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ARTICLE TYPE

Antibiofilm Activity of *tert*-BuOH Functionalized Ionic Liquids with Methylsulfonate counteranion

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The series of varying alkyl chain length substituted *tert*-BuOH-functionlized-imidazolium mesylate salts [alkyl-^{t}OHim][OMs] were synthesized and evaluated for antimicrobial activity and antibiofilm potential on selected pathogenic microorganisms including bacteria (Gram positive and Gram negative), yeast, and fungi. The dodecyl substituted ionic liquid [C_{12}-^{t}OHim][OMs] significantly prevented the biofilm formation of *S. epidermidis* at 100 µM concentration as well as showed noteworthy antimicrobial activity. We conclude that the ionic liquids (ILs) bearing lower than dodecyl chain lengths were found to be less effective against most of tested pathogenic microorganisms.

Introduction

Imidazolium-based ionic liquids (ILs) were widely being applied in academic and industry sector as a greener solvent or catalyst.[1] Their physical, biological and chemical properties such as liquid at room temperature, reasonable chemical stability, low flammability, insignificant vapor pressure and high ionic conductivity of ILs are the main motivating factor behind the vast interest in green chemistry applications. Tuneability nature of ILs introduces an incomparable flexibility in the design of reagents for a specific functional role.[2] Since a decade, task-specific ILs gained vast interest in developing biological applications such as biocatalysis, biomass transformation, biodegradability, drug delivery, and gene delivery vector.[3]

Numerous studies have demonstrated the antimicrobial activity of various task-specific ionic liquids against both environmental and health concern microorganisms.[4] However, appropriate designing and reasonable application of task-specific ILs bearing toxicity evaluation creates valuable information and possibilities of developing new disinfectants, antiseptics and preservatives.[5] The task-specific imidazolium ILs containing an ester functional group with varying alkyl chain length possesses adsorption efficiency due to enhance the hydrophobicity of the amphiphilic nature of cation (A, Figure 1).[6] Similarly the antimicrobial activity of ILs bearing more than C-10 chain length with alkoxymethyl moiety on other side (B, Figure 1) has been studied against clinically important pathogens.[7] However, ILs bearing halogenated counter anion can produce volatile byproduct such as HF.[8]

Pernak and co-worker designed non-halogenated ILs containing alkyl sulfonate[9] and lactate anion[10] was shown antimicrobial and biodegradable character. The active pharmaceutical ingredients (APIs) drugs, such as Lidocaine and Ranitidine drugs tuned with docusate (sulfonate dioctylsuccinate, Figure 1) anion created ILs, exhibited the potential application in longer pain relief and drug delivery.[4,11] API propantheline in combination with acesulfamate anion dramatically changed the physico-chemical properties of resulted ionic liquid.[12]

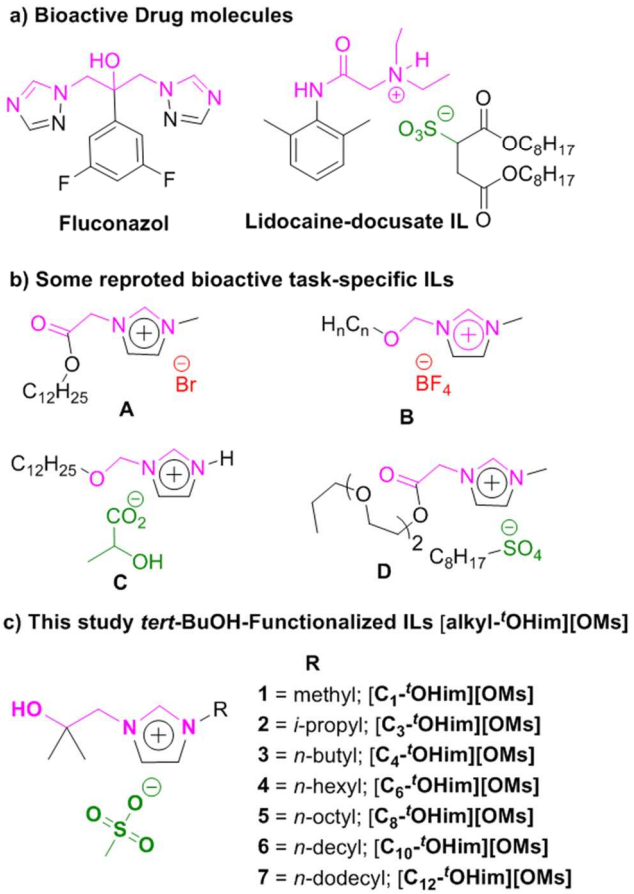

Figure 1. Bioactive active molecules and task-specific-imidazolium ILs

The microbial biofilms represent a major survival mechanism

for microbial populations and are the cause of a host of industrially and clinically relevant complications specially related to medical devices and microbial-influenced biocorrosion.[13] Pathogenic bacterial cells when adhere to each other on a surface and forming a self-produced matrix of extracellular polymeric substance, collectively known as biofilm.[14] Biofilm communities generally exhibit considerable tolerance or resistance to antibiotics and biocidal agents compared to planktonic bacteria of the same species.[14] National Institutes of Health (NIH) estimated upto 80% of all chronic human infections are biofilm mediated and that 99.9% of bacteria in aquatic ecosystems live as biofilm communities.[15]

Our continued interest lies to develop greener protocols using ILs[16] and *tert*-BuOH-functionalized ILs in various organic synthesis reactions.[17] Due to environmental pollution and health concern, the newly designed ILs has to be thoroughly evaluated the toxicity before their potential biological applications. Herein, we report first time the antimicrobial and antibiofilm activities of a series of *tert*-BuOH-functionalized ILs against a panel of clinically relevant pathogens *viz Staphylococcus epidermidis, Staphylococcusaureus, Salmonella typhimurium, Vibrio fischeri, Fusarium moniliforme, Fusarium proliferatum* and *Candida albicans*. Among these *S. epidermidis* is well known for nosocomial infections and many more skin related infections and biofilm formation,[18] while *S. aureus* and *S. typhimurium* are opportunistic pathogens and also involved in biofilm formation.[19] *Vibrio* species that are capable of causing human disease, non-cholera *Vibrio* infections include gastroenteritis, wound infection and septicemia, which is blood poisoning and food borne disease due to consumption of contaminated seafood by *V. fischeri.*[20] The yeast *C. albicans* causes skin and vaginal infections.[21] To our knowledge, this is the first evaluation of the antimicrobial and antibiofilm efficacy of *tert*-BuOH functionalized-imidazolium cation with methylsulfonate counter anion ILs.

