Green Chemistry

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/greenchem

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Structural-functional evaluation of ionic liquid libraries for the design of co-solvents in lipase-catalysed reactions

João V. Rodrigues[a,‡], Diana Ruivo[a,‡], Ana Rodríguez[b], Francisco J. Deive[b], José M.S.S. Esperança[a], Isabel M. Marrucho[a,*], Cláudio M. Gomes[a,*], Luís Paulo N. Rebelo[a]

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX
DOI: 10.1039/b000000x

Using ionic liquids as co-solvents may improve reaction media in enzyme-based biotechnological processes. To establish new conditions, large libraries need to be screened for bio-compatibility and protein stabilisation. Using a lipase model, we herein describe a combination of methods leading to an expedited evaluation of 61 different solvent compositions.

Ionic liquids (IL) are promising alternative solvents for enzymes, due to their high thermostability, low vapour pressure, non-flammable nature and solvation ability towards a wide variety of solutes[1]. Most importantly, these molten salts offer a broad range of different physico-chemical properties which can dramatically influence the stability and function of proteins. Potential applications include the use of ILs to increase the stability and enantioselectivity[2] of enzymes, as refolding and crystallization additives[3,4] and as inhibitors of protein aggregation[5].

However, understanding how ILs act on the structure and function of proteins is still a major challenge. One of the main reasons for this is that proteins respond differently to changes in solvent conditions, due to differences in their own physico-chemical, structural and functional properties. It is, thus, common to find opposing trends for different proteins when the same solvent properties are varied (e.g. hydrophobicity[6]). Moreover, the interpretation of the effects of ILs on proteins may be further complicated as published reports frequently focus solely on either the effects on enzymatic activity, or on those on protein stability (for a review see ref. [6]). This makes it difficult to identify generalized trends predicting how the inclusion of a particular type of ILs as co-solvent will affect protein function and structure. To overcome this limitation, we have established an experimental platform that allows us to evaluate how solvent conditions affect protein structure and function. In particular, we have analysed the functional and structural effects of a library of 61 different ILs over the *Thermomyces lanuginosus* lipase (*Tl*L), which we used as a model. This enzyme has been generating great interest due to its wide range of applicability in the food, energy and pharmaceutical industries[7]. The screened ILs are all water soluble and were used at a concentration of 25% (m/v), and the library included different cation (ammonium, imidazolium, pyrrolidinium,), and anion (halides, alkyl sulfonates, alkyl sulfates, alkyl phosphates and carboxylates) families, (full list in supplementary information, Table S1 and Fig. S1).

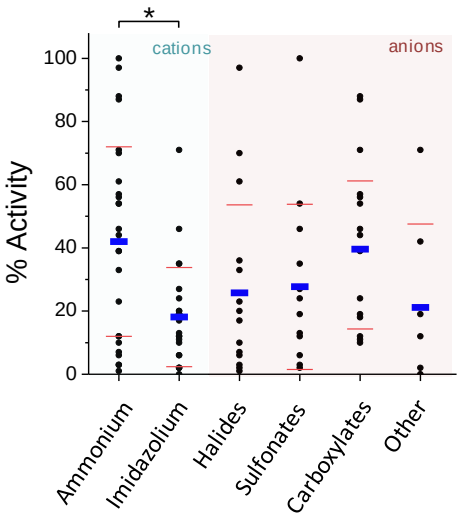

Fig. 1 Distribution of *Tl*L activity in different ILs grouped by ion family. The average activity according to families is as follows: Ammonium (42%, *n*=26), Imidazolium (18.2%, *n*=26), Halides (25.8%, *n*=16, Sulfonates (27.7%, *n*=14), Carboxylates (39%, *n*=18), Other (21%, *n*=7). * $p<0.05$

The function of *Tl*L was evaluated using an adaptation of a colorimetric assay for a 96-well plate, described previously by Choi *et al*[8]. Part of these results is shown in Fig. 1, in which activities are grouped by ion families. On average, the ILs containing ammonium cations lead to relatively higher activities than those based on the imidazolium cation, whereas no statistical difference was observed for the anion series. The high dispersion of data thus suggests that other factors, distinct from mere ion family type, are important in lipase function.

The impact of ILs as co-solvents on *Tl*L was investigated by differential scanning fluorimetry (DSF), which had been previously validated as a rapid method for assessing the stability of proteins in ILs[9]. It is based on monitoring protein thermal denaturation using the Sypro Orange dye, as its fluorescence is greatly increased upon binding to hydrophobic domains exposed to solvent upon protein unfolding[10]. The slope of the thermal unfolding transition denotes the cooperativity of the transition, and its midpoint corresponds to T_m - the protein melting temperature (Figure S2). Since *Tl*L unfolding is irreversible due

to the formation of aggregates, these are not thermodynamic but, apparent protein stability parameters, which serve, nevertheless, to compare different compounds tested under similar conditions. The effects of different co-solvent ILs on lipase stability were thus determined from the difference in the apparent T_m values (ΔT_m) in respect to the condition in which the co-solvent is absent (the T_m of *Tl*L in water is 70°C) (as exemplified in Fig S3). Control experiments in the absence of the protein ruled out any artifactual transitions due to the ILs alone.

Enzymatic activity was then plotted against the T_m for each solvent condition (Fig. 2A). The rationale for this analysis is that structural changes leading to a significant decrease in protein stability result in conformational destabilization impairing function. Nevertheless, a clear-cut correlation was not observed: the distribution of T_m values obtained for those ILs that strongly impair activity (<25%) ranged from T_m = 38 ºC (for $N_{1\ 1}$ Bz (2OH) Cl) to a surprisingly high T_m = 66.5 ºC (for C_2C_1im C_1SO_3), only 3.5 ºC below the control reference. On the other hand, some ILs in which *Tl*L has a moderate activity (25-75 %) also lead to a dramatic decrease in stability, in some cases 30 ºC below the control. Potential explanations for these discrepancies are multiple: they may, for example, result from the fact that some ILs perturb protein stability (decreasing the T_m) while maintaining the catalytic site intact (thus sustaining high activity) at temperatures below the transition midpoint. On the other hand, a stabilizing effect of an IL (increasing the T_m) is not necessarily beneficial, as protein hyper-stabilisation may compromise activity due to a substantial decrease in breathing dynamics required for catalysis[11]. Overall, these examples illustrate the complex interplay between different factors governing protein stability-structure-function relationships, and highlight the need for further structural insights on the effects of ILs.

