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Ionic Liquids as Performance Additives for Electroenzymatic Syntheses

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Abstract: Electroenzymatic syntheses combine oxidoreductase-catalysed reactions with electrochemical reactant supply. The use of ionic liquids as performance additives can contribute to overcoming existing limitations of these syntheses. Here, we report on the influence of different water-miscible ionic liquids on critical parameters such as conductivity, biocatalyst activity and stability or substrate solubility for three typical electroenzymatic syntheses. In these investigations promising ionic liquids were identified and have been used as additives for batch electrolyses on preparative scale for the three electroenzymatic systems. It was possible to improve the space-time-yield for the electrochemical regeneration of NADPH by a factor of three. For an amino acid oxidase catalysed resolution of a methionine racemate with ferrocene-mediated electrochemical regeneration of the enzyme-bound cofactor FAD a 50% increase in space time yield and 140% increase in catalyst utilisation (TTN) were achieved. Furthermore, for the chloroperoxidase-catalysed synthesis of (*R*)-phenylmethylsulfoxide with electrochemical generation of the required cosubstrate H_2O_2 the space time yield and the catalyst utilisation were improved by a factor of up to 4.2 depending on the ionic liquids used.

Keywords: biocatalysis · biotechnology · electroenzymatic synthesis · ionic liquids · sustainable chemistry

Introduction

The combination of catalysed redox reactions with an electrochemical reaction is state of the art for biosensor techniques.[1] However, in synthetic reactions,[2] other methods of supplying redox equivalents are more commonplace due to their often superior productivities.[3] As the productivity of electrochemical reactions is limited by the conductivity of the reaction medium, biocompatible conducting salts could contribute to the solution of this problem. Moreover, biocatalysts used in electroenzymatic synthesis suffer from deactivation at the electrode surface in many cases, leading to insufficient biocatalyst utilisation. Therefore, there is the need for methods that stabilise the catalysts under process conditions in order to make electroenzymatic syntheses competitive. Furthermore, it is also difficult to use electroenzymatic syntheses for the conversion of hardly water soluble substrates, since classical methods such as the addition of an organic co-solvent lead to a decrease in conductivity of the reaction medium, which will result in even lower productivities and biocatalyst utilisations, whereas the use of a second organic phase will complicate the reaction setup tremendously. Thus, co-solvents which do not reduce the conductivity of the reaction medium would be a great advantage for electroenzymatic syntheses.

Ionic liquids (ILs) are promising candidates to overcome all three limitations and improve electroenzymatic systems synergistically. ILs have the following advantages: Firstly; since ILs entirely consist of ions, they have outstanding electrochemical properties.[4] Secondly, it is also demonstrated that they can be advantageous for the performance of biocatalysts.[5] Finally, ILs can act as solubilisers for various substances.[6]

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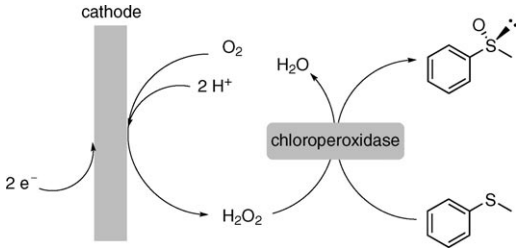
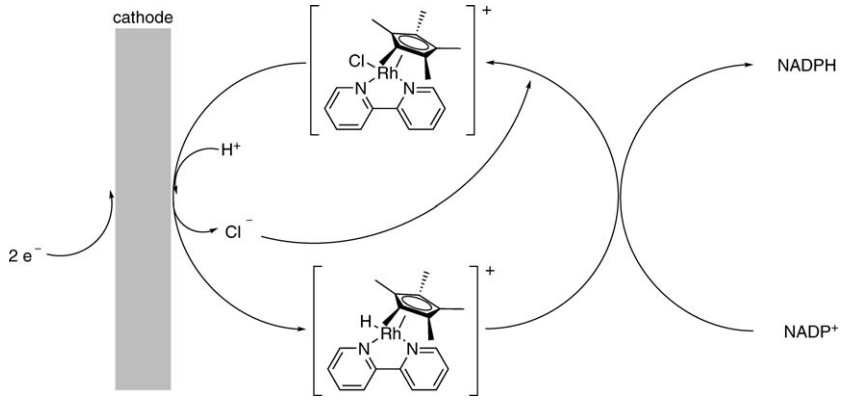
ILs had first been under investigation as electrolytes for electrochemical applications.[7] At present they have caught much attention as alternative reaction media for various catalytic reactions, for example, in biocatalysis.[8] In our work we describe the application of ILs as performance additives in preparative scale electroenzymatic synthesis for the first time.[9] The ILs are not used as pure reaction medium, but rather as additives in volumetric contents of up to 10%. Therefore, the electroenzymatic syntheses were carried out in a homogenous water/IL mixture, which can also be considered as a salt solution containing organic ions. Within the paper the amount of IL is given in volumetric contents (vol%) as this unit is commonly used in biocatalysis. Additionally, diagrams were the results are plotted against the concentration of the IL can be found in the Supporting Information.

