

# Catalysis by flavin-containing polycations, immobilized in polyelectrolyte complexes with poly(methacrylic acid)

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Polycations, containing pendant flavin groups, were immobilized in polyelectrolyte complexes (PECs) with poly(methacrylic acid). The catalytic activity of the flavin was determined by measuring the rate of oxidation of 1-benzyl-1,4-dihydronicotinamide in water-rich medium, as a function of the degree of substitution of the polycation by quaternary ammonium groups ( $\beta$ ) and as a function of the ratio in which the polycation and polyanion were mixed. The influence of complexation on the catalytic activity of the flavin moiety increases with  $\beta$ , and is positive. Flavin-containing PECs could be used many times in batch wise reactions and showed good stability in continuous experiments in a stirred tank reactor and as a sandwich membrane.

(Keywords: polyelectrolyte complex; immobilization of catalyst; flavin; coenzyme-model)

## INTRODUCTION

The immobilization of catalytic and reactive species by binding them to a polymeric support has become an important issue in recent years<sup>1,2</sup>. Especially in the case of expensive or hazardous reagents there is an increasing urge to look for systems that can be easily separated from the reaction mixture and (after work-up) be re-used. In addition, reagents and catalysts immobilized on insoluble supports can be applied in continuous flow systems, which is of technological interest. Several types of polymeric supports can be distinguished, the most widespread ones being lightly crosslinked gel-type resins<sup>3</sup>, macroporous polymers<sup>4</sup> and non-crosslinked macromolecules anchored onto an inert, insoluble material<sup>2,5</sup>. These materials have one important drawback; since they are insoluble, it is often difficult to characterize the modified material<sup>6</sup>. The method described here, immobilization of catalytically active linear polycations by complexation with polyanions, does not show this problem. These ionically crosslinked polyelectrolyte complexes (PECs) are formed from soluble polymers<sup>7</sup>. In a first step the soluble polymers are modified, and can be fully characterized by homogeneous techniques. Finally they are immobilized by complexation. This means that we have a (chemically) well defined catalytic species on a crosslinked support. Furthermore the complexes can be dissolved in ternary solvents. This also makes it possible to characterize the catalytic species completely after usage in a reaction.

Attaching of catalytic species to macromolecules may have an influence upon their (re)activity<sup>8</sup>. This has been shown for the binding of flavin moieties onto linear polycations, where an (up to 17-fold) increase of its activity in the oxidation of 1-benzyl-1,4-dihydronicotinamide (BNAH) was observed<sup>9</sup>. Here we report on the influence of complexation of flavin-containing poly-

cations in polyelectrolyte complexes with poly(methacrylic acid) (PMAA) upon the catalytic activity, and the possibility to re-use them in batchwise and continuous processes.

## EXPERIMENTAL

### Materials

PMAA was atactic ( $M_w = 2.6 \times 10^5$ ). 2-PrOH and KCl (both PA Grade) were obtained from Merck. Flavin-containing polycations **1a-c** (see Figure 1) were prepared, starting from polystyrene ( $M_n = 4.8 \times 10^4$ ,  $D = 1.9$ ), which was chloromethylated following a procedure similar to the one described by Galeazzi<sup>10</sup> (methylal/SOCl<sub>2</sub>/ZnCl<sub>2</sub> = 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-ethylisoalloxazine ('flavin') and finally with NEt<sub>3</sub> as described elsewhere<sup>9</sup>. 1-Benzyl-1,4-dihydronicotinamide (BNAH) was synthesized as described in the literature<sup>11</sup>, and purified three times by recrystallization from EtOH/H<sub>2</sub>O. In all kinetic experiments doubly distilled water was used, and solutions were buffered to pH = 8.0 with HCl/tris(hydroxymethyl)aminomethane (Tris, Biochemical Grade from Jansen Chimica).