The slope of the thermal unfolding transition contains information about how solvent conditions affect protein structure. If solvent composition affects the unfolding pathway causing deviations from two-state transitions, this will result in lower slopes indicative of the accumulation of intermediate forms during the unfolding pathway. Likewise, if a certain solvent condition mildly perturbs the native state it will broaden the conformational landscape of native-like conformers and the thermal unfolding transition of this ensemble will then be monotonic (i.e. with a lower slope than the native state). In fact, the analysis of the variation of the transition steepness versus activity shows that its decrease is associated with a clear compromise of catalytic activity (Fig. 2B, linear fit: Act=2.3+0.84×T_s, r^2=0.64, P<0.0001, where T_s is the transition steepness).

We also noticed that those ILs that strongly impaired the activity of *Tl*L were associated with a strong Sypro Orange fluorescence arising from direct interaction of the IL with the dye alone at 25 ºC (control experiments in the absence of protein). In agreement, Figure 2B shows a marked correlation between *Tl*L activity and the relative fluorescence intensity of Sypro Orange in the presence of the various solvents (Act=5.2 +67.9×$e^{-3.58\times I}$, r^2=0.52, P<0.0001). It is well known that solvent hydrophobicity is a major parameter affecting both stability and activity. Since the fluorescence of Sypro Orange depends on its interaction with hydrophobic environments, it is most likely that the analysis of

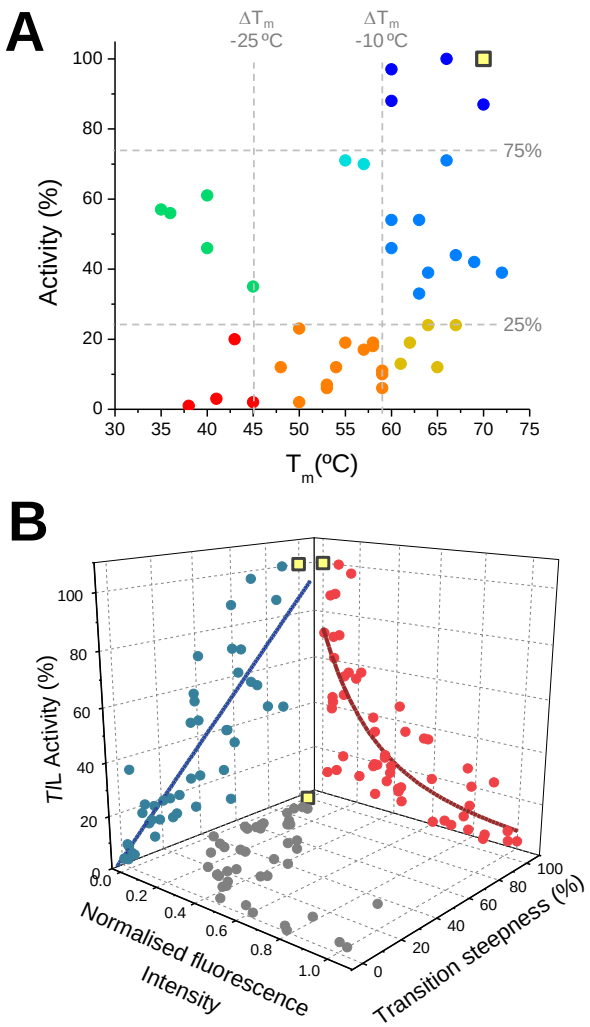

Fig. 2 Relationship between the activity of *Tl*L and the stability parameters determined by DSF. A) Activity as a function of T_m. Data points were grouped and colored according to the effect of ILs on T_m and activity B) Activity as a function of Sypro Orange fluorescence intensity in different solvents and transition slope of the *Tl*L thermal denaturation. The yellow, black-framed squares are the references values in water.

Fig. 2B is related to the effect of the hydrophobic character of the solvent on the activity.

To further address this effect we have analysed our data considering the predicted hydrophobicities values (octanol:water partition coefficient – logP) which were computed for each IL combination using the virtual computational chemistry lab web server[12,13]). Indeed, this analysis also denoted a noticeable dependence of the activity on the solvent logP (Fig. 3, fit: Act=-12+40.7×$e^{-0.25\times LogP}$, r^2=0.41). This further supports that higher hydrophobic character of the IL has a detrimental influence on *Tl*L activity. In agreement, *Tl*L stability is also inversely proportional to total solvent hydrophobicity: T_m=56.9-3.95×logP, r^2=0.66, P<0.0001 (Figure 3B), although some exceptions are found (grouped within dashed lines). These results may be interpreted by taking into account the destabilizing effect of long alkyl chains in the anion as they interact with the hydrophobic residues that are usually solvent-protected inside the protein core. However, it remains to be clarified why the most stable condition

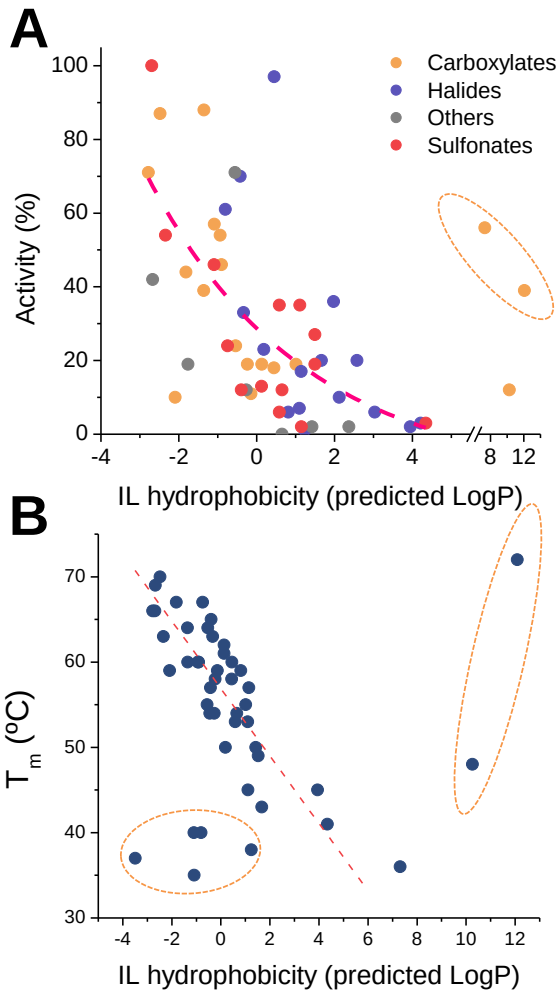

Fig. 3 Effect of hydrophobic character of ILs on the activity and stability of *Tl*L. A) Effect of the hydrophobicity of the molecule on the activity of *Tl*L. B) Effect of total solvent hydrophobicity on the T_m of *Tl*L.

