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Environmentally benign tetramethylguanidinium cation based ionic liquids

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Abstract

Ionic liquids (ILs) are being considered as greener alternatives to conventional organic solvents. Recent applications of ILs in the dissolution and stability of biomolecules have led to an increasing demand for biocompatible ILs. In the present work, we report the synthesis and toxicity study of tetramethylguanidinium [TMG] cation based ILs with benzoate, salicylate, lactate, dihydrogen phosphate, nitrate, formate, acetate, propanoate, butanoate and valerate anions. Results obtained from toxicological evaluation with normal HEK293 and cancerous DLD-1 cell cultures show that all studied ILs are non-toxic. Subsequent biodegradability tests with bacterial cultures *Pseudomonas putida, Bacillus subtilis* and closed bottle test show that these ILs are also biodegradable. Our study suggests that the side-chain of IL anions and anion-cation combination can influence the biocompatibility of the ILs immensely.

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INTRODUCTION

Ionic liquids (ILs) have attracted growing interest in various application owing to their unique characteristics, such as low vapour pressure, low toxicity, non-flammability *etc*.[1] Many of the ILs are being used regularly for various organic synthesis, catalysis, biocatalysis and biomass pre-treatment due to their excellent thermal and chemical stability. They can also dissolve and stabilize a wide range of materials like nucleobases, DNA and proteins.[2,3] Most importantly, by different combinations of cations and anions, a large number of solvents with very distinct properties can be obtained.[4,5] Along with these favourable properties, if it is possible to identify and formulate ILs that are also biocompatible, it would open up great possibilities for various other applications. Hence, the environmental impacts of ILs have been gaining attention in academia and industry, particularly with regard to their toxicity and biodegradability.[6,7,8]

Recent studies have suggested that a large number of reported ionic liquids are no less toxic to the aquatic environment than conventional solvents.[9-13] Hence, toxicity evaluations of ILs are gaining tremendous interest. Pernak *et al.* have done a pioneering study on the ecotoxicity of ILs and found that pyridinium based ILs exhibited strong antibacterial activity.[14] It has also been demonstrated that different combinations of cation and anion and chain length of ions can influence aquatic toxicity.[15,16] Some ILs, based on cations such as morpholinium-[Morp], pyrrolidinium-[Pyr], the non-cyclic quaternary ammonium and choline, have been reported to have lower toxicity.[15,17,18] Cho *et al.* have found that toxicity depends on the type of anion and it increases in the following order: hexafluoroantimonate ($[SbF_6]$) < $[PF_6]$ < $[BF_4]$ < trifluoromethanesulfonate ($[CF_3SO_3]$) < octylsulfate ($[C_8H_{17}OSO_3]$) < [Br] ~ [Cl].[16,19] They also found that microorganisms were more sensitive to ionic liquids when the cation was combined with $[NTf_2]$.[16] Wiedmer *and* co-workers investigated the toxicological effect of seven novel cholinium, guanidinium, and tetramethylguanidinium carboxylate ionic liquids on *Vibrio fischeri marine* bacteria. They found that toxicity of the ILs was mainly determined by the surface-active anions, while cations played a secondary role. They also reported that increasing anion alkyl chain length increases the toxicity, while branching of the chain decreases the toxicity of the ILs.[9] Other reports on imidazolium cation based ILs have also shown that toxicity of ILs increases with alkyl chain length.[10,11,15,20]

In addition, mammalian cells have also been employed in toxicity studies.[11,18,19,21-25] Toxicity of ethyl-, butyl-, octyl-, benzyl-, and allyl-substituted imidazolium, pyridinium, and

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ammonium cations based ILs towards HeLa cells has been studied by Wang *et al.*[26] In agreement with previous reports, they have also found that the length of the alkyl-substituents on the cations had a large effect on the toxicity**.** Kumar *et al.* determined, for the first time, the anti-tumor activity of phosphonium and ammonium-based ionic liquids using MDA-MB human tumor cell lines.[27,28] They found that significant improvement in anti-tumor activity can be achieved with increase in alkyl chain length. Further, Jodynis-Liebert *et al.* investigated the cytotoxicity of didecyldimethylammonium saccharinate [DDA][Sac] on different skin, lung and gastro-intestinal tract derived cell lines.[29] In their study, [DDA][Sac] was found to be more cytotoxic to the skin and lung cell lines compare to other cell lines. Fang *et al.* carried out cytotoxicity study of piperazinium- and guanidinium-based ionic liquids on HEK-293 and C6 cells. They found that ILs with tetrafluoroborate anion and with benzene ring on cation are the ones with relatively high toxicity among the studied ILs.[30]

Further, literature show that ILs might potentially accumulate in the environment after disposal due to their chemical and thermal stability. Hence, there have been recent reports of biodegradation studies on ILs undertaken according to the Organisation for Economic Cooperation and Development (OECD) standards. Reports show that biodegradability seems to increase with the increase of the alkyl chain length and also indicate that biodegradability possibly depends on the type of anion, as well.[31,32] Hou *et. al.* found from their study that choline cation based ILs with amino acid derived anions, incorporating carboxylic acid and amide groups showed excellent degradability. The ILs containing basic amino acids like Lysine, Histidine and Arginine were less susceptible to microbial breakdown, and degradation levels of 65–68% were achieved.[33] Gathergood *et al.* shows that biodegradability seems to increase with the increase in the alkyl chain length. They found that the sulfate anion with octyl chain is considerably more biodegradable than the other commonly used anions.[31]

However, above discussed investigations were limited mostly to the imidazolium and cholinium based ILs, and there are very few reports on other IL classes. Among these lesser studied ILs, tetramethylguanidinium cation $[TMG]^+$ based ILs have received attention in various applications, such as in SO_2 absorption, in the formation of microemulsions and nano particle synthesis, reaction catalysis etc.[34-39] However, no biocompatibility data is available for this novel class of ILs, despite of their huge prospects in biological applications for the very presence of the guanidinium group. Hence, it is of

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great practical importance to assess the biocompatibility of the existing and newly produced [TMG]-based ILs for task-specific applications. Here, we report the synthesis and toxicity study of [TMG] cation based ILs with benzoate [BEN], salicylate [SAL], lactate [LAC], dihydrogen phosphate [DHP], nitrate [NO_3], formate [HCOO], acetate [ACE], propanoate [PRO], butanoate [BUT] and valerate [VAL] anions. The aim of this work is to evaluate the toxicity of these ILs to sensitive and resistant cell lines and to assess their biodegradability through two different methods – closed bottle tests and by incubating these ILs in bacterial cultures, which after four weeks of incubation was monitored by NMR spectroscopy. Based on these methods, we have been able to evaluate the effect of the anions on the toxicity and biodegradability of the [TMG] cation based ILs.

