PCCP

PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/c3cp52845f

Probing the specific ion effects of biocompatible hydrated choline ionic liquids on lactate oxidase biofunctionality in sensor applications

Vincenzo F. Curto,[a] Stefan Scheuermann,[a] Róisín M. Owens,[b] Vijayaraghavan Ranganathan,[c] Douglas R. MacFarlane,[c] Fernando Benito-Lopez*[ad] and Dermot Diamond[a]

Received 8th July 2013,
Accepted 20th November 2013

DOI: 10.1039/c3cp52845f

www.rsc.org/pccp

This paper presents an extended study on the ion effects of a series of biocompatible hydrated choline based ionic liquids (ILs) on lactate oxidase (LOx), an important enzyme in biosensing technology for the *in vitro* detection of lactic acid. Secondary structural analysis revealed changes in the protein conformation in hydrated ILs, while thermal unfolding/aggregation dynamics showed different profiles in the presence or absence of ILs. Moreover, LOx thermally denaturised at 90 °C showed residual activity in the presence of chloride and dihydrogen phosphate anions. Kinetic and lifetime studies were also performed, providing a better understanding of the ion effects of ILs on the biocatalytic activity of the enzyme.

Introduction

Preserving the native state of proteins plays a vital role in maintaining their biofunctionality and activity. Retaining protein functionality over time is a key factor in the pharmaceutical field, as it determines the stability of protein-based drugs.[1] In this regard, a shelf life between 18–24 months is required for economic practicability. At the same time, with the exponential growth of point-of-care technologies, there is an increasing demand for physically and chemically stable enzyme formulations for biosensing, as this determines device lifetime.[2]

In general, irreversible changes to the folded state enzyme conformation can be induced by factors such as temperature, pH and specific ion interactions.[3] Approaches to improve the enzyme stability can be classified into two main categories: (1) the modification of the enzyme state and (2) the optimisation of the solvent environment.[4] The modification of the enzyme state can be achieved in several ways: by isolation of structural variants extracted from natural organisms capable of existing in extreme environments, by rationale-based mutagenesis (bioengineering), by directed evolution of the enzyme or by PEGylation.[5,6] The optimisation of the solvent environment, on the other hand, involves the use of surfactants and additives, changes in pH and/or the ionic strength of aqueous media.[3] Parallel to this, the use of organic (non-aqueous) media in enzymology has been explored in recent years since this can improve enzyme performance in certain cases.[7] Ionic liquids (ILs) have recently emerged as a new class of organic solvents that may have considerable potential for enzyme stabilisation.[8]

ILs are molten salts having melting points below that of water. They have made considerable impact in several research areas, such as catalysis,[9] electrochemistry,[10] biosensing[11] and biochemistry.[12] The properties of ILs depend dramatically on the cation–anion combination and in this sense, properties such as polarity, viscosity, thermal stability and conductivity can be tailored *via* the appropriate cation–anion combination.[13] This is an attractive proposition, since it is possible to design a solvent that has the optimum characteristics to improve, for example, the limited temperature and pH range in which many enzymes are stable when solubilised in water.[8]

A common strategy to improve the stability of proteins is to use a controlled amount of water in the IL (between 20–30% w/w), to produce so-called hydrated ILs (HyILs), which provide the required hydrogen-bonding environment for protein solubilisation.[8] For example, extended stability of cytochrome *C* was observed in hydrated choline dihydrogen phosphate (HyCDHP).[14] Several other studies have reported similar beneficial effects for mushroom tyrosinase,[15] ribonuclease A,[16] interleukin-2[17] and even DNA in hydrated choline ILs.[18] Moreover, ILs based on the choline cation are now commonly used in many applications for which biocompatibility is an essential requirement, *i.e.* cell cultures or *in vivo* applications.[19,20]

[a] *CLARITY: Centre for Sensor Web Technologies, National Centre for Sensor Research, Dublin City University, School of Chemical Sciences, Dublin, Ireland. E-mail: fernando.lopez@dcu.ie*
[b] *Department of Bioelectronics, Ecole Nationale Superieure des Mines, CMP-EMSE, MOC, 880 Rue de Mimet, Gardanne 13541, France*
[c] *School of Chemistry, Monash University, Wellington Road, Clayton, Australia*
[d] *CIC microGUNE, Arrasate-Mondragón, Spain. E-mail: fbenito@cicmicrogune.es; Fax: +34 943739805; Tel: +34 943710237*

The use of ILs in bioelectrochemistry has been summarised in a recent review by Fujita *et al.*,[21] showing how ILs are now widespread in many electrochemical applications, *e.g.* as biofuel cell electrolytes for which more stable matrices are needed. Another growing field for ILs is biosensing, where they are used for the modification of electrodes, acting as a binder for carbon matrix (''Bucky gel'') electrodes or for the immobilisation of bioreceptors.[22] We recently demonstrated the application of ILs as an electrolyte for the development of organic electrochemical transistors (OECTs), for sensing glucose[11] and lactic acid (LA).[23] For the latter, the IL was incorporated into a polymeric matrix (an ionogel) to achieve a solid-state electrolyte.

Lactic acid is an important metabolic analyte involved in many physiological processes[24] and in sports performance.[25] Consequently, there is considerable interest in lactate biosensors, but despite substantial body of research, the relatively poor stability of L-lactate oxidase (LOx) limits the effectiveness of current devices.[26,27] LOx is a flavin mononucleotide (FMN) enzyme that catalyzes the oxidation of L-lactic acid into pyruvate, producing H_2O_2 in the process. LOx from the *Aerococcus viridans* contains two tightly packed tetramers, in which each enzyme monomer holds a non-covalently bound FMN group.[28]

Herein, we investigate the use of hydrated choline cation-based ionic liquids in the stabilisation of lactate oxidase. In particular, this work focuses on the effects of different HyILs on the biofunctionality of LOx, with particular emphasis on the influence of the HyILs on the enzyme secondary structure, and on the biocatalytic activity. The outcome of long-term stability studies of LOx in several HyILs and PBS is also reported.