(T_m = 72 ºC) is observed with the most hydrophobic IL ($N_{8\,8\,8\,H}$(2-C_4)C_7COO) tested in this work. Eventually, an encapsulation-type mechanism in which the protein is surrounded by micelles (possibly of mixed type) could account for a more stable protein structure due to rigidification, as suggested by Pavidis *et al*[14]. Another explanation may reside in the structural similarities between the IL anion and the products of esterase reaction catalysed by *Tl*L. Binding of (2-C_4)C_7COO^- to the active site may stabilize the protein by a pharmacological chaperone-type mechanism[15,16].

Conclusions

Establishing structure/function relationships for proteins in IL media is an extremely valuable tool for increasing our predictive power and creating better solvents for biotechnology. We have simultaneously evaluated the activity and stability of *Tl*L in the presence of 61 ILs using 96-well plate based methods. By combining three parameters obtained by DSF (T_m, transition slope and fluorescence intensity of Sypro Orange in a given solvent) it is possible to gain further structural information about the effect of ILs on the proteins, namely the effects on the native state and unfolding pathways. This type of analysis, in combination with the study of IL physico-chemical properties, allows us to rationalise, at least partially, the impact of ILs on enzymatic function.

This work was partially supported by the strategic grant PEst-OE/EQB/LA0004/2011 (to ITQB Laboratório Associado) and by *Fundacão para a Ciência e Tecnologia* through research grants: PTDC/QUI/70101/2006, PTDC/CTM-NAN/121274/2010, INVESTIGATOR FCT and CIENCIA2007 (I.M.M. and J.M.S.S.E) and fellowships SFRH/BPD/34763/2007 (J.V.R.) and SFRH/BD/74081/2010 (D.R.). F.J.D. wishes to acknowledge *Xunta de Galicia* for the *Isidro Parga Pondal* contract.

Notes and references

[a] *Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. da República, 2780-157 Oeiras, Portugal.*
** Corresponding authors: gomes@itqb.unl.pt and imarrucho@itqb.unl.pt*
[b] *Department of Chemical Engineering, University of Vigo, P.O. Box 36310, Vigo, Spain*

† Electronic Supplementary Information (ESI) available:Table S1 listing ionic liquids and raw data; Fig. S1 - main cation structure of ILs; Fig. S2 – grapic showing examples of DSF curves, Fig. S3 - graphic showing activity and Δ T_m for each IL; Fig. S4 - effect of hydrophobicity of ILs on the fluorescence of Sypro Orange; Table S2 List of ILs used in this work and source. See DOI: 10.1039/b000000x/

‡ Equally contributing authors

1 N. V. Plechkova and K. R. Seddon, *Chem Soc Rev*, 2008, **37**, 123.
2 H. Zhao, S. M. Campbell, L. Jackson, Z. Y. Song, and O. Olubajo, *Tetrahedron-Asymmetry*, 2006, **17**, 377.
3 P. Attri, P. Venkatesu, and A. Kumar, *Org Biomol Chem*, 2012, **10**, 7475.
4 M. Kowacz, A. Mukhopadhyay, A. L. Carvalho, J. M. S. S. Esperanca, M. J. Romao, and L. P. N. Rebelo, *Crystengcomm*, 2012, **14**, 4912.
5 H. Weingartner, C. Cabrele, and C. Herrmann, *Phys Chem Chem Phys*, 2012, **14**, 415.
6 M. Naushad, Z. A. Alothman, A. B. Khan, and M. Ali, *Int J Biol Macromol*, 2012, **51**, 555.
7 R. Fernandez-Lafuente, *J Mol Catal B Enzym*, 2010, **62**, 197.
8 S. J. Choi, J. M. Hwang, and S. I. Kim, *J Biochem Mol Biol*, 2003, **36**, 417.
9 J. V. Rodrigues, V. Prosinecki, I. Marrucho, L. P. Rebelo, and C. M. Gomes, *Phys Chem Chem Phys*, 2011, **13**, 13614.
10 M. W. Pantoliano, E. C. Petrella, J. D. Kwasnoski, V. S. Lobanov, J. Myslik, E. Graf, T. Carver, E. Asel, B. A. Springer, P. Lane, and F. R. Salemme, *J Biomol Screen*, 2001, **6**, 429.
11 A. R. Correia, S. Adinolfi, A. Pastore, and C. M. Gomes, *Biochem J*, 2006, **398**, 605.
12 in 'VCCLAB, Virtual Computational Chemistry Laboratory, http://www.vcclab.org', 2005.
13 I. V. Tetko, J. Gasteiger, R. Todeschini, A. Mauri, D. Livingstone, P. Ertl, V. Palyulin, E. Radchenko, N. S. Zefirov, A. S. Makarenko, V. Y. Tanchuk, and V. V. Prokopenko, *J Comput Aided Mol Des*, 2005, **19**, 453.
14 I. V. Pavlidis, D. Gournis, G. K. Papadopoulos, and H. Stamatis, *J Mol Catal B Enzym*, 2009, **60**, 50.
15 C. M. Gomes, *Curr Top Med Chem*, 2012, **12**, 2460.
16 P. Leandro and C. M. Gomes, *Mini Rev Med Chem*, 2008, **8**, 901.