Experimental

All chemicals were obtained from commercial suppliers and used without further purification unless otherwise stated. Flash chromatography was carried out using Merck silica gel 60 (230-400 mesh). Analytical thin layer chromatography (TLC) was performed with Merck Silica gel-60, F-254 aluminium-backed plates. Visualization on TLC was monitored by UV light. ^{1}H and ^{13}C NMR spectra were recorded using Bruker and calibrated using residual undeuterated solvent or tetramethylsilane as an internal reference.

1. Synthesis of *t*-BuOH-functionlized ILs

1.1. 1-(2-hydroxy-2-methyl-*n*-propyl)-3-methylimidazolium mesylate ([C_1-^{t}OHim][OMs], **1**)

A mixture of imidazole (2.00 g, 29.3mmol) and dimethyloxirane (2.90 mL, 32.3 mmol) was stirred in reaction vial at 55 °C for 12 h. The resulting thick liquid was dried under high vacuum at room temperature, afforded intermediate 1-(2-hydroxy-2-methyl-*n*-propyl)-3-methylimidazole dissolved in 20mL CH_3CN, then methyl methane sulfonate (0.80 mL, 8.8 mmol) was added drop-wise to the solution. The reaction mixture was stirred at 90 °C for 24h and evaporated under reduced pressure to remove. The residue was repeatedly washed with diethyl ether (5 mL × 15) and dried under high vacuum for 12h at room temperature to afford **1**.[17e] ^{1}H NMR (Chloroform-d,400 MHz) δ 1.19 (s, 6H), 2.74 (s, 3H) 3.97 (s, 3H), 4.25 (s, 2H) 7.32 (s, 1H) 7.41 (s, 1H) 9.51 (s, 1H); ^{13}C NMR (Chloroform-d,100 MHz) δ 26.4, 36.4, 39.6, 59.6, 68.7, 121.8, 123.8, 138.6.

1.2. 1-(2-hydroxy-2-methyl-*n*-propyl)-3-isopropylimidazolium mesylate ([C_3-^{t}OHim][OMs], **2**).[17e]

Liquid, ^{1}H NMR (Chloroform-d,400 MHz) δ 1.21 (s, 6H) 1.57 (d, *J* = 6.8 Hz, 6H) 2.76 (s, 3H), 4.26 (s, 2H), 4.57-4.69 (m, 1H), 7.30 (s, 1H), 7.43 (s, 1H), 9.63 (s, 1H); ^{13}C NMR (Chloroform-d,100 MHz) δ 22.7, 26.3, 39.0, 52.8, 59.2, 68.5, 118.8, 124.2, 136.3.

1.3. 1-(2-hydroxy-2-methyl-*n*-propyl)-3-*n*-butylimidazolium mesylate ([C_4-^{t}OHim][OMs], **3**).[17e]

Liquid, ^{1}H NMR (Chloroform-d,400 MHz) δ 0.94 (t, *J* = 7.2 Hz, 3H) 1.20 (s, 6H), 1.32-1.36 (m, 2H), 1.86 (q, *J* = 7.6, 2H), 2.74 (s, 3H), 4.22 (t, *J* = 7.2 Hz, 2H), 4.27 (s, 2H), 7.38 (s, 1H), 7.60 (s, 1), 9.46 (s, 1). ^{13}C NMR (Chloroform-d, 100MHz) δ 13.2, 19.2, 26.2, 31.7, 39.5, 49.4, 59.3, 68.6, 120.7, 124.2, 137.4.

1.4. 1-(2-hydroxy-2-methyl-*n*-propyl)-3-*n*-hexylimidazolium mesylate[17e] **(**[C_6-^{t}OHim][OMs], **4)**.

Liquid,^{1}H NMR (Chloroform-d, 400 MHz,) δ 0.86 (t, *J* = 6.4 Hz, 3H), 1.22 (s, 6H), 1.26- 1.39 (m, 6H), 1.88 (q, *J* = 6.8 Hz, 2H), 2.78 (s, 3H), 4.19 (t, *J* = 7.2 Hz, 2H), 4.33 (s, 2H), 7.21 (s, 1H), 7.40 (s, 1H), 9.77 (s, 1H); ^{13}C NMR (Chloroform-d,100 MHz) δ 13.8, 22.3, 25.8, 26.5, 30.0, 31.0, 39.6, 50.1, 59.6, 68.6, 120.2, 123.8, 138.3.

1.5. 1-(2-hydroxy-2-methyl-*n*-propyl)-3-*n*-octylimadazolium mesylate ([C_8-^{t}OHim][OMs], **5**).

Liquid, ^{1}H NMR (Chloroform-d, 500MHz) δ 0.79 (t, *J* = 6.9 Hz, 3H) 1.26 - 1.14 (m, 16H), 1.81 (bs, 2H), 2.66 (s, 3H), 4.14 (t, J = 7.5 Hz, 2H), 4.23 (s, 2H), 5.02(bs 1H) 7.32 (s, 1H), 7.61 (s, 1H), 9.42 (s, 1H); ^{13}C NMR (Chloroform-d, 125MHz) δ 13.8, 22.2, 25.9, 26.1, 28.9, 28.68, 29.8, 31.3, 39.4, 49.5, 59.1, 68.5, 120.5, 124.3, 137.2. Anal. Calcd for $C_{16}H_{32}N_2O_4S$: C, 55.14; H, 9.26; N, 8.04. Found: C, 55.20; H, 9.29; N, 8.16.

1.6. 1-(2-hydroxy-2-methyl-*n*-propyl)-3-*n*-decylimadazolium mesylate ([C_{10}-^{t}OHim][OMs], **6**).