Materials and methods

Chemicals

Lactate oxidase from *Aerococcus viridans* Grade I (EC 1.13.12.4) for sensor technology was kindly provided by Roche Diagnostics Deutschland GmbH as a lyophilised yellow powder. Choline formate (CF), choline levulinate (CL), choline nitrate (CN), choline tartrate (CT), choline valproate (CV), choline aminoacetate (CAAc) and choline gallate (CG) were synthesised by neutralisation reaction of the selected acidic form of the anion with choline hydroxide, following the procedure described elsewhere.[19,29] Choline dihydrogen phosphate ionic liquid buffer (CDHP/B) was synthesised as described elsewhere.[30] It typically involves the addition of 6.30 g (0.5 mol) of choline hydroxide (20 wt% in water) in a beaker containing 4.02 g of dry CDHP (1 mol), with stirring at room temperature until it becomes a clear solution. The water content (calculated) in this buffer mixture at this stage is 48% and the remaining 52% is ionic liquid. Choline dihydrogen phosphate (CDHP) was purchased from IOLITEC (Denzlingen, Germany). Choline chloride (CCl) was purchased from Sigma-Aldrich® (San Diego, USA). CF, CL and CG are liquids at room temperature while the other ILs are solids under ambient conditions. Horseradish peroxidase (HRP), 4-aminoantipyrine (4APP), hydroxybenzoic acid (HBA), lactic acid, pH phosphate tablets pH 7.4, and monobasic and dibasic potassium phosphate were obtained from Sigma-Aldrich® (San Diego, USA) and used as received.

Preparation of LOx solutions

HyIL solutions for circular dichroism spectroscopy (CD), long-term and residual activity studies were prepared with final water content of 25% w/w, except for CT where a 35% w/w solution was employed. Generally, HyIL–LOx solutions were prepared starting from 20% w/w (30% w/w for CT) water content in HyILs, where the right amount of water with LOx was added in order to reach the desired final water weight percentage and protein concentration. Notice that for CCl and CG it was not possible to obtain similar experimental hydrated conditions (25% or 35% w/w). LOx solutions in the presence of CCl and CG were prepared by dissolving CCl and CG in PBS (pH 7.4; 10 mM) at a final concentration of 1 M. In this case the use of PBS for the CCl and CG solutions was necessary in order to control the pH of the solutions, providing a straightforward comparison of these results with the IL-free solution. Hereafter they will be referred to as CG and CCl solutions. Moreover, LOx–IL-free solution (PBS 7.4; 10mM) was also studied for comparison. PBS is used as a reference system in order to establish variations in the standard behaviour of the LOx enzyme when compared to non-conventional solvents, such as HyILs.

In order to ascertain the effective solubilisation of LOx in the HyIL solutions an UV-vis nanodrop system (Thermo Scientific – NanoDrop 2000c) was employed, following a similar protocol to the one described by Weaver *et al.*[17] These experiments showed a negligible UV absorbance signal in the region of the spectra in which the ILs and the enzyme do not absorb, indicating that LOx was completely solubilised (data not shown).

Circular dichroism spectroscopy

The secondary structure of the enzyme was investigated by circular dichroism spectroscopy in the far-UV region (190–250 nm) using a JASCO CD J-810 spectropolarimeter. CD spectra were recorded using a 0.1 cm path length quartz cuvette loaded with 300 μL of the sample at the final concentration of 0.25 mg ml^{-1} of LOx. Temperature induced denaturation of the protein was performed from 25 °C to 90 °C at the scan rate of 1 °C min^{-1} at 222 nm. Quantitative secondary structure analysis of LOx in solution was performed by fitting the far-UV CD data using the published analysis algorithms CDSSTR (reference set 4) of the online server DICHROWEB.[31,32]

Colorimetric assays

Colorimetric assays for residual activity, kinetic and long-term stability studies were performed using a TECAN Infinite M200 96 well plate reader. All the experiments were run at 25 °C. A red chromogenic dye (λ_{max} = 510 nm) is formed from the following cascade of reactions upon addition of lactic acid:

$$\text{Lactic acid} + O_2 \xrightarrow{\text{LOx}} \text{Pyruvate} + H_2O_2 \quad (1)$$

$$2H_2O_2 + 4\text{AAP} + \text{HBA} \xrightarrow{\text{HRP}} \text{quinoneiminedye} + 4H_2O \quad (2)$$

The composition of the assay was: 0.2 mM of 4AAP, 6 mM of HBA, 0.4 Unit per well (300 μL) of HRP and 50 nM of LOx in PBS at pH 7.0 (0.15 M).

Long-term stability and residual activity. Long-term stability and residual activity studies were performed by adding to the final assay solution only 5 μL of the LOx solution (IL-free solution, CCl, CG and HyILs). The dissolution of a small amount of HyIL–LOx and the relatively high molarity of the PBS of the colorimetric assay guarantee a minimal pH shift from neutrality, which might be caused by the acid/base character of some of the studied ILs. A lactic acid concentration of 10 mM was used to start the cascade of reactions. The long-term stability of the protein was investigated using LOx stock solutions stored at 5 °C and 37 °C, which were periodically tested over a period of 140 days. Residual activity tests upon conditioning of the LOx solutions at 90 °C were similarly performed. Denaturation was induced by incubation of the samples in a thermostatic standard silicone oil bath.

Kinetic studies. To determine the kinetic parameters of LOx, solutions at the concentration of 0.5 M in ILs were employed in all the cases. This IL molarity guarantees the dominance of ion-specific effects on the enzyme rather than electrostatic forces.[33] Moreover, compared to the long-term stability and residual activity studies, a higher HRP concentration was employed (4 units per well) in order to take into account the possible effect of the ILs on the HRP. Under these experimental conditions, the production of hydrogen peroxide from LOx is assumed to be the only limiting step for the cascade of reactions. Furthermore, due to the higher IL concentrations, the pH of the PBS assay was affected and so its variation was recorded using a standard glass membrane pH meter (VWR, sympHony SP70P). Kinetic constants (K_M and V_{max}) were obtained from the data through a nonlinear fitting of the Michaelis–Menten equation using an in-house Excel developed macro.† *Turnover* numbers (k_{cat}) were calculated from V_{max} using the relationship, $k_{cat} = V_{max}/[E]$ where [E] is the molar enzyme concentration, *i.e.* 50 nM.