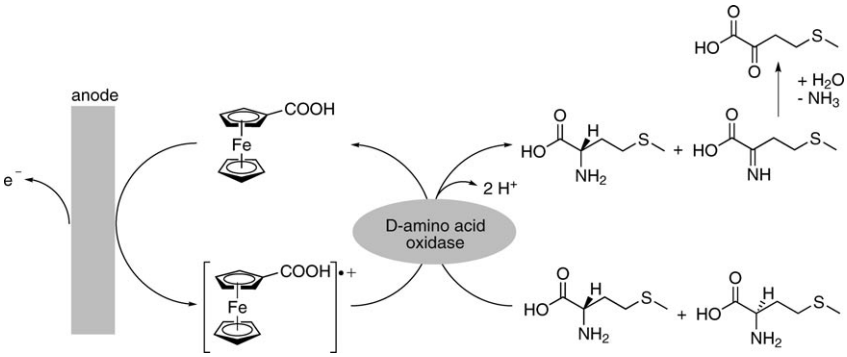
To generally demonstrate the potential of ionic liquids as performance additives three representative electroenzymatic synthesis reactions with different drawbacks were investigated. The first example is the electrochemical generation of NADPH via a rhodium mediator, which can be applied for different NADPH-consuming reactions (Scheme 1).[10] The second example is an amino acid oxidase (D-AAO) catalysed resolution of a methionine racemate with electrochemical regeneration of the enzyme-bound cofactor FAD by ferrocene carboxylic acid (Scheme 2).[11] The third example is the chloroperoxidase (CPO) catalysed synthesis of (*R*)-phenylmethylsulfoxide with electrochemical generation of the required cosubstrate H_2O_2 (Scheme 3).[12] Depending on the reaction, different parameters such as the conductivity of the reaction medium, the activity and the stability of the biocatalyst or the substrate solubility, which can be influenced by IL were investigated in presence of various IL (see Table 1). From those experiments promising ILs were selected and used as performance additive for the corresponding electroenzymatic synthesis.



Scheme 3. CPO-catalysed synthesis of (*R*)-phenylmethylsulfoxide with electrochemical generation of the required cosubstrate H_2O_2.



Scheme 1. Electrochemical generation of NADPH via a rhodium mediator.



Scheme 2. D-AAO-catalysed resolution of a methionine racemate with electrochemical regeneration of the enzyme-bound cofactor FAD by ferrocene carboxylic acid.

Results and Discussion

Electrochemical generation of NADPH: The effective regeneration of NADPH represents one of the major tasks for various oxidoreductase-catalysed reductions. Beside the common enzyme- or substrate-coupled approaches,[13] electrochemical regeneration via a suitable redox mediator presents an interesting alternative, but is often significantly slower than the other approaches. Thus, for this reaction system we tried to achieve higher productivities by increasing the conductivity of the reaction media through addition of IL. The application of other conventional inorganic conducting salts is not favoured, since high concentrations of various salts destabilise the cofactor.[14] For this reason, not only the influence of different ILs on the conductivity of the reaction medium, but also on the stability of the cofactors NADPH and NADP$^+$ were investigated.

Table 1. Investigated cations and anions.

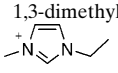
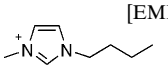
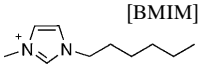
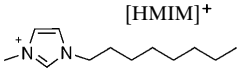
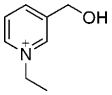
Cations	Anions
 1,3-dimethylimidazolium $[MMIM]^+$	methylsulfate $[MeSO_4]^-$
 1-ethyl-3-methylimidazolium $[EMIM]^+$	ethylsulfate $[EtSO_4]^-$
 1-butyl-3-methylimidazolium $[BMIM]^+$	dimethylphosphate $[Me_2PO_4]^-$
 1-hexyl-3-methylimidazolium $[HMIM]^+$	diethylphosphate $[Et_2PO_4]^-$
 1-methyl-3-octylimidazolium $[OMIM]^+$	2-(2-methoxyethoxy)ethysulfate $[MDEGSO_4]^-$
 1-ethyl-3-methyl-pyridinium $[EMPY]^+$	tetrafluoroborate $[BF_4]^-$
 1-ethyl-3-hydroxymethylpyridinium $[EM(OH)PY]^+$	bromide $[Br]^-$

Figure 1 summarises half lives of NADPH in presence of 2 to 10 vol% contents of different ILs. From these results it is obvious, that in contrast to a lot of inorganic salts, ILs do not have a destabilising effect on NADPH. On the contrary, they are able to stabilise the reduced cofactor.[15]

For each of the IL the stabilising effect increases with increasing amount of IL. Best results were obtained with $[EMPY][EtSO_4]$. With this IL an addition of only 10 vol% leads to an almost tripled half life. $[BMIM][MDEGSO_4]$ showed the lowest potential for cofactor stabilisation, but with 10 vol% still an improvement by the factor of 1.5 was possible. The stability of $NADP^+$ is much higher than the stability of NADPH under the chosen reaction conditions and hence it is not limiting the reaction. Nevertheless, we also investigated the influence of the same IL on the half lives of the oxidised cofactor. Within a measuring time of one week, only minor differences which are in the range of experimental error have been detected. The ILs used seem not to have an effect on the stability of the oxidised cofactor.

Figure 2 depicts the results of the conductivity measurements for the same IL. As expected all ILs have a positive influence on the conductivity of the reaction medium. The conductivities achieved rise with increasing amount of IL. The addition of $[MMIM][Me_2PO_4]$ led to the highest improvement. With 10 vol% an increase by a factor of 3.9 was possible.

Furthermore, experiments concerning the activity and stability of two different alcohol dehydrogenase, which could possibly be used in combination with the electrochemical regeneration of NADPH were conducted. The results are given in the Supporting Information. Since the coupling of the enzymatic and the electrochemical reaction faces stability issues, the process improvements from the IL additive would be overcompensated by enzyme inactivation from the mediator in this setup. Therefore, the preparative electrochemical generation of NADPH was not coupled with an enzymatic reaction.[16]

As the most promising candidate $[EMPY][EtSO_4]$ was chosen for the electrochemical reaction. Preparative scale syntheses of NADPH were carried out with the addition of different amounts of this IL as well as in pure buffer. The results of these syntheses are summarised in Figure 3.

