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Immobilization of 2-Deoxy-D-ribose-5-phosphate Aldolase on Membrane Supports by Spray-Coating: Optimization by Design-of-Experiment

Thilo Fischer,[a] Marcel Sperling,[a] Sophia Rosencrantz,[a] Martin Wäscher,[b] Jörg Pietruszka,[b, c] and Stefan Reinicke*[a]

A strategy for the immobilization of the enzyme 2-deoxy-D-ribose-5-phosphate aldolase (DERA) is presented via spray-coating on membrane support material to generate an enzymatically active membrane which can be used in continuously run synthesis modules. A functional, water-soluble copolymer containing addressable units to covalently bind DERA is mixed with the enzyme, followed by spray-coating of the mixture onto polyacrylonitrile/polyethylene imine- (PAN/PEI) membranes and a subsequent post-processing to stabilize the coating. Confirmation of successful immobilization was achieved by atomic force microscopy (AFM) imaging and assessment of retained enzyme activity. Through variation of relevant parameters, improvements in stability and activity were seen, which formed the basis for the following optimization by Design-of-Experiment (DoE). A first fractional factorial design yielded additional performance improvements and important insights into the impacts of experimental parameters and their interaction. A second full factorial design did not result in further improvements, but validated the results of the first design.

Introduction

Due to the outstanding catalytic properties of enzymes, including high specifity, high reaction rates, reusability, and mild reaction conditions, there is a strong demand for industrial applications, particularly involving chemoenzymatic synthesis.[1] When using isolated enzymes instead of whole-cell-systems, there remains the challenge of retaining enzyme activity and therefore also the viability outside their natural environment. Furthermore, like other species of dispersed or dissolved catalysts, they have to be removed from the product post-synthesis, which often covers up to 80% of the total process costs in biocatalytic fields.[2] Depending on the application, it may therefore be beneficial to use enzymes in an immobilized form. This would not only avoid high purification costs, but also enable an alternative process control (e.g. continuous instead of discontinuous).

For the purpose of the combination of transport and separation processes, membranes are a great option to be used in conjunction with enzymes or enzyme immobilization[3], while synthesis in a flow process also ensures a direct removal of the product from the enzyme. As a result, it prevents a product accumulation in the vicinity of the enzyme, hence enabling to work with enzymes, suffering from product inhibition. This is especially important for industrial applications, since it allows for utilizing relevant substrate and product concentrations.

One biocatalytic process where this aspect is most relevant is the aldol coupling of acetaldehyde with another aldehyde catalyzed by 2-deoxy-d-ribose-5-phosphate aldolase (DERA), an enzyme which is indeed inhibited by its product. On top of that, a flow setup can help to limit the contact time between enzyme and substrate, which allows to isolate the monoaldol product that would otherwise only be an intermediate subjected to another aldol coupling step leading to dihydroxyaldehydes.[4] However, the monoaldol product is an important building block for pharmaceutically active compounds, fragrances and food supplements[5] and therefore of high value.

While there have been efforts to immobilize DERA[6], only one contribution so far had dealt with a continuous flow process, though not focussing on the isolation of monoaldol product.[7] First successful efforts in this direction were recently made by Pietruszka et al., though not yet with a membrane setup but with a conventional packed bed-reactor system.[8] With a membrane setup, however, in which DERA would be immobilized in thin films and enzyme amounts are controlled by the provided membrane surface, a much narrower contact

[a] T. Fischer, Dr. M. Sperling, S. Rosencrantz, Dr. S. Reinicke
Department of Life Science and Bioprocesses
Fraunhofer-Institute for Applied Polymer Research (IAP)
Geiselbergstraße 69, 14476 Potsdam (Germany)
E-mail: Stefan.Reinicke@iap.fraunhofer.de
Homepage: https://www.iap.fraunhofer.de/en/research/life_science_and_bioprocesses.html
[b] M. Wäscher, Prof. J. Pietruszka
Institute of Bioorganic Chemistry
Heinrich-Heine-University Düsseldorf at Forschungszentrum Jülich
Stetternicher Forst, 52426 Jülich (Germany)
[c] Prof. J. Pietruszka
IBG-1: Biotechnology
Forschungszentrum Jülich GmbH
52425 Jülich (Germany)

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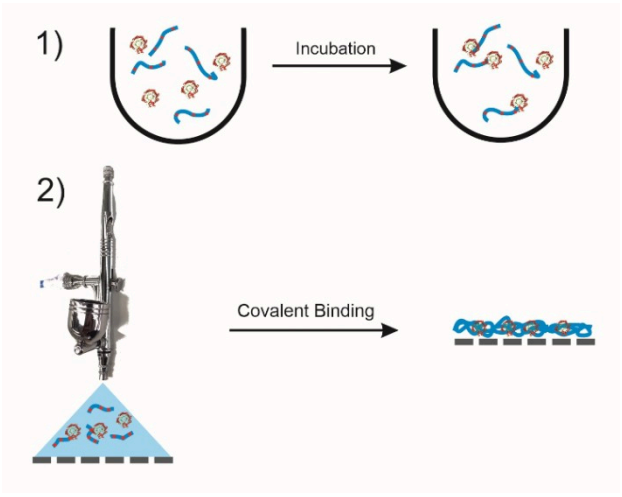
time distribution and thus more control over the process would be possible. Besides, immobilization of DERA within the packed bed-reactor had been done through a Halo-Tag, which is convenient to use but too expensive for larger scale operations.