Results and discussion

Effect of the ionic liquids on LOx structure and conformation

Fig. 1(a) presents the CD spectra at 25 °C (solid line) for lactate oxidase when in IL-free aqueous solution. The spectrum clearly shows the three characteristic peaks at 195 nm, 208 nm and 222 nm originating from the folded secondary structure of the enzyme. However, for CCl, the CD spectrum presents only two of these characteristic peaks at 208 nm and ~220 nm, as shown in Fig. 1(b) (solid line). The high absorbance, due to CCl in solution in the region 200–190 nm, made any quantitative analysis difficult. In the case of the different HyIL–LOx solutions and CG, high absorbance in most of the far-UV region was observed, making any CD spectral recording impractical. Blank experiments using enzyme-free HyILs revealed that the HyILs are responsible for the high absorbance (data not shown).

† Non-linear fitting was performed through minimisation of the sum of the square residuals (SSR) using the *Solver* function.

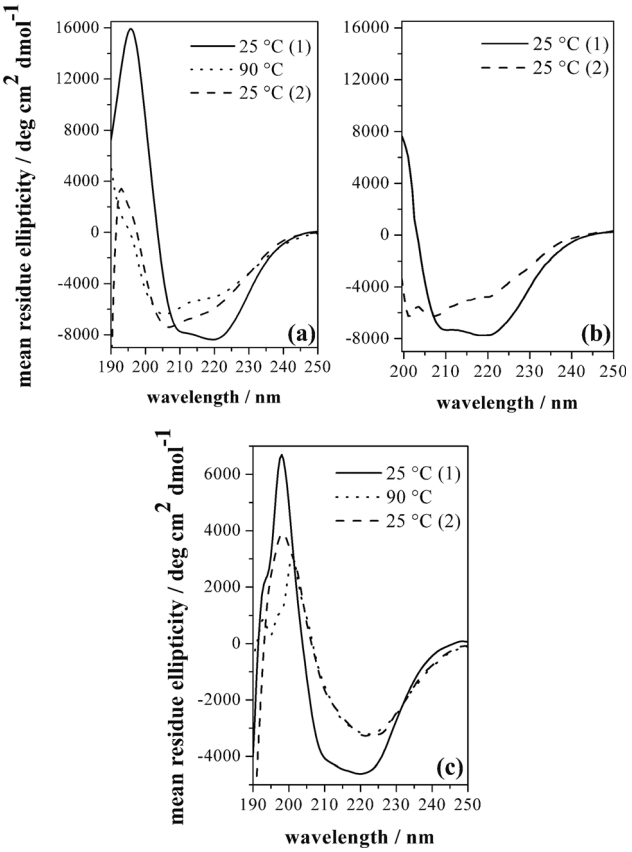

Fig. 1 CD spectra of LOx dissolved in IL-free solution (a), CCl (b) and HyCDHP (c). The spectra were first recorded at 25 °C (1) (—), then thermal denaturation of the LOx was performed at 1 °C min^{-1} till 90 °C, followed by the recording of spectrum at 90 °C (···). Once the sample was cooled down the spectrum at 25 °C (2) (- - -) was recorded. The LOx concentration is 0.25 mg mL^{-1} in all the cases.

Nevertheless, in the case of LOx dissolved in HyCDHP it was possible to record the full spectrum over the entire far-UV region (Fig. 1(c) – solid line), from which useful information about the secondary structure of LOx in HyCDHP can be extracted.‡

Comparing the three spectra in Fig. 1, some differences in the secondary structure of LOx at 25 °C (1) are observed, especially for HyCDHP where a less pronounced peak at 208 nm is visible, which implies that changes are occurring in the secondary structure of LOx. This is verified by the quantitative analysis (Table 1), which shows substantial differences in the α-helix and β-sheet content for HyCDHP compared to IL-free solution. For instance, LOx in HyCDHP presents only 11% of α-helices, compared to 28% in IL-free solution. Conversely, a higher content of β-sheets and turns are observed for LOx when dissolved in HyCDHP. In this case, they represent 34% and 24% of the secondary structure, respectively, suggesting that a change in the conformation of the enzyme in HyCDHP arises due to the different ionic environment.

‡ The less intense CD signal for HyCDHP/LOx is possibly due to the higher refractive index of HyCDHP (RI_{HyCDHP} = 1.49) compared to CCl and IL-free solution.

Table 1 Secondary structure analysis of LOx in IL-free solution and HyCDHP, with reported values in percentage. Quantitative CD analysis was performed for spectra recorded at 25 °C before (1) and after (2) thermal denaturation at 1 °C min^{-1} till 90 °C of the LOx. NRMSD states for normalised root mean square deviation

	25 °C (1)		25 °C (2)	
	IL-free	HyCDHP	IL-free	HyCDHP
α-Helices	28	11	18	8
β-Sheets	23	34	30	37
Turns	20	24	28	25
Unordered	30	30	23	30
NRMSD	0.011	0.087	0.039	0.116

It is known that a stabilising solvent often will produce an increase in the melting point of proteins, which implies that unfolding is less favorable at room temperature.[16,17] Fig. 1(a) and (c) show the CD spectra recorded at 90 °C (no useful data at this temperature were obtained for CCl) compared with equivalent spectra obtained at 25 °C after denaturation, Fig. 1 (dashed lines). At 90 °C, both spectra present less intensive peak signals at around 200 nm, and a substantial wavelength shift for the three peak maxima. Moreover, the peak at ~195 nm in the IL-free spectrum (predominantly given by the α-helices) has significantly reduced at 90 °C. On the other hand, the same peak in the HyCDHP spectrum (Fig. 1c) is still substantially present at 90 °C, implying that recovery of the original structure occurs once the sample is cooled down to 25 °C.

This conclusion is supported by the data in Table 1, where the decrease in the α-helix content of LOx in HyCDHP is only *ca.* 3%, much smaller than the 10% decrease observed in the IL-free sample. In addition, for IL-free samples, the concomitant increment in β-sheets, turns and unordered content that occurs upon thermal denaturation suggests that irreversible aggregation of the enzyme is happening. In fact, high levels of intermolecular β-sheet structures typically indicate aggregation.[3]

The formation of aggregates in the IL-free case is verified by the change in the photomultiplier voltage value (High Tension – HT) of the spectropolarimeter during the thermal denaturation of LOx, Fig. 2(a) (solid line). In fact, Benjwal *et al.*[34] proposed an easy method to monitor real-time aggregation of proteins, by following changes in light scattering, as this correlates with variations in the particle size (aggregates). Alternatively, monitoring the CD-signal at 222 nm can provide information about the unfolding of proteins. Fig. 2(a) shows a sharp transition of the signal (solid black line) starting at 68 °C, with a similar effect occurring at 222 nm (solid light grey line), suggesting that unfolding and aggregation of LOx proceed simultaneously. For CCl, a sharp variation of the MRE (Mean Residual Ellipticity) signal at 222 nm suggests unfolding of LOx but the HT value *versus* temperature does not clearly support aggregation, although it does increase. This behavior can be attributed to aggregation of the unfolded enzyme being less favoured aggregation of the unfolded enzyme due to the presence of CCl. In contrast, both the HT and MRE curves for the HyCDHP/LOx do not present any similar transitions.

