Catalytically active flavin-containing polyelectrolyte complexes
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CHAPTER 2

Catalysis by flavin-containing polycations, immobilized in polyelectrolyte complexes with poly(sodium styrenesulphonate)

SUMMARY

Polycations, containing pendent flavin groups, can form polyelectrolyte complexes (PECs) with Poly(sodium styrene sulphonate) (PSSS), as was shown by conductometric measurements. The catalytic activity of the immobilized flavin-containing polycations was determined by measuring the rate of oxidation of 1-benzyl-1,4-dihydronicotinamide (BNAH) in water-rich medium. The effect of the degree of substitution with quaternary ammonium groups (β) of the polycation and the influence of the ratio in which the polycation and polyanion are mixed were investigated. A negative influence of complexation upon the catalytic activity of the flavin-moiety was found, which is almost instantaneous. In stoichiometric complexes only a small effect (about 5% loss in activity) was found for low β, but for high β the complexation resulted in substantial decrease of catalytic activity (50%). The effect also depended upon the mixing ratio of polyanion and polycation, and became stronger if more polyanion was applied. The flavin-containing PECs could be reused many times in batch wise reactions and showed excellent stability in continuous experiments in a stirred tank reactor and as a sandwich-membrane.

INTRODUCTION

The covalent binding of catalytically active species to polymeric materials has been shown to be a versatile way to control the properties and reactivity of catalysts\textsuperscript{1,2,3,4}. For instance, crosslinked polymers can be used to obtain 'site isolation' of catalytically active moieties. This is especially useful in the case that dimerization or cross-reactions can cause termination of the catalytic cycle\textsuperscript{5}. In most cases however, species immobilized on such crosslinked materials are less (re)active compared to their low molar mass analogs, due to diffusional limitations\textsuperscript{6}. Attention has therefore been focused on linear polymers as carriers for catalysts in recent years. Such polymer-bound catalysts are not likely to show diffusion limitation, because of the large fraction of solvent (more than 95\%) in the polymer domains\textsuperscript{7}. The isolation for re-use of such polymeric catalysts is practically not very convenient however, but it has been shown that they can be immobilized successfully by grafting on suitable carriers with very good preservation of catalytic activity\textsuperscript{1**}. An alternative way of immobilizing linear polymeric catalysts is the adsorption, or rather, complexation to the surface of an insoluble material like silica\textsuperscript{9} or to another soluble polymer. The latter possibility is especially suitable if the catalytically active polymer has acidic, basic or ionic moieties. In that case the combination with a complementary polymer will lead to a physically crosslinked network, or polyelectrolyte complex\textsuperscript{10,11,12} (PEC) in which the polymeric catalyst is immobilized. The cooperativity of these interactions, characteristic for polymeric materials, make such networks quite stable, even though the separate bonds may be very weak. Also, in the presence of a suitable solvent (like water) and/or low molar mass salts the PECs can show enormous swelling, with over 80\% of solvent, and the term 'immobilized water' has been used in this context\textsuperscript{11}. In
certain well defined (ternary) solvent mixtures they can even be solubilized again\textsuperscript{13}. This combination of swelling and the dynamic nature of the crosslinking keeps the immobilized catalyst very easily accessible for its substrates.

The model reaction of the polymer-bound flavin studied here, the aerobic oxidation of an NADH-analog, 1-benzyl-dihydronicotinamide (BNAH) (see figure 1.3), has been examined before. It was shown that the binding of flavin to polycations leads to an increase of reaction rate, due to substrate enrichment and a faster hydride-transfer (increase of $k_2$), which might be ascribed to the favourable influence of the neighbouring cationic groups\textsuperscript{14}. Here we describe the immobilization of such flavin-containing polycations in polyelectrolyte complexes with a strong polyanion, poly(sodium styrenesulphonate) (PNaSS), and the influence of changes in the (polycation-bound) flavin microenvironment due to complexation on its catalytic activity.