Preliminary work on immobilizing DERA in thin polymer layers has been performed in our group already.[9–11] Here, thiolactone containing copolymers were used in which the thiolactone units fulfil a multi-purpose role, including binding of protein, binding to the support material and crosslinking the enzyme accommodating polymer matrix after the immobilization step. While this chemistry has been mainly used in non-scalable high precision immobilization approaches including the Langmuir-Schaefer technique and the self-assembly of DERA-polymer conjugates, a more practical and scalable immobilization procedure is needed to have an immobilization protocol at hand with higher practical relevance for synthetic purposes. This is now achieved by simply mixing enzyme and polymer and subsequently spraying the mixture onto the membrane support of choice, followed by post-treatment to stabilize the generated biocatalytically active layer (Scheme 1). Regardless of this simplification, the benefits of the used chemistry, as described above, stay in full effect, offering a very useful immobilization procedure. The paper is structured as follows: First preliminary investigations are presented in which plane silicon wafers are coated in order to be able to judge on film stability. Then, we switched to PAN/PEI-membrane supports finally implementing them into a basic flow setup for determining relevant parameters for the optimization of the immobilization. As last and main part, a design-of-experiment approach is conducted to maximize the performance of the enzymatically active membranes.



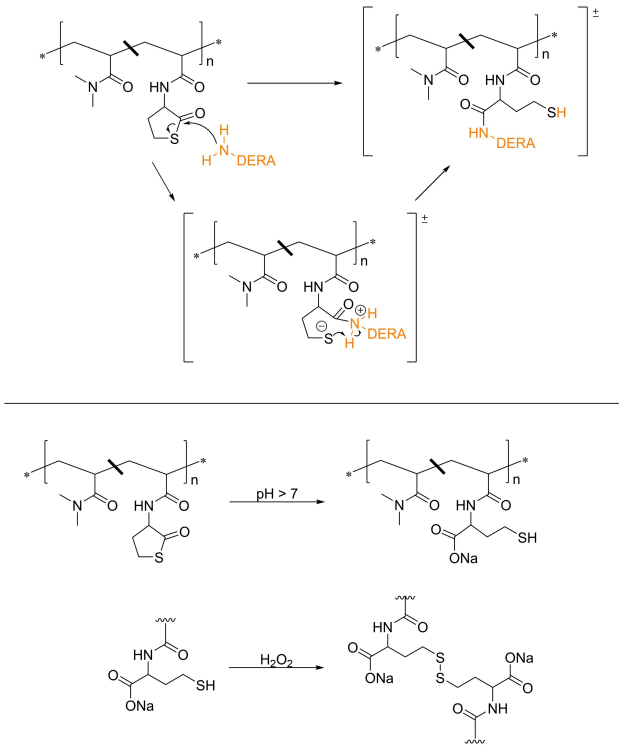
Scheme 1. Depiction of the immobilization procedure. First, the enzyme is incubated with the matrix forming polymer (see Scheme 2) in buffered solution. This solution is then sprayed onto the membrane support.

Results and Discussion

The polymer that is supposed to form the enzyme accommodating layer on the membrane support is based on polydimethylacrylamide P(DMAAm), additionally carrying a defined amount of homocysteine thiolactone units (Tla) along the chain (Scheme 2). This type of polymer appeared to be well compatible with DERA.[9–11] The first step is to mix the polymer with the enzyme. Here, the amino groups of lysine residues of the DERA will partly attack at the carbonyl carbon of the thiolactone rings, thereby connecting the enzyme with polymer. This reaction is considered rather inefficient[12], which, however, is beneficial, since too many anchoring points may disturb the structure of the enzyme too much which may cause its deactivation.

In the same way as the amino groups of lysine residues, amino groups on the membrane surface will react after the spray-coating step, thereby anchoring the polymer matrix on the membrane surface. Finally yet importantly, the thiols released upon reaction with the amines can form disulfide bonds, which crosslinks the matrix and therefore stabilizes it. In order to enhance the thiolactone ring opening and in turn the disulfide formation, a post-treatment step after the spraying is necessary, which includes contact of the surface with a basic, diluted solution of H_2O_2.

It is noted, that the chemical groups formed by the proposed reaction mechanism are hardly detectable as both enzyme and polymer already contain plenty of these groups (amide, carboxyl group, thiols). However, in a previous contribu-



Scheme 2. Structure of the polymer in use and basic reaction mechanisms occurring during the incubation and the post processing step.

tion, we could generate indirect proof by showing that the presence of DERA has a crosslinking effect, which can only stem from the reaction of its lysines with the thiolactone groups of the polymer[11]. Disulfide formation on the other hand was indicated by an enhanced film thickness.

The spraying step takes place after the enzyme has been incubated with the polymer for a defined amount of time. The post-treatment directly follows the spraying step. The exact timing is part of the optimization procedure, which is discussed later.