The decrease of the MRE signal may suggest that some unfolding of LOx is happening, as is also indicated in Table 1

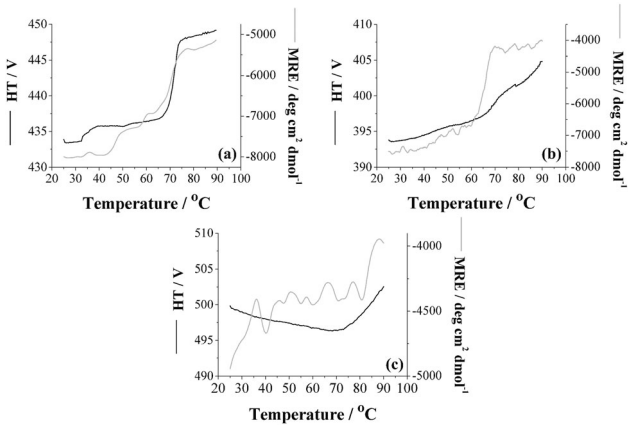

Fig. 2 Thermal denaturation of LOx dissolved in IL-free solution (a), CCl (b) and HyCDHP (c). The (—) HT value and the (—) MRE (Mean Residual Ellipticity) at λ = 222 nm was recorded from 25 °C to 90 °C at 1 °C min^{-1}. LOx concentration is 0.25 mg mL^{-1} in all cases.

from the quantitative analysis of the CD spectra for HyCDHP. With regard to the HT value for HyCDHP (Fig. 2(c)), a less well-defined step increase is observed starting at *ca.* 72 °C, which implies that aggregation of the enzyme is inhibited compared to IL-free and CCl LOx solutions, due to the ionic effect and the greater viscosity of HyCDHP.

The structural analysis does not provide any information on the capability of the enzyme to retain its catalytic activity after thermal denaturation. Therefore, the residual activity was characterised by incubating the LOx solutions at a constant temperature of 90 °C for different times, as explained in the experimental section. Choline valproate, choline aminoacetate and choline gallate were found to be strong denaturating agents for the enzyme. In fact when LOx was dissolved in those HyILs and then tested, no catalytic activity was observed. For this reason CV, CAAc and CG were not studied any further.

In contrast, LOx exhibited catalytic activity when dissolved in HyILs, *i.e.* HyCF, HyCL, HyCN and HyCT. However, upon incubation of these HyIL–LOx solutions at 90 °C, their residual activity was negligible after 5 min. A similar behaviour was also observed for the IL-free–LOx solution. These results indicate that thermal induced denaturation of LOx in those solutions is irreversible, leading to the entire loss of biocatalytic activity. This behaviour is expected for the LOx–IL-free solution, considering the aggregation dynamics shown in Fig. 2(a). Unfortunately, the lack of CD data obtained for the LOx solutions in HyCF, HyCL, HyCN and HyCT do not allow us to predict whether unfolding is occurring, with loss of bioactivity, regardless of enzyme aggregation.

For CCl and HyCDHP the thermal deactivation profiles are quite different. Fig. 3 displays the residual activity of LOx *versus* incubation time, showing how most of the activity loss occurs during the first 5 minutes of incubation (~75%), followed by a progressive decrease, which becomes completely irreversible between 25 and 30 min. These results correlate well with the previous observations on the conformational changes of LOx when dissolved in CCl and HyCDHP. Although enzyme

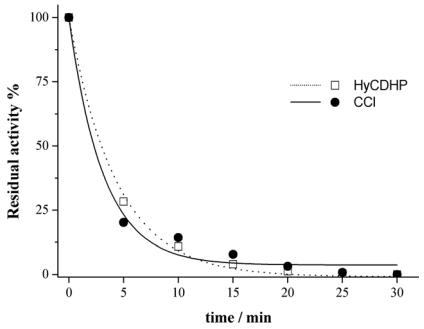

Fig. 3 Residual activity of LOx dissolved in HyCDHP (□) and CCl at 1 M in PBS at pH 7.4 (●) upon thermal denaturation at 90 °C.

unfolding occurs in all cases, it appears that this process is quite reversible when denaturation conditions are applied for a short period of time (5–30 min). This is a clear demonstration that the presence of CDHP and CCl enhances the stability of LOx when it is thermally stressed.

Effect of the ionic liquids on the LOx biofunctionality

In order to obtain more information on the effects of the different ILs on the lactate oxidase function, kinetic characterisation of the enzyme aqueous solutions in the presence of ILs (0.5 M) was carried out. Fig. 4 shows the experimental data points fitted using the Michaelis–Menten non-linear model[35] and Table 2 summarises the calculated kinetic parameters K_M and k_{cat} and the catalytic efficiency given by the ratio k_{cat}/K_M. Moreover, experimental pH measurements of the final assay solutions in the absence and presence of lactic acid are also reported in Table 2. It was found that addition of the IL causes a change in the pH, in some cases producing an increase (*e.g.* CF, pH 7.0 → 7.2) or a decrease (*e.g.* CDHP, pH 7.0 → 6.0) depending on whether the anion behaves as a Brønsted acid (DHP) or base (formate). Addition of lactic acid in the presence of the ILs in all the cases reduces the pH, typically by *ca.* 0.1 pH unit or less.

