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Stable Electroenzymatic Processes by Catalyst Separation

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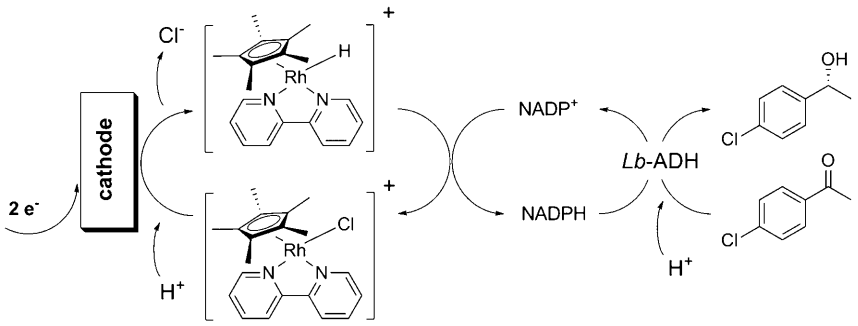
Electroenzymatic synthesis[1] combines the superior selectivity of redox enzymes with the waste-free supply of redox equivalents by electric current. However, for the last two decades, electroenzymatic synthesis has not fulfilled its great potential, because the synthetic value is often diminished by reduced enzyme stability.[2,3] During the coupling of the electrochemical reduction of nicotinamide cofactors (NAD(P)H) by the rhodium complex [Rh(bpy)][4–7] to an enzymatic synthesis reaction (see Scheme 1), the low biocatalyst stability is associated with the presence of the mediator. Since the precise nature of the interaction is not yet known, we describe here the elucidation of the inactivation mechanism as well as an approach to overcome this limitation by separating the two catalysts. This results in an electroenzymatic reactor setup with yet unreported catalyst stabilities and utilisations.



Scheme 1. Electroenzymatic reaction system for the synthesis of chiral alcohols.

To overcome the low enzyme stability in the presence of mediator, three approaches have been described in the literature so far. Firstly, for a comparable, nonelectrochemical application of [Rh(bpy)], the enzyme immobilisation through its nucleophilic side chains (-NH_2 and -SH) was studied to prevent harmful interactions between the side chains and the mediator.[8] Although this permitted work for a period of a few hours, a decreasing reaction rate and incomplete conversion indicate that other factors also play a part in the inactivation.

Secondly, the use of a nucleophilic buffer to saturate the coordination sites at the rhodium complex has been reported to prevent an interaction with the enzyme.[9] In this case as well, the reaction system was stabilised to a certain extent, but product formation collapsed at very low conversion (<10%).

Thirdly, in a previous contribution we reported that considerably increased enzyme stability can be achieved by the addition of a nonreactive protein (bovine albumin).[2] In this way, it was possible to quantitatively convert 17 mmol L^{-1} of substrate, and to achieve total turnover numbers of 35 (ratio of product formed to catalyst consumed) for both mediator and cofactor. However, a reduction of the reaction rate was also found here in the course of the reaction. Since the process is limited by the heterogeneous electrode reaction and thus conforms to a zeroth-order rate equation this also points to an inactivation. These observations have not yet been explained satisfactorily.

We were unable to observe any stabilising effect for any of the enzymes studied using the first two methods men-

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tioned above.[3] It is therefore doubtful whether these three concepts can be generally applied.

Since enzyme activity was still present at the end of the reaction when a nonreactive protein was added, we thus concluded that the termination of the reaction cannot be brought about solely by the inactivated enzyme but also by inactivated mediator. As yet, the stability of the mediator under reaction conditions has received little attention in the literature. To investigate the interactions between the mediator and the amino acids in more detail, we determined the influence of amino acids on the mediator activity by cyclic voltammetry. In the presence of amino acids, an in part drastic reduction in the cathodic peak current was detected, which is directly correlated with the catalytic activity of the mediator. Figure 1 shows the ratio of the peak currents in the presence and absence of amino acids.

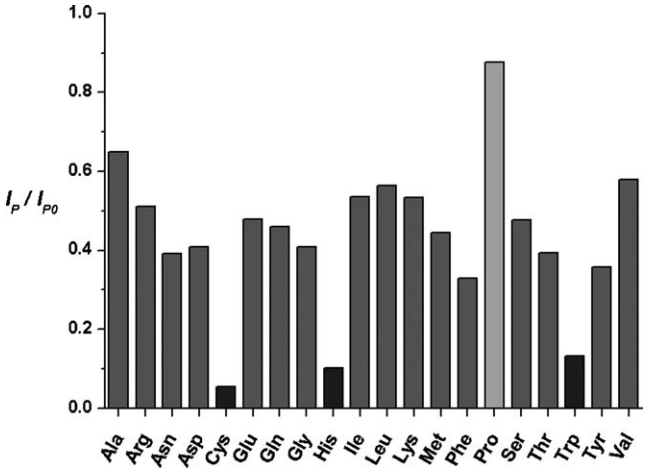


Figure 1. Activity loss of the mediator in the presence of amino acids. Reaction conditions: 50 mmol L^{-1} phosphate buffer, pH of 7; 2 mmol L^{-1} mediator; 5 mmol L^{-1} amino acid; potential 0 mV to −1000 mV versus Ag|AgCl; 100 mV s^{-1}.