### Kinetic experiments

Unless stated otherwise, polyelectrolyte complexes were prepared by mixing together dilute solutions of polycation and polyanion ( $-\text{NEt}_3\text{Cl}$  and  $-\text{COO}(\text{H})$  in the range of  $(5-12) \times 10^{-4}$  M) in the reaction medium, and stirring for 15 min. It was checked that the activity of the immobilized catalyst did not change significantly within 1 h after mixing. In the kinetic experiments the total volume was kept at 8.0 ml by adding appropriate amounts of buffer solution in order to keep the concentration of (polymer-bound) flavin constant at  $5 \times 10^{-6}$  M. All kinetic experiments were performed at 25°C in a mixture of water and 2-PrOH (95/5 v/v) at pH = 8.0 (Tris

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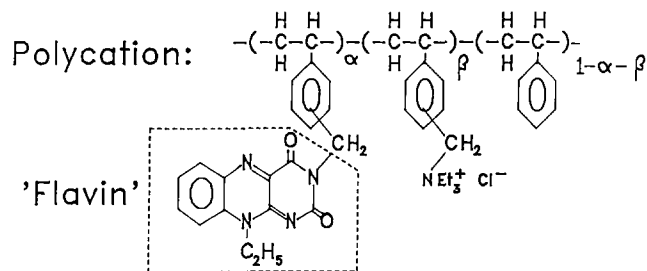


Figure 1 Structure of the applied flavin-containing polymers 1a-c (1a,  $\alpha=0.007$ ,  $\beta=0.23$ ; 1b,  $\alpha=0.017$ ,  $\beta=0.56$ ; 1c,  $\alpha=0.014$ ,  $\beta=0.97$ )

buffer); ionic strength was kept at 0.05 (with KCl). The reaction medium was saturated with oxygen by bubbling with air for 5 min. 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<sup>12</sup> after injection of an aliquot of BNAH. Products were identified by u.v. spectroscopy ( $\text{BNA}^+$ ,  $\lambda_{\text{max}}=263$  nm) and Merckoquant peroxide test strips ( $\text{H}_2\text{O}_2$ ).

*Batchwise and continuous re-utilization of flavin-containing PEC*

All PECs used in re-utilization experiments were prepared by adding solutions of the polyelectrolytes to a large volume (100 ml) of the reaction medium (without BNAH), while stirring vigorously. If necessary, PMAA was added until flavin could not be detected any more by u.v./v.i.s. spectroscopy in the supernatant solution.

Batchwise re-utilization of stoichiometric PEC was achieved by centrifugation of the suspension at 20 000 rpm for 30 min, careful removal of the supernatant, and resuspension of the PEC particles in fresh medium by stirring for at least 15 min.

Continuous experiments were carried out in a thermostated (25°C) tank reactor, fitted with a nitrocellulose membrane (Sartorius, 0.45  $\mu\text{m}$ ) to retain the suspended PEC (see Figure 2). The reaction was monitored by a u.v. detector operating at 365 nm, thus determining the concentration of BNAH remaining in the product stream. Unless stated otherwise, the substrate stream had the same composition as used for kinetic measurements, with  $[\text{BNAH}]_0=1.5 \times 10^{-4}$  M. Sandwich membranes (see Figure 3) were prepared by complete draining of the tank reactor containing a suspension of the PEC, thus forming a thin layer of PEC particles on the nitrocellulose membrane. Another membrane was used to cover the PEC layer.

RESULTS AND DISCUSSION

*Kinetic experiments*

The kinetics and mechanism of the model reaction used here, the oxidation of 1-benzyl-1,4-dihydronicotinamide (see Figure 4), have been studied intensively<sup>13</sup>. Under aerobic conditions the reduced flavin is very rapidly re-oxidized by dissolved oxygen, and can therefore be considered as a true catalyst for the oxidation of the substrate. There is evidence for the formation of a charge-transfer complex between oxidized flavin and dihydronicotinamide<sup>14</sup>, and the rate-determining step of the reaction is considered to be the transfer of a hydride