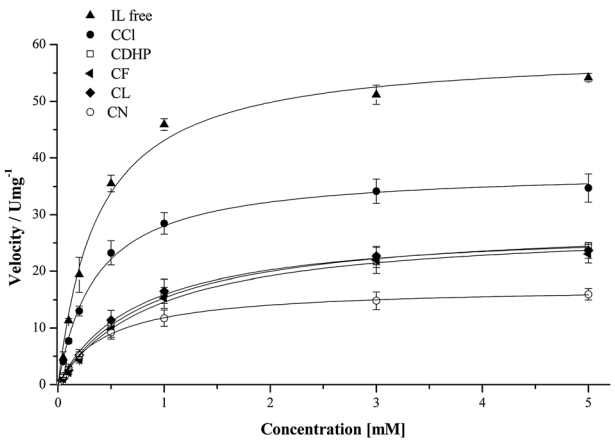

Fig. 4 Initial velocity of LOx *versus* lactic acid concentration (n = 3). CCl and IL concentration in the final assay is equal to 0.5 M. Fitting curves result from exponential Michaelis–Menten non-linear fitting.

Table 2 Kinetic parameters and pH values of LOx solutions in the absence (IL-free) and presence of ILs (concentration 0.5 M)

	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [s^{-1} mM^{-1}]	pH no LA
IL-free	0.370	78.80	210.81	7.0
CCl[a]	0.356	50.66	142.30	6.9
CDHP	0.872	38.26	43.88	6.0
CF	0.926	37.47	40.46	7.2
CL	0.757	37.23	49.18	7.2
CN	0.465	23.10	49.68	6.8

[a] Kinetic parameters in the presence of choline chloride were determined under similar experimental conditions as those of other ILs, with a final concentration equal to 0.5 M.

It should be noted that it was not possible to measure any kinetic parameters for choline tartrate, probably because of the significant degree of dissociation of the dicarboxylic anion, which generates an environment which is too acidic (pH = 3.6) for the enzyme to retain its functionality, as LOx has optimum activity between 6.5 and 7.5; as suggested by the enzyme supplier.

Table 2 presents the results obtained for K_M and k_{cat} values in the various aqueous environment studies. K_M represents the affinity of the enzyme for the substrate, and for a given reaction velocity a low value of K_M indicates a better affinity between the two, whereas k_{cat} represents the *turnover* number of the enzyme (the number of substrate molecules converted into product by the enzyme). The catalytic efficiency (overall effectiveness of the biocatalytic behaviour of the enzyme) is given by the ratio k_{cat}/K_M. Taking the catalytic efficiency first, it is clear that this is significantly reduced (approximately 5 fold) for all ILs compared to IL-free solution (100% water), except for CCl, which is reduced by *ca.* 30%; *i.e.*, the catalytic activity of CCl is about 3 times greater than the other ILs tested. This relates closely to the k_{cat} values, which are always lower for the ILs and CCl compared to IL-free solution. The picture of variation in K_M is little more complex, as the values of IL-free solution and CCl closely match (0.37 mM and 0.36 mM, respectively), for CN it is slightly higher (0.47 mM), while for other ILs they are higher again, in the range 0.8–0.9 mM. This result suggests that in the presence of ILs, the enzyme and substrate interactions are weakened and it is believed that such effect can be mainly attributed to the anions of the tested ILs.

Considering the catalytic efficiency values as the key parameter, the following relationship can be expressed for the influence of the anion on the biocatalytic activity of lactate oxidase in choline-based ILs;

$$Cl^- > nitrate^- > Levulinate^- > DHP^- > Formate^-$$

Since the first observation by Hofmeister, the effect of ions on protein stability has been mostly attributed to their ability to modify the water distribution around and within the protein hydration environment.[36] In order to understand the cause of this effect, the kosmotropic ('structure-making') and chaotropic ('structure-breaking') nature of ions must be considered, as these are important parameters in terms of stabilising or destabilising specific ion effects on proteins. Several studies

have revealed that the best stabilising conditions are normally provided by chaotropic cations and kosmotropic anions.[36–39] Choline is well known as a chaotropic cation for the stabilisation of proteins when coupled with a kosmotropic anion, such as DHP.[37] According to the Hofmeister anion series, DHP is considered a stabilising anion, while nitrate and chloride anions are normally considered as strong and slightly destabilising anions, respectively.[36] No relevant data could be found for the levulinate anion, while several reports suggest that formate ILs can produce positive stabilisation effects,[40,41] although this has not been specifically related to either its stabilising or destabilising nature.

Maeda-Yorita *et al.*[42] described an enhanced stabilisation effect on the bioactivity of LOx from chloride anions, which is broadly in accordance with our findings. However, the results for nitrate and DHP are not so easy to interpret. In fact, in the established anion series, the relative position of DHP compared with nitrate is not in line with previously reported studies.[16,36]

CDHP is known as a slightly acidic IL, due to the acidic character of the DHP anion ($H_2PO_4^-$).[43] This is supported by the pH values reported in Table 2, which show a decrease of the solution pH in the presence of CDHP (pH 6.0 compared to 7.0 for IL-free). The other IL solutions generate a less pronounced pH shift (±0.2 pH units) compared to IL-free solution. Together with the specific ion effects, the pH variation of the solution when adding CDHP could possibly explain the lower catalytic efficiency of lactate oxidase (LOx optimum pH activity is between 6.5 and 7.5).

MacFarlane *et al.*[30] reported the possibility of controlling proton activity in HyILs, obtaining an intrinsic proton buffering behavior of CDHP, denoted here as CDHP/B. Table 3 reports the kinetic parameters when CDHP/B is used in solution instead of CDHP and Fig. 5 shows the kinetic curves for CDHP/B, with CDHP and IL-free solution for comparison. Firstly, the pH increases substantially (~0.80 pH units) when CDHP/B was added to the assay solution, reaching the optimum pH window for LOx; this is due to the inherent equilibrium of the dihydrogen phosphate and mono-hydrogen phosphate groups in CDHP/B. Secondly, K_M decreases and k_{cat} increases in comparison to CDHP, which contributes to a substantial improvement in the catalytic efficiency of LOx, increasing to 130.64 s^{-1} mM^{-1} (*ca.* 3 times greater than CDHP) but still less than IL-free–LOx solution (210.81 s^{-1} mM^{-1}). Considering these results, it would appear that the catalytic behavior of LOx in CDHP is strongly influenced by pH. These results also enable the effect of the choline cation and the DHP anion to be discriminated from that of pH. As LOx is tested at the same pH as the other ILs (7.0 ± 0.2), we can now assert that the specific ion effect of the choline cation and the DHP anion on LOx is analogous to the other ILs, since a similar reduction in the catalytic efficiency of LOx compared to the IL-free solution, with respect to other HyILs, is observed. Following these observations, the previous anion series for LOx can be now amended to:

$$Cl^- > DHP^- > nitrate^- > Levulinate^- > Formate^-$$

Table 3 Kinetic parameters and pH values of LOx in IL-free solution and in the presence of CDHP/B and CDHP at the final concentration of 0.5 M