Experimental Method

Chemicals and Materials

1, 1, 3, 3-tetramethylguanidine (＞99%, CAS No. 80-70-6, *Sigma Aldrich* Co., Ltd.) was used for synthesis without further purification. Benzoic acid (≥99.9%, CAS No. 65-85-0, Rankem Laboratory Chemicals, Ltd), Salicylic acid (≥99%, CAS No. 69-72-7, Sisco Research Laboratories Pvt Ltd), L-(+)-lactic acid (≥ 98%, CAS No. 79-33-4, Sigma), Orthophosphoric Acid (85%,CAS No. 7664-38-2, Merck KGaA, Ltd), Nitric acid (70%, CAS No. 7697-37-2, Merck KGaA, Ltd), Formic acid (≥ 98%, CAS No. 64-18-6, Sigma Aldrich), Acetic acid (≥ 99%, CAS No. 80-70-6, Sigma Aldrich), Propanoic acid (≥ 99.5%, CAS No. 79-09-4, Sigma Aldrich), Butanoic acid (≥ 99%, CAS No.107-92-6, Sigma Aldrich) Pentanoic acid (≥ 99%,CAS No. 109-52-4, Sigma Aldrich) and Ethanol (≥99.9%, CAS No. 64-17-5, Merck KGaA, Ltd) were all of analytical grade**.** RPMI Medium 1640, Fetal bovine serum, L-Glutamine 200mM (100x), and trypsin-EDTA solution were used from GIBCO and the cell proliferation reagent MTT were purchased from Sigma.

Synthesis and characterization of the ILs

Tetramethylguanidinium benzoate [TMG][BEN], tetramethylguanidinium salicylate [TMG][SAL], tetramethylguanidinium lactate [TMG][LAC], tetramethylguanidinium dihydrogen phosphate [TMG][DHP], tetramethylguanidinium nitrate [TMG][NO_3], tetramethylguanidinium formate [TMG][HCOO], tetramethylguanidinium acetate [TMG][ACE], tetramethylguanidinium propanoate [TMG][PRO], tetramethylguanidinium butanoate [TMG] [BUT] and tetramethylguanidinium valerate [TMG][VAL] were

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prepared by the neutralization of 1,1,3,3-tetramethylguanidine with benzoic acid, salicylic acid, lactic acid, phosphoric acid, nitric acid, formic acid, acetic acid, propanoic acid, butanoic acid and valeric acid, respectively. In our synthesis method, 100 ml ethanol and 2.30 g TMG (20.0 mmol) were loaded into a 250 ml flask in a water bath of 25^0C. Then 20.0 mmol acid (carboxylic acid) in 35 ml ethanol was charged into the flask under stirring. The reaction took 2 hours. The product mixture was evaporated under reduced pressure. The crude oily residue was dissolved in 100 ml ethanol, treated with active carbon, filtered, and subsequently evaporated under vacuum. The ^{1}H NMR, ^{13}C NMR spectra were recorded on a Bruker AM 500 spectrometer and shown in Figs. S1-S10 - ^{1}H NMR (500MHz, D_2O) 2.94(s, 12 H), 7.48(m, 2 H) 7.57(m, 1 H), 7.88(m, 2 H) for [TMG][BEN]; 2.87(s, 12 H), 6.93(m, 2 H) 7.45(m, 1 H), 7.81(m, 1 H) for [TMG]SAL]; 4.13(m, 1H), 2.99(s, 12 H), 1.36(d, 3H) for [TMG][Lac]; 3.00(s, 12 H) for [TMG][DHP]; 2.95 (s, 12 H) for [TMG][NO_3]; 2.93(s, 12 H), 8.40(s, 1 H) for [TMG][HCOO]; 2.99 (s, 12 H), 1.95(s, 3 H) for [TMG][ACE]; 2.93(s, 12 H), 2.21(q, 2 H), 1.03(t, 3 H) for [TMG][PRO]; 2.87(s, 12 H), 2.06(t, 2 H), 1.45(m, 2 H), 0.79(t, 3 H) for [TMG][BUT]; and 2.95(s, 12 H), 2.23(t, 2 H), 1.53(m, 2 H), 1.31(m, 2 H), 0.88(t, 3 H) for [TMG][VAL]. HRMS spectra were recorded for all IL cations. The results are as follows: positive ion: m/z: 116 (M+ H+) for all ILs. The obtained ILs also were characterized by differential scanning calorimetry (DSC, Perkin-Elmer DSC-7) and thermogravimetric analysis (TGA, Perkin-Elmer TGA-7). Thermal Properties of these ILs are listed in Table S1."

Cell lines

The normal human embryonic kidney cell line, HEK-293 and human colorectal adenocarcinoma cell line, DLD-1 are obtained from National Centre for Cell Science, Pune, India. HEK-293 was propagated in Dulbecco's Modified Eagle Medium-High Glucose (DMEM-HG), while DLD-1 was grown in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin (Gibco, NY, USA). The cell lines were maintained at 37^0C in a humidified atmosphere having 5% CO_2.

Cell treatment

To investigate the cytotoxicity of ionic liquids, we tested all synthesized ILs on normal human embryonic kidney cell line, HEK-293 and human colorectal adenocarcinoma cell line, DLD-1. These are sensitive and resistant cell lines, respectively, and are commonly used

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model cell lines in toxicological research.[22,29,40,41,42] Moreover, they grow rapidly, can be transfected readily, and are amenable to stringent quantitative assessments.[43,44] The cells have been seeded at 5000 cells/well in a 96-well plate. Stock solutions of the ionic liquids (1M) were prepared in water. To determine the concentration range of ILs in cellular assays, sensitivity test was performed prior to the acute toxicity study. In the sensitivity test, each IL was applied to both cell lines from 0.15 mM up to 10mM for 24, 48 and 72 hrs. The concentration 10mM was deduced as the concentration for acute toxicity analysis. For the IC_{50} assessment, cells were further exposed to ILs up to 20mM concentration.

