Development of Electrochemical Reactors Using Dehydrogenases for Enantiopure Synthon Preparations

Reporting

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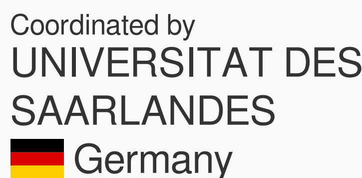
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Final Report Summary - ERUDESP (Development of Electrochemical Reactors Using Dehydrogenases for Enantiopure Synthon Preparations)

Executive summary:

The final goal of the project was the construction and demonstration of a Gas Diffusion Electrode BioReactor with immobilized enzymes, cofactor and mediated electron transfer. Therefore, bioelectrochemical reactions were tested either in a batch- or a flow-cell. The final BioReactor was constructed and optimized based on these results. Also the gas diffusion electrodes (GDE) were developed for the bioelectrochemical reactions either in a batch- or a flow-cell and finally in the demonstrator.

Large scale macroporous electrodes were developed and optimized by the use of the Langmuir-Blodgett

method. They were used as a support for the subsequent modification with silica layers or electrophoretic paint layers. The generation of sol-gel biocomposite films was performed on large electrodes being part of the final reactor. In this case, it was necessary to replace the potentiostatic way of getting electrodeposited films by a galvanostatic operating mode. These layers were successfully used for the immobilization of enzymes, cofactors and mediators. The thin sol-gel films entrapping dehydrogenases can be deposited onto carbon and gold electrode surfaces by drop-coating or electro-assisted deposition (E-AD) and the biomolecules remained highly active when polyelectrolyte additives (PDDA, PEI, PAA) are used. Also an additional redox enzyme, diaphorase (DI), which is known to facilitate cofactor regeneration by a wide range of mediators, can be co-immobilized with DSDH in both matrices in an active form. Effective strategies for the durable immobilization of NAD+ cofactor have been developed, such as its covalent attachment to the silica film via glycidoxypropyl arms, or via the encapsulation of a high molecular weight NAD+-dextran compound within the sol-gel matrix, or even using Ca2+ ions as a supramolecular bridge between the cofactor and the mediator. Alternatively, the electrophoretic paint deposition by the use of a nitrofluorenone mediator was used for the immobilisation of the whole electroenzymatic chain by modification of macroporous electrodes and tested in the demonstrator. Both deposition methods were successfully upscaled and tested in the final BioReactor with satisfying results.

On the basis of the above strategies, several kinds of operational reagent-less all-together bioelectrocatalytic systems, as deposited on carbon nanotube assemblies or onto the internal surface of macroporous gold electrodes, have been elaborated and their performance was further enhanced by the incorporation of gold nanoparticles in the biocomposite matrix to promote charge transfer, thus improving the turnover of the bioelectrocatalytic device.

In parallel a medium throughput multi-cell array was constructed and validated for the electrochemical screening of organometallic rhodium and ruthenium mediators. Several oxidation mediators (ferrocene, phenothiazine, or osmium bipyridine types) have been successfully immobilized in an active form (exhibiting durable regeneration of NAD+ cofactor) in the sol-gel layers.

Rational enzyme engineering led to promising enzyme mutants in a combined effort of microbiology, protein crystallography and computer modelling. Variants of GatDH and DSDH with improved thermal stability were constructed. The most promising enzyme seemed to be an L-sorbitol dehydrogenase (LSDH) from Bradyrhizobium japonicum because of its interesting product range and thermal stability.

For the possible products of the used enzymes, which are rare sugars, a high market potential can be assumed for the near and distant future. In the moment the main advantage of this new technology is strongly correlated to high priced fine chemicals with productions up to 100 kg per year. For bulk productions the reactor cannot compete with existing technologies at this time. However, further development has the potential to open also this field of biotechnology to electrochemical reactors.

Project Context and Objectives:

Summary description of project context and objectives

Six research groups worked together during the whole project period to reach the final goal of the project: the construction and demonstration of a Gas Diffusion Electrode BioReactor with immobilized enzymes

the construction and demonstration of a Gas Diffusion Electrode BioReactor with immobilized enzymes, cofactor and mediated electron transfer.

The large scale macroporous electrodes were developed and optimized in WP1 by the use of the Langmuir-Blodgett method. They were used as a support for the subsequent modification with silica layers or electrophoretic paint layers. Both of these matrix materials were obtained via an electroassisted generation technology leading to mesoporous silica or polymer structures either completely filling the pores or just covering the pore walls. These layers were successfully used for the immobilization of enzymes, cofactors and mediators.

Gas diffusion electrodes (GDE) were developed in WP5 for the bioelectrochemical reactions either in a batch- or a flow-cell and finally in the demonstrator. These electrodes were used as counter electrodes and so, these electrodes were calibrated for the electroenzymatic oxidation as well as for the reduction reactions. The modified macroporous working electrodes and developed GDEs for the construction of the bioelectrochemical reactor were tested successfully for the electroorganic reduction of phenylglyoxylic acid (PGA) to (R,S)mandelic acid and for the electroenzymatic conver-sion of D-sorbitol into fructose in the

presence of D-sorbitol dehydrogenase (DSDH).

The ambitious objective of WP3 was to immobilize all the necessary components in surface layers in a configuration likely to ensure functional enzyme/cofactor/mediator ensembles. This was reached stepwise:

1) Immobilization of dehydrogenases in thin films (sol-gel, electrophoretic paint)
2) Functionalized mediator and cofactor synthesis
3) Mediator immobilization
4) Cofactor immobilization
5) Mediator, enzyme and cofactor immobilization; all-together systems

All these results obtained in WP1, WP3 and WP5 were the basis for the final goal of the construction of and for the electroenzymatic reaction in the BioReactor.