Basic coating stability

A first assessment of the principle applicability of the described procedure was done by spraying and post-treating material on plane silicon wafers. The wafers were equipped with primary amino groups beforehand in order to provide anchoring points for the enzymatically active layer. AFM analysis provides a first idea about the coating stability. Polymers with different Tla contents of 3.5% and 15% were tested in two manners. First, comprising of a submersion approach as a reference, where, instead of using the spraying approach, the pretreated silicon wafers were completely submerged in the coating solution for about 6 h, and second, the intended approach of spray-coating the coating solution onto the pretreated silicon wafers as described. AFM images of these tests can be seen in Figure 1. Furthermore, the influence of different exposure times of the coated silicon wafers to the sprayed post-treatment solution was investigated. The results basically show that higher Tla contents in the P(DMAAm-co-TlaAAm) polymer form better and more stable coatings, while spray-coating yields much better surface coatings than the referential submersion approach. Spraying the material directly onto the surface allows for the use of lower concentrations and solution volumes, due to the accessibility of the material to the surface, whereas in a submersion approach, interaction is much more random and is therefore less likely, resulting in much thinner layers. It also became apparent that after the coating procedure there was still an excess amount of material on the silicon wafers, which was removed during the post-treatment and the following washing procedures. The tests also indicate a certain loss of material upon post-processing, a prolonged processing time, however, not having very much impact.

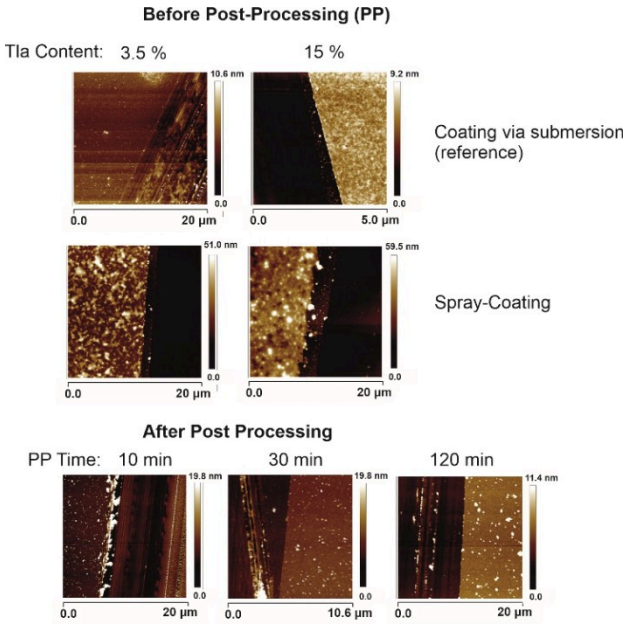


Figure 1. AFM height images of DERA/P(DAAm-*co*-TlaAAm) films on amino functionalized silicon wafers. By scratching over the film surface with a needle, the bare wafer surface was exposed in order to be able to measure film thickness. The average thicknesses are summarized in Table S1 (SI).

Of course, these results refer solely to film building capabilities of the system and not to performance in terms of enzyme activity. Still, this showed that the concept of immobilization in polymer matrices and attachment to surfaces presenting primary amines works and a stable coating was achieved.

Basic investigation of immobilization parameters with focus on activity retainment – batch and flow-through protocol

The success of an enzyme immobilization strongly depends, among immobilization yield and other parameters, on the amount of retained activity. In the first tests for enzymatic activity, PAN/PEI membrane supports were coated instead of Si wafers, as these membranes are the targeted support for the envisioned flow process later on. Supplemental spray-coating experiments using fluorescently labelled DERA show that the enzyme distribution over the membrane surface is fairly uniform (Figure S1A, SI). There is a slight accumulation of enzyme at the edges along one of the two plane axes, which is the result of the concave curvature of the membrane specimens that are used for the immobilization. On the local scale, however, no inhomogeneities in the form of DERA clusters are detectable that could potentially have a detrimental effect on enzymatic activity. Please also note that the spray-coating solution does not really sink in after the spraying act (Figure S1B), so the biocatalyst should settle exclusively on the surface but not inside the pores. Activity measurements, based on a coupled assay using the natural substrate of DERA (see Experimental Part and Scheme S1, SI), were first done in a batch mode with end point detection. The active membranes were immersed in the assay solution for a certain amount of time and before the first immersion as well as after each removal, the respective absorption was measured. The drop of absorption over the immersion time indicates the extent of enzymatic activity.

The first crude measurement showed DERA activity on the coated membranes, although it appeared to be greatly reduced and some enzymatically active material obviously leaked into the solution (Figure 2). The single coated membrane contained about 25× the amount of DERA as the positive sample, while the triple coated membrane theoretically contained 75× the