Cytotoxicity assay

After the treatment of cells with ILs, cytotoxicity of ILs was measured using the MTT assay. According to this assay,[45,46] the cells were incubated with 10µl of MTT (5mg/ml in 1xPBS) for 4 hours. The formazan crystals produced by living cells were solublised with 100µl of solution (20% SDS (w/v) in 50% dimethyl formamide (DFM) (v/v). Then, the absorbance was calculated at 590nm with a reference at 620nm using an ELISA plate reader (Berthold technologies, Germany). All experiments were performed in triplcates and repeated twice. The relative cell viability (%) was expressed as a percentage relative to the untreated control cells. The statistical analysis was performed using GraphPad prism software version 5. In MTT test, one-way ANOVA were used with Bonferroni's post-test. The dose response curves were fitted to a Hill equation using a non-linear least squares method and the IC_{50} values were determined.

Closed bottle test

Closed bottle tests were conducted according to OECD guideline 301 D. The mineral medium was composed of 8.5 mgl^{-1} KH_2PO_4, 21.75 mgl^{-1} K_2HPO_4, 33.4 $mgl^{-1}Na_2HPO_4$ $.2H_2O$, 0.5 $mgl^{-1}NH_4Cl$, 27.5 $mgl^{-1}CaCl_2.2H_2O$, 22.5 $mgl^{-1}MgSO_4.7H_2O$ and 0.25 mgl^{-1} $FeCl_3$. Solutions containing the test substances at $3mgl^{-1}$ were prepared in aerated mineral media and then inoculated with aerated secondary effluent from the wastewater treatment plant, IIT Madras. A control test with microorganisms, but without ILs, was conducted in parallel as the oxygen blank. Sodium benzoate was used as the reference substance. Triplicates of test control, reference samples and test ILs were kept away from sunlight at 298 K. The standard test period was 28 days, and the samples were taken for the determination of dissolved oxygen at seven day intervals. Biodegradability was calculated by dividing the biochemical oxygen demand (BOD; expressed as mg of O_2 per mg of the

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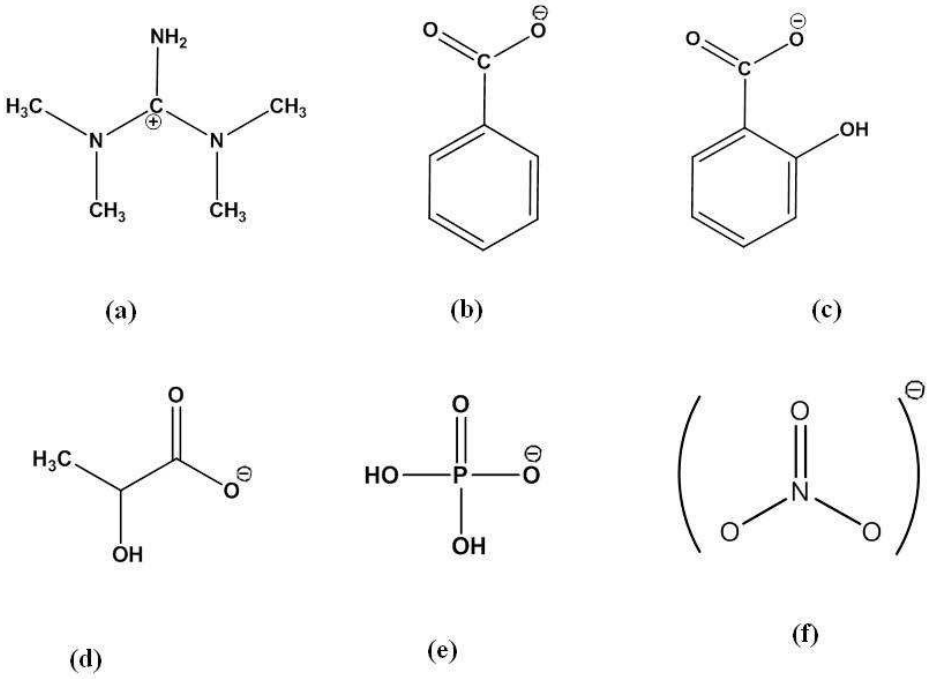
test IL) by the theoretical oxygen demand (ThOD). Biodegradability studies were also carried out with pure bacterial cultures namely, *Pseudomonas putida* MTCC 9782, *Pseudomonas aeruginosa* MTCC 7763, *Pseudomonas aeruginosa* CPCL, *Bacillus subtilis* NCIM 2718 and *Escherichia coli* NCIM 2931 (purchased from NCIM, Pune, India).

Results and Discussion

In this work, we tested the cytotoxicity and biodegradability of [TMG] cation based ionic liquids with different combinations of cation-anion and also with anions of variable side chain lengths (Fig. 1). The linear chains in the alkanoate anions were systematically elongated from formate to valerate (pentanoate). Normal kideny cell line, HEK-293 and colorectal adenocarcinoma cell line, DLD-1were exposed to these [TMG] based ILs for 24, 48 and 72 hours and viability was assessed by MTT assay. The obtained dose-dependent toxicity curves describe very different behaviours: few ionic liquids drastically decreased cellular viability while others allowed cell viability of 80% or above. Thus, the study allowed us to compare a large set of different ionic liquids and infer general conclusions for the designing of more biocompatible ILs in future.



(a) (b) (c) (d) (e) (f)

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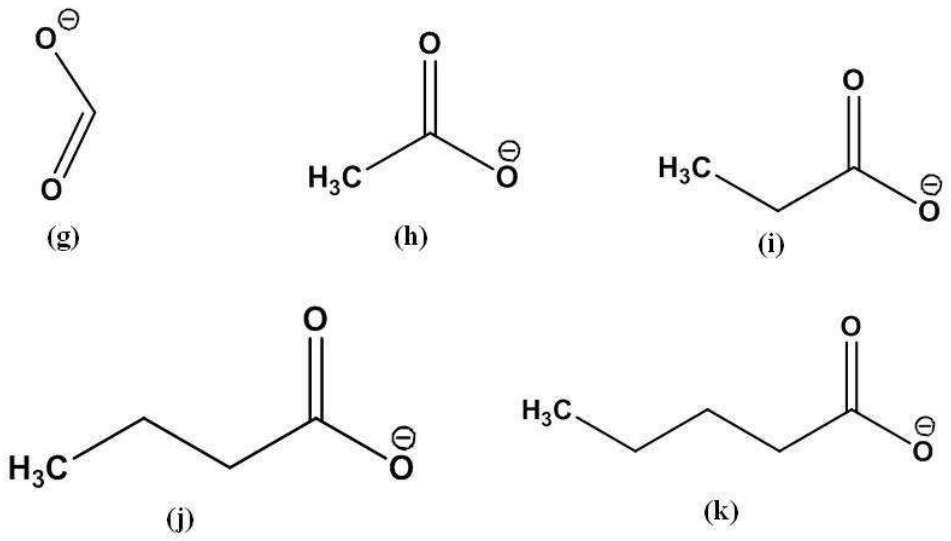