The electrodeposition method to get sol-gel-based functional layers (with enzyme, cofactor and mediator), as developed in WP3, has been adapted to the particular case of electrochemical cell array developed in WP2. This has required a 'technology transfer' from Partner 3 to Partner 1a (successfully achieved by exchanges of post-docs). The upscaling and the electro-assisted deposition of sol-gel films, including dehydrogenase-doped sol-gels, were successfully performed under galvanostatic conditions. Also the immobilisation of DSDH, diaphorase and cofactor in an active form in polyelectrolyte-doped sol-gel films by spin-coating was successfully upscaled and tested in the demonstrator. This method yielded the best electroenzymatic results.

Also an alternative strategy for the immobilisation of the whole electroenzymatic chain was found and tested in the demonstrator by modification of macroporous electrodes with an electrophoretic paint deposition by the use of a nitrofluorenone mediator ((4-carboxy-(2,5,7-trinitro-9-fluorenylidene)-malonitrile), with satisfying results.

Beside the experiments with the demonstrator, it was necessary to design, construct and validate an

Beside the experiments with the demonstrator, it was necessary to design, construct and validate an electrochemical single cell due to the multiplication to a 16-fold cell array during the first period of the project. A suitable measuring protocol was applied and after the satisfying validation of this single cell the multiplication to the 16-fold cell array has been accomplished. This cell array allows purging the electrolyte with nitrogen. The electrochemical screening of the received redox mediators of Partner 5 occurred in three steps: started with the electrochemical behaviour of the mediators at three different pH values, followed by electrocatalytic experiments in the presence of the cofactor. Finally, the suitable redox mediators were used for the electroenzymatics in the presence of the model enzyme DSDH by cyclic voltammetry and chronoamperometry. The product formation was analysed by HPLC and also UV/Vis spectroscopy was consulted to prove that the redox mediators lose their activity and that the enzyme did not work in the presence of the organometallic rhodium redox mediators.

In WP4 Galactitol dehydrogenase (GatDH) and D-sorbitol dehydrogenase (DSDH) are used as model enzymes in the project. The enzymes were cloned, fused with a His(6)tag, expressed in sufficient amounts, purified, and distributed to the partners. Substrate conversion of 100% to interesting products with ee values of 99.5% were shown by the industrial partner. Computational protein modelling tools were utilized to suggest mutations conferring optimized application of the enzymes DSDH and GatDH. The modelling work was focused on improved thermal stability, altered substrate specificity, and enzyme surface attachment. The proposed variants were constructed by directed mutagenesis and the derived proteins have been characterized and used for crystallization studies. Initial attempts to crystallize the His-tagged variant of DSDH showed that the His-tag interferes with crystallization, thus laborious purification of untagged DSDH had to be carried out in order to crystallize ligand complexes. Small diffracting crystals of a putative complex with cofactor and D-sorbitol have been obtained and analyzed. Two variants of GatDH and DSDH expressed slightly improved stability versus higher temperature or more favoured pH values. It has also been shown that DSDH was stable in the presence of solvents, which increased the number of potential substrates. For improving the applicability of GatDH and DSDH, a Cys-tag has been added in front of the His(6)-tag. This constructed variants were catalytically active enzymes which could be immobilized with the thiol group directly to the gold surface. Furthermore diaphorase from Clostridium kluyveri could be heterologously expressed as active enzyme which can be used for better electron transfer in the sol-gel materials.

Small diffracting crystals of L-sorbitol dehydrogenase (LSDH) from Bradyrhizobium japonicum have been obtained through extensive crystallization screening and a data set for LSDH could be collected to 3.55 A resolution leading to a preliminary structure by Molecular Replacement showing a tetrameric arrangement. LSDH expressed thermal stability and can be used to produce the high priced rare sugar L-sorbose, which let it appear as an interesting enzyme in synthesis applications.

The industrial partner has escorted the evolution of the project and advised the partners from an industrial point of view for suitable directions of reactor development (change from reduction to oxidation). A business survey of the biotechnology market was established, focusing on the expected demand for chiral hydroxylated compounds. They provided the partners with information about economic requirements for the envisaged reactors and the demands for the development of competitive processes.

The project-used enzymes were adapted to the industrial used license-free expression system. Efficient production of the biocatalyst in high space-time yield was achieved.

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Project Results:

Description of the main S&T results/foregrounds

The main goals in this work package can be summarised as follows:

1. to immobilize active dehydrogenase enzymes in sol-gel films in a way that could be extended to macroporous electrodes;
2. to prepare functionalized mediators and cofactors, to check their electrochemical response, and to evaluate their possible immobilization in an active form on electrode surfaces (as self-assembled monolayers or within silica films);
3. to build an optimal electrode/mediator/cofactor/enzyme configuration for effective bioelectrocatalysis;
4. to develop strategies to increase the active area of electrodes and to promote charge transfer reactions.

The main activities and results can be summarised as follows:

1) Immobilization of dehydrogenases and other enzymes in thin layers (mainly sol-gel)

this has required a huge amount of work as the extension of existing pH-triggered sol-gel and polymer deposition methods to dehydrogenase immobilization in an active form in thin films was not straightforward. After many investigations and optimization steps, it was shown that DSDH (and GatDH) can be immobilized by physical entrapment into thin films (chitosan, sol-gel, or Reshydrol) films deposited onto carbon and gold electrode surfaces by drop-coating or electro-assisted deposition (E-AD). While few or no activity was observed without additive, the use polyelectrolyte additives (poly(diallyl-dimethyl ammonium), PDDA, poly(ethylene imine), PEI, or poly(allyl amine), PAA) enabled the biomolecules to remain highly active, as checked by cyclic voltammetry experiments realized using cofactors (and mediator, if necessary) in solution, upon addition of the respective enzymatic substrates. Both DSDH and GatDH were active in oxidation and reduction.