Liquid, ^{1}H NMR(Chloroform-d, 500MHz) δ 0.82 (t, *J* = 6.9 Hz, 3H) 1.28 - 1.16 (m, 20H), 1.82 (bs, 2H), 2.68 (s, 3H), 4.20 - 4.13 (m, 3H), 4.23 (s, 2H), 7.29 (s, 1H), 7.56 (s, 1H), 9.41 (s, 1H); ^{13}C NMR (Chloroform-d,125MHz) δ14.0, 22.6, 26.2, 26.4, 29.0, 29.3, 29.4, 29.5, 30.0, 31.9, 39.6, 50.0, 59.6, 68.6, 120.4, 125.0, 138.1. Anal. Calcd for C_{18} $H_{36}N_2O_4S$: C, 57.41; H, 9.64; N, 7.44. Found: C, 57.43; H, 9.74; N, 7.61.

1.7. 1-(2-hydroxy-2-methyl-*n*-propyl)-3-*n*-dodecylimadazolium mesylate ([C_{12}-^{t}OHim][OMs], **7**).

Liquid, ^{1}H NMR (Chloroform-d, 500MHz) δ 0.85 (t, J = 6.8 Hz, 3H) 1.39 - 1.23 (m, 24H), 1.90 (bs, 2H), 2.78 (s, 3 H), 4.14(bs, 1H) 4.23 (t, J = 7.3 Hz, 2 H), 4.32 (s, 2 H), 7.36 - 7.28 (m, 1 H), 7.60 (s, 1 H), 9.54 (s, 1 H); ^{13}C NMR (Chloroform-d, 125MHz)δ 13.8, 22.4, 26.93, 26.17, 28.69, 29.02, 29.08, 29.09, 29.15, 29.29, 29.85, 31.59, 39.47, 49.61, 59.14, 68.53, 120.56, 124.28, 137.27.

Anal. Calcd for $C_{20}H_{40}N_2O_4S$: C, 59.37; H, 9.97; N, 6.92. Found: C, 59.40; H, 10.03; N, 6.69.

Antimicrobial activity

Bacterial Strains and growth media

For antimicrobial activity of all [alkyl-^{t}OHim][OMs] the micro-organisms used in this study were Gram positive bacteria (*Staphylococcus epidermidis* NCIM 2493 (biofilm forming), *Staphylococcus aureus* NCIM5021), Gram negative bacteria (*Salmonella typhimurium* NCIM 2501*, Vibrio fischeri* NCIM 5269), Fungi (*Fusarium moniliforme* NCIM 1100*,* and *Fusarium proliferatum* NCIM 1103) and yeasts (*Candida albicans* NCIM 3471, *Candida albicans* NCIM 3628). All microbial strains were procured from National Collection of Industrial Microorganisms (NCIM), Pune, India. All bacterial strains were grown in Muller Hinton (MH) Broth (Hi Media, India), whereas fungi were grown in Potato Dextrose Broth (Hi Media, India), and yeast were grown in MGYP (Malt extract-Glucose-Yeast extract-Peptone) medium.

MIC/MBC determination for antibacterial activity

Broth microdilution tests were carried out according to CLSI. [22]Different concentrations of [alkyl-^{t}OHim][OMs] **1-7** were prepared in MH broth (bacteria), PD broth (fungi) and MGYP (yeast) and passed through 0.22 µm filter (Millipore, Ireland). Microorganisms under were grown over 18-24 h at 37 °C in MH Broth (bacteria) and over 48h at 30 °C in PD Broth (Fungi and yeast), from which an inoculum was taken and this suspension was further diluted to give a final inoculum density of 2 x10^6 CFU mL^{-1}, as verified by total viable count. The microtitre plate for determination of MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) was performed as described here. A negative control (growth medium without microorganism) was included. All [alkyl-^{t}OHim][OMs] with different concentrations along with controls and test concentrations were prepared in three replicates. The microtitre plates were then incubated for 24 h at 37 °C for bacteria and 48 h at 30 °C for fungi and yeast in a stationary incubator. Presence and absence of growth of the micro-organisms was determined visually after incubation. The lowest concentration at which there was no visible growth (turbidity) was taken as the minimal inhibitory concentration (MIC) and the minimum bactericidal concentrations (MBC) derived by transferring 20 µL of the suspension from the wells, which displayed no signs of growth to specified agar plates (as per growth condition). Then plates were then incubated in a stationary incubator at 37 °C for 24 h (bacteria) and 30 °C for 48 h (fungi) and examined for 99.9% killing. Fluconazole used as a standard antifungal drug was purchased from Hi-media, India.

Antibiofilm Activity

The ability of bacteria to form biofilm was assayed as described.[23]In brief, The fresh colony of *S. epidermidis* was inoculated in Trypticase soya broth (Hi-Media, India) (TSB) incubated it at 37 °C for overnight, next day 1:100 dilution of culture was made in TSB supplemented with 0.5% glucose. In sterile 24-well tissue culture plates (Non-treated, Eppendorf, USA) was filled with 1.5 mL of TSB broth per well (containing *S. epidermidis* cells) and kept it for 24 h at 37 °C. After incubation, the content of each well was gently removed by pipette. The wells were washed three times with 1.5 ml of sterile PBS (Phosphate buffer saline, pH 7.6) to remove free-floating bacteria and other cell debris. After that 1 ml of different concentrations (10, 50, 100, 250 and 500 µM) of [alkyl-^{t}OHim][OMs] **1-7** in PBS was added in each well separately along with control (without ILs). Then the plate was kept for 4 h at 37 °C. After 4h incubation biofilm was mixed properly and each replicate of culture in [alkyl-^{t}OHim][OMs] **1-7** treated was serially diluted to 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} in sterile saline solution. Then 100 µL of each dilution was plated on LB (Luria Bertani agar, hi-media India) agar plates. Plates were allowed to grow for 18h at 37 ºC and CFU mL^{-1} (Colony forming unit per mL) was calculated.The percentage (%) inhibition of biofilm activity was calculated using the following equation: [CFU per mL of cells treated with ILs / CFU per mL of control cells (non-treated)] × 100. Experiments were performed in triplicate. The data are expressed as means ± SD.The morphological changes was observed under optical microscope (Nikon Eclipse LV150NL) after growing biofilm on silicon substrate and incubated with 100µM ILs for 4h.