Figure 1. Molecular structures of (a) 1,1,3,3-tetramethylguanidinium cation, (b) benzoate anion, (c) salicylate, (d) lactate (e) dihydrogen phosphate, (f) nitrate, (g) formate, (h) acetate, (i) propanoate, (j) butanoate, and (k) valerate anions.

Functionalized groups in IL anions reduce toxicity

Fig. 2 shows the effect of increasing IL concentrations on the cell viability of HEK-293 cell line after 72 hours of treatment. Cytotoxicity results after 24 and 48 hours of exposure of HEK-293 cell lines to ILs are shown in Fig. S11 and Fig. S12, respectively. As these figures clearly show, the proliferation of HEK-293 cells was affected by these ILs in a dose-dependent manner. Moreover, different combinations of IL cation and anions affected the cell viability differently (Fig. 2a). More interestingly, as Fig. 2b depicts, the increasing length of side-chains of the IL anion reduced the cell viability significantly. Thus, while [TMG][HCOO] and [TMG][ACE] show minimal toxicity even at 10mM concentration, [TMG][PRO], [TMG] [BUT] and [TMG][VAL] started showing certain level of cytotoxicity on this cell line.

To examine the effects of these ILs at relative high concentration, we have exposed the HEK-293 cells to 10 mmol L^{-1} of ILs for 72 hrs. As evident from Fig. 3a, the viability of HEK-293 cells was more than 70%, except in the case of [TMG] [PRO], [TMG] [BUT] and [TMG] [VAL]. Nevertheless, the cell viability was > 50% even at this highest tested

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concentration of [TMG][PRO] and [TMG][VAL]. Taken together, the normal HEK-293 cells were seen to have good viability in the presence of these ILs. To further confirm the non-toxic nature of these ILs, we subsequently exposed the colorectal adenocarcinoma cell line, DLD-1 to 10 mmol L^{-1} concentration of ILs for 72 hours and the results are shown in Fig. 3b. It is clear from Fig. 3b that the viability of tested human cells is more than 70 % in our [TMG] based ILs up to the tested concentration of 10 mM, except for [TMG][PRO], [TMG][BUT] and [TMG][VAL]. Hence, we conclude that most of our synthesized ILs are nontoxic. It is worth mentioning here that for cholinium based ILs with perfluorobutanoate, perfluoropentanoate, perfluorohexanoate, and perfluoroheptanoate anions, Marrucho *et al.* noted < 30% cell viability towards HEK-293 cell line at 10mM incubated for 72 hrs.[22] Frade *et al.*[24] reported that dimethyl-guanidinium cation with longer alkyl chain shows cell viability < 20% towards human colon carcinoma cell line (CaCo-2) at 6mM incubated for 4 hrs. In comparison to these studies, our all [TMG] based ILs exhibited much less toxicity with >50% cell viability at 10mM for 72 hrs, in both the normal and cancer cell lines.

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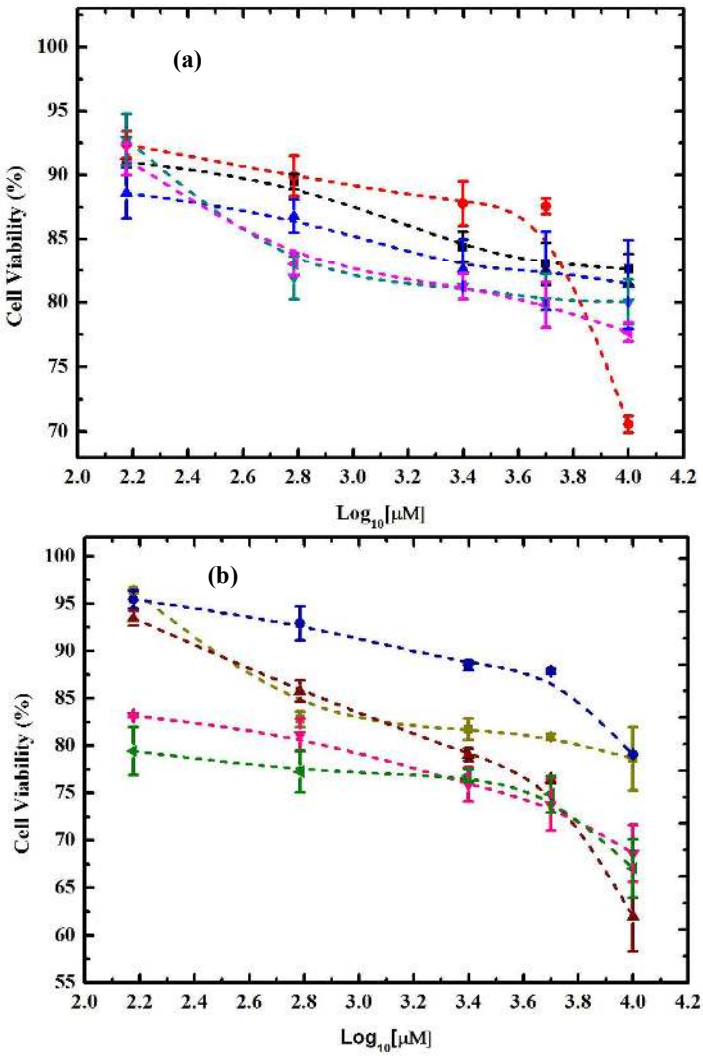

Figure 2. Effect of increasing concentration of [TMG] based ILs on the viability of HEK-293 cell line after 72 hours of exposure. Results are shown for ILs with (a) varying cation-anion combination, and (b) varying anion side-chain. Color scheme: (---■---) [TMG][BEN], (---●---) [TMG][SAL], (---▲---) [TMG][LAC], (---▼---) [TMG][DHP], (---◀---) [TMG][NO_3], (---■---) [TMG][HCOO],(---●---) [TMG][ACE], (---▲---)[TMG][PRO], (---▼---) [TMG][BUT], and (---◀---) [TMG][VAL].