The E-AD approach was then extended to coat the walls of macroporous gold electrodes (with various half-layers, HL) while maintaining open the interconnections between the macropores. It is now established that the entrapped biomolecule remained active once immobilized with this process and that its redox signal gets more intense with the increasing number of HL. This has been performed for both sol-gel silica and electrophoretic paint matrices, but one advantage of using a silica matrix instead of the electrophoretic paint is that the current densities are higher (by ca. 20-30%), which tends to prove that the silica layer contributes to a better environment for enzymatic activity and/or electron transfer (via a redox mediator).

The E-AD method can be also extended to the co-encapsulation of both DSDH and diaphorase in the silica gel layer, which was possible in one step electrolysis and both proteins were found to keep their operational behaviour once entrapped. The interest of diaphorase is to extend the choice of mediators that are likely to regenerate the cofactors, which was very useful in the context of this project (see below).

2) Synthesis and electrochemical screening of functionalized mediators and cofactors

A wide range of functionalized mediators have been successfully synthesized, including numerous derivatives from the [Cp*Rh(bpy)Cl]+ family (bearing different substituents likely to be exploited for immobilization within the sol-gel matrix or as SAM onto gold surfaces), several substituted bipyridine, bis(bipyridine), phenanthroline, and terpyridine Ru, Rh and Os complexes, as well as [Cp*Rh(bpy)Cl]+-functionalized 2,5-Di(2-thienyl)pyrrole (SNS). In the cathodic mediator series ([Cp*Rh(bpy)Cl]+ family), some of them were really good mediator in solution to regenerate NADH, but the presence of thiol or nitrogen-containing groups totally prevents their electrocatalytic activity of this mediator, so that after immobilization as SAM on gold or within sol-gel films, they lost their capacity to regenerate the NADH cofactor. An explanation has been given on the basis of unwanted interaction between such amine or thiol ligands with the Rh center, preventing the formation of the active hydride rhodium complex necessary to induce the electrocatalytic activity. In the anodic mediator series, thanks to the contribution of several partners (P5, of course, but also P2 and P3), many active systems have been generated, which were able to regenerate the NAD+ cofactor, both in solution and after immobilization into the sol-gel matrix or as

SAM onto gold surfaces. They include alkoxysilane-functionalized ferrocene derivatives, ferrocene moieties covalently linked to poly(ethylene imine (Fc-PEI), an osmium polymer (Os-polymer, polymer bearing osmium bipyridine moieties), poly(methylene green) (PMG), or carboxytrinitrofluorenone (CTNTF).

On the other hand, partner 5 investigated several strategies to prepare functionalized cofactor derivatives that are likely to be further immobilized onto electrode surfaces and/or within the functional layers. Among them, a ferrocene-bis boronic acid NAD+ derivative was first prepared by exploiting the reaction of NAD+ with diol part of ferrocene with boronic acid. Immobilization of NAD+ with boronic acid is indeed applied to several systems in the literature. In doing so, the mediator and NAD+ are in the same molecule. This gave rise to some electrochemical activity but no long-term stability when incorporated into a silica layer (leaching out in solution). An alkoxy-silane derivative bearing NAD+ moieties was then prepared from the reaction of NAD-aminoboronic acid and epoxysilane. This reagent was successfully grafted within the functional sol-gel layers. In parallel, partner 3 showed that this chemistry can be also exploited for the in situ incorporation of NAD+ by covalent attachment to the sol-gel film using glycidoxypropyl-trimethoxysilane (GPS) in the synthesis medium. Finally, another strategy based on building blocks was proposed by partner 5 to immobilize cofactors (and mediators as well, see task 3.7.) via 2,5-Di(2-thienyl)pyrrole (SNS) derivatives. SNS derivatives consist of thiophene and pyrrole rings and are of interest for covalent bonding of NAD+ to the pyrrole center via a phenylboronic acid intermediate to give the expected SNS-NAD complex. Attempts to electropolymerize this SNS-NAD complex were made but no stable electrochemical response can be obtained to date.

3) Co-immobilization of enzymes and cofactors in an active form in sol-gel films

In the way of getting a final reagent-less electrochemical reactor, following enzyme entrapment, the second step was to immobilize the cofactor in an active form in the sol-gel layer. The experiments have been made with DSDH-doped sol-gel films prepared either by drop-coating or via E-AD. In order to check the co-enzyme activity, the biocomposite films were deposited onto carbon electrodes, enabling the direct oxidation of the NADH cofactor generated by the enzymatic transformation of D-sorbitol into fructose. The best systems were then transferred to gold (macroporous) electrodes by using an appropriate mediator

best systems were then transferred to gold (macroporous) electrodes by using an appropriate mediator (mostly ferrocene methanol) in solution, the mediator immobilization methods being described in the next section (4) of this report.

Briefly, several strategies have been applied to immobilize the NAD+ cofactor. The simple entrapment of NAD+ in silica layers was not satisfactory because of fast leaching out of the film by diffusion in solution, resulting in poor long-term stability. Among other strategies that have been tested, three of them were successful: (a) the encapsulation of a high molecular weight compounds made of NAD+ covalently linked to dextran (the big volume of dextran (compared to NAD) allows indeed a durable immobilization by physical entrapment into the sol-gel layer, preventing from cofactor leaching in solution); (b) the direct in situ functionalization of NAD+ with a glycidoxypropyl-trimethoxysilane, GPS, precursor (i.e. in the sol) before film deposition onto the electrode surface, which led to a satisfactory stable response; © the resort to NAD+ functionalized by a boronic acid connected to an alkoxy-silane, which was then introduced in the sol-gel layer by co-condensation. Details on these various approaches can be found in deliverable 3.4. Note that unprecedented operational long-term stability (>10 hours) have been observed with the NAD+-GPS system.