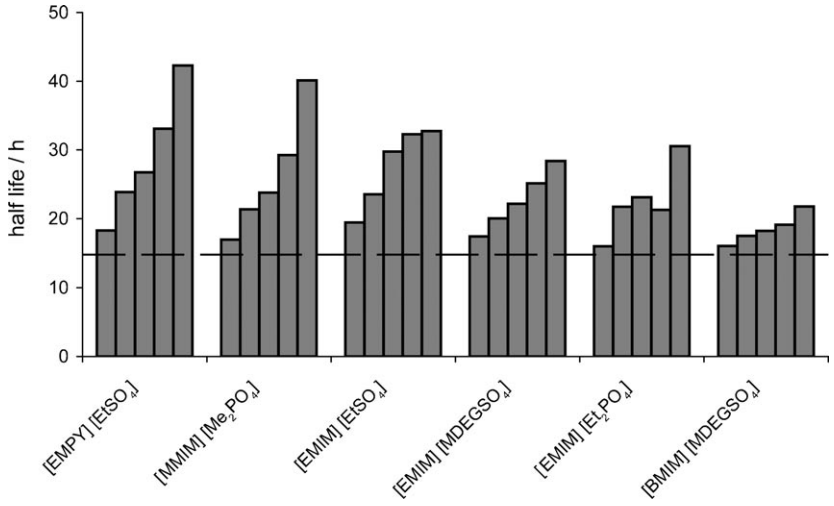


Figure 1. Half life of NADPH in the presence of different ILs. For each IL 2, 4, 6, 8 and 10 vol% of IL were measured and grouped together (from left to right). The dashed line represents the half life in pure buffer (~14.4 h).

The addition of $[EMPY][EtSO_4]$ to the reaction medium significantly increases the amount of $NADP^+$ reduced per time and the turnover frequency (TOF = number of regeneration cycles within a defined time frame) of the used rhodium mediator. Batch experiments in pure buffer led to a space time yield (STY = amount of product per reaction volume and time) of 27 $mmol\,L^{-1}\,d^{-1}$ and a TOF_{Rhbpy} of 62 h^{-1} while with 10 vol% of $[EMPY][EtSO_4]$ the STY and the TOF_{Rhbpy} was nearly tripled. The STY and the TOF_{Rhbpy} increased linearly with increasing amount of IL, which is a clear evidence for

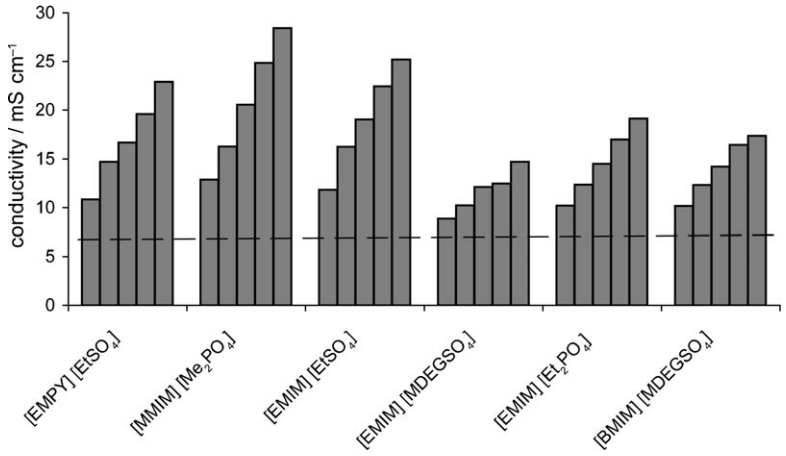


Figure 2. Influence of different ILs on the conductivity of a 50 mmol L^{-1} phosphate buffer pH 7. For each IL an addition of 2, 4, 6, 8 and 10 vol% of IL was investigated and these results are grouped together (from left to right). The dashed line represents the conductivity of the pure buffer (~7.3 mS cm^{-1}).

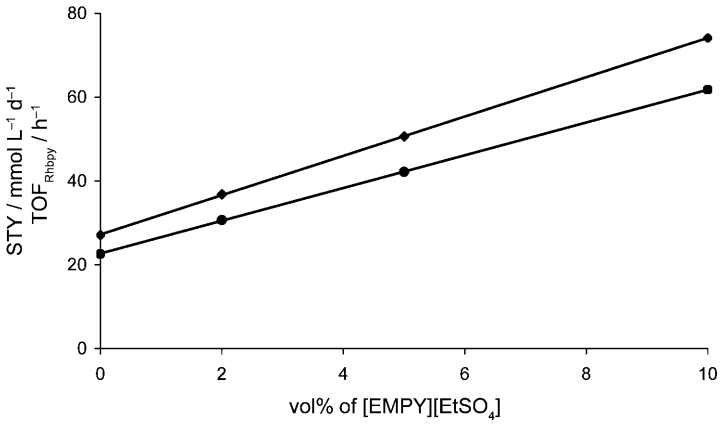


Figure 3. Influence of [EMPY][EtSO$_4$] on the STY (◆) and TOF$_{Rhbpy}$ (●) of NADPH production.

the limitation of this reaction by the conductivity of the reaction medium. Increasing the conductivity by using higher concentration of the buffer salt could result in reduced cofactor stabilities. This underscores, that ILs are synergistic performance additives. However, the obtained productivities are still not high enough to compete with other established cofactor regeneration methods.[13]

D-AAO-catalysed resolution of a methionine racemate with electrochemical regeneration of FAD: FAD-dependent enzymes (e.g., oxidases) can be regenerated by molecular oxygen. However, H_2O_2 is formed as a by-product of this reaction, which deactivates the enzyme (for the influence of H_2O_2 on the used D-AAO, see Supporting Information). Typically, catalase is added to degrade the peroxide produced. However, in some cases the stability of the flavo enzyme is still limited.[17] Also, the reaction medium has to meet the conditions for both enzymes; additionally, product separation or catalyst recycling might be more difficult. Electrochemical cofactor regeneration via a ferrocene mediator represents an anaerobic approach, which avoids the formation of H_2O_2. However, during the reaction deactivation of the enzyme at the electrode surface occurs. Consequently, to improve this reaction system there is need for a method to stabilise the biocatalyst, as well as an appropriate conducting salt to achieve higher productivities. Again, these limitations were overcome by using ILs as performance additives.