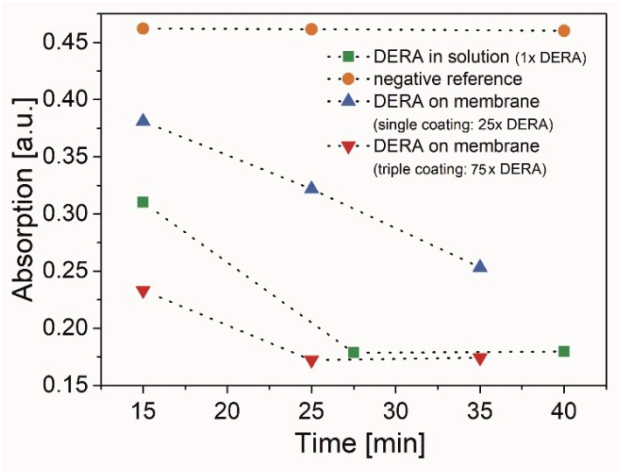


Figure 2. Assessment of DERA activity after first immobilization trials. Drop of absorption with time indicates activity. For the assessment, membranes were simply immersed in the assay solution and taken out after 15 min followed by start of the measurement. All dissolved, enzymatically active material was in contact with the assay solution over the whole measuring time of 40 min. Starting value at 0 min for each case is indicated by the negative reference, where absorption hardly changed over time. Immobilization parameters: enzyme/polymer ratio = 1; total solids concentration = 2 mg/mL; pH of post processing solution = 9; exposure to spray-coating solution = 5 min; exposure to post-processing solution = 2 min; Tla fraction polymer = 31 %.

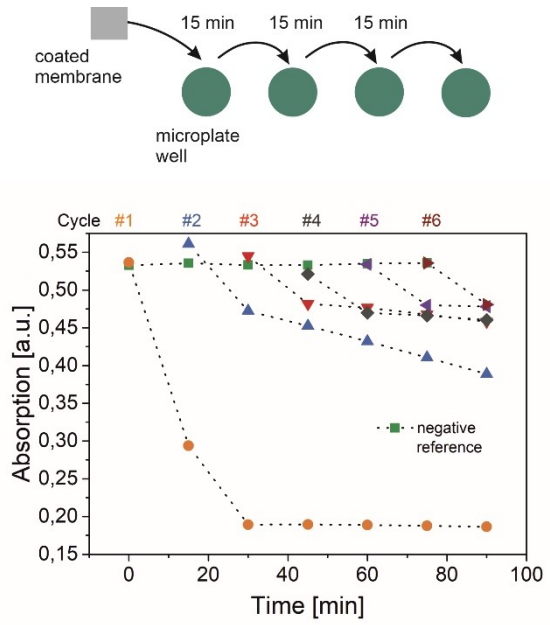


Figure 3. Assessment of DERA activity in multicycle style. For each cycle, a starting value for the absorption was recorded, followed by immersion of the membrane for 15 min. After the membrane was taken out again, absorption was started to be recorded continuously.

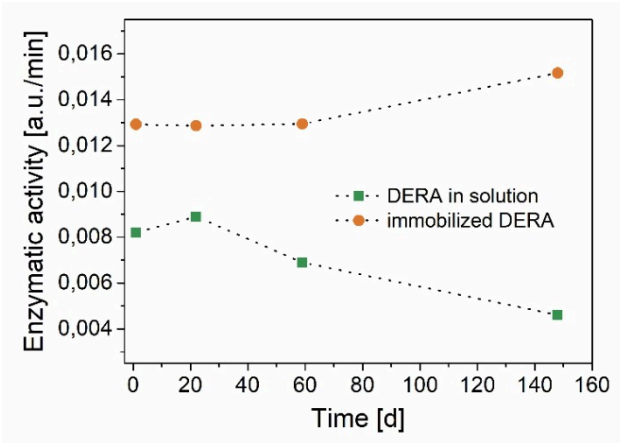


Figure 4. Development of enzymatic activity with long-term storage of the DERA containing membrane in comparison with DERA in solution. Potassium phosphate buffer (pH 7, 20 mM) was used as storage medium.

amount of DERA, leading to an estimated activity reduction by a factor of 50.

A multicycle procedure for activity testing then allowed for much more detailed analysis of membrane activity, stability and now also reusability, the latter being of great importance for future applications. In this procedure the membrane is removed from a first well containing the assay solution after a certain time, and then placed in another, fresh well to initiate the second cycle. This is repeated several times and after each removal, absorption is started to be measured continuously in the respective well. The first absorption value respectively indicates whether there was activity during the immersion of the membrane in the particular well, while a further drop of absorption from this point on shows that active enzyme has been leaked into the assay solution.

A typical multicycle measurement is shown in Figure 3. The plot indeed indicates the initial loss of material from the membrane showing the presence of residual soluble material after the immobilization procedure. However, it also reveals a consistent and stable layer remaining on the membranes after a few operation cycles. This proved the immobilization concept to be valid, but also indicated the need for further improvements. The next requirement comprises a consistent coating for multiple uses, also remaining intact for a prolonged period of time, which was assessed via long-term measurements. Indeed, this revealed the coating to show unchanging activity during the test period of over six weeks, while the activity of free DERA in solution was constantly decreasing. This indicates a meaningful stabilization of DERA and increased enzyme viability, as a desired side effect of the immobilization (Figure 4).

In an attempt to generally improve the extent of activity retention over the immobilization, which has been rather poor during the first trials as mentioned earlier, several immobilization parameters were varied. However, neither the shortening of the exposure time of the post-treatment solution, nor the further increase of the Tla fraction in the polymer or polymer concentration showed much of an improvement in activity or stability (Figure S2, SI).