4) Strategies to immobilize mediators operating with enzymes entrapped in sol-gel films

The next step was to immobilize the necessary electrocatalyst (mediator) in the dehydrogenase-doped sol-gel layers in a configuration enabling them to regenerate the cofactor (for this first screening/optimization approach, the cofactor was added in solution). The experiments have been made with DSDH-doped sol-gel films prepared either by drop-coating or via E-AD, but one rapidly realizes that an additional redox protein, diaphorase (DI), was necessary to ensure the safe regeneration of the cofactor (the direct electron transfer between the mediator and the cofactor can lead to rapid deactivation of the process). Again, the enzymatic interconversion of D-sorbitol and fructose was the monitored reaction.

Reduction reactions. Many functionalized [Cp*Rh(bpy)Cl]+/[Cp*Rh(bpy)H]+ derivatives have been tested as mediators likely to regenerate NADH but, as mentioned above, their immobilization on electrode surfaces totally prevents any electrocatalytic activity. In attempting to overcome this limitation, we have thus evaluated another strategy based on their adsorption on carbon nanotubes (CNTs), demonstrating that this strategy offers a unique opportunity to immobilize the Rh(III) complex in an active form, which was indeed likely to reduce NAD+ but not in biocomposite sol-gel films. This is the reason why one mostly worked in oxidation afterwards.

Oxidation reactions. Many mediators operating in oxidation (i.e. NAD+ regeneration) have been successfully immobilized in an active form onto various electrode surfaces. They include Ferrocene-silane compounds that can be introduced in a silica gel layer containing DSDH and diaphorase, deposited into macroporous electrodes, showing good electrocatalytic activity but poor control of the incorporated quantities because of the limited solubility of the component in the sol. Even more stable systems were interpenetrated networks of sol gel silica and mediator-bearing polymers (Fc-PEI, Osmium polymer), or ferrocene moieties grafted to the silica network, which needed however diaphorase to operate well. Polymerized methylene green (Poly-MG) films on carbon nanotube assemblies gave rise to a significantly improved electrochemical response for the mediated oxidation of NADH without requiring the additional DI protein. Another well-operating system was designed on the basis of self-assembly of carboxy

protein. Another well-operating system was designed on the basis of self-assembly of carboxy-trinitrofluorenone (CTNFM) on gold.

5) Reagent-less devices and strategies to enhance the bioelectrocatalytic performance

The final step was to immobilize all the necessary components in a configuration ensuring good electronic connection and thereby satisfactory bioelectrocatalytic cycles, likely to transform D-sorbitol into fructose (the model reaction used here) without adding any component into the solution. This has been achieved using DSDH-doped functional layers (containing most often diaphorase and always a polyelectrolyte) prepared either by drop-coating or via E-AD. The functional layers were based either on sol-gel matrices (deposited by drop-coating or via electro-assisted deposition) or on electrophoretic paints. They were usually first tested on flat glassy carbon electrodes and then applied to macroporous gold electrodes, if possible.

Without entering in details, the simple combination of configurations as described above (i.e. operating well for immobilized mediator alone or immobilized cofactor alone) was not straightforward and only some all-together systems were found to behave satisfactorily. They include: silica+polyelectrolyte+DSDH+diaphorase+NAD+-GPS+mediator (mediator = ferrocene-silane, ferrocene-PEI, or osmium polymer) deposited by drop-coating onto flat glassy carbon electrode (GCE) or macroporous gold electrodes; silica+polyelectrolyte+DSDH+NAD+-GPS drop-coated onto Poly-MG mediator on carbon nanotube assemblies on GCE; silica+polyelectrolyte+DSDH+diaphorase+NAD+-GPS electrodeposited onto carbon nanotube assemblies wrapped with an osmium polymer; an Reshydrol-based electrophoretic paint layer comprising a mixture of polyelectrolyte+DSDH+diaphorase+NAD+ (in this last case, the NAD+ cofactor was immobilized by using Ca2+ ions acting as a supramolecular bridge between the CTNF mediator and the cofactor).

Two approaches have been evaluated to increase the active area of electrodes. The first one involves the use of carbon nanotubes to enhance the effectiveness of charge transport. In addition, it allows efficient immobilization of the mediators for NADH regeneration (i.e. NAD+ reduction by Rh(III) complex) and for NAD+ regeneration (i.e. NADH oxidation by poly-MG). The second approach was directed to overcome the insulating character of silica or electrophoretic paint via the incorporation of metal nanoparticles (gold in this case, AuNPs) in the encapsulating layer. A two-fold increase in the electrode surface area was observed in comparison to a silica film free of AuNPs deposited on the same flat Au support. Moreover, it could be shown that there is efficient electrochemical communication between the enzymes and the electrode surface and furthermore the electrocatalytic currents for the oxidation of D-sorbitol into fructose could be enhanced by one order of magnitude when incorporating mediator modified gold nanoparticles into the polymer layer. The best enhancement factors were observed when incorporating such nanoparticles into the sol-gel matrix (in comparison to the electrophoretic paint).