**EXPERIMENTAL**

*Materials*

PSSS was obtained from Jansen Chimica, and was purified by dialysis against aqua bidest, and reprecipitation from methanol in dry acetone. KCl and $^3$PrOH (both p.a. grade) were obtained from Merck. mavin-containing polycations la-c (see figure 2.1) were prepared, starting with polystyrene (M=4.8 x 10$^4$, D=1.9) which was chloromethylated following a procedure similar to the one described by Galeazzi\textsuperscript{15} (dirnethoxymethane: SOCl$_2$: ZnCl$_2$ = 1: 1: 0.1, using a solution of 5 g. polystyrene in 100 ml. of 1,2-dichloroethane at 40°C),
and modified with 10-ethyl-isoalloxazine ('flavin') and finally with NEt₃ as described elsewhere¹⁴. 1-Benzyl-1,4-dihydronicotinamide (BNAH) was synthesized as described in literature¹⁶, purified three times by recrystallization from EtOH/H₂O, and stored at -18°C under dry nitrogen. In all kinetic experiments doubly distilled water was used, and solutions were buffered to pH=8.0 with HCl and tris(hydroxymethyl)aminomethane (TRIS, biochemical grade from Janssen Chimica).

**Conductometric titrations**

Conductivity measurements were performed with a Wayne Kerr Autobalance Universal Bridge B642 and a Philips PW9512/01 conductivity cell equipped with two platinum electrodes. Measurements were performed at 25°C in a mixture of doubly distilled water and ¹PrOH (95/5). Polyelectrolyte solutions had concentrations in the order of 1 x 10⁻³M based upon ionic groups.
Kinetic experiments

Polyelectrolyte complexes were prepared by adding solutions of polycation and polyanion (both in the hereafter mentioned medium) together, and stirring for 15 minutes. In these experiments the total volume was kept at 8.0 ml by adding appropriate amounts of buffer solution, and the total amount of flavin was kept constant at $5 \times 10^{-6}$ M. All kinetic experiments were performed at 25°C in a mixture of water and $^1$PrOH (95/5) at pH=8.0 (TRIS-buffer); ionic strength was kept at 0.05 M (with KCl). The reaction medium was saturated with oxygen by bubbling with air for five minutes. Reactions were carried out in a reaction tube fitted with a Clark type electrode containing an oxygen probe (YSI 5331) connected to a recorder. Reaction rates were determined by monitoring the oxygen-concentration after injection of an aliquot of BNAH. The initial substrate concentration [BNAH], was $3.0 \times 10^{-4}$ M.

Batch-wise and continuous reutilization of flavin-containing PEC

Batchwise re-utilization of stoichiometric PEC was achieved by centrifugation of the suspension at 20000 rpm for 15 minutes, careful removal of the supernatant, and resuspension in fresh medium by stirring for at least 15 minutes. Continuous experiments were carried out in a continuous stirred tank reactor (CSTR) kept at 25.0°C, fitted with a nitrocellulose membrane (Sartorius, 0.45 μ) to retain the suspended PEC (see figure 2.2). The reaction was monitored by a UV-detector operating at 365 nm, thus determining the concentration of BNAH remaining in the product stream. The substrate stream had the same composition as with batch-wise kinetic measurements; [BNAH]₀=1.4 x 10^{-4} M. Sandwich membranes (see figure 2.3) were prepared by draining the tank
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Figure 2.2 Schematic representation of the experimental setup for the steady-state flow stirred tank reactor (CSTR) in continuous reactions

Figure 2.3 Schematic representation of a sandwich membrane, used for continuous oxidation experiments
reactor, containing a suspension of the PEC, completely, thus forming a thin layer of PEC-particles on the nitrocellulose membrane. Another nitrocellulose membrane was used to cover the PEC-layer.

**RESULTS AND DISCUSSION**

*Complexations*

The conductivity of aqueous electrolytic solutions depends upon the concentration of the electrolyte and upon the mobility of the separate ions\(^\text{17}\). For polyelectrolyte solutions some complications occur, as the mobility of the low molar mass counterions is strongly limited due to the connectivity of the polyionic charges\(^\text{18}\). Therefore polyelectrolyte solutions show a relatively low conductivity. Complexation of two oppositely charged polyelectrolytes however, is accompanied by the release of the low molar mass counterions and thus by an increased conductivity of the solution\(^\text{13,19}\). This effect can be used to study the complexation reaction. Figure 2.4 shows the conductivity of a mixture of polycations la-c and PSSS as a function of the ratio of polycationic and polyanionic groups. It clearly indicates that the mobility of the low molar mass counterions is highest if equal amounts of both polyelectrolytes are present, especially if both polyelectrolytes have a high charge density. There is only a small deviation toward an excess of polyanion in the PEC from the 1:1 stoichiometry at optimum complex formation if \(\beta\) (loading of the polycation with quaternary ammonium groups) is lowered. Obviously, the steric restrictions placed upon the polycation during complexation will be larger as the charge density of the chains lowered, since more and more loops will have to be
formed. A (small) excess of the polyanion will therefore be favoured for low $\beta$.