Haemolysis Assay

In order to scrutinize any lysis of the RBC membrane by alkyl ter-alcohol TMS ionic liquids a haemolytic assay was performed. The [alkyl-^{t}OHim][OMs] **1-7** were spectro-photometrically assayed for their ability to induce hemoglobin release from blood erythrocytes method of Shin and group[24] was applied. Essentially, fresh goat blood was collected in a heparinised tube and centrifuged for 20 min at 3000 rpm (503g). After centrifugation, the erythrocytes were washed with PBS (pH 7.4). To obtain a 5% haematocrit, the packed erythrocytes were re-suspended in phosphate buffered saline (PBS) and rinsed three times with equal volumes of PBS, following centrifugation for 15 min at 3000 rpm (503 g). Equal volumes (100 µL) of the erythrocyte suspension were added to each well of a 96-well microtitre plate. Erythrocytes were subsequently exposed to [alkyl-^{t}OHim][OMs] **1-7** at various concentrations (50, 100, 250 and 500 µM) was incubated at 37 °C for 1 h and After incubation the cells were kept in an ice bath for 60 sec. followed by centrifugation at 3000 rpm (503 g) for 5 min. Aliquots of the supernatant were transferred to a fresh 96-well microtitre plate, and haemoglobin release measured spectrophotometrically at 405 nm. As a positive control (100% haemolysis), erythrocytes were treated with 0.1% Tween 80, whilst PBS (0% haemolysis) acted as a negative control. All samples (and controls) were assayed in quadruplicate. Percentage haemolysis was calculated as follows.

$$\%\ \text{Haemolysis} = \frac{(\text{Abs. Ionic liquid sample}) - (\text{Abs. Erythrocytes PBS})}{(\text{Abs. 0.1 \% Tween 80}) - (\text{Abs. Erythrocytes PBS})} \text{ X } 100$$

Result and discussion

The various alkyl chain length such as methyl, *i*-propyl, *n*-butyl, *n*-hexyl, *n*-octyl, *n*-decyl and *n*-dodecyl group into the side chain of *tert*-BuOH functionalized imidazolium-based cation with methylsulfonate anion IL (Figure 1) were synthesized according

to our previously reported procedure of **1**.[17] The precursorr *tert*-BuOH group substituted imidazole was achived by reaction ofisobutylene oxide with imidazole,then the series of various length of alkyl chain were introduced by *N*-alkylation reaction ofvariousalkyl chain length of methylsulfonateesters to afforded the series of IL:1-alkyl-3-*tert*-alcohol substituted imidazolium mesylate anion salts: 1-(2-hydroxy-2-methyl-*n*-propyl)-3-methylimidazolium mesylate ([C_1-^{t}OHim][OMs],**1**), 1-(2-hydroxy-2-methyl-*n*-propyl)-3-isopropylimidazolium mesylate ([C_3-^{t}OHim][OMs], **2**), 1-(2-hydroxy-2-methyl-*n*-propyl)-3-*n*-butylimadazolium mesylate ([C_4-^{t}OHim][OMs], **3**), 1-(2-hydroxy-2-methyl-*n*-propyl)-3-*n*-hexylimadazolium mesylate ([C_6-^{t}OHim][OMs], **4**), 1-(2-hydroxy-2-methyl-*n*-propyl)-3-*n*-octylimadazolium mesylate ([C_8-^{t}OHim][OMs], **5**), 1-(2-hydroxy-2-methyl-*n*-propyl)-3-*n*-decylimadazolium mesylate ([C_{10}-^{t}OHim][OMs], **6**), 1-(2-hydroxy-2-methyl-*n*-propyl)-3-*n*-dodecylimadazolium mesylate ([C_{12}-^{t}OHim][OMs], **7**). All of these IL-^{t}OH are in liquid state at room temperature and were characterized by ^{1}H, ^{13}C NMR spectroscopy and elemental analysis.

Table 1. MIC and MBC in μM[a] of microorganisms with respect to [alkyl-^{t}OHim][OMs].

Strains		1(C_1)	2(C_3)	3(C_4)	4(C_6)	5(C_8)	6(C_{10})	7(C_{12})	IL-C[b]	Fluconazole
S. epidermidis NCIM 2493	MIC	>2000	>2000	>2000	775	287	9.5	3.5	12	-[c]
	MBC	>2000	>2000	>2000	1550	587	26.75	15.2	96	-
S. aureus NCIM 5021	MIC	>2000	>2000	>2000	>2000	>2000	107.5	17.45	24	-
	MBC	>2000	>2000	>2000	>2000	>2000	215	50.2	192	-
S.typhimurium NCIM 2501	MIC	>2000	>2000	>2000	>2000	>2000	107.5	81.5	-	-
	MBC	>2000	>2000	>2000	>2000	>2000	240	157.5	-	-
V. fischeri NCIM 5269	MIC	>2000	>2000	>2000	>2000	>2000	107.5	81.5	-	-
	MBC	>2000	>2000	>2000	>2000	>2000	240	157.5	-	-
F.moniliforme NCIM 1100	MIC	>2000	>2000	>2000	>2000	>2000	160.4	17.45	-	417.95
	MBC	>2000	>2000	>2000	>2000	>2000	267.5	25.1	-	835.9
F. oxysporum NCIM 1103	MIC	>2000	>2000	>2000	>2000	>2000	160.4	17.45	-	417.95
	MBC	>2000	>2000	>2000	>2000	>2000	267.5	25.1	-	835.9
C. albicans NCIM 3471	MIC	>2000	>2000	>2000	>2000	>2000	267.5	17.45	11	417.95
	MBC	>2000	>2000	>2000	>2000	>2000	1335.5	25.1	88	835.9
C. albicans NCIM 3628	MIC	>2000	>2000	>2000	>2000	>2000	667.6	17.45	-	>2000
	MBC	>2000	>2000	>2000	>2000	>2000	1335.5	25.1	-	>2000

[a]The standard deviation error was 0.25 ± 6.5. [b]MIC and MBC values of lactate-IL (**C**, Figure 1) taken from ref no. 10. [c]- Not determined..