Promoting the charge transfer reactions in the non conducting biocomposite layer can be also achieved by optimizing the composition of the film as well as the way of incorporation of each component occurring in the electron transfer chain (i.e. mediator and cofactor). In order to find the best conditions ensuring the most effective electron hopping, mediators and cofactors were immobilized to the matrix, either via bonding to the silica network with different spacers or via their linkage to the polyelectrolyte, as already mentioned above. Most effective systems were those based on mediator-bearing polymers and silica

mentioned above. Most effective systems were those based on mediator-bearing polymers and silica-attached cofactors.

Computational protein modelling tools were utilized to suggest mutations conferring optimized application of the enzymes Galactitol dehydrogenase (GatDH) and D-sorbitol dehydrogenase (DSDH). The modelling work was focused on improved thermal stability, altered substrate specificity, and enzyme surface attachment. The enzymes were cloned, fused with a His(6)tag, expressed in sufficient amounts, purified, and distributed to the partners. Substrate conversion of 100% to interesting products with ee values of 99.5% were shown by the industrial partner.

The proposed mutations by partner 4b were constructed as well as the variants with stabilizing sulphur bonds at the subunit interface and mutations in the iterative saturation mutagenesis. All enzyme variants were tested for activity and temperature stability: 18 GatDH mutants were constructed with the method of rational design, from which A57E appeared to be the best variant from bioinformatics prediction, expressing 2.6-fold activity and the highest stability of all tested mutations. From the predictions with the WHAT-IF program for introduced sulphur bonds in the subunit interface, the mutant Q168M exhibited a slight increase in stability at 50°C, but the decreased activity downgraded the variant in respect to utilization in the electrochemical reactor. The variant could be purified to a specific activity of 3.1 U/mg as compared to 12 U/mg of the purified native enzyme. With iterative saturation mutagenesis 1950 clones were obtained, three of them exhibited slightly increased thermal stability.

DSDH was shown to be stable in the presence of solvents, which increased the number of potential substrates. The disadvantage of shorter enzyme half-life compared to buffer conditions is accompanied by new access to a number of additional substrates. The presence of a solvent like 1-octanol could enable or increase the reduction of several ketones up to 10-fold, which expands widely the possible application of DSDH.

Among the mutations proposed from bioinformatics analyses, the triple-mutant A71E/L74R/A127K expressed slightly improved thermal stability. For improving the applicability of GatDH and DSDH, a Cys-tag has been added in front of the His(6)-tag. These constructed variants were catalytically active enzymes which could be immobilized with the thiol group directly to the gold surface. LSDH from Bradydhizobium japonicum appeared to be a promising candidate for future use in the reactor with electrochemical cofactor regeneration, because of the broad substrate spectrum, the efficient production of the rare sugar D-sorbose and its thermal stability.

Results from native polyacrylamide gels and gel filtrations indicate that the active enzyme is composed of three identical subunits. However, crystallographic data from partner 4a suggest a tetrameric structure. These different findings still have to be examined in more detail. The enzyme exhibited oxidation activity with several sugar alcohols and also reasonable activity with L-sorbitol oxidation to D-sorbose, which was confirmed by HPLC-analysis and depicted in the graph above.

The Tm for different protein variants has been determined by monitoring melting curves by circular dichroism in collaboration with Prof. Morten Bjerrum (University of Copenhagen). Further measurements were carried out and it was suggested that LSDH was more thermo stable if the His-tag is cleaved. The DSDH variants analyzed were not stabilized in terms of Tm.

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Major efforts were made in crystallization and crystal testing. Finally a crystal was obtained from which useful data could be collected at the state of the art ESRF (Grenoble, France) beamline ID29. Macroseeding and microseeding approaches were used to improve crystal quality. Crystallites grown using 1.8 M NaH_2PO_4/ K_2HPO_4, pH 5.6 (UID 183) as precipitant were used to streak-seed in trays set up with 1.4 M NaH_2PO_4/ K_2HPO_4, pH 5.6 in the reservoir. Before crystallization 100 mM D-sorbitol and 0.4 mM NAD^+ (final concentrations) were added to the protein solution. Larger crystals grew after 6 months and although data collection statistics showed a limited resolution, they were still usable for structure determination.

The structure was determined by molecular replacement. The search model used was the monomer of Clavunolic Acid Dehydrogenase (PDB ID: 2JAP; MacKenzie, 2007). The structure revealed a tetrameric arrangement as in the search model, with the significant difference that in the case of LSDH the tetramer is generated by the crystal symmetry. Refinement is slow because of the limited resolution and current statistics are R-work 32.7% / R-free 48%.

In the earlier stages of the project, a 3D structure of LSDH was not available, but Partner 1b provided the sequence of LSDH. A blast search [Altschul et al: J. Mol. Biol. 215:403-410, 1990] in the Protein Data Bank showed that the 10 top matches had relative low sequence similarity (33-28 % identity), however, relatively high structural similarity. Thus, they were used for preparing the homology model. The building of the homology model and subsequent optimisation of side-chain conformations was carried out using Prime [Prime, version 2.0 Schrodinger, LLC, New York, NY, 2008] in the Schrodinger Suite.

Suggestions to mutations stabilising the LSDH monomer

Based on the homology model of the LSDH monomer, PROPKA3.0 [Olsson et al: J. Chem. Theo. Comp., 7: 525-537, 2011] was applied for the prediction of stabilising mutations in the LSDH monomer by comparison to sequential and structural similar protein molecules. Stabilising interactions in similar protein molecules are detected and, when these interactions are not present in the LSDH molecule, the appropriate mutations are modelled to assess the stabilising contribution of the mutations. It turned out that often several residues are clustered to form stabilising interactions. PROPKA3.0 applies a combinatorial approach where all possible combinations of mutations in a mutation set are modelled to assess which mutations confer the greatest stability gain. The following Table 3 contains a list of mutations suggested to improve the stability of LSDH.