Therefore, before the final optimization of the immobilization procedure via a DoE approach, the simple solid-liquid contact in the activity multicycle procedure was replaced by

more realistic membrane module conditions. This was achieved by using a *flow-through approach* that involved pumping the substrate solution right through the membrane, which in this way is exposed to pressure and active substrate flow for the first time. The activity assessment protocol was altered in that the coenzymes TPI/GDH as well as NADH were added to the permeate and were not part of the solution initially passing through the membrane. Therefore, the measured DERA activity in this case is not represented by the slope of the curve, but by the extent of the drop in absorption. As in the case of the multicycle procedure, however, further decline of absorption points to ongoing enzymatic activity in the permeate due to DERA leakage.

Prior to measurement the membranes were now flushed with buffer solution each time until a stable enzymatically active coating remained. This step was skipped in all the previous activity assessment experiments in order to visualize and assess the extent of material loss due to incomplete crosslinking. Now, however, focus was put more on the performance of the remaining stable layer. To improve retained enzyme activity, post-processing associated parameters such as pH, duration and whether post-processing is necessary at all, were studied in further detail. A higher pH of the post-processing solution would speed up the crosslinking reaction within the coating, but would negatively impact DERA viability, as was the case when increasing pH above 10 (Figure S3, SI). When skipping post-processing altogether, to avoid any decrease in enzyme activity, the activity was better initially, but decreased rapidly, since the coating was significantly less stable without it (Figure S4, SI).

To enhance activity per area unit of membrane the concentration of the spray-coating solution was increased by a factor of 3 and 10 respectively. The initial flushing procedure was here skipped again to better visualize the influence of increased concentration on the film stability. Again, the actual activity of the membrane is indicated by the initial absorption drop while the steady slope afterwards represents ongoing activity stemming from DERA in solution, which was washed off from the membranes (Figure 5). Surprisingly, the increase in activity was the same for 3× and 10× increased concentration with 10× also containing increased amounts of DERA in the assay solution, indicating insufficient stability of the thick coating obtained from the enhanced material concentration. This result answers an important question, which is how much biocatalytically active material is actually applicable per area unit of membrane. The applied spraying parameters translate to a maximum amount of material on the membrane of 1.7 mg/cm^2 that can be reasonably applied, although this value only refers to the total amount of solid material. The fraction of enzyme within this material can be varied.

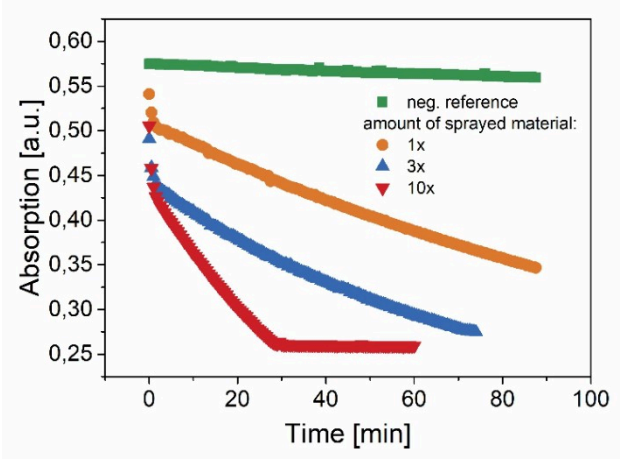


Figure 5. Assessment of DERA activity in flow-through-style with variation of the amount of sprayed material (conc. solid material in spray-coating solution = 3 mg/mL, enzyme/polymer = 0.5). The extent of the initial drop of absorption signals the extent of activity on the membrane while ongoing decrease of absorption points to enzymatically active material in the permeate.

Optimization by Design-of-Experiment

General aspects

At this point, the retained enzyme activity after the immobilization procedure of DERA on PAN/PEI membranes was improved to 10% of the natural activity of DERA in solution instead of 2% measured during initial activity tests at the membrane interface. It also became apparent, which of the multitude of parameters influence the immobilization procedure, which are already optimized and which need further investigation, also taking interactions into account. Parameters that could be regarded as optimized are the incubation time of the coating solution, airbrush pistol settings, PAN/PEI membrane, polymer composition of 31% TlaAAm and the hydrogen peroxide concentration of the post-processing solution. Design-of-Experiment protocols could now be created using the software Cornerstone 7.2 by camLine. Five potentially significant factors were determined and implemented in a fractional factorial design (see table 1) with a subset consisting of cEnz/cPoly (E-P_R) and cSpraySol. These two factors were chosen, since they were already

Table 1. Factors considered for the optimization of the immobilization procedure via DoE.

Factor	Type	Range	Description
E-P_R	Continuous	*1/6–1*	Ratio of enzyme concentration to polymer concentration in the spray-coating solution
$C_{SpraySol}$	Continuous	*1–3 mg/ml*	Total concentration of spray-coating solution
$t_{ExSpray}$	Continuous	5–30 min	Exposure time of spray-coating solution on membrane
t_{ExPost}	Continuous	5–30 min	Exposure time of post-processing solution on membrane
pH_{Post}	Continuous	7–10	pH of post-processing solution on membrane

correlated and their effects and interactions could therefore be estimated the easiest and with the highest expected precision by the software. The DoE was optimized for activity over mass, referring to the increase of membrane mass through the immobilization procedure. The implementation of one subset into the design cuts the number of experiments from $2^5 = 32$ in half to $2^{(5-1)} = 16$ experiments (see Table S2, SI). Therefore, the experimental effort remained reasonable.