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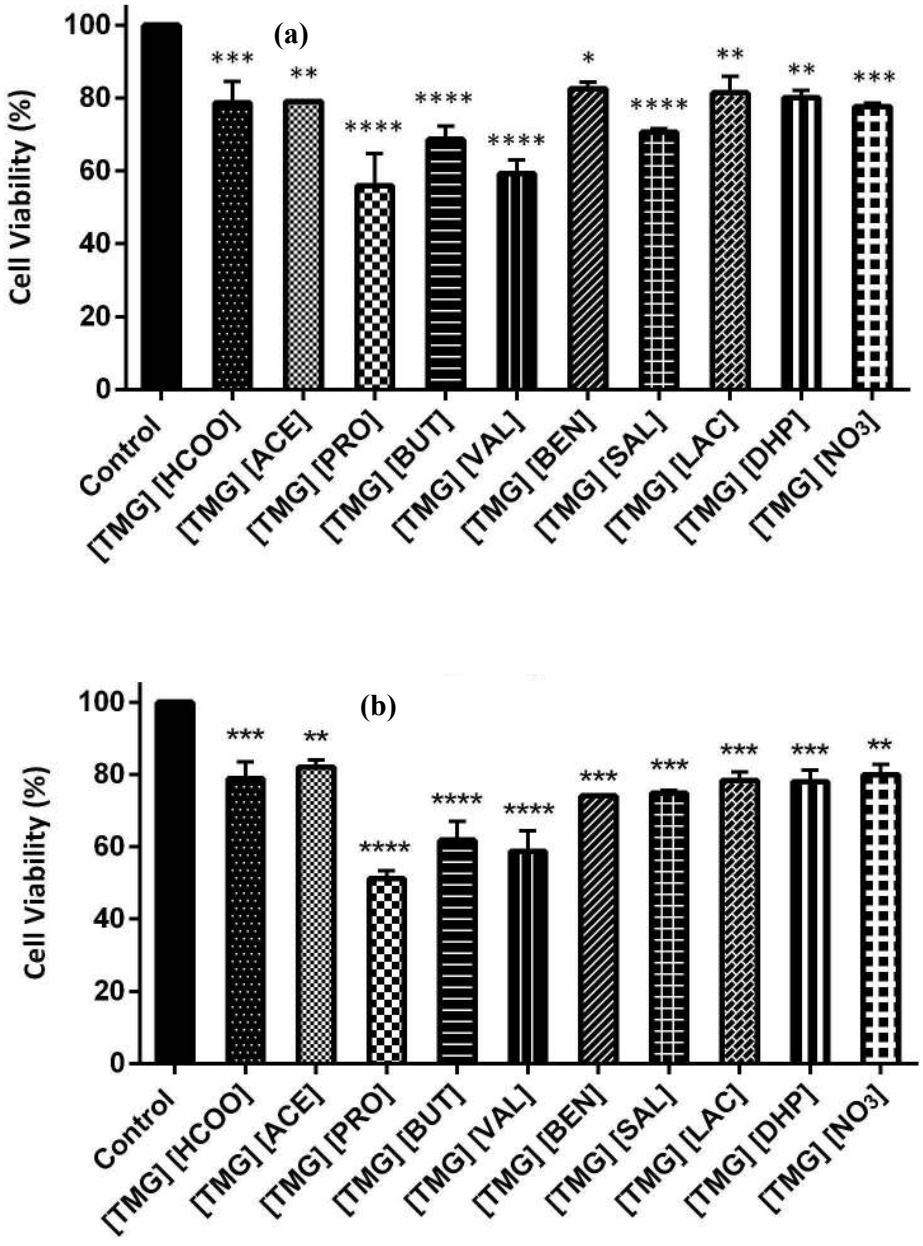

Figure 3. Viability of (a) HEK-293 cells, and (b) DLD1cells exposed to diffrent ILs at 10 mM concentration for 72 hours. Data represents mean ± SD of two independent experiments. The t-test was performed to obtain a level of significance from control. Statistical significance between various ILs were denoted by **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$ with respect to control for each IL.

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We also determined the half inhibitory concentrations (IC_{50}) of these ILs against both cell lines. IC_{50} values for all ILs are listed in Table 1. Values in the table indicate that most of the ILs inhibit the DLD1 cell line less efficiently, compared to the HEK-293 cells. This can be ascribed to the specific nature of more rapid division and proliferation of DLD1 cancer cells than the normal HEK-293 cells, and therefore the former exhibits a higher resistance to the ILs. It is also evident from the table that ILs with long alkyloates (*e.g.* [TMG][PRO], [TMG][BUT], [TMG][VAL]) have IC_{50} values in the range of 7mM to 12mM for both the cell lines. On the contrary, the other ILs show IC_{50} > 15mM, in consistent with the cytotoxicity data in Figs. 2 and 3. Recently, Marrucho *et al*. determined the IC_{50} values for cholinium-based ionic liquids with the long perfluoroalkanoate anions in the same HEK-293 cells and reported the range of IC_{50} from 4.5mM to 4.9mM.[22] The reported range of IC_{50} for guanidinium-based ionic liquids by Fang *et al.* was from 7 mM to 22 mM in HEK 293 cells for 48 hr incubation.[30] In comparison to these results, our [TMG] cation based ILs have shown less inhibitory activity in the studied cell lines.

Table 1. The IC_{50} (mM)[a] values of TMG based ILs against HEK-293 and DLD1 cells after 72 hrs incubation.

Ionic Liquids	HEK-293	DLD1
[TMG][BEN]	>20.00	> 20.00
[TMG][SAL]	13.24	13.70
[TMG][LAC]	>20.00	> 20.00
[TMG][DHP]	19.10	> 20.00
[TMG][NO_3]	14.26	15.94
[TMG][HCOO]	18.24	> 20.00
[TMG][ACE]	>20.00	> 20.00
[TMG][PRO]	11.27	7.07
[TMG][BUT]	12.94	12.35
[TMG][VAL]	12.88	11.83

Experimental error (in Standard Deviation): ≤ 5%.
[a] Compounds that show very weak inhibition in the tested range of concentrations are denoted as: IC_{50} > 20 mM.