The measurements support the hypothesis that the activity of the mediator is reduced in the presence of proteins. Above all, the amino acids cysteine, histidine and tryptophan drastically reduce the activity of the mediator.[3] Even if the nucleophilicity of the amino acids can vary as part of a protein, nevertheless it is to be expected that proteins that have these amino acids at accessible positions will inactivate themselves and the mediator within a short period by reaction with the mediator.

This is thus the first time that a systematic study has been conducted to investigate the aspect of mediator stability in connection with an enzyme reaction. These results enabled us to assign the inactivation to certain amino acids and to correct the previous hypothesis, which generally stated that the basic amino acids and cysteine are involved in the inactivation.[8] Whereas, as expected, cysteine and histidine led to high losses of mediator activity, the effect was less pronounced for the other two basic amino acids lysine and arginine. A possible reaction with the mediator had already been proposed for cysteine[8] and a high reactivity between cysteine and heavy metals had also already been observed for other enzymes.[10] Moreover, the inactivating effects of tryptophan and histidine are very strong, since the heteroaromatic rings can insert into the rhodium coordination sphere. Other amino acids have less influence on the catalytic activity of the mediator, although in this case the α-amino groups may also interact with the mediator. In a protein, these groups would not be reactive as part of the peptide bonds. This becomes apparent on the basis of the high residual activity with proline, which does not contain a free amino group.

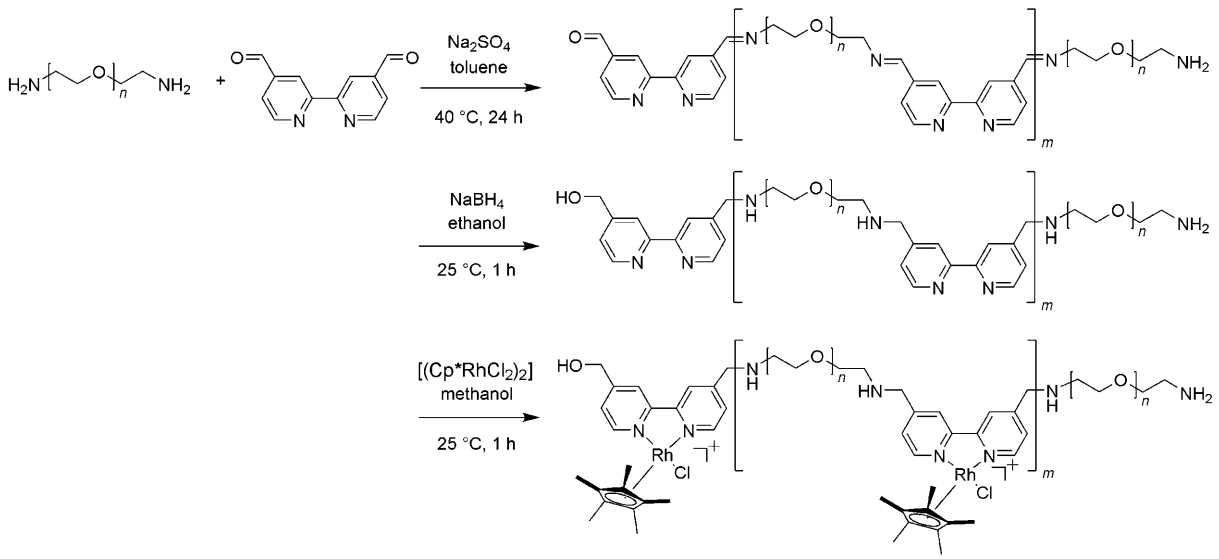
In the alcohol dehydrogenase from *Lactobacillus brevis* (*Lb*-ADH), which is frequently used in both laboratory scale and commercial scale, eight of these inactivating amino acids are present[11] (2 Cys, 4 His and 2 Trp), so that inactivation is inevitable.