1st DoE round

The response graphs generated by the DoE software are shown in Figure 6A with the factors and their respective ranges on the x-axis and adjusted activity, activity-over-mass and mass on the y-axis, respectively, indicating whether minimum or maximum of a respective range is favorable by rising to that side. An effects pareto was also generated (Figure 6B), indicating the impact of each individual factor. For this assessment it is irrelevant, whether the values are negative or positive. The results generated via *Cornerstone* had a coefficient of determination of $R^2 = 0.733$.

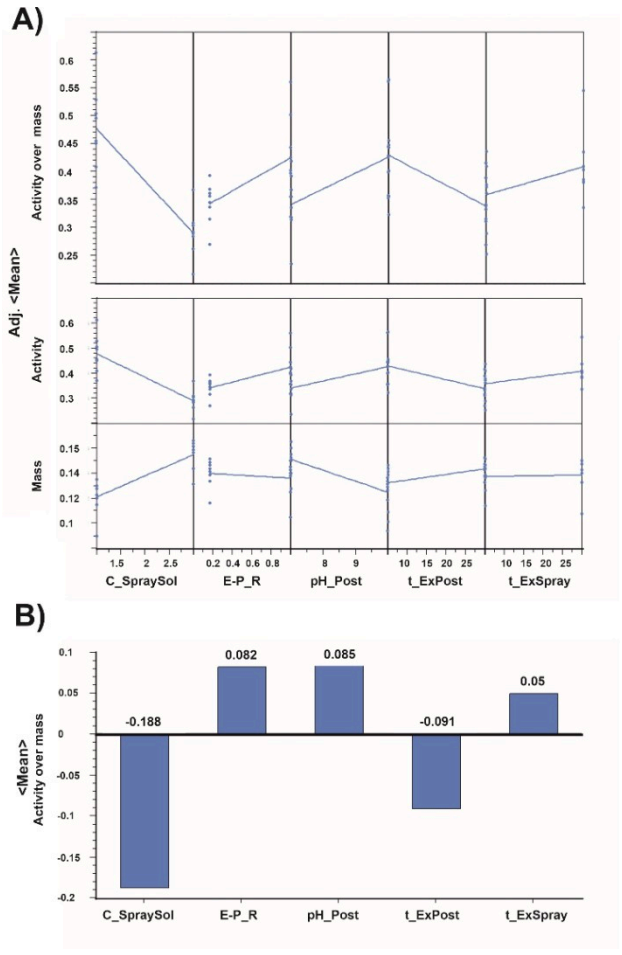


Figure 6. First optimization round via Design-of-Experiment. A) Activity over mass, Activity and Mass Response graphs. B) Activity over mass effects pareto. The meanings of the depicted codes are summarized in Table 1.

The most impactful factor was the concentration of the spray-coating solution $c_{SpraySol}$. Surprisingly, the lower concentrated solution with the mathematical factor 1 (1 mg/mL DERA, 1 mg/mL polymer) yielded better results than the one being three times more concentrated. This implies that using more overall material does not necessarily result in more activity. Since the DoEs optimization strategy towards activity over mass might have influenced this result significantly, a new DoE-fork was created (a separate DoE analysis based on the same factors and data as the original one) with an optimization strategy in regard to activity (independent from mass). As can be seen in Figure S5 (SI), this did not change the result in that using more material does not translate into more activity. The reasoning behind this might be more sterical requirements for successful coordination, the formation of too densely packed coatings, eventually leading to diffusion limitations, or too much structural interference with DERA itself. Also surprising were the results of the mass ratio of enzyme to polymer in the spray-coating solution c_{Enz}/c_{Poly} (named E-P_R in the Figure). There, the results improved for a ratio of 1 instead of the sixfold amount of polymer to enzyme. A previous test with this ratio did not show many changes when increasing polymer concentration in the spray-coating solution. However, during these tests, only one factor was varied and the experiment was not optimized towards activity over mass. This was good news, since it meant that less polymer is needed for a successful immobilization, further reducing overall costs. The pH value of the post-processing solution was the third most impactful factor and showed that a higher pH, in this case a pH of 10, is beneficial. It could have been possible for lower pH values to outperform higher ones, due to milder conditions and in combination with longer exposure durations, but the original concept of higher pH to initialize openings in the TlaAm rings has been proven the best option. The next factor concerning the exposure time of the post-processing solution to the membranes t_{ExPost} was actually the second most impactful factor and as the response graphs revealed, a lower duration of 5 min is superior over a longer duration of 30 min. It seems that the combination of a higher pH with lower post-processing exposure times were sufficient for a successful crosslinking of the coated layer and therefore for the immobilization process. At low exposure times of the post-processing solution, negative side effects of the solution itself were avoided. These negative side effects were previously proven to be minimal, but reduced exposure was still beneficial. The last factor, the exposure time of spray-coating solution $t_{ExSpray}$, was also the least impactful, but still significant enough to be considered for the immobilization procedure and worth the extra expense. Here, a longer exposure time (time between spraying and application of the post-processing solution) of 30 min yielded better results than a lower one of 5 min, which is probably due to increased time requirement for coordination of the enzyme-polymer conjugates to the functional membrane surface to initiate the thiolactone ring opening reaction and therefore covalent bonding as well as a spatial alignment to each other.