Optimisation of low-resolution X-ray structure of LSDH

The X-ray structure of LSDH had been solved by partner 4a. This structure contained LSDH in a tetrameric conformation, as seen in crystals of other DHs. However, the resolution of 3.5 A slows down refinement considerably and limits the final accuracy, especially of the side-chains. Therefore, in order to apply the LSDH tetrameric structure in computer simulations within the time-frame of ERUDESP, the structure was energy-minimised to optimise the side-chain conformations. We used Gromacs [Berendsen et al: Comp. Phys. Comm. 91: 43-56, 1995] with the OPLS-AA force field [Jorgensen et al: J. Am. Chem. Soc. 118 (45): 11225-11236, 1996] for the energy minimisation of the tetrameric LSDH structure. The

Soc. 118 (45): 11225-11236, 1996] for the energy minimisation of the tetrameric LSDH structure. The 'steepest descent' algorithm was run until the convergence criteria were reached after 2088 iterations.

Analysis of cross-monomer interactions in the LSDH tetrameric structure

The inter-monomer interactions in the LSDH tetramer were analysed using PROPKA3.0. Comparison to the inter-monomer interactions of clavulanic acid dehydrogenase (PDB id 2jap) showed that two inter-monomer salt bridges were present in clavulanic acid dehydrogenase, but not in LSDH. Mutations introducing these salt-bridges into LSDH could be a starting point for the introduction of an even increased thermostability in LSDH. Stabilising interactions across monomer-interfaces found in Clavulanic acid dehydrogenase but not in wild-type LSDH.

Clavulanic acid dehydrogenase (right panel)

It has been shown earlier that diaphorase can support and enhance the electron flow in the different sol gels. For further experiments it would be convenient if the enzyme can be produced in larger amounts and

so the 690 bp DiaA gene from Clostridium kluyveri, coding for a 230 aa protein of about 25 kDa, was cloned into E. coli BL21GOLD(DE3). The heterologously expressed protein was shown to be a flavin protein with high DCPIP reduction activity. In case of His(6)-tagged enzymes the C-terminal His-Tag variant exhibited a 3 times higher activity then the N-terminal His-tag variant and was therefore produced for experiments in WP3.

Demonstrator

The ambitious goal of WP5 was the design and construction of a bioreactor demonstrator based on the results received for the flow-cell experiments.

The thickness of the working electrode was changed for a better stability and due to the breaking strength. Also the Nafion membrane of the MEA was a little bit smaller due to the cost saving. This membrane was now threaded on four spikes for a better handling and the spikes are embedded into the middle part of the cell. So, the cell was closed in two steps: first, electrolyte cell and cap with embedded MEA and second, the cap with the biofunctionalized working electrode in the bottom part. This guaranteed a better stability for the MEA. The main results for the upscaled biofunctionalized electrodes are shown in the following:

A) Immobilization of DSDH by sol-gel electrodeposition on large-area electrodes

The experimental set-up for sol-gel electrodeposition on large-area electrodes was first tested with a TEOS/PDDA sol (in the absence of enzyme) in order to find suitable conditions for electrodeposition onto such large surface electrodes. Several parameters were found to affect the quality of the films (the deposition mode, potentiostatic versus galvanostatic, the deposition potential/current and the deposition time). The best system was obtained for sol-gel films prepared by galvanostatic electrodeposition, by applying a current density of 0.72 mA cm2 for 20 s, from a sol consisting of 0.5 M TEOS.

Then, the optimal galvanostatic deposition procedure was applied in the presence of the enzyme (DSDH immobilized in sol-gel) and the results of electroenzymatics indicate a nice bioelectrocatalytic response to

immobilized in sol-gel) and the results of electroenzymatics indicate a nice bioelectrocatalytic response to D-sorbitol.

B) Immobilization of DSDH, DI and NAD+ in sol-gel films prepared by spin-coating

Biocomposite films made of silica-polyelectrolyte encapsulating DSDH (and diaphorase, DI) along with immobilized cofactor (via NAD+ binding to GPS organosilane, see WP3 for details) have been deposited by spin-coating onto large carbon electrodes. The deposition conditions have been optimized and the rate of spinning was found to be critical with respect to the performance of the film in terms of electroenzymatic activity.

This biocomposite film was also tested directly in the flow cell system; E/V vs. 'O2/H2O' or pseudo 'H+/H2), in the presence of 0.1 mM mediator (ferrocene dimethanol) and 10 mM D-sorbitol. After 2 hours of continuing use, the TEOS/PEI/NAD+-GPS/DI/DSDH film was still mechanically stable on the electrode.

C) Immobilisation of a complete bioelectrocatalytic chain in macroporous electrodes

Two different methods have been tested by Partner 2 for the immobilisation of DSDH and DI in the presence of gold nanoparticles (AuNP) modified with CTNFM:

1) The enzymes and the cofactor were entrapped in an electrodeposited paint (EDP) called Resydrol ® dopped with AuNP and CTNFM. The redox mediator required an activation step before starting the experiments and it was found that the system could be simplify by the replacement of DI to Ca2+ ions due to ionic bridges between the mediator and the cofactor. The complete immobilization system was tried on flat and macroporous electrodes (1/2 layer and 3/2 layer) in the prototype of the reactor at a flow of 3 mL/min and a potential of 0 V. As expected an increase in current was recorded between the flat electrode and the one with 1/2 pore layer. The signal obtained for the 3/2 layer (3 HL) electrode was lower compared to the 1/2 layer (1HL) one due to an inefficient electroprecipitation step that did not embed enough biomolecules or that led to a too dense layer not appropriate for substrate diffusion. As the quantity of enzyme required for one encapsulating layer is quite high, fresh Resydrol/biomolecules mixtures cannot be prepared for each electrode. The 3 HL electrode was elaborated with a mixture that had already undergone 2 electrodeposition processes, on the flat and 1HL porous electrodes, so we assume that another hypothesis for the low 3HL electrodes intensity might be due to the ageing of the mixture.
2) The enzyme DSDH as well as GPS-NAD+ was entrapped to the silica network in the presence of AuNP functionalized with CTNFM. The resulting silica ultra-thin layer presents NAD+ groups at the interface between pure polycondensed silica and the reactant medium. A flat electrode was modified with a GPS-NAD+/PDDA/Silica layer containing DSDH and Ca2+ and was also recoreded at 0 V. The increasing recorded current suggests that substrate diffusion is occurring. The more it penetrates the silica layer, the more redox spots provided by the functionalized AuNP become involved in the global catalysis carried out on the working electrode.