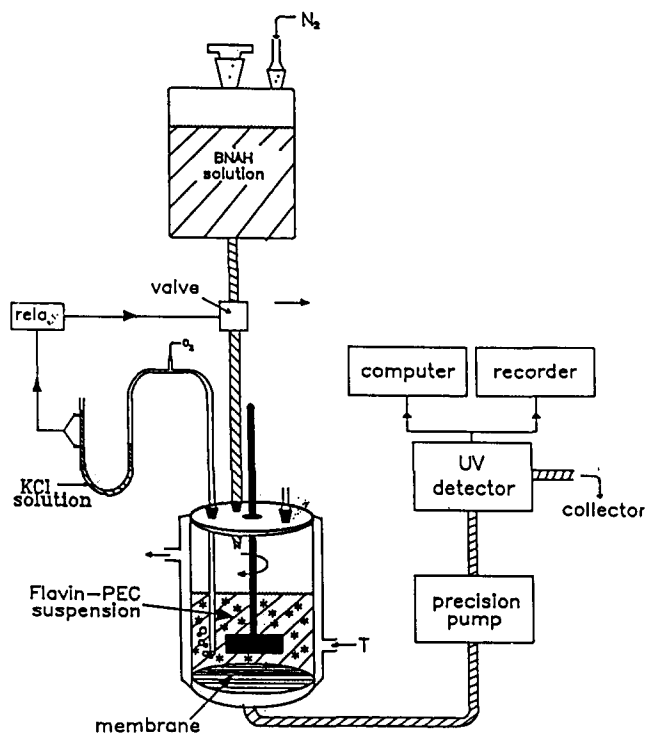


Figure 2 Schematic representation of the experimental set-up for the steady-state flow stirred tank reactor used in continuous oxidation of BNAH by flavin-containing PECs

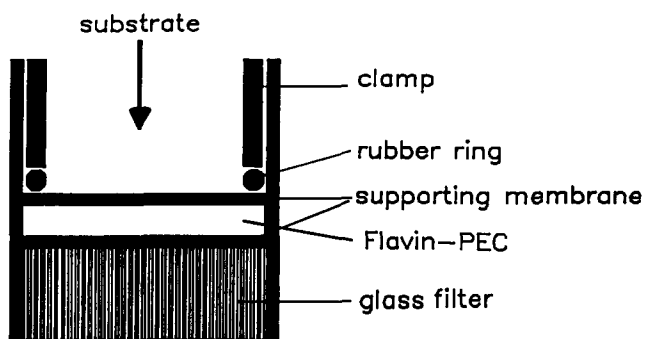


Figure 3 Schematic representation of a sandwich membrane

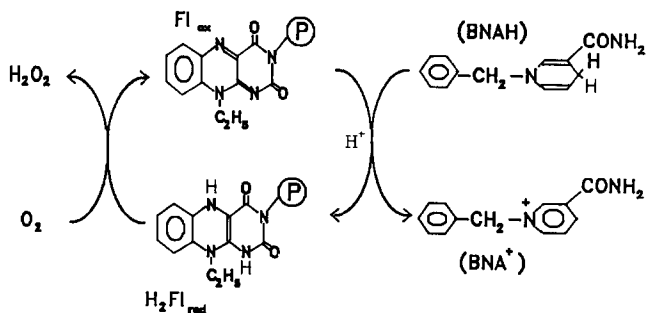
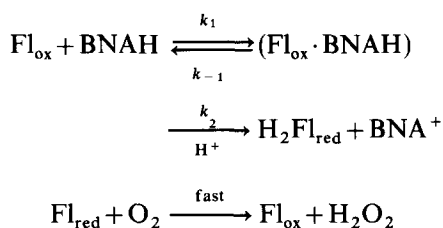


Figure 4 Test reaction for immobilized flavins: aerobic oxidation of 1-benzyl-1,4-dihydronicotinamide

anion in a single step<sup>15</sup>. The reaction can be described by the Michaelis–Menten scheme, and saturation kinetics were reported for flavin-containing polyelectrolytes<sup>9</sup>. The Michaelis–Menten scheme is as follows:



Thus:

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

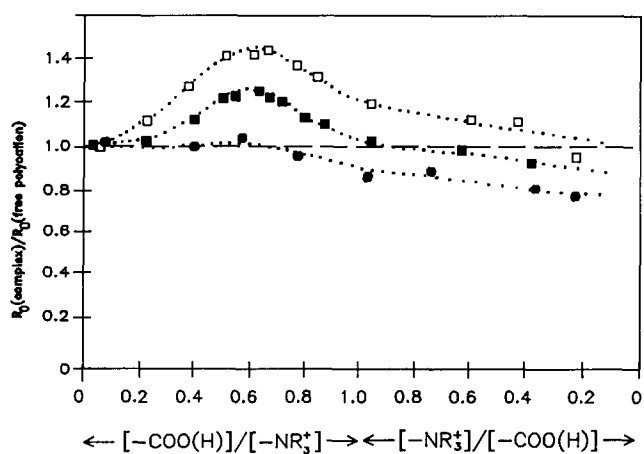
and if  $K_M \gg [\text{BNAH}]_0$ :

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

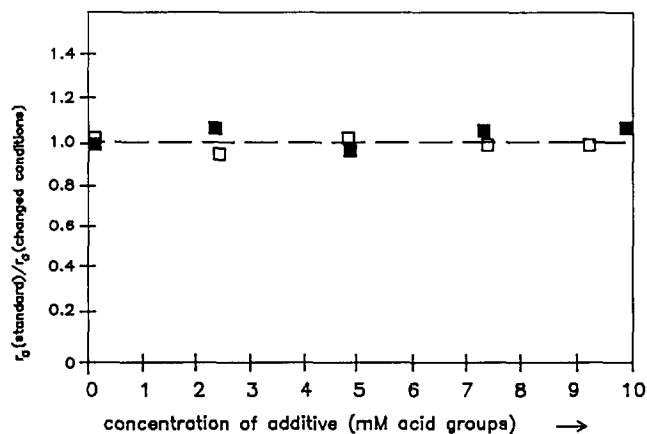
It has been shown that the binding of flavin to polycations leads to an increase of reaction rate<sup>9</sup>, due to substrate enrichment and faster hydride transfer (increase of  $k_2$ ), which might be ascribed to the favourable influence of the neighbouring cationic groups. Recently we showed<sup>16</sup> that complexation of these polycations to poly(sodium styrene sulphonate) leads to a lower reaction rate due to a decrease of  $k_2$ . Here we describe the influence of complexation with a weak polyacid, poly(methacrylic acid).

In *Figure 5* the initial reaction rates of several flavin-containing PECs relative to that of the free polycations are depicted. There is a definite influence of the complexation of the polycation with a polyanion upon the catalytic activity of the flavin moieties. The activity of the complexed flavin-containing polycation appears to depend on the degree of quaternization ( $\beta$ ) of the polycation and on the mixing ratio of the two polyelectrolytes.

In the case of complexation with PMAA, a rise in activity with increasing ratio  $[\text{COO}(\text{H})]/[\text{NR}_3^+]$  is observed at first. If more PMAA is added, the activity decreases again. Obviously there are two opposite effects



**Figure 5** Initial reaction rates of BNAH oxidation catalysed by flavin-containing polycations (●, **1a**,  $\beta=0.23$ ; ■, **1b**,  $\beta=0.56$ ; □, **1c**,  $\beta=0.97$ ) immobilized in PECs with PMAA, relative to the initial reaction rate with free polycations, as a function of the mixing ratios of polycation and polyanion (in water/2-PrOH (95:5, v/v), pH 8 (Tris/HCl),  $I=0.05$  M (KCl))