Structural-functional evaluation of ionic liquid libraries for the design of co-solvents in lipase-catalysed reactions

ELECTRONIC SUPPLEMENTARY INFORMATION

Materials and methods

Lipolytic activity assay method - 2,3-dimercapto-1-propanol tributyrate (Sigma Aldrich), DMPTB, was dissolved in Triton X-100 (Sigma Aldrich) and in 50 mM trizma base buffer (Sigma Aldrich), pH 7.2. The final stock solution is 10 mM DMPTB and 2% Triton X-100 and was stored at -20 ºC. A 40 mM solution of 5,5'-dithiobis(2-nitro benzoic acid) (Sigma Aldrich), DTNB, in isobutanol was prepared daily. Thermomyces lanuginosus lipase (Sigma Aldrich, L0777), TlL, was dissolved in 50 mM trizma base buffer pH 7.2 with 0.0025% of triton X-100. The standard reaction mixture contained 4.44 mM DMPTB, 0.88 mM DTNB, 0.0013% Triton X-100, and 26.70 mM Trizma base, pH 7.2. The concentration of enzyme used was 70 nM. Each well of the 96-well microplate was filled with 100 µl of a 50% IL (prepared in water), 50 µl of the standard reaction mixture and 50 µl of the enzyme sample. Controls without the enzyme and/or the IL were also performed.

The time-dependent absorbance change was monitored at 405 nm for 10 min of reaction in a Biotek Synergy 2 multi-mode microplate reader. The lipolytic activity was calculated based on the initial velocity of the reaction (Abs/s). Lipolytic activity is calculated as a percentage of the activity of TlL in buffer in the presence of the selected ionic liquids and represents the average of at least 2 replicates.

Differential Scanning Fluorimetry (DSF)- Differential scanning fluorimetry, runs were performed by monitoring the fluorescence of the exogenous probe Sypro Orange (Life Technologies, 5000x concentrate in DMSO) in a Bio Rad IQ5 Multicolor Real-Time PCR detection system equipped with a charge-coupled device (CCD) camera and a Cy3 filter with excitation and emission wavelengths of 490 and 575 nm, respectively. 75 µL of 6.67 µM enzyme solution in 20x DMSO concentrated sypro orange probe was left in contact with 25 µL of 25% (w/w) IL solution for 15 minutes. Each well of the 96-Well PCR plate was filled with 20 µL of this mixture. The assays were performed in a Bio Rad IQ5 Multicolor Real-Time PCR detection system with heating rates of 1.0 ºC min-1 from 20 to 90 ºC. Controls without the enzyme and/or the IL were also performed.

Table S1 – List of ionic liquids used in this work and respective activity and stability data for *Tl*L

Compound	Activity (%)	Normalized intensity	Apparent T_m by DSF (°C)	ΔT_m (°C)	Transition steepness (%)	logP (Cation)	logP (Anion)	LogP (IL)
Control (Water)	100	0	70	0	100	-	-	-
$(CH_3)_2N_2CNH_2$ $N(CN)_2$	19	#N/D	#N/D	#N/D	#N/D	-2.043	0.282	-1.761
$N_{1\,1\,1\,(2OH)}$ Bicarbonate	10	3	59	-11	68.3	-1.574	-0.516	-2.09
$N_{1\,1\,1\,(2OH)}$ NO_3	12	8	54	-16	55.1	-1.574	1.31	-0.264
$N_{1\,1\,1\,(2OH)}$ C_3COO	39	5	64	-6	67.2	-1.574	0.215	-1.359
$N_{1\,1\,1\,(2OH)}$ $(C_1)_2PO_4$	42	6	69	-1	93.6	-1.574	-1.094	-2.668
$N_{1\,1\,1\,(2OH)}$ C_2COO	44	6	67	-3	80.2	-1.574	-0.241	-1.815
$N_{1\,1\,1\,(2OH)}$ C_4COO	46	12	60	-10	36.5	-1.574	0.672	-0.902
$N_{1\,1\,1\,(2OH)}$ C_2SO_3	54	12	63	-7	74.0	-1.574	-0.768	-2.342
$N_{1\,1\,1\,(2OH)}$ Pivalate	54	19	60	-10	38.9	-1.574	0.639	-0.935