Cooperation between Partner 1a, 2 and 3 allowed the elaboration of upscaled enzymatically modified flat and macroporous electrodes and their test in the flow reactor.

Business survey of the biotechnology market

The demand for chiral ingredients is constantly rising. Approximately 40% of drugs on the market today are sold as single stereoisomers, and approximately 80% of all products currently in development for the pharmaceutical industry are based on chiral building blocks. In 2002 55% (on a value basis) of chiral products was generated by traditional technologies (Diastereometric Crystalisation, Chromatography & Chiral Pool), 35% by asymmetric (Synthesis & Chemocatalysis), and 10% by biocatalysis. By 2009, the share of single-enantiomer compounds produced by traditional technology will drop to 42%, the share of chemocatalysis would rise to 36% and the share of biocatalysis to 22%.

According to industry experts, the market of chiral compounds is said to grow with a CAGR of 11% to 13% (depending on source) from 2009 through 2012 and will reach a market value of approximately USD 20 bn. The United States would remain the leading market, while Asia-Pacific and Japan are the fastest growing markets for chiral chemicals with an expected CAGR of over 14%. Demand for enantiopure chiral compounds continues to rise, primarily for the use in pharmaceuticals but also in other sectors, in particular flavour and aroma chemicals, agricultural chemicals, and speciality materials.

The formation of chiral alcohols can be performed with enzymes from different classes:

a. Oxidoreductases
b. Dehydrogenases (oxidation or reduction of existing oxygen function in target molecule)
c. Hydroxylases (specific introduction of oxygen in target molecule)
d. Hydrolases
e. Lipases, Esterases (resolution of racemic alcohol via selective esterification or selective hydrolysis of racemic ester
f. Lyases (formation of carbon-carbon bonds, which results in chiral hydroxyketone)
g. Donor specific aldolases

Depending on the used enzymes (reaction type), several problems have to be solved to get an economic efficient process.

The project-used enzymes, namely galactitol dehydrogenase (GatDH) and D-sorbitol dehydrogenase (DSDH), have a broad substrate spectrum including sugars and other hydroxylated compounds. They belong to the class of alcohol dehydrogenase, which are widespread used in industrial application for production of enantiopure alcohols. For the oxidation/reduction reaction they need a cofactor (NAD[H]), as hydrogen acceptor/donor. Because of its relative high costs an efficient regeneration system has to be applied to the process.

In general it is assumed that (theoretical) any other alcohol dehydrogenase can be applied to the electrochemical enzyme reactor, which should be developed during the project. So the products of the reactor are depending on the applied enzyme. The GatDH and DSDH are acting on different sugars and can produce via oxidation of e.g. galactitol the rare sugars L-tagatose or D-tagatose, which is from economic point of view the preferred reaction. That's why it was strongly recommended to prefer the oxidation reaction instead of the reduction, which is dictated by the actual prices of the substrate/ product.

The strategy developed by IZUMORI [J. Biosci. Bioeng. 89-94, 2004] is the basis for the biotechnological interconversion of aldoses, ketoses and alditols and allows for the first time convenient access to rare monosaccharides.

Only minimal information is available about the physiological behavior/function of such new produced rare sugars, so that the potential market (volume) can't be estimated to date. One rare sugar (D-Tagatose) is used to date as low-caloric sweetener and will be used in the future for treatment of diabetes type 2. So a high market volume of this kind of sugar can be assumed.

Furthermore sugars are part of highly active compounds (=glycosides) like digitoxin, glycosidic antibiotics like kanamycin or erythromycin, for which it was shown, that the aglycon didn't shown antibiotic activity. Other fusion partner can be anthracyclines (used in cancer therapy) or proteins (cell-cell interaction - immunotherapy). So new (rare) sugars, which would be commercially available in sufficient amount for investigations of their (physiological) properties themselves or as glycosides, can open the door for new formation of API (active pharmaceutical ingredients) based on (new) sugar molecules (glycol-life science).

Based on the known substrate spectrum of the project-used enzymes the following beneficial reaction should be possible. The added value factor of the product compared to the substrate is around 1,380!

Potential of technology

The needed cofactor (amount) have a great impact on overall process costs. Therefore a potent recycling system has to be applied to the process which uses normally a second substrate for the same enzyme (substrate-coupled regeneration) or a second enzyme (with specific substrate) for cofactor regeneration (enzyme-coupled regeneration). So the needed amount of cofactor can be lowered to 1 g cofactor per kg substrate, which would result in cofactor costs 1 - 2 per kg substrate calculated with bulk prices of the cofactor. Compared to other process costs the cofactor costs are negligible (depending on price difference of substrate/product).