	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [s^{-1} mM^{-1}]	pH no LA
IL-free	0.370	78.80	210.81	7.0
CDHP/B	0.452	59.05	130.64	6.8
CDHP	0.872	38.26	43.88	6.0

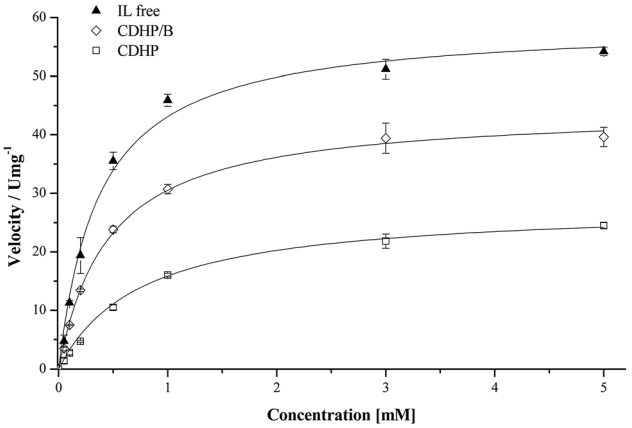

Fig. 5 Initial velocity of LOx *versus* substrate concentration for IL-free solution, CDHP and CDHP/B (n = 3). IL-free solution and CDHP curves are reported from Fig. 4 just for comparison. IL concentration in the final assay is equal to 0.5 M. Fitting curves results of exponential Michaelis–Menten non-linear fitting.

Effect of the ionic liquids on LOx lifetime

Enzyme lifetime/stability is critically important for the development of a practically useful lactate biosensor.[23] In order to test this, the catalytic function of the enzyme in the various ILs was tested and compared to similar experiments performed under IL-free conditions and CCl. The experiments were performed by periodical testing of LOx solutions stored at 5 °C and 37 °C, which correspond to standard storage temperature of home chilling units and average body temperature, respectively. Fig. 6 shows the variation in initial velocity v of LOx over a period of 140 days. The y-axis reports the percentage ratio (*i.e.*, $v/v_0 \times 100$), where v_0 is the initial velocity of the enzyme immediately (day 0) after dissolution in HyILs, CCl or IL-free solutions (pre-incubated at 25 °C for 2 h).

The inset graphs of Fig. 6 shows the trend during the first 21 days at each temperature. In both cases, the fastest loss occurs with HyCL, HyCN, HyCT and HyCF. The decrease is particularly pronounced at 37 °C, with the enzyme activity in these ILs collapsing to almost zero within a few days.

Over a longer time period, enzyme activity was best preserved in CCl, when stored at 5 °C, with over 90% of the original day 0 activity retained for 100 days, dropping to *ca.* 80% after 140 days. It is worth noting that this is significantly better than the results obtained in IL-free solution under the same conditions, which were *ca.* 65% (day 100) and *ca.* 40% (day 140), suggesting a pronounced stabilisation of the enzyme when in the presence of CCl. Next to CCl, the best results at 5 °C were obtained with HyCDHP, for which the performance was very similar to IL-free solution. For the other ILs, the decrease in

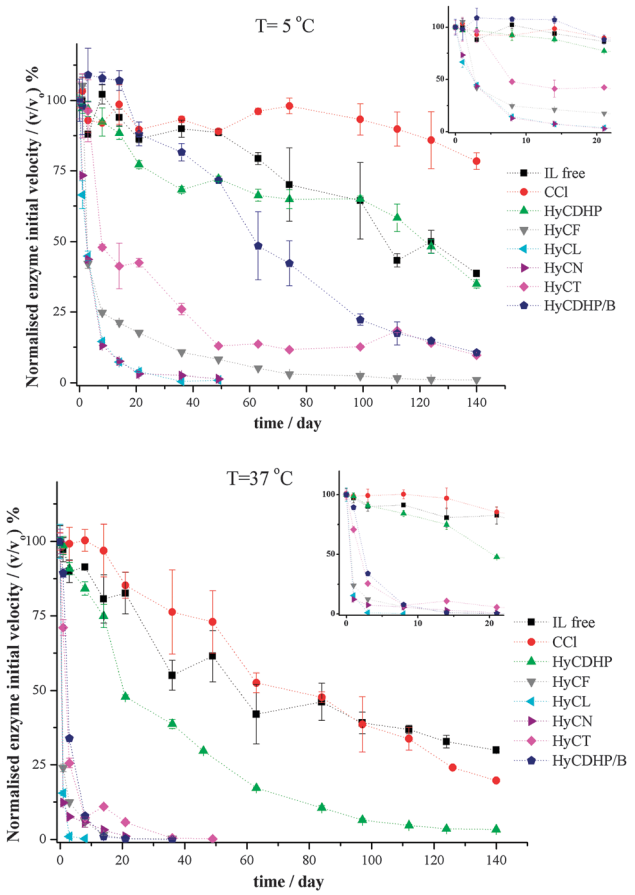

Fig. 6 Long term stability of LOx when stored at 5 °C (top) and 37 °C (bottom) in the HyILs, IL-free and CCl solutions. The normalised enzyme initial velocity on the *y*-axis represents the ratio of the initial velocity *v* of the tested sample to the initial velocity v_0 of the freshly made LOx stock solutions. Inset graphs show the collected data points during the first 21 days of the study (n = 3).

stability was much more marked, the best being HyCDHP/B, in which the LOx was still reasonably active (*ca.* 85%) after 30 days.

At 37 °C, the decrease in enzyme stability was more pronounced, as would be expected because the energetic barrier for the enzymatic deactivation is lower compared to 5 °C. The best performing solution was again CCl, with results very similar to those obtained under IL-free conditions, with both retaining *ca.* 80% of the day 0 activity for 20 days (inset). Next best was HyCDHP (*ca.* 50% day 0 activity after 20 days). Over a longer period, the trend was similar, with CCl ≈ IL-free solution (both retaining *ca.* 25% day 0 activity), followed by CDHP (effectively reaching zero after *ca.* 120 days).