Additional insights can be gained when looking at the influence of the factors on activity and mass gain separately

(Figure 6A, lower two lines). Going from left to right, it can be seen that an increase in the concentration of spray-coating solution $c_{SpraySol}$ is directly disproportional in its effects on mass and activity. Logically, the gained mass increased, while the activity decreased and thereby validating prior assumptions. The ratio of enzyme to polymer in the spray-coating solution c_{Enz}/c_{Poly} showed only a slight increase in mass when a ratio of 1/6 (1 mg/mL DERA, 6 mg/mL polymer) was used, but an increase in activity when equal ratios were used, as stated before. This again proves that in fact less enzyme is favourable. The influence of the pH of the post-processing solution pH_{Post} on the mass was higher than expected. It seems that for lower pH values a higher gain in mass was observed, but a reduction in activity, while a higher pH resulted in lower mass gain, but higher activities. The original assumption would have predicted a gain in mass as well as activity for higher pH values, as stated before. When looking at the exposure time of the post-processing solution *tExPost*, the results matched the expectations. A longer exposure to the crosslinking initiating post-processing solution led to more mass ultimately remaining on the membrane, but also increased the effect of the slight enzyme viability reduction through the solution. At lower exposure times, the factor had less impact while still enabling a sufficient crosslinking within the coating. The last factor concerning the exposure time of the spray-coating solution $t_{ExSpray}$ on the membrane had little to no effect on the mass, but slightly increased activity for a longer exposure duration, as stated above.

2nd DoE round

The results from the first DoE are taken as a base to define the optimum immobilization protocol. Table 2 shows the immobilization parameters that are extracted from the DoE process as the most optimal ones, both for the activity and activity-over-mass optimization branch. It is emphasized that these parameters are deduced from the effects pareto plots (Figure 6) as an act of experimental reasoning and do not result from a fit of an underlying model. This is due to the experimental constraints of some of the main parameters of influence, which rendered further optimization rather unreasonable. In order to support this, we did another DoE around said optimum (Table S4 and S6 SI). For the second DoE round, only three factors, the pH of the post-processing solution, the enzyme/polymer ratio and the sprayed volume of the post-processing solution, were considered. The remaining factors did not promise further optimization potential in our view. With three factors a fractional factorial design was not necessary and therefore a full factorial design could be realized. The subsequent calculation (Figure S6, SI) established that all results were within standard deviation and therefore insignificant. Further evidence for this resides in the very low R-squared value of $R^2 = 0.164$, meaning that only 16.4% of all variation in data can be contributed to the factor/activity relationship, while the remaining 83.6% originate from experimental noise. This means, that the total data variation for this second DoE round was within the expected margin of error. The resulting model was not able to describe a significant trend, which indicated our initial assumptions about the optimum parameters resulting from DoE 1.

Table 2. Final immobilization parameters extracted from the DoE experiments.

Factor	Value
$\frac{c_{Enz}}{c_{Poly}}$	*1*
$c_{SpraySol}$	*1 mg/ml*
$t_{ExSpray}$	30 min
t_{ExPost}	5 min
pH_{Post}	10

The optimal immobilization parameters

The suggested immobilization parameters appear to coincide with the ones used for experiment 11 of the first DoE round (Table S2), which indeed stands out from the other experiments in terms of activity-over-mass (Table S3). At the same time, however, the absolute activity does not stand out so much, which means that mass gain was rather low in this case. Yet, this indicates, that the settings in experiment 11 lead to a rather high retention of specific activity. In other words, even if other settings lead to higher absolute activity, much more enzyme is needed in these cases. Finally, we estimate that when using the parameters summarized in Table 2 for immobilization, the specific activity of the immobilized DERA can be raised from initially 10% (before optimization) to about 17% compared to its counterpart in solution. This is a fair value if one takes the very good long term stability of the enzymatically active membranes into account, which in principle allows for long-term use of one single membrane.

Conclusion

This paper discussed the complete process from the idea to the optimized immobilization procedure of the aldolase DERA for the preparation of biocatalytically active PAN/PEI membranes for the use in membrane modules. Thanks to a combination of applied analytic methods, such as AFM, activity multicycle measurements of coated membranes and simulating membrane module conditions in a flow-through setup, successful immobilization with a tolerable loss of enzyme activity could be achieved while gaining long-term stabilization. The results now justify the beginning of investigations in applied chemo-enzymatic synthesis, involving the aldol reaction of acetaldehyde using variants of DERA with improved acetaldehyde tolerance. After another optimization round, involving the new, industrially relevant substrate acetaldehyde, the next step would be the upscaling of the coated membranes and utilization in real membrane modules with the goal of eventually being used and scaled for industrial application.

Experimental Section

Materials

Poly(dimethylacrylamid-*co*-N-thiolactone acrylamide) [P(DMAAm-co-TlaAAm)] was synthesized by reversible addition fragmentation chain transfer (RAFT) polymerization according to a procedure described elsewhere.[13] The aimed degree of polymerization was 200 respectively. Molecular weight and homocysteine thiolactone (Tla) content were confirmed by SEC using NMP as eluent and nuclear magnetic resonance- (NMR) spectroscopy. Silicon wafers were equipped with amino groups according to a procedure described elsewhere.[11] PAN/PEI blend membranes were synthesized according to our patent protocol.[14] 2-Deoxy-D-ribose-5-phosphate aldolase (DERA) was expressed in E. coli and purified via immobilized metal ion affinity chromatography (IMAC). The samples were subsequently freed from imidazole with PD10 desalting columns (cytiva).[15] The protein was diluted in KP_i-buffer (50 mM, pH 7) to a concentration of ca. 5 mg/mL, and lyophylized for storage and transport.