$N_{1\,1\,1\,(2OH)}$ Salicylate	57	22	35	-35	38.6	-1.574	0.489	-1.085
$N_{1\,1\,1\,(2OH)}$ Cl	61	7	40	-30	63.1	-1.574	0.77	-0.804
$N_{1\,1\,1\,(2OH)}$ OTf	71	9	55	-15	59.9	-1.574	1.019	-0.555
$N_{1\,1\,1\,(2OH)}$ C_1COO	87	4	70	0	86.1	-1.574	-0.908	-2.482
$N_{1\,1\,1\,(2OH)}$ $(C_1)_2CCOO$	88	7	60	-10	59.9	-1.574	0.222	-1.352
$N_{1\,1\,1\,(2OH)}$ C_1SO_3	100	9	66	-4	89.7	-1.574	-1.117	-2.691
$N_{1\,1\,1\,(2OH)}$ C_5COO	#N/D	92	54	-16	8.0	-1.574	1.128	-0.446
$N_{1\,1\,1\,(2OH)}$ H_2PO_4	#N/D	2	37	-33	27.0	-1.574	-1.911	-3.485
$N_{1\,1\,2\,(2OH)}$ Br	33	29	63	-7	60.1	-1.225	0.89	-0.335
$N_{1\,1\,1\,(2O1)}$ Cl	70	6	57	-13	65.0	-1.189	0.77	-0.419
$N_{1\,1\,3\,(2OH)}$ Br	23	35	50	-20	53.8	-0.702	0.89	0.188
$N_{1\,1\,4\,(2O1)}$ Cl	97	15	60	-10	71.2	-0.316	0.77	0.454
$N_{1\,1\,4\,(2OH)}$ Br	6	30	59	-11	16.2	-0.072	0.89	0.818
C_1C_1im C_1SO_3	46	42	40	-30	40.7	0.025	-1.117	-1.092
$N_{1\,1\,5\,(2OH)}$ Br	7	75	53	-17	2.8	0.211	0.89	1.101
C_2C_1im $N(CN)_2$	0	85	No transition	No transition	No transition	0.374	0.282	0.656
C_2C_1im HCOO	11	42	59	-11	25.7	0.374	-0.516	-0.142
C_2C_1im C_2SO_3	12	21	65	-5	19.1	0.374	-0.768	-0.394
C_2C_1im Cl	17	38	57	-13	10.5	0.374	0.77	1.144
C_2C_1im Pivalate	19	29	55	-15	21.2	0.374	0.639	1.013
C_2C_1im C_2COO	19	12	62	-8	29.4	0.374	-0.241	0.133
C_2C_1im C_1COO	24	35	64	-6	40.6	0.374	-0.908	-0.534
C_2C_1im C_1SO_3	24	88	67	-3	35.8	0.374	-1.117	-0.743
$C_2C_1imC_6SO_3$	27	76	No transition	No transition	No transition	0.374	1.124	1.498
C_2C_1im C_4SO_3	35	57	No transition	No transition	No transition	0.374	0.212	0.586
C_2C_1im Gala	71	1	66	-4	41.5	0.374	-3.153	-2.779
C_2C_1im C_4SO_4	#N/D	50	No transition	No transition	No transition	0.374	0.058	0.432
C_2C_1im C_8SO_4	#N/D	2	No transition	No transition	No transition	0.374	0.97	1.344
$N_{1\,1\,Bz\,(2OH)}$ Cl	1	60	38	-32	2.9	0.469	0.77	1.239
C_4C_1pyrr Lactate	19	31	58	-12	24.6	0.755	-0.989	-0.234
C_4C_1pyrr Cl	#N/D	23	49	-21	12.4	0.755	0.77	1.525
C_3C_1im C_3SO_3	12	44	54	-16	27.8	0.898	-0.245	0.653
C_3C_1im C_2SO_3	13	36	61	-9	15.0	0.898	-0.768	0.13
C_3C_1im Cl	20	38	43	-27	12.0	0.898	0.77	1.668
C_3C_1im C_4SO3	35	55	45	-25	3.5	0.898	0.212	1.11
C_4C_1pip Cl	36	45	No transition	No transition	No transition	1.211	0.77	1.981
C_4C_1im $N(CN)_2$	2	78	50	-20	1.0	1.354	0.069	1.423
C_4C_1im C_2SO_3	6	44	53	-17	3.4	1.354	-0.768	0.586
C_4C_1im Cl	10	42	No transition	No transition	No transition	1.354	0.77	2.124
C_4C_1im C_1COO	18	29	58	-12	16.6	1.354	-0.908	0.446
C_6C_1pyrr Cl	#N/D	34	No transition	No transition	No transition	1.667	0.77	2.437
C_5C_1im Cl	20	78	No transition	No transition	No transition	1.81	0.77	2.58
C_6C_1im C_1SO_3	2	69	No transition	No transition	No transition	2.267	-1.117	1.15
C_6C_1im Cl	6	96	No transition	No transition	No transition	2.267	0.77	3.037
C_6C_1im C_2SO_3	19	59	No transition	No transition	No transition	2.267	-0.768	1.499
C_8C_1im Cl	2	96	45	-25	5.9	3.179	0.77	3.949
$N_{1\,1\,4\,8}$ Cl	3	66	No transition	No transition	No transition	3.448	0.77	4.218
$N_{8\,8\,8\,H}$ C_7COO	12	78	48	-22	13.7	8.22	2.04	10.26
$N_{8\,8\,8\,H}$ C_1COO	56	15	36	-34	70.4	8.22	-0.908	7.312
$N_{8\,8\,2\,1}$ C_2SO_4	3	100	41	-29	4.8	5.272	-0.922	4.35
$N_{8\,8\,8\,H}$ $(2\text{-}C_4)C_7COO$	39	28	72	2	55.7	8.22	3.871	12.091
C_4C_1im OTf	2	85	No transition	No transition	No transition	1.354	1.019	2.373