These results confirmed the beneficial effect of CCl for LOx, especially when it is stored at 5 °C with less than 25% loss in activity after the period of test. This result confirms the stabilising effect of CCl observed in the residual activity study (Fig. 3). HyCDHP is also confirmed as the best stabilising of the tested HyILs, although it does not give any better conditions compared to the IL-free solution. For the latter, LOx showed an unexpectedly extended lifetime and further investigation will be necessary to elucidate the cause.

With regard to CCl and HyCDHP, it is worth mentioning here that the chosen storage conditions of the enzyme in these two systems were not identical, since CCl was a 1 molar solution of the salt in PBS while CDHP was used as a hydrated salt with a final water content of 25% w/w. In light of these differences, the resulting long-term stability of LOx would be influenced by the different salt contents. Therefore, the data in Fig. 6 should be interpreted as two sets of results in which the presence of CCl in aqueous solution provides LOx more favorable and stabilising conditions compared to conventional PBS (IL-free). On the other hand, the use of HyCDHP as unconventional solvent for the enzyme did not lead to a better lifetime stability when compared to the IL-free solution, although it provides the most suitable stabilising environment with regard to all the other tested HyILs.

It is also worth noting that, although HyCT cannot be considered to stabilise LOx as well as HyCDHP, at 5 °C an improved stabilisation effect on LOx was observed compared to the HyILs of CL, CN and CF. These results suggested that the specific ion effect of ILs on proteins can be, in general, quite different when in HyILs or in solution at high concentration of ILs, *e.g.* 0.5 M concentration.

For instance, it was not possible to obtain any kinetic parameters in the presence of CT, due to the low pH given by the tartrate anion in solution, as explained before; however, in HyCT lactate oxidase retained about 10% of its initial activity after 140 days, as shown in Fig. 6 at 5 °C. The origin of this result is not particularly clear but it might be attributed to the different water activity (a_w) of the two systems, which can influence the overall effective-pH of solution. In fact with the variation of a_w the dissociation equilibrium of the tartrate anion can shift, with the final result being significant differences in the enzyme environment where it is dissolved. In this regard, in addition to the specific ion effects of CT, a_w/effective-pH can play a crucial role in the preservation of the biofunctionality of the enzyme. Very recent findings from Thompson *et al.*[44] showed for HyCDHP the influence of a_w on the effective-pH in solution, which here was proven to be an important parameter for the retention of the enzyme biofunctionality.

Finally, the stability of LOx when stored in HyCDHP/B (52% w/w ionic liquid) was also investigated. At 37 °C the loss of activity has a similar trend to the one obtained with HyCN, HyCF and HyCL, reaching zero just after 14 days. Nevertheless, at 5 °C, the LOx deactivation during the first 20 days was found to be less pronounced, perhaps indicating the beneficial effect of the choline DHP. Thereafter, a faster loss occurred, retaining only 10% of its initial velocity after 140 days.

Conclusions

This work reports the influence of several biocompatible choline HyILs on the native structure and bioactivity of lacate oxidase, an important enzyme for the realisation of lactate biosensors. The study of the secondary structure of the enzyme showed differences in the α-helix and β-sheet content in HyCDHP; with a marked

decrease of α-helices and increase of β-sheets. However, this does not seem to affect the enzyme activity, which overall is more stable when thermal denaturation is induced. Moreover, irreversible enzyme deactivation is diminished when using HyCDHP and CCl solutions.

The kinetic parameters also revealed a significant IL effect on the catalytic efficiency, which is strongly influenced by the nature of the anion. Interestingly, the acid character of the DHP anion had a negative influence on the biocatalysis of lactic acid, most likely due to the associated increased acidity, a critical parameter for optimising enzyme stability and performance. However, by using an IL ''buffer'', such as CDHP/B, it was possible to study the real ion effects of the choline cation and the DHP anion on the enzyme performance, as this allowed precise control of the pH of the solution.

In addition, long-term stability studies showed a remarkable specific ion effect on the enzyme, especially for choline chloride, for which *ca.* 80% of the initial (day 0) activity is retained after 140 days when stored at 5 °C. Finally, HyCDHP performs as well as IL-free (PBS buffer) solutions in terms of LOx stabilisation. In this regard, future studies will focus on the establishment of a better correlation between the water content in HyILs and the protein lifetime.

Acknowledgements

VFC, SS, FBL, and DD acknowledge Science Foundation Ireland (grant 07/CE/I1147) and the Research Career Start Programme 2010 fellowship from Dublin City University. SS is grateful to the Erasmus placement programme of the European Lifelong Learning Programme (LLP). RMO acknowledges funding from the European Research Council (ERC grant agreement no. 258966). VFC and FBL want to thank Dr. Niamh Gilmartin for her advice during enzyme characterisation. DRM is grateful to the ARC for his Federation Fellowship. The authors of this work are grateful to the MASK, a Marie Curie International Research Staff Exchange Scheme. Thanks to Roche Diagnostics Deutschland GmbH for providing the LOx protein. Thanks also to the Conway Institute (UCD – Dublin) for access to the Circular Dichroism Spectropolarimeter.