Initial screening of these synthesized ILs was examined by minimum concentration required for growth inhibition of microorganisms (MIC) and minimum bactericidal concentrations (MBC) were estimated, results are summarized in Table 1.The examined ILs exhibited significant biological activity against all of the microorganisms at lower concentration in [C_{10}-^{t}OHim][OMs] (**6**) and [C_{12}-^{t}OHim][OMs] (**7**) and at higher concentrations in ILs **1-5** *i.e.* methyl, propyl, butyl, hexyl and octyl chain length. These results indicates that the shorter chain lengths ILs were less pronounced inhibitory effects than decanol chain bearing **6** and dodecane bearing **7**. This was agreement to previously studied alkyl chain length dependence antimicrobial activity of other ILs. Compounds **6** and **7** manifested a more prominent bacteriostatic activity, *i.e.* lower MIC values than microbiocidal activity measured by MBC. Out of all the examined salts, the most pronounced microorganism growth-inhibiting effect on *S. epidermidis* was shown by ILs containing carbons lengths 6, 8, 10 or 12 in side chain. Interestingly, IL bearing longer than 10-carbon chain length were shown remarkable activity (MIC) against tested other bacteria and fungi strains. To compare the efficiency of our synthesized [alkyl-^{t}OHim][OMs], we compare the obtained MIC and MBC with

previously reported non-halogenated IL bearing lactate anion[10] (structure **C,** Figure 1) microbial activity with similar type of microbial strains, results suggested that our synthesised IL bearing *tert*-BuOH moiety and mesylate anion has superior antimicrobial actvity. In case of fungi, ILs **6** and **7** showed similar activity (MIC and MBC) on *F. moniliforme* and *F. oxysporum* as they belonging from the same genus. Further we compare the antifungal activity with Fluconazole, is well know antifungal drug which inhibit the ergosterol biosynthesis pathway by targeting 14-α-lanosterol demethylase enzyme and also disturb the fungal plasmatic membrane.[25] Imidazolim salts has similar ability to disturb the membrane regeneration by decreasing the quantity of sterol in the fungal cell.[26] Its noteworthy that, **7** shown more than 20 folds antifungal activity comparatively to Fluconazole. Finally in case of two strains of *C. albicans* has shown similar MIC to that of fungi. Surprisingly, strain NCIM 3628 was resistant to Fluconazole as well as most of tested ILs except **6** and **7**. Due to the neutral character of Fluconazole, it does not adjust to the surroundings with different hydrophilic-hydrophobic conditions it may trapped into the biofilm framework, thus it may have less effective antifungal activity compare to the [C_{12}-^{t}OHim][OMs] (**7**). This highly potential antifungal activity due to the unique physicochemical properties of [alkyl-^{t}OHim][OMs] perfectly matches the amphipathicity of the fungi. Our obtained result completely agreement with previously reported, role of imidazolium ILs in antifungal activity.[27]Moreover, antifungal data clearly suggesting that *tert*-BuOH functionalized **6** and **7** may consider as alternative to Fluconazole.

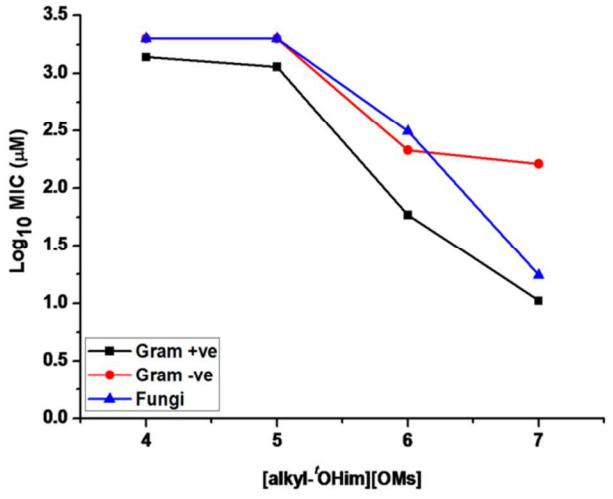

Figure 2. Comparison of Mean MIC values (Log_{10}) of [alkyl-^{t}OHim][OMs] **4-7** against Gram positive and Gram negative bacteria and fungi.

The calculated average MIC values for ILs such as [C_6-^{t}OHim][OMs] (**4**), [C_8-^{t}OHim][OMs] (**5**), [C_{10}-^{t}OHim][OMs] (**6**) and [C_{12}-^{t}OHim][OMs] (**7**) for Gram positive, Gram negative pathogens and fungi are plotted in Figure 2 as the relationship between log_{10} MIC (μM) and ILs. The Gram positive bacteria and fungi showed most sensitive to [alkyl-^{t}OHim][OMs] whilst Gram negative bacteria was less susceptible, it also relevant to previous studies.[7, 28]

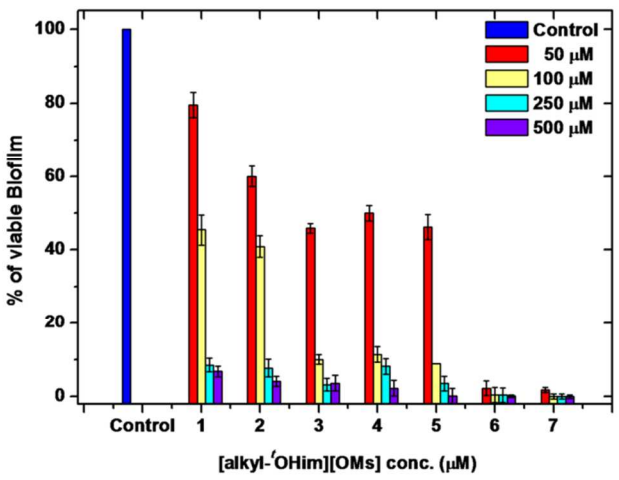

Figure 3. Percent (%) of Viable Biofilm of *S. epidermidis* after 4h treatment of all [alkyl-^{t}OHim][OMs].Value % Calculated on the basis of Colony Forming Units (CFU mL^{-1}). Error bars denotes the standard deviation error.