#N/D = not determined

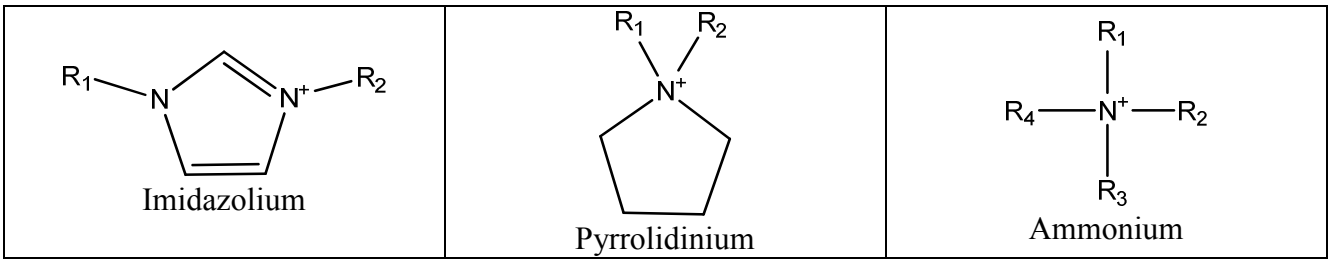

Figure S1 –Main cation structure of ILs

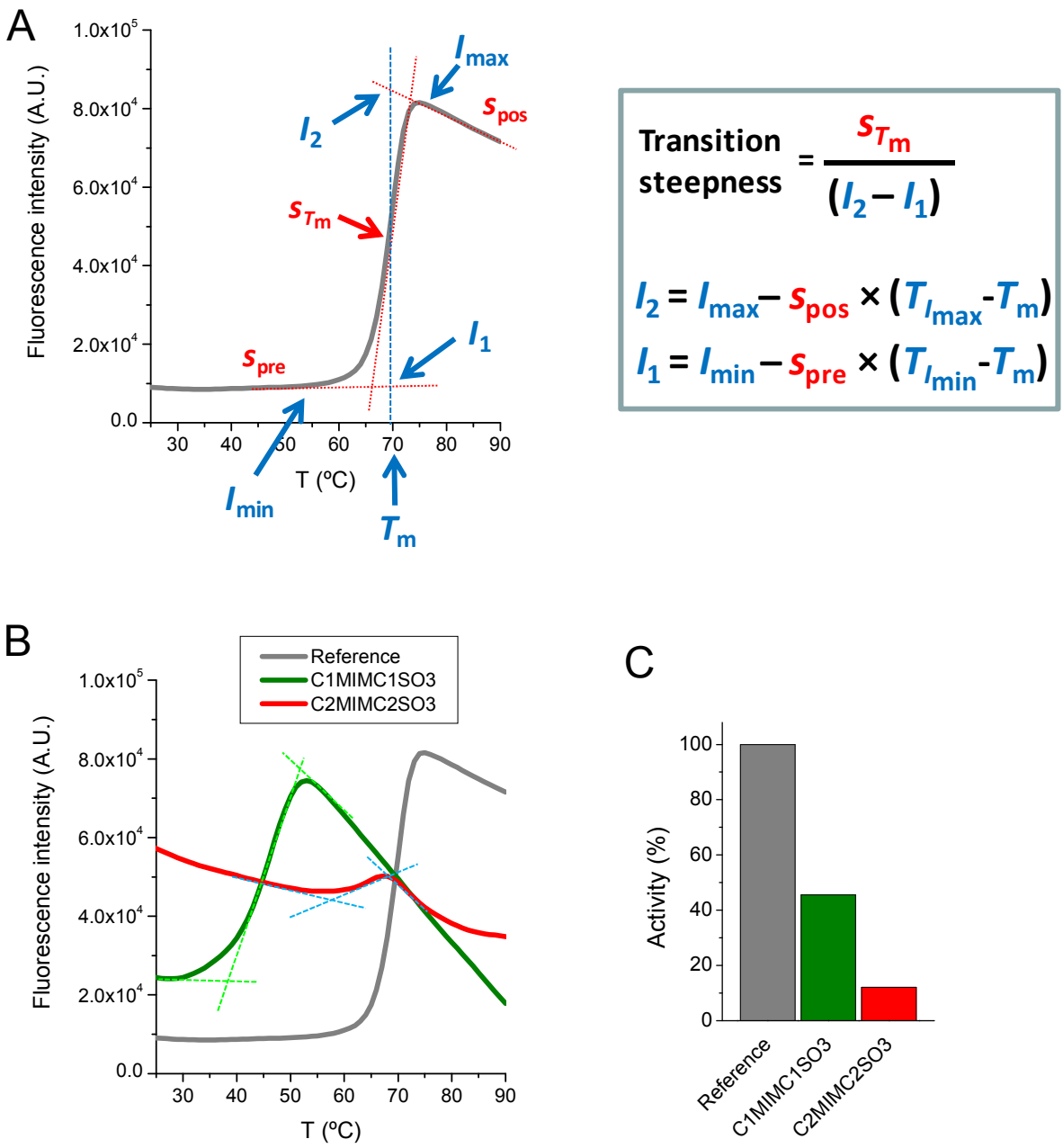

Figure S2 –Examples of DSF curves and parameter determination. A) The melting temperature (T_m) is the temperature at midpoint transition, and the transition steepness is the slope of the intensity change at T_m, normalized by the amplitude of the transition, which is calculated by the difference in maximum and minimum intensities extrapolated through T_m using slopes for the post- and pre-transitions, respectively. B) Different types of curves obtained by DSF using distinct solvents and C) activity data for the corresponding examples.

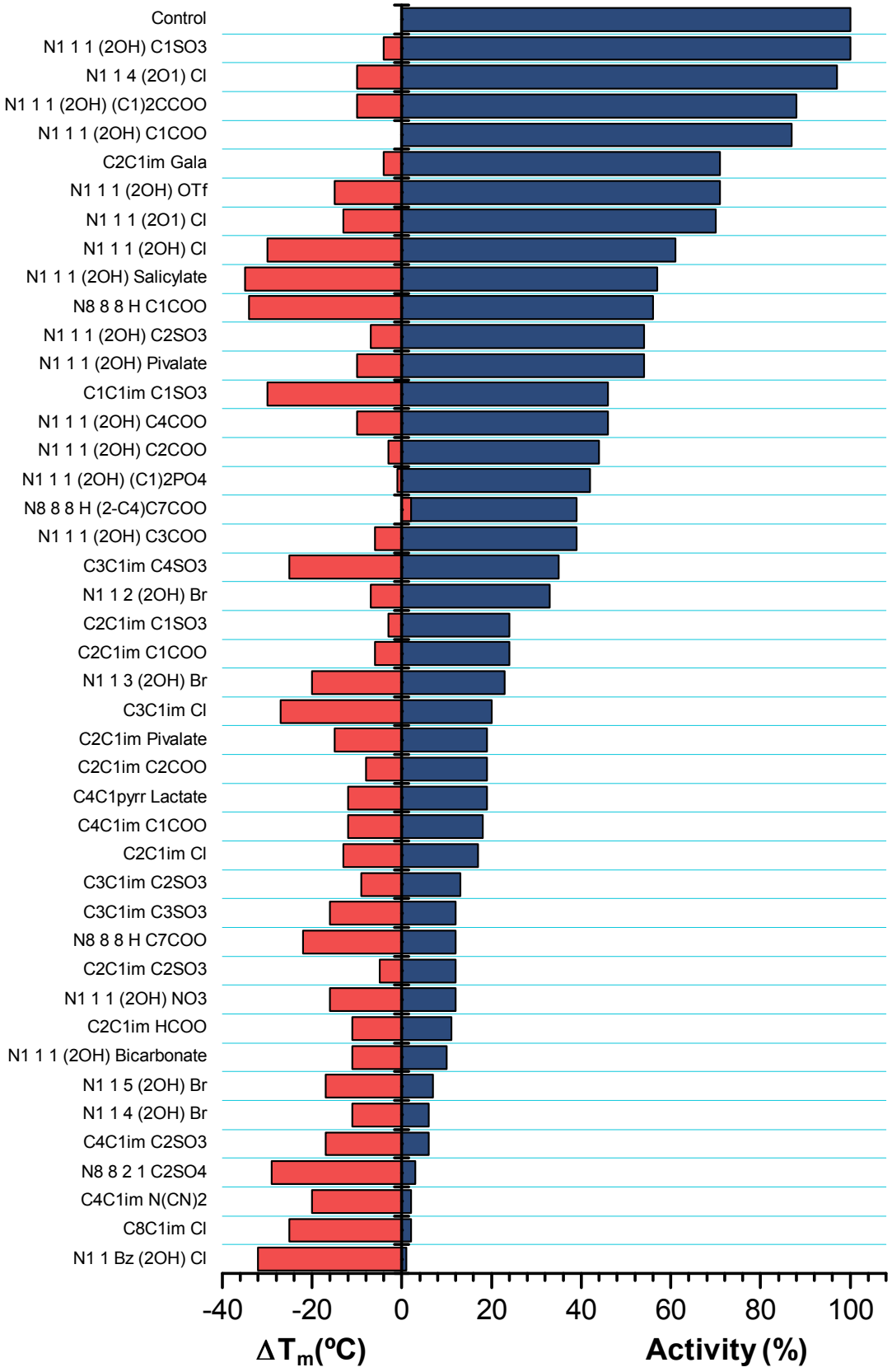

Figure S3 –Comparison between the activity of *Tl*L and the T_m determined by DSF