These findings make it clear why a stable electroenzymatic reaction system is so difficult to establish. The inactivation of the mediator by tryptophan cannot be prevented by modifying the thiol and amino groups.[6] Nucleophilic additives cannot stabilise the reaction system either since the free coordination site at the mediator, which is necessary for catalytic activity, can always also react with the above-mentioned amino acids.[9] The addition of a nonreactive protein leads to the retention of some of the enzyme activity but only by completely inactivating the mediator, which likewise leads to a termination of the reaction.[2] However, restricting the choice of enzymes to those without these amino acids present at accessible sites clearly negates a general applicability of the process.

The only possibility of stabilising the enzyme and mediator and of establishing an efficient process is to create a spatial separation. A compartmentalisation of the electrochemical activation of the mediator and cofactor reduction, on the one hand, and the enzymatic synthesis reaction, on the other hand, prevents direct contact between the enzyme and mediator and should thus completely prevent inactivation. This separation is made more difficult by the fact that the cofactor must be able to reach both reaction chambers for electron transfer. Since the mediator and cofactor are very similar with respect to molecular weight and polarity,[3] the molecular weight of the mediator has to be increased to achieve selective membrane retention.

The water-soluble polymer bonding of a catalyst for retention in the reactor chamber has already become well established for a large number of processes.[12,13] [Rh(bpy)] has already been polymer-bound but only with a very high loss of activity.[5,14] Since polymer bonding has to date only focused on the retention of the mediator in the reactor chamber, in which the enzyme was also present, the mediators only displayed minor stabilities.[15]

In contrast, we have developed a new synthesis route leading to a hydrolysis-stable block polymer by the polycondensation of 2,2′-bipyridine-4,4′-di-aldehyde and α, ω-functionalised amino polyethylene glycol ($M = 6000$ g mol^{-1}) with subsequent imine reduction by sodium borohydride (see



Scheme 2. Synthesis route for polymer bonding of the mediator.

Scheme 2). Complexation with rhodium then yields the polymeric mediator.[16]

The polymeric mediator displays 26% electrochemical activity in comparison to the low-molecular-weight mediator and is thus superior to the previously described [Rh(bpy)] polymeric mediators with respect to rhodium activity.[3,15] The loss of activity is mainly due to the steric shielding of the rhodium centres by the nonreactive polymer, which hampers contact to the electrode surface. Retention at a membrane of regenerated cellulose (cut-off 3 kDa) is quantitative (>99.9%), and also the leaching of rhodium out of the complex is negligible.[3,13] This excellent retention means that the reduced activity can be readily compensated by higher catalyst loading.

To demonstrate the stabilisation of the reaction system, an electroenzymatic reactor was constructed in which the partial reactions took place at a distance from each other (see Figure 2). The polymeric mediator is activated in an electrochemical cell[17] and reduces the cofactor $NADP^+$ to

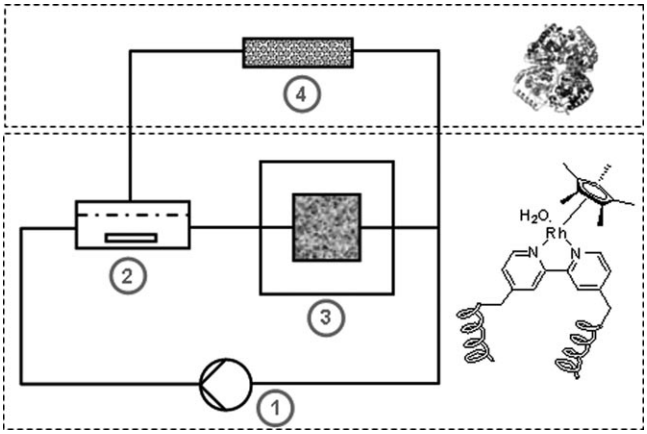


Figure 2. Reactor setup: 1) pump, 2) membrane reactor, 3) electrochemical cell, 4) immobilised enzyme.

NADPH. The mediator is subsequently selectively retained at a membrane, whereas NADPH and the *p*-chloroacetophenone substrate pass through the membrane and react at the immobilised *Lb*-ADH[18] to form *p*-chloro-*(R)*-phenylethanol and $NADP^+$. In this setup, substrate dosage is possible as is the separation of product solution, which is free of the mediator and enzyme, thus facilitating processing and saving costs.

A linear production formation of 0.42 mmol $L^{-1}h^{-1}$ was achieved for six hours (see Figure 3). Subsequently, at a conversion of 90% it was possible to recover the immobilised enzyme without any loss of activity (activity at the start 5.5 ± 1 U mg^{-1}, and at the end 6 ± 1 U mg^{-1}). Due to the complete avoidance of enzyme inactivation, this process represents a new quality in the field of electroenzymatic synthesis. Additionally, of the initially charged 0.1 mmol L^{-1} mediator (based on Rh-units), which achieved a turnover number of 30, the majority (86%; 0.086 mmol L^{-1}) could be recovered. This translates into a mediator utilisation of total turnover number of 214. This value represents the highest mediator utilisation reported so far for an electrochemical activation of [Rh(bpy)] coupled to an enzyme reaction, once again giving clear evidence of the superiority of the new process. Side reactions were also effectively prevented, an enantiomeric excess of more than 97.3% could be achieved.