First, the influences of different ILs on the activity of D-AAO were investigated. As indicated by Figure 4, the enzyme was sensitive to these additives; only in three ILs an activity higher than 75% was observed in the presence of only 2 vol% IL. Best results were obtained with IL-containing phosphorylated anions. These results are in agreement with investigations on the choice of the buffer medium; in phosphate buffer the highest activities have been measured (data not shown). In contrast to the results obtained by Lutz–Wahl and co-workers,[18] who reported an increased activity for isolated AAO in presence of 20 vol% of [MMIM]-[Me_2PO_4] no improvement was measured.

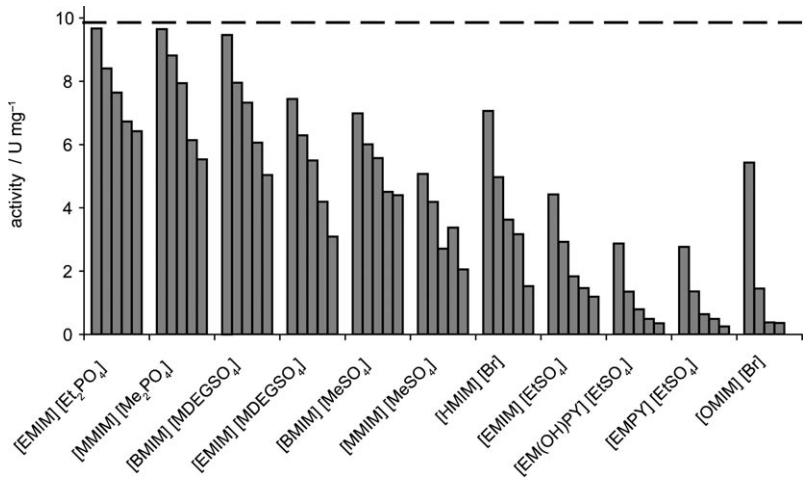


Figure 4. Relative activity of D-AAO in presence of different ILs compared to the activity in pure buffer. For each IL addition of 2, 4, 6, 8 and 10 vol% were measured and grouped together (from left to right). The dashed line represents the activity in pure buffer (~9.9 U mg^{-1}).

For those six ILs which showed highest remaining activities also stability investigations were carried out; results of these experiments are presented in Figure 5. A clear destabilisation was seen for both $[MDEGSO_4]^-$ IL; even for only 4 vol% the half life was much lower than in pure buffer and with higher amounts of IL the value decreased further. In presence of increased amounts of [BMIM][$MeSO_4$] the stability decreased, too. However the half life reached was higher than for the $[MDEGSO_4]^-$ IL. For [MMIM][$MeSO_4$], [EMIM][Et_2PO_4] and [MMIM][Me_2PO_4] a contrary trend was observed; the stability increased with increased amount of IL. Generally, the enzyme was more stable in these ILs than in pure buffer.

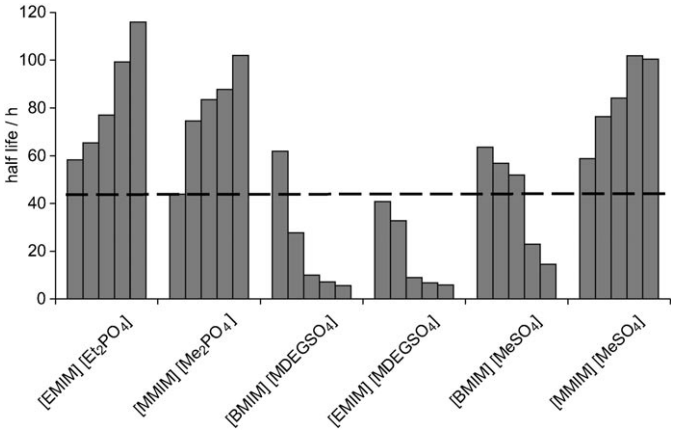


Figure 5. Half life of D-AAO in the presence of different ILs. For each IL 2, 4, 6, 8 and 10 vol% of IL were measured and grouped together (from left to right). The dashed line represents the half life in pure buffer (~45 h).

Since it was only possible to obtain a sufficient compromise for activity and stability with [EMIM][Et_2PO_4] and [MMIM][Me_2PO_4] conductivity measurements were carried out for these two ILs. Figure 6 summarises the findings. It can clearly be seen, that [MMIM][Me_2PO_4] has a stronger influence on the conductivity. With addition of 10 vol% the conductivity was almost doubled.

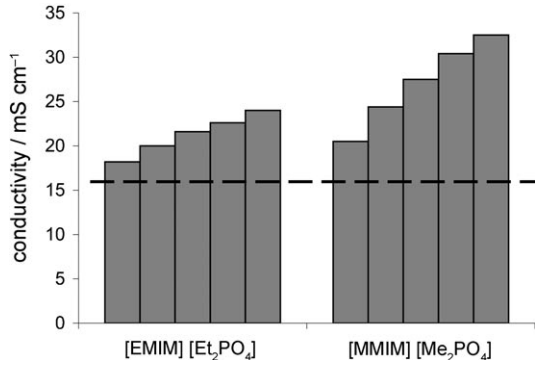


Figure 6. Influence of two ILs on the conductivity of a 100 mmol L^{-1} phosphate buffer pH 8. For each IL an addition of 2, 4, 6, 8 and 10 vol% of IL were investigated and these results are grouped together (from left to right). The dashed line represents the conductivity of the pure buffer (~16.2 mS cm^{-1}).

Therefore it was obvious that [MMIM][Me_2PO_4] showed the best compromise in terms of enzyme performance and conductivity. Thus, batch electrolyses were carried out in presence of different amounts of this IL and compared with the results obtained in pure buffer solution (see Figure 7).