**Figure 6** Initial reaction rates of BNAH oxidation catalysed by flavins plus additive (□, polycation **1c** + isobutyric acid; ■, free flavin 3-methyl-10-ethylisoalloxazine + PMAA), relative to the initial reaction rate without additive, as a function of the amount of additive (in water/2-PrOH (95:5, v/v), pH 8 (Tris/HCl),  $I=0.05$  M (KCl))

of complexation upon activity in this case. A decrease in activity is expected as more cationic charges are shielded, as was found for complexes with poly(sodium styrene sulphonate)<sup>16</sup>. Besides local polarity (which is known to influence flavin-catalysed BNAH oxidation<sup>17</sup>), the positive effect might be connected with the fact that PMAA is a weak polyacid. This means that part of the carboxylic acid groups are not ionized. These COOH groups may form hydrogen bridges with N(1) of the flavin moiety, promoting formation of  $\text{H}_2\text{Fl}_{\text{red}}$  by subsequent hydride shift to N(5) of flavin in the charge-transfer complex with BNAH (see *Figure 4*). It was suggested that a comparable activation effect of flavin exists in flavoproteins<sup>17</sup> and in certain low-molar-mass flavin models containing acidic –OH groups, capable of (intra-molecular) hydrogen bonding with N(1)<sup>18</sup>. In the present work, these effects occur only in the case in which flavin is bound in a polyelectrolyte complex. As can be seen in *Figure 6* there is no effect upon activity if PMAA is added to a solution of the non-polymer-bound flavin (3-methyl-10-ethylisoalloxazine). Furthermore there is no change in activity of polycation-bound flavin (**1c**) if isobutyric acid is added as a low-molar-mass model of PMAA.

It can be seen (*Figure 5*) that the effect of complexation upon catalytic activity increases with the degree of quaternization ( $\beta$ ) of the polycation. This can be explained in terms of the mean distance between the flavin moieties and the cationic groups (see *Figure 7*). 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/acidic groups of the polyanion, so there will be a more pronounced positive effect upon catalytic activity as  $\beta$  increases.

In order to obtain more information about the cause of the activity change upon complexation, we performed a kinetic analysis according to Michaelis–Menten kinetics, which applies to this system. *Table 1* shows  $k_2$  and  $K_M^{-1}$ , obtained from Lineweaver–Burk plots, for free polycations (**1a**, **c**) and their complexes with PMAA. In the case of polycation **1a** ( $\beta=0.23$ ), only minor effects are found upon complexation, showing that the flavin moieties are hardly influenced by the remote PMAA units. On the other hand it is found that, upon complexation of a flavin-containing polycation with high  $\beta$  ( $\beta=0.97$ ),  $k_2$  shows a distinct increase, where  $K_M^{-1}$

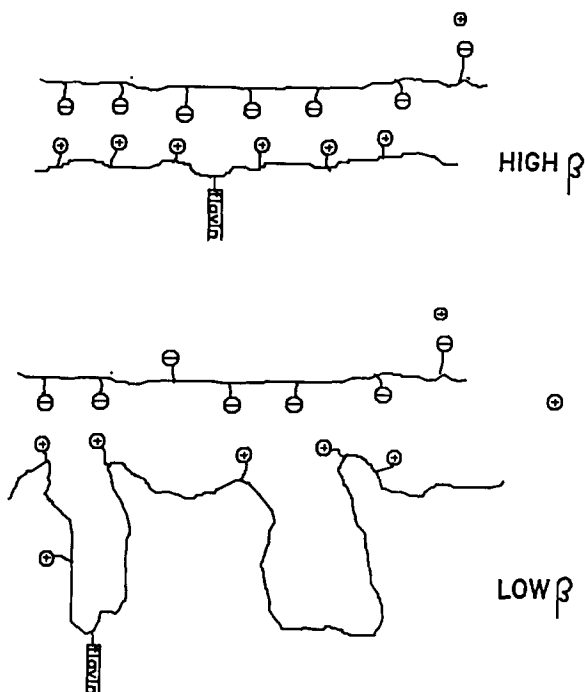


Figure 7 Visualization of the effect of  $\beta$  on the catalytic activity of polycation-bound flavin

Table 1 Kinetic data for BNAH oxidation by polymer-bound flavin and complexes with PMAA at 25°C in water/2-PrOH (95:5, v/v), pH 8 (Tris/HCl),  $I=0.05$  M (KCl)

