Flavin-Containing Polyanions: Synthesis, Activity, and Immobilization in Polyelectrolyte Complexes

Herman F. M. Schoo and Ger Challa*

Laboratory of Polymer Chemistry, University of Groningen, Nijenborgh 16, 9747 AG Groningen, The Netherlands

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ABSTRACT: Linear polymers containing both flavin units and carboxylic acid groups were synthesized by copolymerization of the appropriate styrene derivatives. The catalytic activity of the resulting polyanions in aqueous media was determined for the aerobic oxidation of 1-benzyl-1,4-dihydronicotinamide (BNAH). A 69-fold (maximum) increase of the activity of the flavin moieties was found after binding to the polyanions as compared to a low molar mass analogue, which may be attributed to a higher polarity of the microenvironment of the catalyst and to hydrogen bonding of the flavin units to the carboxylic acid groups. The activity of the polyanions was found to be dependent on the medium pH, with an optimum at pH $\approx$ 8. Complexation of the polyanions with polycations containing pendent quaternary ammonium groups leads to a small decrease in activity, the effect being larger with a higher charge density of the polycation. The resulting polyelectrolyte complex gel particles are quite stable and can be used in a continuous reaction for many days without loss of activity. Finally, homopolymerization of the flavin-containing monomer resulted in the first "flavin homopolymer".

Introduction

For a long time the immobilization of reactive and/or catalytically active molecules has been motivated mainly by practical advantages like the reduced contamination of the environment and the reusability of (expensive) chemicals.[1] Another aspect of this concept, which has led to many interesting developments, lies in the (positive) influence of the polymeric carrier upon (covalently) bound catalysts.[2] Substrate enrichment,[3] stabilization of reactive intermediates, and optimization of catalytically active metal complex formation[4] can lead to more reactive and more stable systems. It also has been shown that carefully designed (cross-linked) polymeric materials can be used to establish "site isolation" in systems where dimerization of catalytic moieties would lead to inactive species.[5] Finally, one of the most remarkable concepts is to prepare a (cross-linked polymeric) three-dimensional array in which a stereoselective binding site is situated.[6,7] The incorporation of a catalyst in the immediate vicinity of such a site may lead to systems with the high selectivity known only for enzymes now.

In the case of the flavin coenzyme, the chemically active part of many redox enzymes, it is known that its activity is tuned (in enzymes) by the delicate cooperation of several interactions, like steric hindrance, coulomb interactions, and hydrogen bonding, as has been shown in detailed NMR studies.[8] Also, covalent binding of flavin to a polyelectrolyte has been previously described.[9,10] It was shown that, by binding it to a polycation, the activity toward a model compound, 1-benzyl-1,4-dihydronicotinamide (BNAH), is increased up to 17-fold, compared to the non-polymer-bound flavin.[11] Also, complexation of these polycations with a weak polyacid induced a further increase in activity.[12] The strongest effect was found if the polyacid is constrained close to the polycation and hence close to the flavin moieties. Consequently, it seemed to be very interesting to bind both the acid and the flavin groups to the same polymer. Here we describe the increased reactivity of flavin after covalent binding to a weak polyacid and the immobilization of such a polyanion by complexation with a polycation.

Experimental Section

Measurements. UV measurements were performed with a Pye Unicam SP8-200 UV/vis spectrophotometer. NMR spectra were recorded at ambient temperature at 300 MHz (^{1}H) or 75 MHz (^{13}C) using a Varian 300 spectrometer. Reactions of BNAH with the flavin-containing polyanions were carried out in a reaction tube fitted with a Clark-type electrode containing an oxygen probe[13] (YSI 5331) connected to a recorder.

Materials. Lumiflavin was synthesized from commercial riboflavin by UV irradiation (λ_{max} = 254 nm) at 10 °C of strongly basic solutions,[14] containing 5 g of riboflavin and 40 g of NaOH in 2 L of H_2O. Repeated extraction of the acidified solution with CH_2Cl_2 yielded the raw material. The product was recrystallized twice from a saturated, slightly basic solution which was in contact with a HCl vapor. The gradual acidification of the solution yielded a crystalline material, showing no impurities in the ^{1}H NMR spectra (yield 45%). *p*-(Chloromethyl)styrene was synthesized as described elsewhere[15] and distilled before use. 1-Benzyl-1,4-dihydronicotinamide was synthesized as described in the literature[16] and recrystallized three times (EtOH/H_2O) before use.