**Kinetic experiments**

As a model reaction we used the oxidation of 1-benzyl-1,4-dihydronicotinamide (see figure 1.3), which has been studied intensively\textsuperscript{20,21}. The reduced flavin is very rapidly reoxidized by dissolved oxygen, and can therefore be considered as a true catalyst for the oxidation of the substrate. The reaction is considered to take place via a charge-transfer complex between oxidized flavin and dihydronicotinamide\textsuperscript{22,23}. In this complex a hydride anion is transferred from the substrate to the flavin N(5) (see figure 1.2), which is the rate-determining step of the reaction\textsuperscript{24}. The reaction can be described by the
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\[
\begin{align*}
\text{Fl}_{\text{ox}} + \text{BNAH} & \xrightleftharpoons[k_1^\text{off}]{k_1^\text{on}} (\text{Fl}_{\text{ox}}^{\text{red}} \cdot \text{BNAH}) \\
& \xrightarrow{k_8} \text{H}_2\text{Fl}_{\text{red}} + \text{BNA}^+ \\
\text{Fl}_{\text{red}} + \text{O}_2 & \xrightarrow{\text{fast}} \text{Fl}_{\text{ox}} + \text{H}_2\text{O}_2 \\
\end{align*}
\]

Thus:

\[
[r_0] = \frac{k_2[\text{Fl}_{\text{ox}}][\text{BNAH}]_0}{K_M + [\text{BNAH}]_0}
\]

and if \( K_M \gg [\text{BNAH}]_0 \):

\[
[r_0] \approx \frac{k_2[\text{Fl}_{\text{ox}}][\text{BNAH}]_0}{K_M} = k_{\text{obs}} [\text{BNAH}]_0
\]

**Figure 2.5 Michaelis-Menten kinetic scheme**

Michaelis-Menten kinetic scheme (figure 2.5), and saturation kinetics was reported for flavin-containing polycations\(^{14}\).

The effect of complexation upon the catalytic activity of the flavin-containing polycations can be seen in figure 2.6, where the relative reaction rate as a function of the time after combining the two polyelectrolyte solutions is depicted. If the mixing caused a phase separation or precipitation by the formation of a PEC, this was found to occur almost immediately. Figure 2.6 shows that the decrease in activity is also quite fast; no significant change was found after the first measurements at about 5 minutes, and one might assume that the change of catalytic activity is almost instantaneous too. Some rearrangement of the PEC may occur subsequently of course, but this does not
appear to have any effect upon the catalytic activity of the immobilized flavin.

In figure 2.7 the activity of several flavin-containing PECs, relative to that of the free polycations is depicted. As can be seen, formation of a PEC influences the activity of the flavin-units. After complexation with PSSS there is a decrease in activity as the ratio \([\text{SO}_3^-]/[\text{NR}_4^+]\) increases. This effect may be attributed to the shielding effect of the complexed polyanion upon the positive charges on the polycation. This in turn leads to a lowering of the rate of hydride-anion transfer. This idea is supported by the kinetic data (Table 2.1) which were calculated according to the Michaelis Menten scheme (see figure 2.5). Upon complexation of PSSS, \(k_2\), which stands for the rate of the hydride anion shift, decreases. However, \(K_m^{-1}\), which is a measure for the complexation of the substrate with the flavin units, and thus for the accessibility of the immobilized catalyst, remains virtually the same. This clearly shows that the
Table 2.1 Kinetic data for BNAH oxidation by polymer bound flavin and for their complexes with PSSS at 25°C, in water/2-PrOH (95:5, vlv), pH 8 (Tris/HCl), I=0.05 M (KCl)

<table>
<thead>
<tr>
<th>Flavin</th>
<th>$k_2$ (s$^{-1}$)</th>
<th>$K_{M}^{-1}$ (M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymer 1a ($\beta=0.23$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>free</td>
<td>0.055±0.002</td>
<td>2600±20</td>
</tr>
<tr>
<td>1:1 complex</td>
<td>0.043±0.002</td>
<td>2690±20</td>
</tr>
<tr>
<td>Polymer 1c ($\beta=0.97$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>free</td>
<td>0.32±0.005</td>
<td>1390±10</td>
</tr>
<tr>
<td>1:1 complex</td>
<td>0.14±0.005</td>
<td>1410±10</td>
</tr>
</tbody>
</table>