Flavin	$k_2$ (s <sup>-1</sup> )	$K_M^{-1}$ (M <sup>-1</sup> )
Polymer 1a		
free	0.055	2600
1:1 complex	0.053	2610
Polymer 1c		
free	0.30	1410
1:1 complex	0.34	1450
0.6:1 complex <sup>a</sup>	0.41	1440

<sup>a</sup> Ratio [-COO(H)][-NR<sub>3</sub><sup>+</sup>]

increases only slightly. The largest increase of  $k_2$  is found for the non-stoichiometric PEC of the polycation with high  $\beta$ , which was also the complex that gave the highest initial reaction rate (see Figure 5). These data show that the increased activity is caused mainly by the acceleration of the rate-determining step (hydride-anion transfer). Furthermore, accessibility of the immobilized flavin moieties is not affected since there is no decrease of  $K_M^{-1}$ , which is a measure of substrate binding.

Finally, the effect of the pH at which the PECs are formed on the activity of the immobilized catalyst was investigated. To this end PECs were prepared by slowly adding the polyelectrolyte solutions to large volumes of buffered solutions with different pH values. It is important to emphasize that PMAA was added until no flavin could be detected in the supernatant solution. The PEC particles formed were centrifuged and resuspended in the standard reaction medium buffered at pH=8.0. PECs prepared at different pH showed different catalytic activity in the oxidation reaction of BNAH (measured under standard conditions at pH=8.0). Table 2 shows that preparation of the PEC at a lower pH leads to a lowering of flavin activity (compared to PEC prepared at pH=8), whereas preparation at higher pH gives an

increase of activity. This effect is related to the fact that PMAA is a weak polyacid. The way in which we prepared PECs results in complexes with oppositely charged groups compensating each other completely. This means that in the PEC particles the number of -COO<sup>-</sup> groups and of -NEt<sub>3</sub><sup>+</sup> groups is the same, but the number of non-ionized -COOH groups present in the PEC particle will depend on the pH of the medium in which the PEC is formed. If the complexation is performed at lower pH, there will be a larger number of protonated acid groups present in the PEC (compared to complexes made at pH=8). If the pH is then brought to 8.0, at least part of these acid groups will be ionized, thus leading to a complex with excess anionic groups, which has been shown to have a lower activity. For complexes prepared at pH >10 a comparable explanation can be given, leading to a somewhat higher activity at pH=8.0.

#### Stability of PEC in batchwise and continuous reactions

In order to investigate the stability of the flavin-containing PEC, we monitored the oxidation of BNAH as a function of the cycle number in batchwise reactions, and as a function of time in continuous reactions. As can be seen from Figure 8, the initial reaction rate of batchwise reactions remains constant for at least six cycles for all  $\beta$  values. Work-up is, however, very tedious.

Much easier and more reliable ways of testing the stability of the PEC and the immobilized flavin are continuous flow experiments. Here we applied a continuous stirred tank reactor (CSTR, Figure 2), in which a suspension of the PEC is retained by a membrane filter, or a sandwich-membrane system (SMS, Figure 3),

Table 2 Relative activities of flavin-containing PECs (with polymer 1c) formed at different pH in BNAH oxidation at standard conditions