Flavin-containing polyanions were synthesized by a multistep synthesis as depicted in Figure 1. The flavin-containing monomer (1) was synthesized starting with *p*-(chloromethyl)styrene (40 mmol). In a typical experiment this was added to very finely ground (steel mill) lumiflavin (10 mmol), K_2CO_3 (30 mmol), NMe_4I (40 mg), and 10 mg of *tert*-butylcatechol in 80 mL of dry DMF. After ultrasonic treatment for 30 min, in order to obtain a stable suspension of the insoluble reactants, the mixture was allowed to react for 1 week with vigorous stirring. The resulting dark brown, green fluorescent solution was poured into water and centrifuged. Flash chromatography of the dried sediment on an Al_2O_3 column (5% w/w water added before use) with a CH_2Cl_2/THF mixture (1/1) yielded 1 as the second fraction. After partial removal of the solvent, orange crystals of pure 1 were formed in the strongly fluorescent solution (yield 52% based on lumiflavin, characterized by ^{1}H and ^{13}C NMR and UV/vis spectroscopy).

The comonomer 2 was synthesized starting from *p*-chlorostyrene (Merck), which was converted to *p*-vinylbenzoic acid.[17] After conversion to the acid chloride,[18] this was reacted with KO^{t}Bu in ^{t}BuOH. Distillation at reduced pressure gave 2 in high yield (69% based on *p*-chlorostyrene, characterized by ^{1}H and ^{13}C NMR).[19]

Polymers 3 were prepared by (co)polymerization of 1 and 2 (total monomer: 10–25% solution) in $CHCl_3$ with AIBN (1 mol %) at 70 °C for 16 h. The resulting solutions were precipitated in MeOH. After drying the polymer was characterized by ^{1}H NMR ($CDCl_3$) to determine its flavin content (α). GPC mea-

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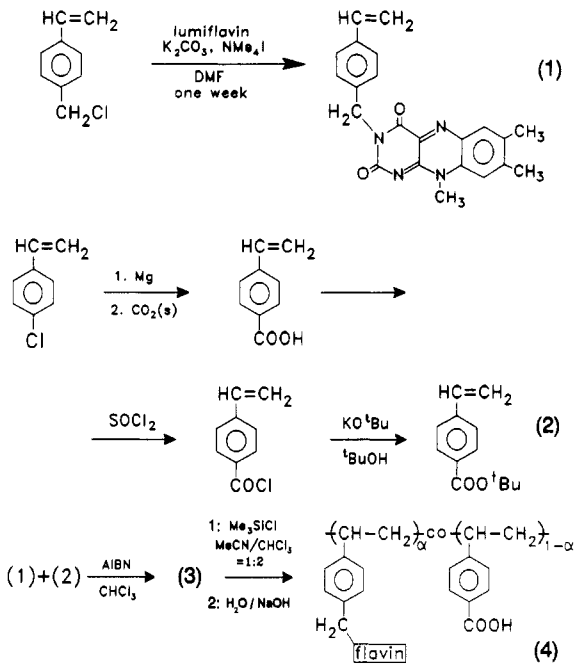

Figure 1. Schematic representation of the synthesis of the flavin-containing polyanions.

Table I
Results of (Co)polymerizations and Reaction Kinetics of BNAH Oxidation

f_1[a]	yield of 3, %	polymer	α	$k/k_{(6)}$[b]
0	70	**4a**	0	0
0.01	68	**4b**	0.008	65
0.03	65	**4c**	0.03	67
0.12	63	**4d**	0.07	69
0.25	59	**4e**	0.14	39
0.50	66	**4f**	0.26	16
0.75	60	**4g**	0.41	*c*
1.00	44	**4h**	1	*c*
6				1
6 + **4a**[d]				1.5
7				0.8

[a] $f_1 = [1]_0/([1]_0 + [2]_0$. [b] In a standard reaction medium at pH = 8. [c] Not soluble in the reaction medium. [d] 10 equiv of COOH groups (**4a**) per flavin (**6**).