Monobasic and dibasic potassium phosphate (Sigma-Aldrich, $\geq$99.0%), hydrogen peroxide (Roth, 35% in H_2O), nicotinamide adenine dinucleotide (NADH) (Roche, ~100%, grade I), 2-deoxy-D-ribose phosphate (DRP) (Sigma-Aldrich, $\geq$95.0%) and triosephosphate isomerase/glycerol-3-phosphate dehydrogenase (TPI/GDH) [Sigma-Aldrich, type III, ammonium sulfate suspension, TPI 750–2000 units/mg protein, GDH 75–200 units/mg protein (biuret)] were used as received.

Spray-coating procedure and post-treatment

For the preparation of the spray-coating solution, DERA was dissolved in KP_i buffer (20 mM, pH 7) and then added to P(DMAAm-*co*-TlaAAm), followed by stirring at room temperature for 1–2 h. For the spray-coating procedure an airbrush pistol (AFC-101A) was used, operating with 0.5 bar nitrogen gas pressure, maximum flow rate, 1.25 turns of airbrush pistol top valve, 0.75 turns of airbrush pistol bottom valve and a sample distance of about 2 cm. For each spray-coating, 500 μL of the previously incubated solution were filled into the airbrush pistol tank. Spraying accured three times in equal intervals, spraying a total volume of about 300 μL of which about 150 μL actually reached the sample (diameter ~ 1–1.8 cm). For the post-processing, KP_i buffer (20 mM) was set to a defined pH and hydrogen peroxide was added to reach a concentration of 0.1%. This solution was also sprayed three times, however with a slightly lower total volume of 90 μL (if not otherwise stated) reaching the sample. After that, the samples were gently waved in Milli-Q water for half a minute for washing. Silicon wafers were then air-dried, while membranes were stored in KP_i buffer (20 mM, pH 7) until measurement.

Flow-through setup

For simulating flow-through conditions, a cap of a 15 ml plastic reaction vessel (Falcon™) was perforated and equipped with the enzymatically active membrane. The cap was then screwed onto the tube and the latter was filled with substrate solution while its bottom was cut away to attach a hand-held pumping ball for pressure application. Later, an improved setup was used by placing the membrane into a Swinnex filter holder which was attached to a disposable syringe that was operated with a syringe pump (kd scientific, KDS-200-CE). In a pre-conditioning step, each membrane was flushed with 5 mL KP_i buffer at a flow rate of 0.5 mL/min. After that, substrate solution was pumped with a rate of 0.2 mL/min respectively. 800 μL of the collected permeate were transferred to a microplate well respectively and subjected to absorption measurement (see next paragraph ‚Activity assay')

Activity assay

The standard activity assay consists of a two-step enzymatic reaction during which the consumption of NADH is observed via photometric measurement by a microplate reader (Infinite M200 Pro, Tecan). Each well of a Greiner 24 flat bottom transparent polystyrol microplate was filled with the substrate DRP (30 μL, 25 mM), NADH (10 μL, 15 mM), TPI/GDH (15 μL, 1 U/10 U, diluted in phosphate buffer (KP_i, 20 mM, pH 7) with a ratio of 1/10) and phosphate buffer (935 μL, KP_i, 20 mM, pH 7). After waiting for about 10 minutes to achieve equilibrium, DERA (10 μL, 0.2 mg mL) was added and the measurement was started immediately. Absorption ($\lambda_{abs.} = 340$ nm, T = 25 °C) was measured in intervals of 30 seconds, until an absorbance plateau was reached. The slope of the resulting absorbance-curve is used as a measure for enzyme activity.

In case of enzymatically active membranes, the latter were immersed in the well and taken out after a certain time interval. Absorption in this case was recorded once before the immersion and in a continuing fashion after the membrane has been removed again. For samples collected via the flow-through setup, a substrate solution only consisting of buffer and DRP was pumped through the membrane. After transfer to the microplate well, which contained already buffer and NADH, TPI/GDH was added followed by continuous monitoring of the absorption. In order to avoid premature full consumption of NADH, the concentration of DRP was halved where needed.

Gravimetric analysis

Gravimetric analysis was performed using an analytic balance (Mettler Toledo XA205 Dual Range).

Atomic force microscopy

AFM analysis was performed on a Bruker Dimension Icon using NanoScope 9.1 software for measurements and NanoScope Analysis 1.5 for image processing. Measurements were conducted in tapping mode in air using a silicon tip with a spring constant of 40 N/m at 0.5 Hz scan rate. The coated wafers were scratched using a syringe tip and measured perpendicular to that scratch to determine layer thickness.

Design-of-Experiment analysis

DoEs were created using the software Cornerstone 7.2 by camLine. For reliable results, samples were measured in triplicates.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: DERA · Design-of-Experiment · enzyme immobilization · membrane

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