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As shown above, this study produced the cytotoxicity trend of several [TMG] based ILs that had different anions. It is evident that [TMG] based ionic liquids exhibit a very complex behaviour towards cell viability that cannot be explained exclusively by the properties of constituent ions. However, certain patterns have been observed such as how the presence of functionalized group in anions led to minimal cell loss at the highest studied doses. It appears that the addition of -COOH has a positive impact for non-toxicity of ILs, as exemplified from very high viability of [TMG][BEN], [TMG][ACE], [TMG][LAC] *etc*. Interestingly, Frade *et al.* reported that the toxicity of [C_{10}MIM] was reduced considerably by the addition of a carboxylic group in the end of the C_{10} alkyl chain, which supports our conclusion.[24] Moreover, the higher cellular viability of [TMG][DHP] and [TMG][LAC] implies that the presence of -OH group in the anion can improve the non-toxic nature of the ILs. This is again in accordance with previous reports that suggested, decreased toxicity of imidazolium based ILs in presence of the hydroxyl groups.[17,47] Thus, our results show that the presence of - COOH and -OH groups in anions can make the ILs less toxic. The effect of linear chains in the alkanoate anions on the toxicity will be discussed in next section.

Lipophilicity of the IL anions increase toxicity

The side-chain variation of the IL anion also can affect the cell viability. As shown in Figs. 2b, S11b and S12b the presence of [PRO], [BUT] and [VAL] anions showed a certain level of cytotoxic effect of the ILs on the tested cell lines, particularly at higher concentrations. However, at lower concentrations, the cell viability in these two ILs was very similar to that of the other ILs. This phenomena of low-dose stimulation (see Fig. 2b for [TMG][PRO]) and high-dose inhibition (Fig. 3) is known as "hormesis".[48] A similar hormetic effect of 1-n-octylmethylimidazolium tetrafluoroborate [C_8MIM][BF_4] was observed in IPC-81 leukaemia cells[11] and of 1-n-butyl-3-ethylimidazolium tetrafluoroborate [BEIM][BF4] in HeLa cells.[49] Nevertheless, it is evident from Fig. 3 that the length of n-alkyl chain correlates very well with the toxicity. Thus, [TMG][PRO], [TMG][BUT] and [TMG][VAL] are more toxic in all cell lines studied compared to other ILs, due to their long alkyl chain. However, [TMG][ACE] is the least toxic IL in this series, as formate is known to produce free radicals.[50] The increasing toxicity with increased alkyl chain length can be explained as follows. The longer alkyl chains lead to higher lipophilicity in the IL anions, which thereby can induce leakage in cell membrane

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for easier penetration to the cells. Lastly, to study the biocompatibility of these ILs, we also carried out the biodegradability assessment of these [TMG] based ILs.

Biodegradability assessment of the ILs

Along with the toxicity studies, the biodegradability of the above discussed [TMG] cation based ILs were also investigated. The thermophysical properties of some of these ILs have been reported previously[51,52,53,54] but these novel solvents are seldom used for any biological applications due to the lack of well characterized biodegradability data. Hence, we studied the biodegradability of these ILs with different types of bacteria to explore growth susceptibility in media containing ILs. Out of five tested bacterial species (*Pseudomonas putida* MTCC 9782, *Bacillus subtilis* NCIM 2718, *Pseudomonas aeruginosa* MTCC 7763, *Pseudomonas aeruginosa* CPCL, and *Escherichia coli* NCIM 2931), *Pseudomonas putida* MTCC 9782 and *Bacillus subtilis* NCIM 2718 were found to exhibit good growth susceptibility in media containing our ILs. Hence, we have chosen these two bacteria for studies on the biodegradability of our [TMG] based ILs. These above discussed bacteria were able to grow in media containing relatively high concentrations of [TMG] based ILs and yet showed very distinct behaviours, e.g. minimum inhibitory concentration (MIC) values varied from 15 mM to 125 mM (see Table 2). The listed values also show the influence of anion on the MIC of ILs. To test the biodegradability, concentrations of all tested compounds used for incubating the bacteria were below the determined MICs, as tabulated in Table 3. Bacterial cultures of 20 cm^3 were incubated in the dark, at 25°C, under agitation (90 rpm), for 28 days. The aliquot of 1 cm^3 was taken from the cultures, filtered (0.2 mm) and analysed by ^{1}H NMR spectroscopy.

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Table 2. Minimal inhibitory concentrations (MIC) of TMG based ILs on the bacterial growth of *Pseudomona putida* MTCC 9782* and *Bacillus subtilis* NCIM 2718.

Ionic Liquids	MIC (mM)
[TMG][BEN]*	31.25
[TMG][SAL]*	31.25
[TMG][LAC]	62.25
[TMG][DHP]	125.00
[TMG][NO3]	125.00
[TMG][HCOO]	15.60
[TMG][ACE]	31.25
[TMG][PRO]	15.60
[TMG][BUT]	31.25
[TMG][VAL]	15.60

Table 3. Concentrations of the tested ILs used in the biodegradability assay.

Ionic Liquids	Tested conc. (mM)
[TMG][BEN]	15
[TMG][SAL]	15
[TMG][LAC]	31
[TMG][ACE]	15
[TMG][PRO]	7
[TMG][BUT]	15
[TMG][VAL]	7

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IL alkanoate anions degrade faster than aromatic anions

The biodegradation of ionic liquids in bacterial cultures was monitored by NMR spectroscopy on seven days interval after the incubation. The anions are demonstrated to be highly biodegradable. Very distinct degradation was observed in the anions, as shown in Fig. 4, Fig. 5 and Fig. S14 by the disappearance of the anion peaks with time in the spectra. Notably, out of four bacteria, *Bacillus subtilis* and *Pseudomonas aeruginosa* were able to degrade all the anions expect [BEN] and [SAL], and *Pseudomonas putida* was able to degrade [BEN] and [SAL]. For clarity, results of degradation by *Pseudomonas aeruginosa* have been omitted, as they were similar to *Bacillus subtilis*. Fig. 4 depicts that acetate and lactate anions were degraded significantly after 7 days. On the other hand, for benzoate and salicylate anions, the biodegradability was observed only after 14 days in the presence of *Pseudomonas putida*, as shown in Fig. 5. Thus, the IL anion's biodegradability was observed to be highly dependent on concentration as well as on the type of anion, e.g., while acetate (15 mM) and lactate (31 mM) were degraded after one week, benzoate and salicylate (15 mM) were more resistant to bacteria attack up to 2 weeks. It is also worth mentioning here that the peak attributed to the [TMG] cation were intact in the spectral analyses (Fig. 4 and Fig. 5), thus suggesting that it was only partially degraded with these bacteria. Hence, the biodegradability of the [TMG] cation was evaluated using the 'Closed Bottle' test (OECD 301D and ISO 14593) as described in the next section.