Antibiofilm activity

In order to measure the antibiofilm activity of series of [alkyl-^{t}OHim][OMs] against of clinically significant nosocomial pathogen and biofilm forming *S. epidermidis* strain was grown in 24 well plate as described in experimental sections. This applied method can permit reproducible and quantitative assaying of biofilm susceptibility to antimicrobial and biocidal agents include imidazolium ILs. Biofilms were grown for 24 h in TSB medium supplemented with 0.5 % glucose as described, and 24 h biofilm was treated with six different IL's with concentrations of (0, 50, 100, 250 and 500 μM) for 4h. After 4h of treatment, viable cells of biofilm were evaluated by determining average viable cell counts (CFU mL^{-1}) for each concentration along with control (without ILs). The percentage (%) of viable biofilm has shown in Figure 3, as alkyl chain length increases the activity of ILs against biofilm also increases. Above the concentration of 100 μM showed more than 55±4.5 % and 85±5.5 % preventing biofilm formation in ILs **1-2** and **3-5** respectively. In other hand, **6** and **7** have showed excellent death effects on biofilm, at lowest concentration (50 μM), killed biofilm more than 97±2.7 %, which showed promising activity among the all tested ILs. We believe that the significantly influenced antimicrobial and biofilm activity of IL-C12 could be due to amphipathic nature of IL, in which longer alkyl chains possess high lipophilicity properties and the cationic *tert*-butanol contained imidazolium moiety may increase membrane permeability properties of the molecule. Once membrane become permeable, the ionic liquid will enter into the cells and thereby lead to killing phenotype.

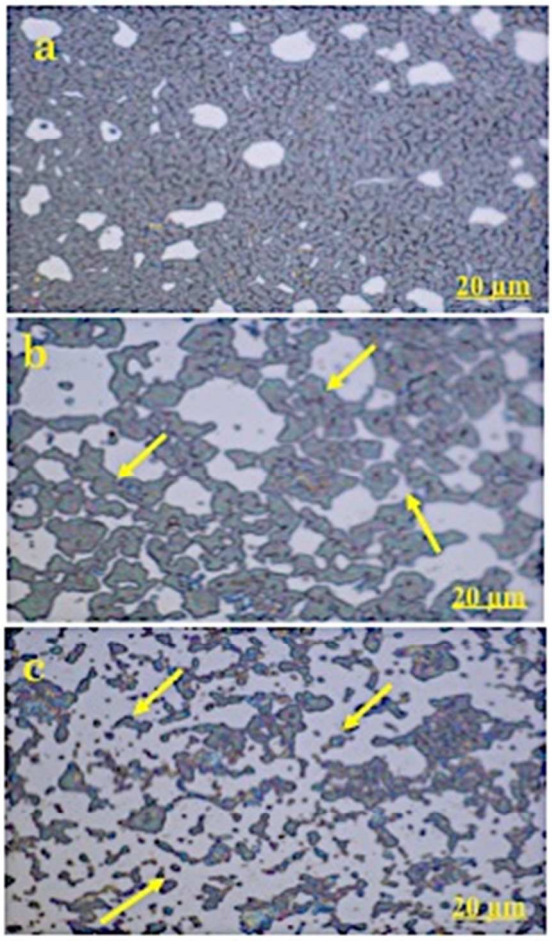

Figure 4. Optical microscopic images distorted morphology (indicated by yellow arrows) of *S. epidermidis* biofilmafter exposure of 100 µMconcentration ILs; a) Control (without ILs), b) [C_{10}-^{t}OHim][OMs] (**6**), c) [C_{12}-^{t}OHim][OMs] (**7**).

Figure 4 shown the optical microscope images of morphological changes in the biofilm formation after treatment with ILs **6**-**7** (100 µM). As see in biofilm grown control image **a** of Figure 4 was more significantly disrupted by **7** than **6** (compare image **c** to **b** of Figure 4). As the alkyl chains increases the bacterial biofilm cell membrane was highly disrupted as indicated by yellow arrow in figure 4. Biofilm disrupted was not limited to **6**-**7** but also seen in lower alkyl chain length ILs **1**-**5** (see SI,Figure 2).

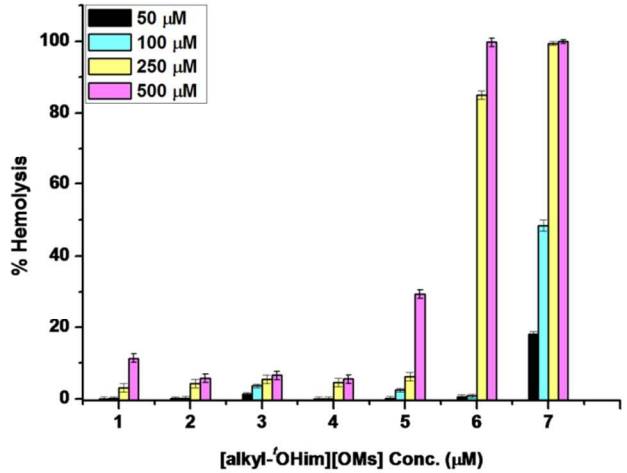

Figure 5. Haemolytic activity(%) of [alkyl-^{t}OHim][OMs] ILs against fresh erythrocytes. Each value is expressed as the mean of six replicates.

Haemolytic activity

Finally, the haemolytic activity of [alkyl-^{t}OHim][OMs] (**1**-**7**) was evaluated against fresh goat erythrocytes and results depicted in Figure 5. Haemolytic assay indicated that the tested ILs did not show significant haemolytic activity up to using 100 µM concentration, except [C_{12}-^{t}OHim][OMs] (7) caused 48.4 ± 2.5% haemolysis. This was expected considering the high antimicrobial activity of **7**, which is most likely due to the ability to disrupt cellular membranes. Interestingly, the increased concentration of ILs **1**-**5** at 500 µM didn't exhibit the haemolytic activity. Overall, the concentrations of all ILs at MIC would not be expected to produce haemolysis, since these concentrations are inhibitory to microbial growth rather than producing a killing phenotype (of erythrocytes) which was observed at and above the MIC values. Our observations are in accordance with Busetti *et al.*, wherein quinolinium bromide ILs also exhibited similar relationship between of haemolyis and MIC.[29] In case of both IL **6** and **7**, observed haemolysis at above 250 µM concentration, which is not surprising given the average MICs for the range of tested microorganisms was below 250µM concentration (Figure 2). This haemolysis data clearly suggested that [alkyl-^{t}OHim][OMs] are highly membrane active and showed cidal activity *via* disruption of the cell membrane.