pH (of PEC formation)	Relative reactivity (at pH=8.0)
7.0	0.81
8.0	1.0
9.0	1.09

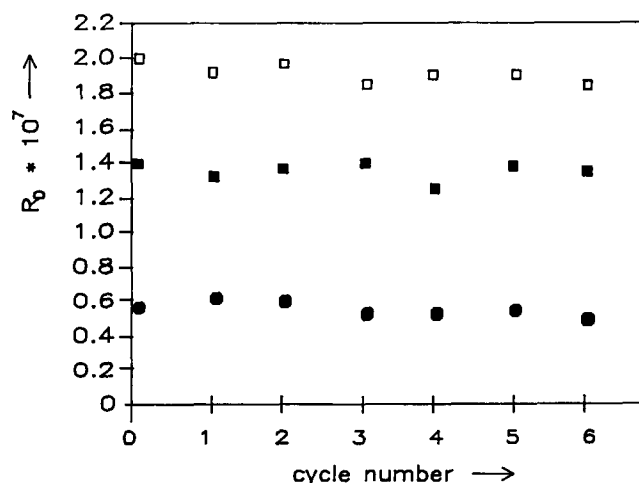
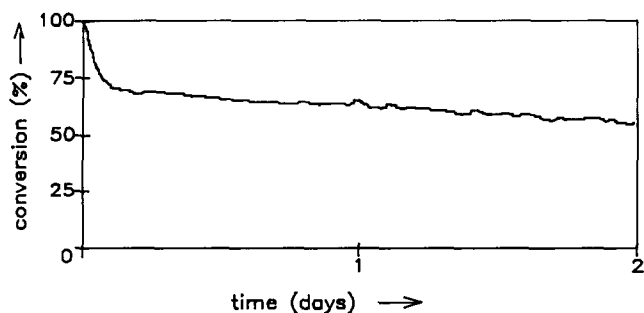
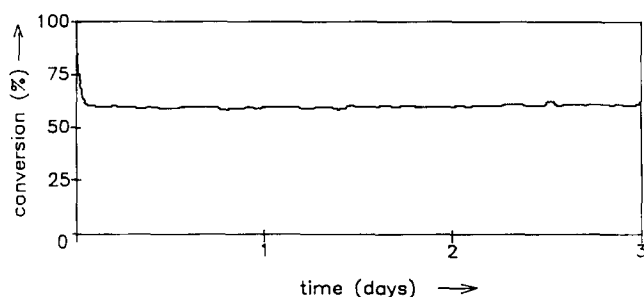


Figure 8 Initial reaction rates of BNAH oxidation catalysed by flavin-containing polycations (●, 1a,  $\beta=0.23$ ; ■, 1b,  $\beta=0.56$ ; □, 1c,  $\beta=0.97$ ) immobilized in PECs with PMAA, as a function of the cycle number (in water/2-PrOH (95:5, v/v), pH 8 (Tris/HCl),  $I=0.05$  M (KCl))



**Figure 9** Conversion of BNAH by flavin-containing polycation **1b** immobilized in a stoichiometric PEC with PMAA, in a continuous-flow reactor as a function of time ( $1.2 \times 10^{-7}$  mol flavin,  $[\text{BNAH}]_0 = 1.5 \times 10^{-4}$  M, flow rate =  $33 \text{ ml h}^{-1}$ , reaction volume = 50 ml, water/2-PrOH (95:5, v/v), pH 8 (Tris/HCl),  $I = 0.05$  M (KCl))



**Figure 10** Conversion of BNAH after passing a sandwich membrane containing a stoichiometric PEC of flavin-containing polycation **1b** and PMAA, as a function of time ( $3.0 \times 10^{-7}$  mol flavin,  $[\text{BNAH}]_0 = 1.5 \times 10^{-4}$  M, flow rate =  $33 \text{ ml h}^{-1}$ , water/2-PrOH (95:5, v/v), pH 8 (Tris/HCl),  $I = 0.05$  M (KCl))

consisting of a layer of PEC particles between two nitrocellulose membranes. As was the case in previously described work<sup>16</sup>, a small decrease in activity was found when a CSTR was used (Figure 9), due to sedimentation of PEC particles. Much more interesting is the SMS, because of the fact that possible loss of activity can no longer be caused by sedimentation. Here we find that the conversion of BNAH as catalysed by flavin-containing polycations immobilized in PECs with PMAA is constant for at least three days (Figure 10). This shows the usefulness of this method in the immobilization of catalytically active polyelectrolytes.

## CONCLUSIONS

We have shown that complexation of flavin-containing polycations to PMAA influences the catalytic activity

of the flavin units favourably. The size of this effect was found to increase with the degree of substitution with quaternary ammonium groups of the polycation, and depends on the ratio of polycation/polyanion. Complexation of the flavin-containing polycations with poly(methacrylic acid) appears to be an effective way to immobilize the catalyst. The material obtained can be re-used many times batchwise, and in continuous processes without loss of activity after several days.

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