surements of the polymers indicated values for M_n of about 10^5 for polymers **3a–e** and somewhat lower values for **3f–h**. The final step consists of the hydrolysis of the *tert*-butyl ester groups. In a typical experiment a mixture of 1 g of polymer and 5 g of NaI in 75 mL of $CHCl_3$/MeCN (2/1) was cooled to −20 °C. Then 5 mL of Me_3SiCl was added to the stirred solution, which immediately turned dark red/brown. After warming up slowly to room temperature the solvent was evaporated in vacuo. The resulting polymers (**4a–e**; see Table I) were dissolved in a slightly basic aqueous solution, thus completing the hydrolysis of the *tert*-butyl ester groups. For purification the solution was dialyzed against 0.05 M HCl, during which the bright yellow polymer precipitated. This procedure was repeated twice. ^1H NMR did not show any *tert*-butyl ester groups left on the polymer. The other polymers (**4f–h**; see Table I) were reprecipitated from DMF in MeOH twice. For the polymers with the lower α (**4b–e**), the flavin content was determined again after the ester hydrolysis, using the anaerobic oxidation of BNAH as was described elsewhere.[20]

Polycations (**5a–c**; see Figure 2), used for complexation, were synthesized by reacting (partly) chloromethylated polystyrene[12] ($M_n = 4.8 \times 10^4$, $D = 1.9$) with NEt_3 (5 g of polymer in 100 mL of 20% (v/v) amine in MeOH) for 2 days. After precipitation in dry acetone the polycations were dialyzed against aqua bidest and finally reprecipitated in dry acetone. The degree of quaternization of the polymer (β) was controlled by adjusting the degree of chloromethylation of the prepolymer.

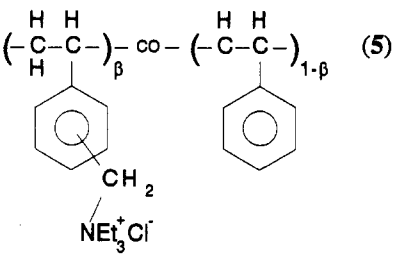

Figure 2. Polycations used for the formation of polyelectrolyte complexes (**5a**, $\beta = 0.24$; **5b**, $\beta = 0.44$; **5c**, $\beta = 1$).

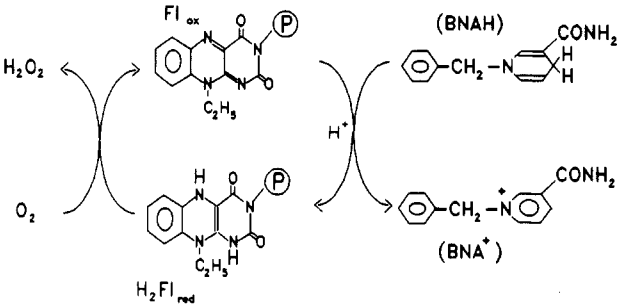

Figure 3. Test reaction for immobilized flavin: aerobic oxidation of 1-benzyl-1,4-dihydronicotinamide (BNAH).

Determination of the Catalytic Activity and of the Stability in a Continuous-Flow System. The catalytic activity of the flavin-containing polyanions was determined, using the aerobic oxidation of 1-benzyl-1,4-dihydronicotinamide (BNAH) as a model reaction (see Figure 3). All kinetic experiments were performed at 25 °C in a mixture of doubly distilled water and 2-PrOH (95/5 v/v); the ionic strength was kept at 0.05 (with KCl). Solutions were buffered with KH_2PO_4/NaOH (pH = 6), HCl/tris(hydroxymethyl)aminomethane (pH = 7–9), or $NaHCO_3$/NaOH (pH = 10, 11). 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 the (polymer-bound) flavin constant at 5×10^{-6} M. The reaction medium was saturated with oxygen by bubbling with air for 5 min. Reaction rates were determined by monitoring the oxygen concentration[21] after injection of an aliquot of BNAH (solution in 2-propanol). Products were identified by UV/vis spectroscopy (BNA^+, λ_{max} = 263 nm) and Merckoquant peroxide test strips (H_2O_2).