Conclusions

In conclusion, this study provided a detailed account of *tert*-BuOH-functionlized-imidazolium mesylate ionic liquids evaluation on pathogenic microbial system. The ILs displayed excellent, broad spectrum antimicrobial activity against microorganisms belonging to diverse groups included Gram positive, Gram negative bacteria, yeast, and fungi. More specifically, dodecyl substituted IL demonstrated improved antibiofilm and antimicrobial activity than the other C_{12} less alkyl chain length of [alkyl-^{t}OHim][OMs]. These [alkyl-^{t}OHim][OMs] are non-halogenated make them environmentally-friendly and greener material character, also methylsulfonate anion stated by its non-toxic and pharmaceutically acceptable moiety. Taken into consideration the structural and biological parameters of ionic liquids evaluated in this study is expected to the various sectors

like pharmaceutical, drug delivery, or nano-biotechnology and possible role in environmental science and in clinical applications.

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Notes and references

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†Electronic Supplementary Information (ESI) available: [^{1}H and ^{13}C NMR of new IL, optical images of biofilm and ILs]. See

DOI: 10.1039/b000000x/

‡ Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

1 For recent reviews on ILs, see: (a) J. P. Hallett, T. Welton, *Chem Rev.* 2011, **111**, 3508-3576. (b) J. Dupont, P. A. Z. Suarez, *Phys. Chem. Chem. Phys.* 2006, **8**, 2442–2452. (c) R. Sheldon, *Chem. Commun*. 2001, 2399-2407. (d) P. Wasserscheid, W. Kein, *Angew. Chem., Int. Ed.* 2000, **39**, 3772-3789. (e) T. Welton, *Chem. Rev.* 1999, **99**, 2071-2083.

2 For recent reviews on task-specific ILs, see: (a) P. Chandra, S. S. Shinde, A.V. Biradar, *Curr. Org. Chem.*, 2015, **19**, 728-742. (b) R. Giernoth, *Angew. Chem., Int. Ed.* 2010, **49**, 2834-2839. (b) G. Imperato, B. Ko¨nig, C. Chiappe, *Eur. J. Org. Chem.* 2007, 1049-1058. (c) S.-G. Lee, *Chem. Commun.* 2006, 1049-1063. (d) J. H. Devis, *Chem. Lett.* 2004, **33**, 1072-1077.

3 (a) F. V. Rantwijk, R.A. Sheldon, *Chem. Rev.*, 2007, **107**, 2757-2785. (b) W. Li, Z. Zhang, B. Han, S. Hu, J. Song, Y. Xie, X. Zhou, *Green Chem.*, 2008,**10**,1142-1145. (c) Y. Zhang, X. Chen, J. Lan, J. You, L. Chen, *Chem. Bio. Drug Des*., 2009, **74**, 282-288. (d) D. Davies, *Nat. Rev.Drug Discov.*, 2003, **2**, 114-122.

4 W. L. Hough, M. Smiglak, H. Rodriguez, R. P. Swatloski,S. K. Spear, D. T. Daly, J. Pernak, J. E. Grisel,R. D. Carliss, M. D. Soutullo, J. H. Davis, R. D. Rogers, *New J. Chem.*, 2007, **31**, 1429-1436.

5 (a) M. Zakrewsky, K. S. Lovejoy, T. L. Kern, T. E. Miller, V. Le, A. Nagy, A. M. Goumas, R. S. Iyer, R. E. Del-Sesto, A. T. Koppisch, D. T. Fox, S. Mitragotria, *Proc Natl.Acad.Sci. USA*, 2014, **37**, 13313-13318. (b) B. L. Hahn, P.G. Sohnle, *Microb. Pathog.*, 2013, **63**, 24-29. (c) M. R. Prausnitz, R. Langer, *Nat. Biotechnol*, 2008, **26**, 1261-1268. (d) B. S. Baker, *Clin. Exp. Immunol*, 2006, **144**, 1-9.

6 M. T. Garcia, I. Ribosa, L. Perez, A. Manresa, F. Comelles, *Langmuir*, 2013, **29**, 2536−2545.

7 J. Pernak, J. Sobaszkiewicz, I. Mirska, *Green Chem.*2003, **5**, 52-56.

8 R. P. Swatlowski, J. D. Holbrey, R. D. Roger, *Green Chem.* 2003, **5**, 361-363.

9 R. G. Gore, L. Myles, M. Spulak, I. Beadham, M. Teresa Garcia, S. J. Connon, N. Gathergood, *Green Chem.*, 2013, **15**, 2747-2760.

10 J. Pernak, I. Goc, I. Mirska, *Green Chem.*, 2004, **6**, 323-329.

11 D. Dobler, T. Schmidts, I Klingbenhonfer, F. Runkel, *Int. J. Pharm.*, 2013, **441**, 620-627.

12 P. M. Dean, J. Turanjanin, M. Yoshizawa-Fujita, D. R. Mac Farlane, J.L. Scott, *Cryst. Growth Des.*, 2009, **9**, 1137-1145.

13 (a) M. Donlan and J. W. Costerton, *Clin. Microbiol. Rev*., 2002, **15**, 167-193. (b) L. Hall-Stoodley, J. W. Costerton, P. Stoodley, *Nat.Rev. Microbiol*., 2004, **2**, 95-108. (c) M. M. Tunney, S. P. Gorman, S. Patrick, *Rev. Med. Microbiol*., 1996, **74**, 195-205. (d) I. B. Beech, J. Sunner, *Curr. Opin. Biotechnol*., 2004, **15**, 181–186. (e) H. Ceri, M. E. Olson, C. Stremick, R. R. Read, D. Morck, A. Buret, *J.Clin.Microbiol*., 1999, **37**, 1771–1776.