Figure 2.7 Initial reaction rates of BNAH oxidation, catalysed by flavin-containing polycations immobilized in polyelectrolyte complexes with PSSS, relative to the initial reaction rates with free polycations, as a function of the mixing ratios of polycation and polyanion (in standard medium)
accessibility of the flavin remains very good after complexation, and that the reduced reaction speed is caused mainly by the slower hydride-anion shift.

We also see that the negative effect of complexation upon catalytic activity increases with the degree of quaternization ($\beta$) of the polycation. This can be explained in terms of the distance between the flavin-moieties and the cationic groups (figure 2.8). As $\beta$ increases, the mean distance between flavin and cationic groups decreases. This also means that, after complexation, the flavins will be forced closer to the anionic groups of the polyanion, so these will have a more pronounced effect upon catalytic activity as $\beta$ increases.

![Figure 2.8](image)

**Figure 2.8** Schematic visualization of the effect of $\beta$ on the catalytic activity of polycation-bound flavin
Stability of polyelectrolyte complexes in batchwise and continuous reactions

In order to investigate the stability of the PEC, we monitored the oxidation of BNAH as a function of the cycle number in batchwise reactions. The PEC was centrifuged and resuspended in fresh medium between reactions. As can be seen from figure 2.9, only with the first workup a small decrease in activity was found. This is probably due to the loss of some non-stoichiometrically complexed or free polycation during centrifugation, since their electrostatic charge will impede sedimentation. In subsequent experiments the initial reaction rate of the batchwise reactions remained constant for at least ten cycles and for all B's. Workup is however very tedious.

The promising results with the re-utilization of the PECs prompted us to test the catalytic activity of the hydrogel particles as a function of time in

![Figure 2.9](image)

**Figure 2.9** Initial rate of BNAH-oxidation catalysed by flavin-containing polycations, immobilized in stoichiometric PECs with PSSS, as a function of the cycle number
Figure 2.10 Conversion of BNAH (in standard medium) by a stoichiometric PEC of \( l_a \) and PSSS (1.5x10^4 mmol flavin), in a CSTR-reactor as a function of time.

Continuous reactions. A continuous stirred tank reactor (CSTR, figure 2.2), equipped with a membrane to prevent leakage of the catalyst was used to test the stability of the flavin-containing PEC. Figure 2.10 shows the result for a stoichiometric PEC of polymer \( l_a \) over a period of 70 hours. A small decrease of the conversion was found. This was not caused by the leakage of catalyst from the reactor, but by the gradual settlement of the catalytic gel particles in 'dead corners' of the reactor as was found by careful inspection after the reaction. This problem was overcome by allowing the PEC-particles to aggregate prior to the reaction, in a so called sandwich membrane (figure 2.3). The observed activity of the immobilized flavin is, lower as compared to the suspended PEC, but there was no sign of a decrease in activity any more for a period of at least one week. Shown here (figure 2.11) is the conversion of the substrate over a period of five days with intermediate changes in flow rate, followed immediately by a change in conversion as expected. This shows conclusively that the PEC is very stable under the conditions applied, and that
the formation of polyelectrolyte complexes is a very effective way to immobilize a catalytically active polyelectrolyte.

CONCLUSIONS

It appeared to be possible to immobilize flavin-containing polycations in polyelectrolyte complexes with poly(sodium styrenesulphonate). Conductometric measurements showed that a maximum release of low molar mass counterions is found if equal amounts of the polymer-bound ionic groups are present. Only minor deviations of this stoichiometry are found if the charge density of the polycation is decreased, showing that complex formation and composition is governed mainly by the electroneutrality of the PEC formed. The activity of the flavin is decreased by 5 to 50%, depending upon the degree of quaternization (β) of the polycation, but remains much higher (5 to 8 times) compared to the...
non-polymer bound flavin. Immobilization of a catalytically active polyelectrolyte by complexation in a PEC appeared to be a very easy and versatile method to obtain a system that can be re-used batchwise or in a continuous system.

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