The rather high cofactor concentration of 1 mmol L^{-1} $NADP^+$ was deliberately chosen so that it was negligible for the reactor stability. However, after successfully demonstrating that the spatial separation of enzyme and mediator enormously increases the process stability, this concentration can easily be reduced.[18]

Herein, we have elucidated that the low enzyme stability, the main limiting factor in electroenzymatic syntheses, is due to the reaction of certain amino acids with the mediator and that both the enzyme and the mediator are inactivated in this process. We can now explain why all previous at-

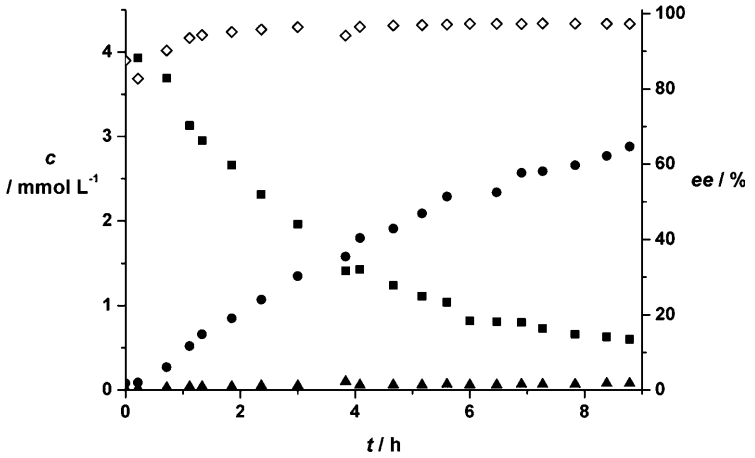


Figure 3. Synthesis of *p*-chloro-(*R*)-phenylethanol by immobilised *Lb*-ADH with electrochemical cofactor regeneration. Reaction conditions: 100 mL 100 mmol L^{-1} phosphate buffer, pH 7; 4 mmol L^{-1} *p*-chloroacetophenone (squares); 0.1 mmol L^{-1} polymeric mediator (relative to Rh units); 1 mmol L^{-1} $NADP^+$; 1 g *Lb*-ADH-immobilisate[18]; −800 mV versus Ag|AgCl; circles: *p*-chloro-*(R)*-phenylethanol; triangles: *p*-chloro-*(S)*-phenylethanol; open diamonds: enantiomeric excess *ee*.

tempts to stabilise the reaction system during the last twenty years experienced limited success since only the prevention of direct contact between the mediator and enzyme permits a stable reaction system. We verified this hypothesis by coupling the mediator to a water-soluble polymer, and separating enzyme and mediator by a membrane.

The stable product formation rate and the high catalyst stabilities demonstrate the robustness of the optimised system and its superiority to systems previously reported in the literature with respect to enzyme and mediator utilisation.[2,8,9,15] This is the first electroenzymatic process that employs [Rh(bpy)] for which the major obstacle of low catalyst stabilities is overcome and for which both mediator and enzyme could be recovered with very high residual activities. Furthermore, the system described offers more optimisation potential, whereby special attention should be focussed on higher product concentrations and improved cofactor utilisation.

Experimental Section

Synthesis of the polymeric mediator: α,ω-Aminated poly(ethylene glycol) (1.0 g, 0.167 mmol) and 2,2′-bipyridine-4,4′-di-aldehyde (35.4 mg , 0.167 mmol) were dissolved in dry toluene (50 mL) and dry sodium sulfate (1 g) was added. The reaction mixture was stirred by a magnetic bar at 40 °C for 24 h. After filtration, the solvent was removed under reduced pressure and the product was dissolved in absolute ethanol (50 mL). Sodium borohydride (31.4 mg, 0.83 mmol) was added and the mixture was stirred for one hour at room temperature. After water (50 mL) was added, the product was purified by ultrafiltration (membrane cut-off 3 kDa). Lyophilisation yielded white flakes (1.0 g, 96 %). This polymer was then dissolved in dry methanol (50 mL) and $[\{Cp^*RhCl(\mu\text{-}Cl)\}_2]$ (61.8 mg, 0.1 mmol, synthesised according to Spika.[15]) was added. After the solution had been stirred for one hour at room temperature, the solvent was removed under reduced pressure and the product was purified by aqueous ultrafiltration. Lyophilisation yielded orange flakes (1.01 g, 97 %).

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