Polyelectrolyte complexes were prepared by mixing together semidilute solutions of the polycation and polyanion ([$-NEt_3Cl$] and [$-COO(H)$]) in the range of $(0.5–1.2) \times 10^{-3}$ 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 order to determine the stability of the flavin-containing PEC's, sandwich membranes were prepared consisting of a thin layer of PEC particles (containing 1.0×10^{-7} mol of flavin) between two nitrocellulose membranes. The conversion of the substrate ($[BNAH]_0 = 1.5 \times 10^{-4}$ M) after passing this membrane was measured as a function of time, by monitoring the UV absorption of the outcoming stream at λ = 365 nm (BNAH). A complete description of the procedures concerning these experiments can be found elsewhere.[12]

Results and Discussion

Functionalization and (Co)polymerization. The synthesis of the flavin-containing polyanion (**4**; see Figure 1) was performed by radical copolymerization of the flavin-containing styrene derivative (**1**) and the *tert*-butyl-protected 4-vinylbenzoic acid (**2**). This route was chosen since preliminary experiments had shown that direct copolymerization of **1** with 4-vinylbenzoic acid did not yield any (soluble) polymer. A typical ^1H NMR spectrum of the protected acid copolymer (**3**) is shown in Figure 4I. It clearly shows the presence of the flavin subunits (marked peaks) and of the *tert*-butyl moieties (1.5 ppm). From such spectra the amount of incorporated flavin (α) could

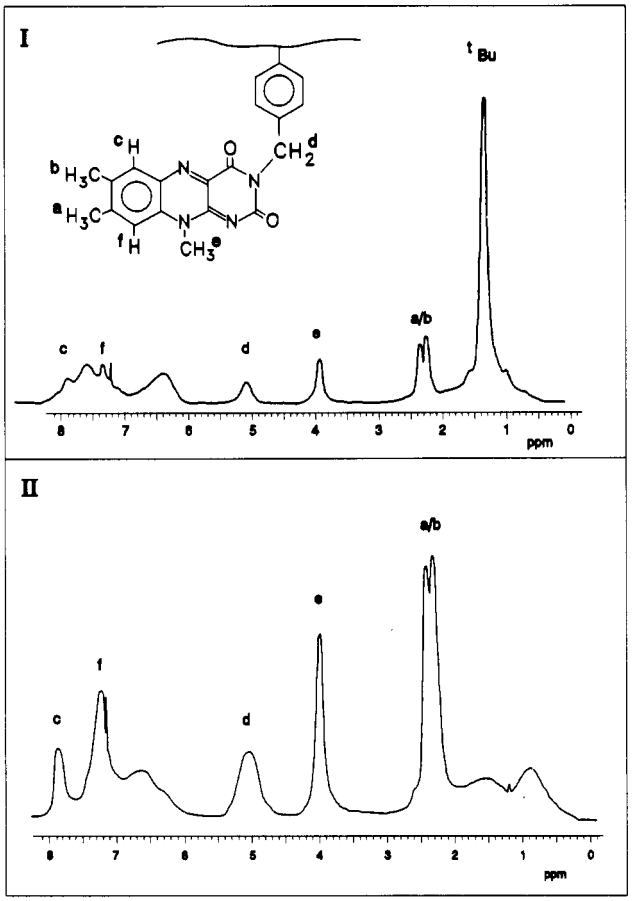

Figure 4. ^{1}H NMR of a flavin-containing copolymer (**3f**, top) with $\alpha = 0.26$ and of a flavin homopolymer (**4h**, bottom). Flavin peaks are marked.

be determined by integration. After hydrolysis the *tert*-butyl signal could not be detected anymore. The flavin moieties are quite stable under the reaction conditions applied, since the α values obtained by ^{1}H NMR of the prepolymers **3b–e** did not show significant deviations from the values determined (by anaerobic oxidation of BNAH) for the polymers after hydrolysis (**4b–e**).

A range of copolymers with varying α (fraction of flavin-containing units) was synthesized from the functionalized monomers **1** and **2**. As can be seen in Table I, the conversion of the monomers was about 60%, except in the case of the homopolymerization of **1**. Figure 4II shows the ^{1}H NMR spectrum of the homopolymer. To our knowledge, this is the first homopolymer of a flavin-containing monomer, and the high loading with a strongly dipolar,[22] redox-active group, capable of one- and two-electron transport, may give it very interesting properties.