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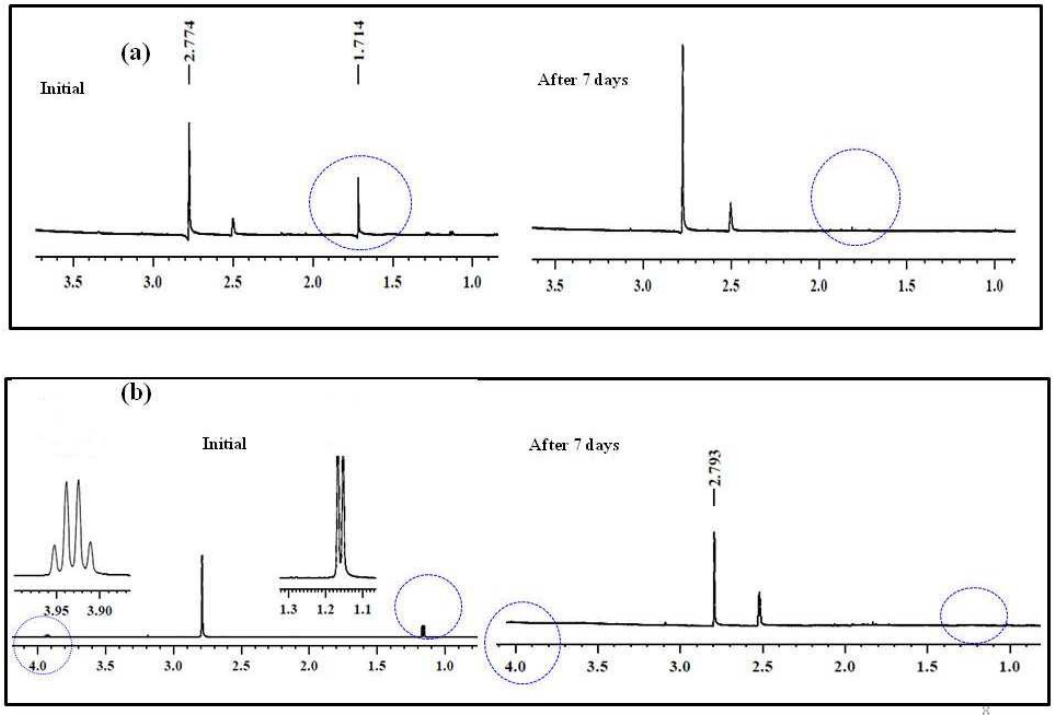

Figure 4. Biodegradability assessment of the selected ILs using *Bacillus subtilis* NCIM 2718. ^{1}H NMR spectra at the beginning and after 7 days of incubation for (a) [TMG][ACE] (15 mM) and (b) [TMG][LAC] (31 mM). ^{1}H NMR (500 MHz, DMSO-d_6) spectra show the following peaks: (a) 2.77 (s, 12H) for [TMG] and 1.71 (s, 3H) for [ACE] in [TMG][ACE]; (b) 2.79 (s, 12H) for [TMG] and 3.93(q, 1H), 1.15(d, 3H) for [LAC] in [TMG][LAC].

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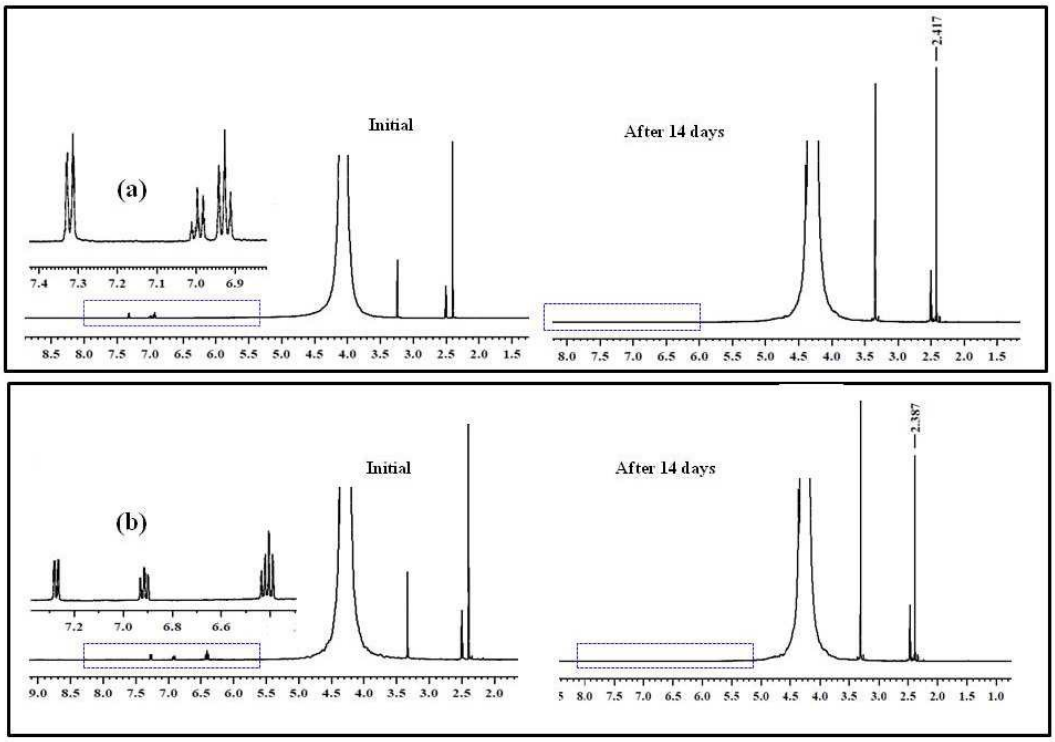

Figure 5. Biodegradability assessment of the selected ILs using *Pseudomonas putida* MTCC 978*2*. ^{1}H NMR spectra at the beginning and after 14 days of incubation for (a) [TMG][BEN] (15 mM) and (b) [TMG][SAL] (15 mM). ^{1}H NMR (500 MHz, DMSO-d_6) spectra show the following peaks: (a) 2.41(s, 12H) for [TMG] and 6.95(m, 2H), 7.00(m, 1H), 7.35(m, 2H) for [BEN] in [TMG][BEN]; (b) 2.38 (s, 12H) for [TMG] and 6.50(m, 2H), 6.90 (m, 1H), 7.25(m, 1H) for [SAL] in [TMG][SAL].