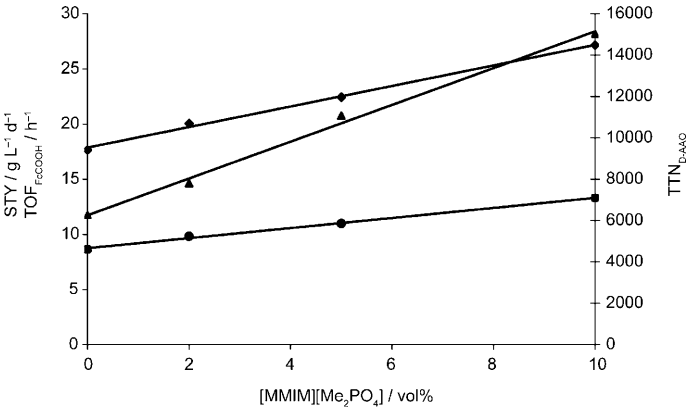


Figure 7. Influence of [MMIM][Me_2PO_4] on STY (◆), TOF_{FcCOOH} (●) and $TTN_{D\text{-}AAO}$ (▲) during D-AAO-catalysed resolution of a methionine racemate with electrochemical regeneration of FAD by ferrocene carboxylic acid.

In pure buffer the STY was approximately 18 g L^{-1} d^{-1} and the TOF_{FcCOOH} for the ferrocene carboxylic acid was 8.6 h^{-1}, whereas with 10 vol% [MMIM][Me_2PO_4] a 1.5-fold increase of these numbers was found. The selectivity of the enzyme was not influenced by the IL; D-methionine was converted quantitatively resulting in an optical purity of $ee > 99.99\%$ for the L product. During the experiments improved TTNs (TTN = produced amount of product in mol per mol biocatalyst) for the biocatalyst were observed with increasing amounts of IL. With an addition of 10 vol% an increase of $TTN_{D\text{-}AAO}$ by a factor of 2.4 was possible. These findings are in line with the experiments showing that the enzyme was also more stable during incubation in the presence of increasing amount of this IL. Therefore, [MMIM][Me_2PO_4] does not only improve the storage stability of the enzyme, but is also able to stabilise D-AAO under process conditions. Thus, ILs can act as conducting salts and biocatalyst stabilisers, although the electroenzymatic approach is not yet competitive to the aerobic regeneration in terms of STY and TTN.

CPO-catalysed synthesis of (*R*)-phenylmethylsulfoxide with electrochemical generation of H_2O_2: Even if the CPO requires H_2O_2 to be able to catalyse various reactions, high concentrations of the co-substrate have to be avoided, since CPO is both inhibited and deactivated by H_2O_2. The electrochemical reduction of O_2 is a promising approach to gently introduce the co-substrate, because H_2O_2 is dosed via a relatively large surface, thus avoiding highly concentrated oxidant.[12] However, the poor solubility of attractive substrates

limits the performance of the synthesis. The application of an organic co-solvent as solubiliser for the substrate showed a drastic reduction of the conductivity of the reaction medium and was harmful to the CPO at the same time.[19] The solubility of the substrate is crucial, since CPO (compound I) is deactivated rapidly in the absence of substrate.[20] For these reasons, there is need for a method to stabilise the biocatalyst by adding an appropriate solubiliser for the substrate and a conducting salt to improve this reaction system. Once more, these limitations are overcome by using ILs as performance additives.

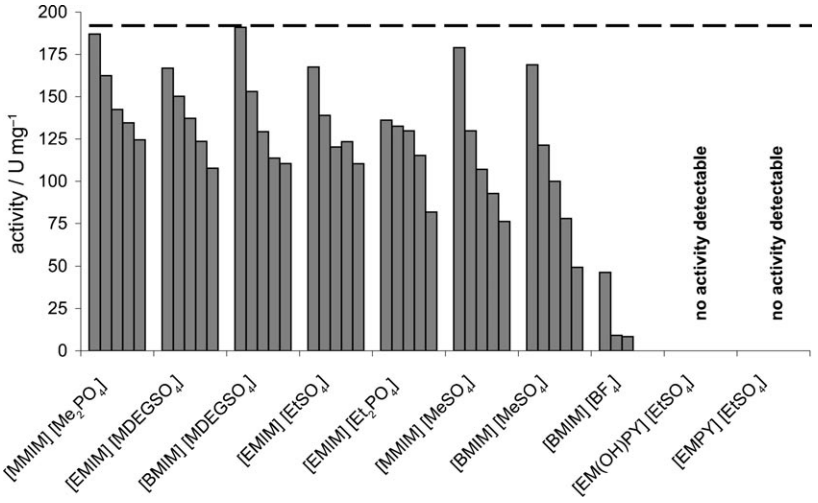


Figure 8. Relative activity of CPO in the presence of different ILs compared to the activity in pure buffer. For each IL 2, 4, 6, 8 and 10 vol% of IL were measured and grouped together (from left to right). The dashed line represents the activity in pure buffer (~191 U mg^{-1}).

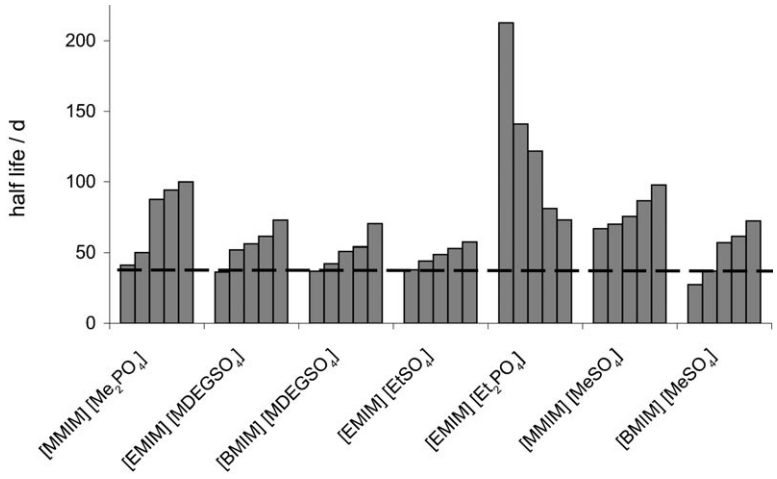


Figure 9. Half life of CPO in the presence of different ILs. For each IL 2, 4, 6, 8 and 10 vol% of IL were measured and grouped together (from left to right). The dashed line represents the half life in pure buffer (~45 d).