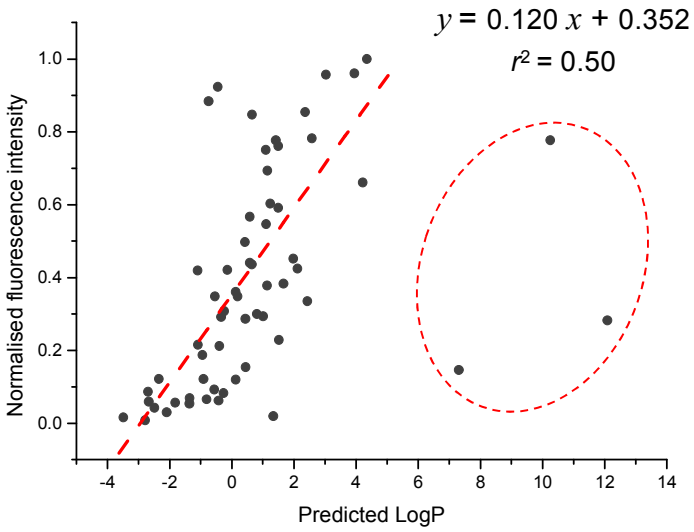

Figure S4 – Effect of the hydrophobic character of IL on the fluorescence intensity of Sypro Orange in the corresponding solvent condition.

Table S2 – List of ionic liquids used in this work and source

Ionic liquids	Source
$(CH_3)_2N_2CNH_2$ $N(CN)_2$	GVSM Carrera, RFM Frade, J Aires de Sousa, CAM Afonso, LC Branco, *Tetrahedron* 2010, **12**, 643
$N_{1\,1\,1\,(2OH)}$ Bitartrate	Sigma Aldrich
$N_{1\,1\,1\,(2OH)}$ H_2Citrate	Sigma Aldrich
$N_{1\,1\,1\,(2OH)}$ Bicarbonate	Sigma Aldrich
$N_{1\,1\,1\,(2OH)}$ NO_3	Iolitec
$N_{1\,1\,1\,(2OH)}$ C_3COO	M Petkovic, JL Ferguson, HQ Nimal Gunaratne, R Ferreira, MC Leitão, KR Seddon, LPN Rebelo, C Silva Pereira, *Green Chem* 2010, **12**, 643
$N_{1\,1\,1\,(2OH)}$ $(C_1)_2PO_4$	Iolitec
$N_{1\,1\,1\,(2OH)}$ C_2COO	M Petkovic, JL Ferguson, HQ Nimal Gunaratne, R Ferreira, MC Leitão, KR Seddon, LPN Rebelo, C Silva Pereira, *Green Chem* 2010, **12**, 643
$N_{1\,1\,1\,(2OH)}$ C_4COO	M Petkovic, JL Ferguson, HQ Nimal Gunaratne, R Ferreira, MC Leitão, KR Seddon, LPN Rebelo, C Silva Pereira, *Green Chem* 2010, **12**, 643
$N_{1\,1\,1\,(2OH)}$ C_2SO_3	A.J.L. Costa, M.R.C. Soromenho, K. Shimizu, J.M.S.S. Esperança, J.N. Canongia Lopes, L.P.N. Rebelo, *EUCHEM* 2012, Wales, UK, August 5-10, 2012
$N_{1\,1\,1\,(2OH)}$ Pivalate	S Shahriari, LC Tomé, JMM Araújo, LPN Rebelo, JAP Coutinho, IM Marrucho, MG Freire, *RSC Adv* 2013, **3**, 183
$N_{1\,1\,1\,(2OH)}$ Salicylate	Sigma Aldrich
$N_{1\,1\,1\,(2OH)}$ Cl	Sigma Aldrich
$N_{1\,1\,1\,(2OH)}$ OTf	Iolitec
$N_{1\,1\,1\,(2OH)}$ C_1COO	M Petkovic, JL Ferguson, HQ Nimal Gunaratne, R Ferreira, MC Leitão, KR Seddon, LPN Rebelo, C Silva Pereira, *Green Chem* 2010, **12**, 643
$N_{1\,1\,1\,(2OH)}$ $(C_1)_2$CCOO	M Petkovic, JL Ferguson, HQ Nimal Gunaratne, R Ferreira, MC Leitão, KR Seddon, LPN Rebelo, C Silva Pereira, *Green Chem* 2010, **12**, 643
$N_{1\,1\,1\,(2OH)}$ C_1SO_3	A.J.L. Costa, M.R.C. Soromenho, K. Shimizu, J.M.S.S. Esperança, J.N. Canongia Lopes, L.P.N. Rebelo, *EUCHEM* 2012, Wales, UK, August 5-10, 2012
$N_{1\,1\,1\,(2OH)}$ C_5COO	M Petkovic, JL Ferguson, HQ Nimal Gunaratne, R Ferreira, MC Leitão, KR Seddon, LPN Rebelo, C Silva Pereira, *Green Chem* 2010, **12**, 643
$N_{1\,1\,1\,(2OH)}$ H_2PO_4	Iolitec
$N_{1\,1\,2\,(2OH)}$ Br	Sigma Aldrich
$N_{1\,1\,1\,(2O1)}$ Cl	AJL Costa, P Papis, PM Reis, K Shimizu, J Szydłowski, JN Canongia Lopes, JMSS Esperança, LPN Rebelo, *COIL-5*, Vilamoura, Portugal, April 21-25, 2013
$N_{1\,1\,3\,(2OH)}$ Br	AJL Costa, MRC Soromenho, K Shimizu, IM Marrucho, JMSS Esperança, JN Canongia Lopes, LPN Rebelo, *ChemPhysChem* 2012, **13**, 1902
$C_{2OH}C_1$im Cl	Iolitec
$N_{1\,1\,3\,(2O1)}$ Cl	AJL Costa, P Papis, PM Reis, K Shimizu, J Szydłowski, JN Canongia Lopes, JMSS Esperança, LPN Rebelo, COIL-5, Vilamoura, Portugal, April 21-25, 2013
$N_{1\,1\,4\,(2OH)}$ Br	AJL Costa, MRC Soromenho, K Shimizu, IM Marrucho, JMSS Esperança, JN Canongia Lopes, LPN Rebelo, ChemPhysChem 2012, 13, 1902
C_1C_1im C_1SO_3	Blesic et al. *Phys Chem Chem Phys* 2009, **11**, 8939
$N_{1\,1\,5\,(2OH)}$ Br	AJL Costa, MRC Soromenho, K Shimizu, IM Marrucho, JMSS Esperança, JN Canongia Lopes, LPN Rebelo, ChemPhysChem 2012, 13, 1902
C_2C_1im $N(CN)_2$	Iolitec
C_2C_1im OTf	Iolitec
C_2C_1im SCN	Iolitec
C_2C_1im HCOO	M Petkovic, JL Ferguson, HQ Nimal Gunaratne, R Ferreira, MC Leitão, KR Seddon, LPN Rebelo, C Silva Pereira, *Green Chem* 2010, **12**, 643
C_2C_1im Br	Iolitec
C_2C_1im C_2SO_3	Blesic et al. *Phys Chem Chem Phys* 2009, **11**, 8939
C_2C_1im Cl	Iolitec
C_2C_1im Pivalate	It was prepared by several of the authors in accordance with the protocol cited in J Blath, N Deubler, T Hirth, T Schiestel, *Chem Eng J* 2012, **181**, 152
C_2C_1im C_2COO	B Zhao, L Greiner, W Leitne, *RSC advances* 2012, **2**, 2476
C_2C_1im C_1COO	Sigma Aldrich
C_2C_1im C_1SO_3	Blesic et al. *Phys Chem Chem Phys* 2009, **11**, 8939
C_2C_1imC_6SO_3	Blesic et al. *Phys Chem Chem Phys* 2009, **11**, 8939
C_2C_1im C_4SO_3	Blesic et al. *Phys Chem Chem Phys* 2009, **11**, 8939
C_2C_1im Galactate	They were prepared by some of the authors in QUILL centre (Queen's University Ionic Liquids Laboratory, Belfast, UK).
C_2C_1im C_2SO_4	Merck
C_2C_1im C_4SO_4	Merck
C_2C_1im C_8SO_4	Merck
$N_{1\,1\,Bz\,(2OH)}$ Cl	Sigma Aldrich
C_4C_1pyrr Lactate	They were prepared by some of the authors in QUILL centre (Queen's University Ionic