Notes and references

1 S. Frokjaer and D. E. Otzen, *Nat. Rev. Drug Discovery*, 2005, **4**, 298–306.
2 T. D. Gibson, *Analysis*, 1999, **27**, 630–638.
3 E. Chi, S. Krishnan, T. Randolph and J. Carpenter, *Pharm. Res.*, 2003, **20**, 1325–1336.
4 H. Weingartner, C. Cabrele and C. Herrmann, *Phys. Chem. Chem. Phys.*, 2012, **14**, 415–426.
5 V. G. H. Eijsink, S. Gaseidnes, T. V. Borchert and B. Van den Burg, *Biomol. Eng.*, 2005, **22**, 21–30.
6 F. M. Veronese, *Biomaterials*, 2001, **22**, 405–417.
7 A. M. Klibanov, *Nature*, 2001, **409**, 241–246.
8 M. Armand, F. Endres, D. R. MacFarlane, H. Ohno and B. Scrosati, *Nat. Mater.*, 2009, **8**, 621–629.
9 T. Welton, *Chem. Rev.*, 1999, **99**, 2071–2083.
10 D. R. MacFarlane, M. Forsyth, P. C. Howlett, J. M. Pringle, J. Sun, G. Annat, W. Neil and E. I. Izgorodina, *Acc. Chem. Res.*, 2007, **40**, 1165–1173.
11 S. Y. Yang, F. Cicoira, R. Byrne, F. Benito-Lopez, D. Diamond, R. M. Owens and G. G. Malliaras, *Chem. Commun.*, 2010, **46**, 7972–7974.
12 N. Jain, A. Kumar, S. Chauhan and S. M. S. Chauhan, *Tetrahedron*, 2005, **61**, 1015–1060.
13 H. Ohno, *Electrochemical Aspects of Ionic Liquid*, John Wiley & Sons Inc., 2005.
14 K. Fujita, M. Forsyth, D. R. MacFarlane, R. W. Reid and G. D. Elliott, *Biotechnol. Bioeng.*, 2006, **94**, 1209–1213.
15 J.-Q. Lai, Z. Li, Y.-H. Lu and Z. Yang, *Green Chem.*, 2011, **13**, 1860–1868.
16 D. Constatinescu, C. Herrmann and H. Weingartner, *Phys. Chem. Chem. Phys.*, 2010, **12**, 1756–1763.
17 K. D. Weaver, R. M. Vrikkis, M. P. Van Vorst, J. Trullinger, R. Vijayaraghavan, D. M. Foureau, I. H. McKillop, D. R. MacFarlane, J. K. Krueger and G. D. Elliott, *Phys. Chem. Chem. Phys.*, 2012, **14**, 790–801.
18 R. Vijayaraghavan, A. Izgorodin, V. Ganesh, M. Surianarayanan and D. R. MacFarlane, *Angew. Chem., Int. Ed.*, 2010, **49**, 1631–1633.
19 R. Vijayaraghavan, B. C. Thompson, D. R. MacFarlane, R. Kumar, M. Surianarayanan, S. Aishwarya and P. K. Sehgal, *Chem. Commun.*, 2010, **46**, 294–296.
20 K. D. Weaver, H. J. Kim, J. Sun, D. R. MacFarlane and G. D. Elliott, *Green Chem.*, 2010, **12**, 507–513.
21 K. Fujita, K. Murata, M. Masuda, N. Nakamura and H. Ohno, *RSC Adv.*, 2012, **2**, 4018–4030.
22 M. Opallo and A. Lesniewski, *J. Electroanal. Chem.*, 2011, **656**, 2–16.
23 D. Khodagholy, V. F. Curto, K. J. Fraser, M. Gurfinkel, R. Byrne, D. Diamond, G. G. Malliaras, F. Benito-Lopez and R. M. Owens, *J. Mater. Chem.*, 2012, **22**, 4440–4443.
24 B. Phypers and J. T. Pierce, *Contin. Educ. Anaesth. Crit. Care Pain*, 2006, **6**, 128–132.
25 J. M. Green, R. C. Pritchett, T. R. Crews, J. R. McLester and D. C. Tucker, *Eur. J. Appl. Physiol.*, 2004, **91**, 1–6.
26 B. Lillis, C. Grogan, H. Berney and W. A. Lane, *Sens. Actuators, B*, 2000, **68**, 109–114.
27 H. Minagawa, Y. Yoshida, N. Kenmochi, M. Furuichi, J. Shimada and H. Kaneko, *Cell. Mol. Life Sci.*, 2007, **64**, 77–81.
28 I. Leiros, E. Wang, T. Rasmussen, E. Oksanen, H. Repo, S. B. Petersen, P. Heikinheimo and E. Hough, *Acta Crystallogr., Sect. F: Struct. Biol. Cryst. Commun.*, 2006, 1185.
29 O. Winther-Jensen, R. Vijayaraghavan, J. Sun, B. Winther-Jensen and D. R. MacFarlane, *Chem. Commun.*, 2009, 3041–3043.
30 D. R. MacFarlane, R. Vijayaraghavan, H. N. Ha, A. Izgorodin, K. D. Weaver and G. D. Elliott, *Chem. Commun.*, 2010, **46**, 7703–7705.
31 L. Whitmore and B. A. Wallace, *Biopolymers*, 2008, **89**, 392–400.

32 L. Whitmore and B. A. Wallace, *Nucleic Acids Res.*, 2004, **32**, W668–W673.
33 S. Weibels, A. Syguda, C. Herrmann and H. Weingartner, *Phys. Chem. Chem. Phys.*, 2012, **14**, 4635–4639.
34 S. Benjwal, S. Verma, K.-H. Röhm and O. Gursky, *Protein Sci.*, 2006, **15**, 635–639.
35 J. M. Berg, J. L. Tymoczko and L. Stryer, *Section 8.4 - The Michaelis-Menten Model Accounts for the Kinetic Properties of Many Enzymes*, W H Freeman, 5th edn, 2002.
36 Z. Yang, *J. Biotechnol.*, 2009, **144**, 12–22.
37 K. Fujita, D. R. MacFarlane, M. Forsyth, M. Yoshizawa-Fujita, K. Murata, N. Nakamura and H. Ohno, *Biomacromolecules*, 2007, **8**, 2080–2086.
38 H. Zhao, *J. Chem. Technol. Biotechnol.*, 2010, **85**, 891–907.
39 H. Zhao, O. Olubajo, Z. Song, A. L. Sims, T. E. Person, R. A. Lawal and L. A. Holley, *Bioorg. Chem.*, 2006, **34**, 15–25.
40 J. P. Mann, A. M c Cluskey and R. Atkin, *Green Chem.*, 2009, **11**, 785–792.
41 D. Hekmat, D. Hebel, S. Joswig, M. Schmidt and D. Weuster-Botz, *Biotechnol. Lett.*, 2007, **29**, 1703–1711.
42 K. Maeda-Yorita, K. Aki, H. Sagai, H. Misaki and V. Massey, *Biochimie*, 1995, **77**, 631–642.
43 D. R. MacFarlane, J. M. Pringle, K. M. Johansson, S. A. Forsyth and M. Forsyth, *Chem. Commun.*, 2006, 1905–1917.
44 B. C. Thompson, O. Winther-Jensen, B. Winther-Jensen and D. R. MacFarlane, *Anal. Chem.*, 2013, **85**, 3521–3525.