In the beginning the influence of ten different ILs on the activity of the CPO was investigated. The results of these experiments are summarised in Figure 8. As for the D-AAO all ILs tested had a negative influence on the activity of the CPO. With increasing amount of IL the activity decreases. Moreover, total inactivation of the CPO occurred in presence of pyridine cations as well as a very drastic drop in activity for [BMIM][BF_4]. Investigations on the performance of CPO-catalysing sulfoxidations in the presence of different ILs have also been carried out by Chiappe et al.[21] In contrast to their work, we found that the enzyme was active in presence of [MMIM][$MeSO_4$]. One of the reasons explaining this fact can be that they investigated higher contents of IL. However, Sanfilippo et al. reported a tolerance of up to 30 vol% [MMIM][$MeSO_4$] for the CPO catalysing the oxidation of 1,2-dihydronaphthalene.[22] The main reason might be that Chiappe et al.[23] did not always adjust pH values of the IL/buffer mixture. In accordance to their work we also found, that the enzyme tolerates higher amounts of IL while catalysing the sulfoxidation of thioanisole than during chlorination reactions (data not shown).

Since seven of the tested ILs showed moderate CPO activities, stability investigations were carried out for these ILs. All results from these measurements are illustrated in Figure 9. The stability of the CPO was increased with increased amounts of IL for all investigated additives, only for [EMIM][Et_2PO_4] the contrary effect was observed, nevertheless even with small amounts of [EMIM][Et_2PO_4] a drastic increase in half life was measured. With more than 4 vol% of IL the half life of CPO was higher than in pure buffer for all additives.

Since all ILs showed good results for CPO stability, also conductivity and solubility measurements were carried out for these ILs. Figure 10 summarises the results from the conductivity measurements. As expected, all ILs had a positive influence on the conductivity. With increasing amount of IL the conductivity rises. Best results were obtained with [MMIM][$MeSO_4$]; the addition of 10 vol% of this IL increased the conductivity by the factor of 7.

Moreover, all ILs had a positive effect on the solubility of the substrate thioanisole (see Figure 11). The addition of merely 5 vol% IL led to an increase of at least 40% for

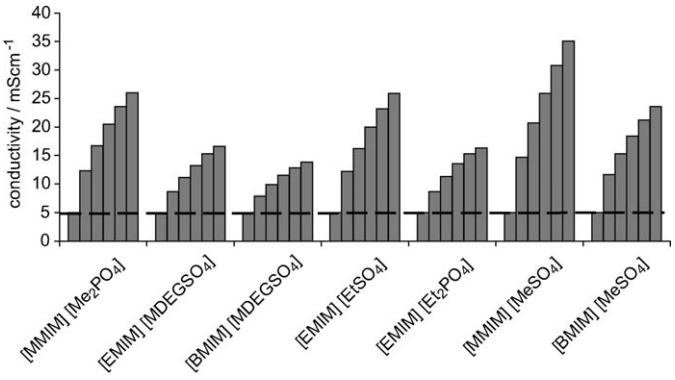


Figure 10. Influence of different ILs on the conductivity of a 100 mmol L^{-1} acetate buffer pH 5. For each IL an addition of 2, 4, 6, 8 and 10 vol% of IL were investigated and these results are grouped together (from left to right). The dashed line represents the conductivity of the pure buffer (~5.0 mS cm^{-1}).

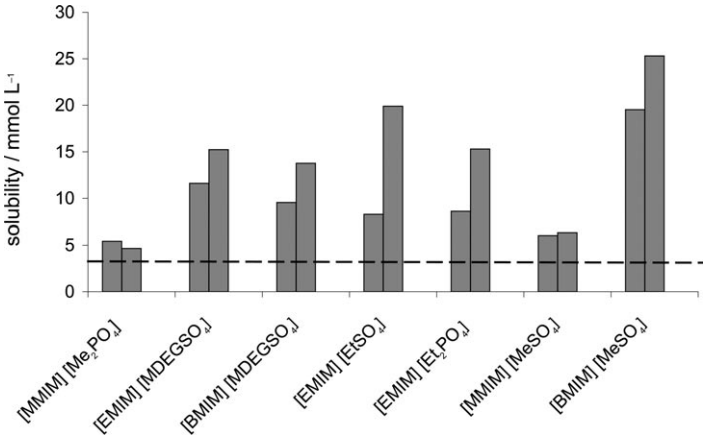


Figure 11. Influence of different ILs on the solubility of thioanisole in 100 mmol L^{-1} acetate buffer pH 5. For each IL an addition 5 and 10 vol% of IL were investigated and these results are grouped together (from left to right). The dashed line represents the solubility in pure buffer (~3.8 mmol L^{-1}).

[MMIM][Me_2PO_4]. Especially, [BMIM][$MeSO_4$] was able to improve the solubility; 5 vol% of this IL led to an improvement by a factor of 5.

Taking all investigations into account, ILs are appropriate performance additives for the CPO-catalysed oxidation of thioanisole with electrochemical generation of the required cosubstrate H_2O_2. Even if the chosen IL had a negative influence on the activity of the enzyme, some of them are able to stabilise it tremendously. Furthermore, the IL can act as solubilisers for thioanisole and have a positive influence on the conductivity of the reaction medium at the same time. Since the different ILs had different advantages, it was not possible to select one IL which was superior. Therefore electroenzymatic syntheses with an addition of 2 vol% IL have been carried out for five of these ILs.

