATS was evaluated by monitoring the changes in foam height (h) as a function of time (t).

Figure 10 shows the foam height of aqueous solution of the QUATS at different times (0 min, 5 min, 30 min, 1 h, 24 h). The foaming ability of the QUATS was found to increase with the hydrocarbon chain length. QUATS with longer alkyl chains tend to form more stable monolayers compared to those with shorter chains, giving rise to stable foams.

Comparing the effect of the head group on the foaming abilities of the different proline derivatives (**1d**, **2d** and **3d**) of the same chain length, it was found that the foamability increases in the order of QUATS > free amine ester > ester hydrochloride. A lower foaming ability was observed for the ester hydrochloride derivative compared to the free amine ester derivative. This might be attributed to the molecular arrangement of the surfactants in water. In the case of the proline ester hydrochloride, the presence of the positively charged headgroup causes electrostatic repulsion between the molecules, which hinders the formation of foams. However, quaternising the nitrogen center caused an increase in foaming ability which might be due to an increase in hydrophobic character of the surfactant and the screening of the positively charged nitrogen by the methyl groups, hence decreasing the electrostatic repulsion between the molecules. From the biological



**Fig. 10** Effect of chain length of proline QUATS on foam height at different time intervals



**Fig. 11** Foaming ability of the different SDS-*3d* binary mixtures as a function of mole fraction of *3d*

studies, it was found that the SDS-QUAT binary mixture showed enhanced properties over the individual surfactants. In view of their potential applications as cosmetics or detergents, the foaming properties of the different binary mixtures were studied (Fig. 11).

Foamability of the mixed system was found to decrease with increasing the mole fraction of **3d** up to a mole fraction of 0.6, and then increases with further addition of the QUAT. The decrease in foaming ability may be due to the dimer formation between the anionic SDS and QUAT **3d** as observed in the mixed micelle system, causing a decrease in the concentration of the monomers (SDS and QUAT **3d**) that account for the good foamability.

### Hemolytic Activity

The *in vitro* red blood cell method (RBC) is used to evaluate the eye irritation potential of detergents and surfactants. This assay determines hemolysis ( $HC_{50}$ ), denaturation index (DI) and the ratio of both parameters ( $L/D$  ratio), which is used to characterize *in vitro* effects of test substances. Red blood cell assays provide reliable results, reduces and even avoids testing on animals [33].

The results obtained via hemolysis and denaturation tests of the different mixed micelle solutions as well as their *in vitro* classification are shown in Table 5.

**Table 5** Hemolytic activity and *in vitro* classifications of SDS-*3d* mixed system

$\alpha_{\text{QUAT}}$	$HC_{50}$ ( $\mu\text{g/mL}$ )	DI %	$L/D$ ratio	<i>In vitro</i> classification
0	43.6	100	0.44	Irritant
0.2	331.6	81.3	4.08	Moderate irritant
0.4	332.4	69.1	4.81	Moderate irritant
0.6	>790	–	$\geq 7.90$	Moderate- mild irritant
0.8	464	98.9	4.69	Moderate irritant
1	373	82.9	4.50	Moderate irritant

The results showed that pure SDS ( $\alpha_{\text{QUAT}} = 0$ ) is an irritant, and upon increasing the mole fraction of the QUAT **3d**, the mixed micelle system changes from an irritant to a moderate irritant, showing that the presence of the QUAT decreases the irritancy of SDS. The mixed surfactant mixture consisting of  $\alpha_{\text{QUAT}} = 0.4$  and  $0.6$  showed the least irritation index among the mixed surfactant system studied.

## Conclusion

The proline surfactants showed good foaming and antibacterial properties with the QUAT  $C_{14}$  derivative displaying the best activity among the series. Studies on the surfactant-DMPC mixed system revealed that the presence of the quaternary ammonium moiety enhances the antibacterial activity due to the formation of a more favourable mixed micelle with the phospholipids. The SDS-QUAT  $C_{14}$  mixed system exhibited interesting physicochemical properties whereby positive  $\beta$  values were observed in both deionised water and NaI solution. This is assumed to be due to dimerization of the SDS and QUAT, causing a decrease in the concentration of these monomers and, hence, increasing the CMC of the mixed system higher than the ideal values. The SDS- QUAT  $C_{14}$  mixed system displayed good antibacterial activities with an optimum activity observed for the  $\alpha_{\text{QUAT}} = 0.4$  and  $0.6$ . These surfactant mixtures also showed moderate irritancy compared to the pure SDS. Overall, evidence from this study shows that the SDS-QUAT  $C_{14}$  mixtures with  $\alpha_{\text{QUAT}} = 0.4$  and  $0.6$  can be employed as potential ingredients in detergent-like formulations due to their good antibacterial property and relatively lower irritancy.

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