Comparison of technologies

The existing technology (stirred batch reaction) used by partner 6 to produce enantiopure synthons is compared with the electrochemical reactor by theoretical calculations (D 6.1.3). Here it could be clearly demonstrated that the reactor can only compete in the high priced product sector with only small market volume around 1 - 100 kg per year. The production of API (active pharmaceutical ingredients), which is the interest of partner 6, has a market volume of > 1 tons - several 100 tons per year. The price per kg is < 100 per kg. Caused by the limited space-time yield (g - kg per day) and the high 'hardware' investment of the electrochemical reactor the production of such compounds with this technology is from economical point of view out of range. The main advantage of a functional electrochemical reactor is that any downstream process is needless, assuming 100% conversion in outflow. All of the enzymatic (and electrochemical) needed compounds are covalently attached on the electrode and will not contaminate the reaction solvents. This would legitimate the high input in reactor upstream costs.

At this point the main advantage of this new technology is strongly correlated to high priced fine chemicals. For bulk productions no further advantage can be seen to existing technologies in the moment. However

For bulk productions no further advantage can be seen to existing technologies in the moment. However, further development might also open this field of biotechnology to electrochemical reactors.

The space-time yields of the electrochemical reactors are up to now not comparable to existing technologies - so the question about market potential and marketing strategy can't be answered. To date it is assumed that in the near future from an economic point of view the electrochemical cofactor regeneration will not be competitive with existing methods producing bulk chemicals, which is reported by Hollmann et al. [ChemCatChem. 2: 762-782, 2010] in an excellent review about 'nonconvential regeneration methods'. They give a summary about the existing knowledge about nonconvential (=nonenzymatic) cofactor regeneration, but they end with the conclusion, that, however, at present, they are not compatible with the highly developed whole-cell approaches or even with enzymatic regeneration methods under cell-free conditions.

Enzyme production

For an industrial enzyme production process very easy and cheap systems have to be chosen. That's why

license free techniques are preferred to maximize the benefit. The candidate enzymes within this project (GatDH and DSDH) were transferred to the IEP (partner 6) used industrial expression system (E. coli RB791 with pQE70). This E. coli strain is less sensitive against phages than E. coli BL21 which could be a big problem in high cell density fermentation. This system is used by partner 6 in vessels up to 40 m3 with a yield of 80 - 100 g / l biowet weight. The Cys2His2DSDH variant could be produced with an activity around 600 U per gram biowet weight. From 10 Liter fermenter 970 g biomass could be harvested. So a volumetric productivity of 65,000 Units per Liter (calculated on 9 Liter endvolume) of the N-terminal modified sorbitol dehydrogenase could be produced.

Potential Impact:

The potential impact (including socio-economic impact and the wider societal implications of the project so far) and the main dissemination and exploitation of results

The results of the project have been already disseminated in the past three years, first of all in the traditional scientific way, that means by publication in major peer reviewed international journals and by oral and poster presentations at national and international meetings. At these occasions two of the involved PhD students have been awarded with best poster prizes. This type of dissemination will be continued also in the next few months because there are five more manuscripts that have been already submitted or that will be submitted in the near future. These publications are listed in the dissemination section.

A further way of dissemination of partner 2 is to make the results accessible to young students via guided tours of the laboratory at the occasion of the traditional visits in the frame of the French 'Fete de la Science' and the open door days organised once a year at the engineering school ENSCBP.

The third and last dissemination activity concerns the incorporation of the main results of the ERUDESP project into a fifteen hours lecture about 'Engineering of surfaces and interfaces', which is dedicated to the graduate students of ENSCBP who are specialising in the Master of Micro- and Nanotechnology

graduate students of ENSCBP who are specialising in the Master of Micro- and Nanotechnology.

Furthermore the obtained results inspired us the conduct further experiments that go beyond the ERUDESP project, namely studying flexible macroporous electrodes (see picture). This new type of electrodes will be used in the future not only as a working electrode in electrochemical set-ups but also to explore their interesting optical properties.

All partners used part of the know-how that has been acquired during the project, in order to apply for new grants. ENSCBP is focussing on the use of the macroporous electrodes for the direct conversion of light into electric energy. This grant proposal has been submitted to the French research agency ANR and the results of the call for proposals will be known in fall 2011. Another joint proposal of Partners 1a, 1b and 3 has been send to ANR and DFG for further development of the reactors with soluble and membrane bound mandelate dehydrogenases.

UCPH1, UCPH2 and USAAR1b will continue to use the ERUDESP catalysts as an example in which study of enzyme structure and function contributes to an interdisciplinary project in which industrial

enzymes are used. A higher resolution structure will be pursued to better explain the properties of the enzymes, as more crystals produced during the project period are available for testing. As well as a PhD thesis, joint publications of Parners 1b, 4a and 4b are planned on the structure. Partner 4a and 4b plan to envisage possibilities of continuing collaboration with Partner 1b on structure/function studies of LSDH, which could involve additional grant application and/or spin off Master or Bachelor student projects involving the University of Copenhagen.

The results of the project increased the knowledge in several areas such as electrochemistry, NAD-NADH conversion and its applications in several areas, enzymatic transformation with dehydrogenase application in various areas. All the knowledge's will also be used as topic of graduate courses. The gained experience of this project enabled to write new projects proposal in the area of environmentally acceptable energies projects, sensors, development and application of new chemical and biocatalysts in several areas. The results of these topics can further give some industrial applications which are the most important topics of the future.

All partners included the results in courses and lectures for the education of undergraduate and graduate students. The electrochemical reactor has been introduced to the biotechnology members of the 'University of the Greater Region' an association of the following Universities: Saarbrucken, Trier, Kaisereslautern, Metz, Nancy, Luxembourg, Liege. This association is supported by the EU Program INTERREG IVA.

Project website:
http://www.erudesp.eu

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