Figure 12 summarises the results for the syntheses in pure buffer and with the different ILs. Depending on the IL an improved STY of at least 35 g L^{-1} d^{-1} for [MMIM][Me_2PO_4]

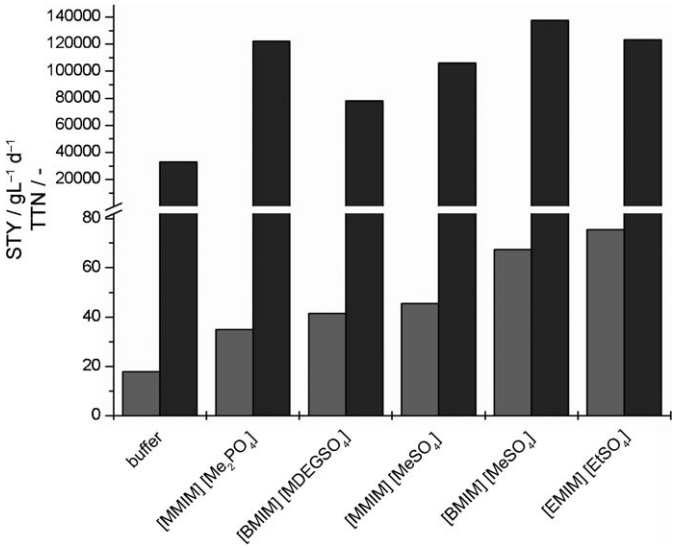


Figure 12. Influence of different ILs on the STY (light grey) and TTN (dark grey) of CPO-catalysed syntheses of (*R*)-phenylmethylsulfoxide with the electrochemical generation of H_2O_2.

and a maximum of 75 g L^{-1} d^{-1} for [EMIM][$EtSO_4$] were observed. In pure buffer, only a STY of 18 g L^{-1} d^{-1} could be achieved. Also the catalyst utilisation was improved; in buffer only a TTN_{CPO} of 3300 was observed, while in the presence of IL a minimum TTN of 78000 for [BMIM][$MDEGSO_4$] and a maximal TTN of 138000 for [BMIM][$MeSO_4$] was possible. This compares well with the maximal TTN for CPO reported for other H_2O_2 supply systems.[24]

The influence on STY and TTN strongly depends on the IL, since each IL leads to a different compromise between enzyme performance, substrate solubility and conductivity. While comparing the results from the syntheses with the investigations concerning enzyme performance, conductivity and solubility, it becomes obvious, that especially the solubility of thioanisole influences the reactions. For example, these investigations showed relatively good results concerning enzyme performance and conductivity, but only little effect on the substrate solubility for [MMIM][Me_2PO_4]. Applying this IL as performance additive for the electroenzymatic synthesis relatively high TTN were observed, but the STY is still quite low, leading to the assumption, that the reaction is not only limited by the electrochemical generation rate of H_2O_2, but also by the solubility of the substrate. This statement can be supported by the results obtained with [BMIM][$MeSO_4$]. For this IL the highest improvement for the substrate solubility was observed, leading to very good TTN and STY. Taking all these results into account, ILs are excellent performance additives for the reaction under investigation.

Conclusion

We were able to successfully apply IL as performance additives for electroenzymatic syntheses for the first time. They ideally combine the function of conducting salts, enzyme stabilisers and co-solvents at the same time. The performance of three completely different electroenzymatic reactions was enhanced by small amounts of IL in the reaction medium. Their addition led to higher conductivities of the reaction media, stabilised biocatalysts and nicotine amide cofactors, as well as to improved substrate solubility resulting in increased productivities and improved catalyst utilisations. IL as performance additives for electroenzymatic synthesis seem to be able to overcome the main challenges of these reactions and their application can be beneficial for various syntheses. They define a new state of the art for electroenzymatic syntheses.

Experimental Section

Chemicals: All IL were supplied by Solvent Innovation (Cologne, Germany). Chloroperoxidase from *Caldariomyces fumago* (E.C. 1.11.1.10) and horseradish peroxidase (E.C. 1.11.1.7) were purchased from Fluka (Taufkirchen, Germany). D-Specific amino acid oxidase from *Trigonopsis variabilis* (E.C. 1.4.3.3), NADPH and $NADP^+$ were provided by Jülich Chiral Solutions (Jülich, Germany). All other reagents were purchased from Sigma Aldrich (Schnelldorf, Germany) and were of analytical grade or better.

Methods: All results represent the average of at least two experiments. The standard deviation was always less then 5%.

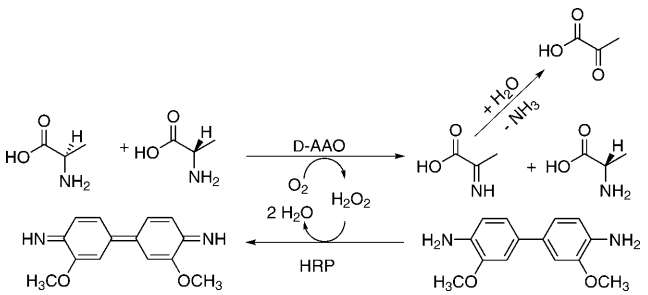
Preparation of IL/buffer mixtures: Depending on the reaction different buffers were used: All investigations concerning the electrochemical generation of NADPH were carried out in phosphate buffer (50 mmol L^{-1}, pH 7). Investigations regarding the D-AAO-catalysed resolution of a methionine racemate with electrochemical regeneration of FAD were carried out in phosphate buffer (100 mmol L^{-1}, pH 8). In all experiments for the CPO-catalysed synthesis of (*R*)-phenylmethylsulfoxide with electrochemical generation of H_2O_2 acetate buffer (100 mol L^{-1}, pH 5) was used.

The addition of some IL to the three buffers led to shifts in the pH of ± 0.3 pH units. Therefore after addition of IL the pH was controlled and, if necessary, fixed by addition of small amounts of phosphoric or acetic acid respectively or the corresponding bases to maintain the desired pH.

Conductivity measurements: The conductivity was measured with a conductivity meter (Cond 315i, WTW Weinheim, electrode TetraCon 325) at a temperature of 298 K (25°C).