14 (a) P. T. Gilbert, T. Maira-Litran, A. J. McBain, A. H. Rickard, F. Whyte, *Adv. Microbial. Phys.*, 2002, **46**, 203–256. (b) P. S. Stewart, J. W. Costerton, *Lancet*., 2001, **358**, 135–138. (c) P. S. Stewart, *Int. J. Med. Microbiol*., 2002, **292**, 107–113.

15 (a) K. Lewis, *Antimicrob. Agents Chemother.*, 2001, **45**, 999-1007. (b). J. W. Costerton, M. Wilson, *Biofilms*, 2004, **1**, 1-4.

16 (a) P. S. Shinde, S. S. Shinde, S. A. Dake, V. S. Sonekar, S. U. Deshmukh, V. V. Thorat, N. M. Andurkar, R. P. Pawa*r, Arab. J. Chem.,* 2014, **7**, 1013-1016. (b) P. S. Shinde, S. S. Shinde, A. S. Renge, A. B. Rode, R. P. Pawar, *Lett. Org.Chem.*, 2009, **6**, 8-10.

17 (a) S. S. Shinde, S. N. Patil, A. Ghatge, P. Kumar, *New J. Chem.*, 2015, **39**, 4368-4374. (b) S. S. Shinde, S. N. Patil, *Org. Biomol. Chem.*, 2014, **12**, 9264-9271. (c) S. S. Shinde, H. M. Chi, B. S. Lee, D. Y. Chi, *Tetrahedron Lett.,* 2009, **50**, 6654-6657. (d) S. S. Shinde, B. S. Lee, D. Y. Chi, *Tetrahedron Lett.* 2008, **49**, 4245-4248. (e) S. S. Shinde, B. S. Lee, D. Y. Chi, *Org. Lett.*, 2008, **10**, 733-735.

18 M. Otto, *Nat. Rev. Microbiol*. 2009, **7**, 555-567.

19 A. Krarup, U.B. S. Sorensen, M. Matsushita, J. C. Jensenius, S. Thiel, *Infect. Immun*. 2005, **73**, 1052-1060.

20 M. F. R. Mizan, I. K. Jahid, S. Ha, *Food Microbiol*. 2015, **49**, 41-55.

21 R. A Calderone, W. A Fonzi, *Trends Microbiol.*, 2001, **9**, 335-327.

22 Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; 18thinformational supplement. CLSI document M100-18. Wayne, PA: Clinical and Laboratory StandardsInstitute, 2008.

23 (a) S.M. Kwasny, T. J Opperman, *Curr. Protoc. Pharmacol.* Chapter 13: Unit 13A, 2010. (b) G. A. O'toole, R. Kolter, *Mol. Microbiol*., 1998, **28**, 449-461.

24 S. Y. Shin, S. H. Lee, S. T. Yang, E. J. Park, D. G. Lee, M. K. Lee, S. H. Eom, W. K. Song, Y. Kim, K. S. Hahm, J. I. Kim, *J. Pept. Res.*, 2001, **58**, 504-514.

25 M. Nucci, A. L. Colombo, Diagn Microbial Infect Dis, 2007, 58, 77-82.

26 E. I. Nweze, A. Ghannounm, J. Chandra, M. A. Channounm, P. K. Mukherjee, *J. Antimicrob. Chemother.*, 2012, **67**, 149-153.

27 (a) V.Z. Bergamo, R.K. Donato, D.F. Dalla Lana, K.J.Z. Donato, G.G. Ortega, H.S. Schrekker, A.M. Fuentefria, *Letters Appl. Microbiol.*, 2014, **60**, 66-71.(b) H. S. Schrekker, R. K. Donato, A.M. Fuentefria, V. Bergamo, L.F. Oliveira, M.M. Machado, *Medchemcomm.*, 2013, **4**, 1457-1460.

28 L. Carson , P. K. W. Chau , M. J. Earle, M. A. Gilea, B. F. Gilmore, S. P. Gorman , M. T. McCann, K. R. Seddon, *Green Chem.*, 2009, **11**, 492-497.

29 A. Busetti,D. E. Crawford,M. J. Earle,M. A. Gilea,B. F. Gilmore, S. P. Gorman,G. Laverty,A. F. Lowry,M. McLaughlin, K. R. Seddon, *Green Chem.,* 2010, **12**, 420–425.

TOC

Antibioflim Activity of *tert*-BuOH Functionalized Ionic Liquids with Methylsulfonate counteranion

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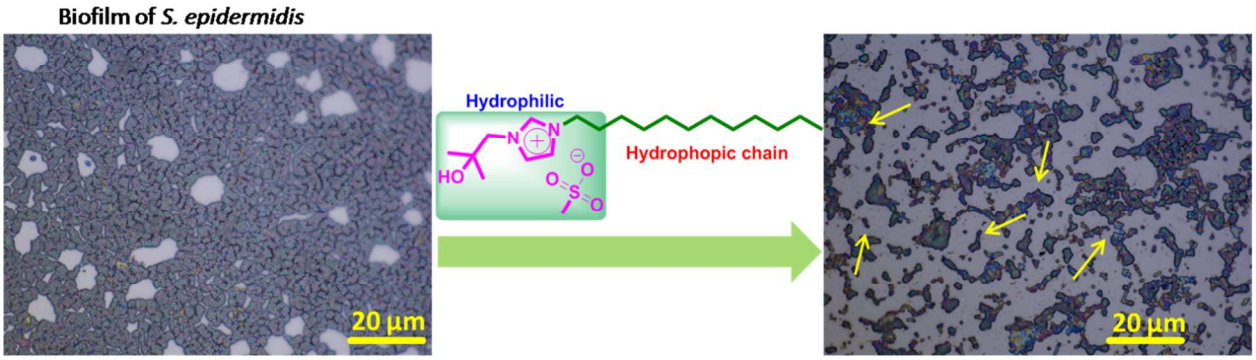

The *tert*-BuOH functionalized and dodecyl alkyl chain bearing imidazolium ionic liquid [C_{12}-^{t}OHim][OMs] significantly prevented the bioflim formation of *S. epidermidis* and shown potential antimicrobial activity.