[TMG] cation is also biodegradable

Aerobic biodegradability of representative IL, [TMG][DHP] was evaluated by standard methods - the closed bottle test- in which ILs were added to aerobic aqueous media inoculated with microorganisms from waste water, and the depletion of dissolved O_2 was determined periodically. The biodegradability of [TMG][DHP] was compared with the reference compound sodium benzoate (Fig.6). Generally, the biodegradation test methods are considered to be successful if 60% of the reference compound is biodegraded within 14 days. In this study, the biodegradability of sodium benzoate was measured as 80% in 14 days by this method, thus confirming the validity of the test method. According to the Organization for Economic Cooperation and Development (OECD), the compounds which

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reach a biodegradation level of 60% after 28 days are considered to pass the biodegradation test.[31,33] It was seen that [TMG][DHP] underwent a significant level of degradation (62%) within 14 days by the closed bottle test, thus achieving the pass level for this test. The [TMG] cation showed greater susceptibility to aerobic biodegradation than other cations, such as 1-Butyl-3- methylimidazolium and 1-methyl-3-hexaly imidazolium *etc* which showed 10% - 25% biodegradation in 28 days.[31,32] The methyl branches in [TMG] cation could be responsible for the slight increase in resistance to aerobic biological breakdown by microorganisms (60-62% in 14 days). Petkovic *et al.* had noted that ILs with branched chain alkanoate anions usually are more resistant to fungal attack than their linear isomers[55] Thus, [TMG] cation can also be referred as 'readily biodegradable'. In the rational design of environmentally benign compounds, the pursuits for minimizing toxicity and maximizing biodegradability are often seen to conflict with each other.[56] For example, the elongation of side chain that resulted in enhanced biodegradation of pyridinium-based ILs correlated with an increase in their toxicity.[57] Interestingly, such a conflict between the toxicity and biodegradability did not occur in these [TMG] based ILs. For example, both [TMG][ACE] and [TMG][BUT] are found to be nontoxic (Fig. 3) and easily biodegradable (Figs. 4, S14b), in spite of the longer chain that the latter IL possesses.

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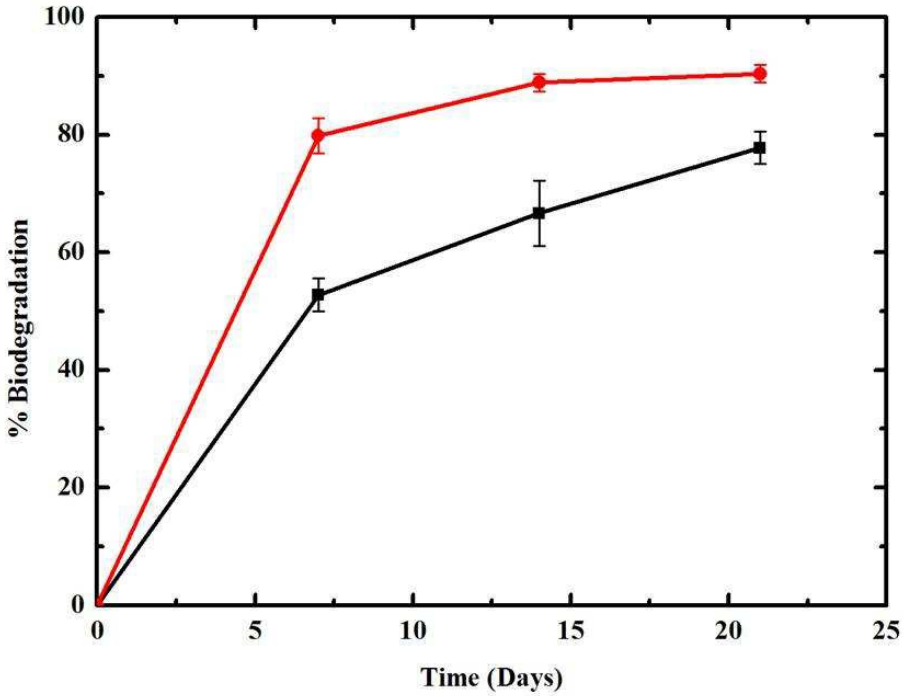

Figure 6. Biodegradation curves (Closed Bottle test) with mixed culture from waste water treatment plant: (■) [TMG][DHP] and (●) reference substance; sodium benzozte.

Conclusions:

Tetramethylguanidinium cation based ILs have received recent attention in various applications. However, no biocompatibility data is available for this novel class of ILs. In this study, we synthesized a series of tetramethylguanidinium [TMG] cation based ILs with a variety of anions such as benzoate [BEN], salicylate [SAL], lactate [LAC], dihydrogen phosphate [DHP], nitrate [NO_3], formate [HCOO], acetate [ACE], propanoate [PRO], butanoate [BUT] and valerate [VAL]. Subsequently, the biocompatibilty tests of the synthesized ILs were performed by measuring their toxicity and biodegradability. Toxicological evaluation with HEK-293 and DLD1 cell lines showed that almost all of the studied [TMG] based ILs are non-toxic. Only [TMG][PRO], [TMG][BUT] and [TMG][VAL] showed a mild decrease in cell viability at moderate to high concentrations. Nevertheless, the measured cell viability is well above 30% - the threshold above which an IL is considered to be non-toxic (Frade *et al.*).[24] Subsequent biodegradability tests with bacterial cultures and closed bottle test show that [TMG] cations and all anions with this cation are biodegradable. To the best of our knowldege, this is the first study to assess the

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cytotoxicity and biodegradability of these ILs. Results also suggest that there is a scope to improve the "greenness" of this novel class of solvents by a judicial choice of functionalized groups, such as -COOH, -OH *etc* in the anion and/or cation. Work is in progress along this direction.

Supporting Information

Supporting Table and Figures are provided as Table S1and Figs. S1 – S14.

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