	Liquids Laboratory, Belfast, UK).
C_4C_1pyrr OTf	Iolitec
C_4C_1pyrr Cl	Iolitec
C_3C_1im C_3SO_3	Blesic et al. *Phys Chem Chem Phys* 2009, **11**, 8939
C_3C_1im C_1SO_3	Blesic et al. *Phys Chem Chem Phys* 2009, **11**, 8939
C_3C_1im C_2SO_3	Blesic et al. *Phys Chem Chem Phys* 2009, **11**, 8939
C_3C_1im Cl	Iolitec
C_3C_1im C_4SO3	Blesic et al. *Phys Chem Chem Phys* 2009, **11**, 8939
C_2py Cl	Iolitec
C_4C_1pip Cl	Iolitec
C_4C_1im C_1SO_3	Blesic et al. *Phys Chem Chem Phys* 2009, **11**, 8939
C_4C_1im $N(CN)_2$	Iolitec
C_4C_1im C_2SO_3	Blesic et al. *Phys Chem Chem Phys* 2009, **11**, 8939
C_4C_1im BF_4	Iolitec
C_4C_1im Cl	Iolitec
C_4C_1im Alalinate	Ohno H, Fukumoto K, *Acc Chem Res* 2007, **40**, 1122
C_4C_1im C_1COO	Sigma Aldrich
C_6C_1pyrr Cl	Iolitec
C_5C_1im C_1SO_3	Blesic et al. *Phys Chem Chem Phys* 2009, **11**, 8939
C_5C_1im Cl	They were prepared by some of the authors in QUILL centre (Queen's University Ionic Liquids Laboratory, Belfast, UK).
C_4py Cl	Iolitec
C_6C_1im C_1SO_3	Blesic et al. *Phys Chem Chem Phys* 2009, **11**, 8939
C_6C_1im Cl	Iolitec
C_6C_1im C_2SO_3	Blesic et al. *Phys Chem Chem Phys* 2009, **11**, 8939
C_8C_1pyrr Cl	They were prepared by some of the authors in QUILL centre (Queen's University Ionic Liquids Laboratory, Belfast, UK).
C_4C_1py Cl	Iolitec
C_8C_1im Cl	They were prepared by some of the authors in QUILL centre (Queen's University Ionic Liquids Laboratory, Belfast, UK).
N_{1148} Cl	Cheng Chen, Guang Xian Zhang, Feng Xiu Zhang, Hui Zheng, *Adv Materials Res* 2012, **549**, 278
C_6C_1py Cl	Iolitec
C_6C_1py C_1SO_3	AB Pereiro, A Rodríguez, M Blesic, K Shimizu, JNC Lopes, LPN Rebelo, *J Chem Eng Data* 2011, **56**, 4356
C_8C_1py Cl	AB Pereiro, A Rodríguez, M Blesic, K Shimizu, JNC Lopes, LPN Rebelo, *J Chem Eng Data* 2011, **56**, 4356
N_{888H} C_7COO	Bioniqs
N_{888H} C_1COO	Bioniqs
P_{4442} $(C2)_2PO_4$	Cytec
N_{8821} C_2SO_4	Bioniqs
N_{888H} (2-C_4)C_7COO	Bioniqs
C_4C_1im OTf	Iolitec