Enzyme activity investigations

D-Amino acid oxidase from Trigonopsis variabilis: As activity assay the conversion of D-alanine to the corresponding imino acid 2-iminopropanoic acid, followed by the spontaneous hydrolysis to α-keto-γ-(methylthio)-butyric acid was investigated with a coupled peroxidase assay (Scheme 4).[24] As H_2O_2 was formed during the amino acid oxidase catalysed reaction, the H_2O_2 could be used to oxidise *o*-dianisidine by horse radish peroxidase (HRP) leading to an auburn colouration. Enzymatic activity was determined spectrophotometrically measuring the difference in absorption at a wavelength of 436 nm during one minute reaction time. Alanine and dianisidine solution were aerated with oxygen for 30 min before use and incubated at 25°C for 5 min afterwards. One milliliter cuvette volume contained D,L-alanine (10 mmol L^{-1}), *o*-dianisidine·2HCl (0.2 mg mL^{-1}), horse radish peroxidase (~25 U) and D-amino acid oxidase (~5 U). We also checked the activity of the HRP in presence of the used IL to ensure, that the HRP was more active than the D-AAO.



Scheme 4. Assay for the investigation of D-AAO activity.

Chloroperoxidase from Caldariomyces fumago: As activity assay the oxidation of thioanisole to (*R*)-phenylmethylsulfoxide was investigated (Scheme 3). Enzymatic activity was determined spectrophotometrically measuring the difference in absorption at a wavelength of 284 nm during one minute reaction time.[20] In the cuvette thioanisole solution (2 mmol L^{-1}, 980 μL) and H_2O_2 solution (200 mmol L^{-1}, 10 μL) were mixed and incubated at 25°C for 5 min; with the addition of enzyme solution (10 μL) the reaction was started. As enzyme solution a combined stock of three different commercial batches of CPO were used. The combined enzyme stock had a protein content of 34.5 mg mL^{-1} and an R_Z value of 0.92 (1.44 for pure CPO) corresponding to a total CPO content of 22 mg mL^{-1}. In the standard assay without IL the activity was 191 U mg^{-1}_{CPO} regarding thioanisole as substrate and 930 U mg^{-1}_{CPO} regarding the MCD assay.[23]

Stability investigations

Cofactor stability: To measure the stability of NADPH, cofactor (1.5 mg) was diluted in 2 mL of the corresponding IL/buffer mixture and stored at 25°C. Samples were withdrawn in defined time periods and the remaining absorption at 340 nm was measured. Additionally, for some of the samples the remaining amount of cofactor was investigated by a photometric enzyme coupled assay. For this assay, the absorption of a mixture from acetophenone (30 mmol L^{-1}, 970 μL) and cofactor solution (20 μL) was determined at 340 nm. Then alcohol dehydrogenase solution (50 mg mL^{-1}, 10 μL) was added. After reaching a stable value for the adsorption at 340 nm the amount of enzymatically active NADPH was calculated from the difference of these two values.

To measure the stability of $NADP^+$, cofactor (2 mg) was diluted in 2 mL of the corresponding IL/buffer mixture and stored at 25°C. Samples were withdrawn in defined time periods and the remaining amount of enzymatically active cofactor was investigated by a photometric enzyme assay. For that reason, the absorption of a mixture from sodium formate (240 mmol L^{-1}, 970 μL) and cofactor solution (20 μL) was determined at 340 nm. Then formate dehydrogenase solution (10 μL) was added. After reaching a stable value for the adsorption at 340 nm the amount of enzymatically active $NADP^+$ was calculated from the difference of these two values.

Enzyme stability: The different enzymes were stored in the corresponding IL/buffer mixtures at 25°C; samples were withdrawn in defined time periods and analysed for activity via the photometric assays described previously (see above).

Electroenzymatic syntheses

Reaction setup for all electroenzymatic syntheses: Reactions where carried out in a thermostated glass reaction vessel at 25°C (workshops Forschungszentrum Jülich, Jülich, Germany). A graphite fleece on a stainless steal fixation was used as working electrode, a platinum net separated by a dialysis sack functioned as counter electrode and as reference an Ag|AgCl electrode was used. The electrodes were connected to a potentiostat (263 A, Princeton Applied Research).

Electrochemical generation of NADPH: The reaction medium (*x* vol% 50 mM phosphate buffer pH 7 + *y* vol% [EMPY][$EtSO_4$]; 200 mL) was degassed with argon before use. $NADP^+$ (2.5 mM) and rhodium mediator (0.05 mM; prepared by the method of Kölle and Grätzel)[25] were added and the reaction was started by applying a potential of −750 mV vs. Ag|

AgCl. Samples were withdrawn and analysed for concentration spectrophotometrically at 340 nm.

D-*AAO-catalysed resolution of a methionine racemate with electrochemical regeneration of FAD*: The reaction medium (*x* vol% 100 mM phosphate buffer pH 8 + *y* vol% [EMIM][Me_2PO_4]; 300 mL) was degassed with argon before use. Ferrocene carboxylic acid (0.5 mM), D,L-methionine (20 mM) and D-AAO were added and the reaction was started by applying a potential of 350 mV vs. Ag|AgCl. Samples were withdrawn and analysed by HPLC (column: Crownpack CR+, perchloric acid pH 2, 0.9 mL min^{-1}, 40 °C).

*CPO-catalysed synthesis of (*R*)-phenylmethylsulfoxide with electrochemical generation of H_2O_2*: The reaction medium (98 vol% 100 mM acetate buffer pH 8 + 2 vol% IL; 300 mL) was saturated with O_2 before use and aerated with O_2 during the whole experiment. Thioanisole (20 mM) and CPO were added and the reaction was started by applying a potential of −650 mV vs. Ag|AgCl. Every 30 min thioanisole (6 mmol) was injected. Samples were withdrawn and analysed by GC (column: Lipodex G, hydrogen, 0.6 bar, 160 °C (10 min) 20 °C min^{-1} to 180 °C (7 min)).

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