Catalytic Activity. The catalytic activity of the flavin-containing polyanions described here was determined, using the aerobic oxidation of 1-benzyl-1,4-dihydronicotinamide (BNAH) as a model reaction (Figure 3). This reaction is supposed to take place via a charge-transfer complex of the catalyst and the substrate,[23] in which a hydride anion is transferred from the BNAH to the N(5) (see Figure 5) of the flavin unit[24] (Fl_{ox}) in the rate-determining step. The resulting flavin anion then takes a proton from the solution, and H_2Fl_{red} can be reoxidized by molecular oxygen. It has been suggested that this hydride transfer is promoted by the formation of a hydrogen bond to the N(1) (see Figure 5) of flavin,[25,26] prior to or during the hydride transfer. In that case the transfer of both the hydride anion and the proton in a concerted step does not lead to the unstable, high-energy flavin-anion species.

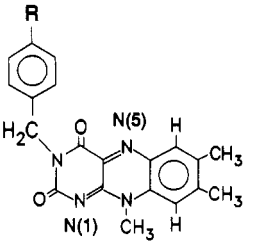

Figure 5. Low molar mass flavin models, with R = H (**6**) and R = COOH (**7**).

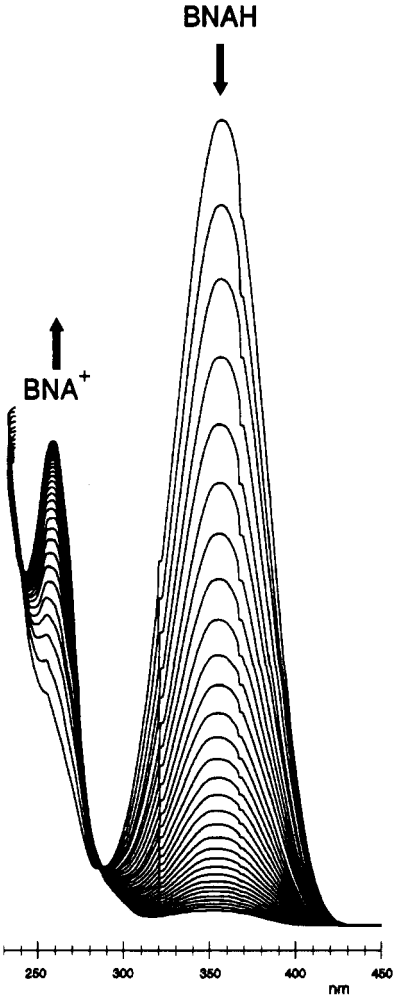

Figure 6. UV/vis spectra taken at regular intervals (3 min) showing the formation of BNA^+ (λ_{max} = 263 nm) from BNAH (λ_{max} = 358 nm) catalyzed by **4d** (in standard medium).

In aqueous media substituted dihydronicotinamides, like the substrate used here (BNAH), are known to undergo an acid-catalyzed addition of water.[27] This reaction, leading to a 6-hydroxy-1,4,5,6-tetrahydronicotinamide[28] (with λ_{max} = 290 nm), might complicate the model reaction. Figure 6 shows a series of UV/vis spectra for a reaction mixture of BNAH and a catalytical amount of the polyanion-bound flavin (**4**), at regular time intervals (3 min, at pH = 6). The spectra show only the conversion of BNAH (λ_{max} = 358 nm) into BNA^+ (λ_{max} = 263 nm). The sharp isosbestic point at 292 nm clearly demonstrates the selective conversion of the substrate by the flavin-containing polyacid, and there is no indication for acid-catalyzed addition of water (on the time scale of the experiments).

An investigation of the kinetics of this reaction demonstrated the large positive effect of the binding of flavin to a polyanion. The flavin-containing polyanions showed

second-order overall kinetics (first order in both flavin and BNAH), which may be seen as a limiting case of the Michaelis–Menten kinetics which was found earlier for flavin-containing polycations.[10] Obviously, in the present case the concentration of the charge-transfer complex between flavin and BNAH is small (K_m and/or k_2 large), leading to simple first-order kinetics not only in flavin but also in BNAH. Table I shows the (apparent) second-order rate constant for the polymeric flavins **4a–f** and for low molar mass analogues 3-benzyl-7,8,10-trimethylisoalloxazine (**6**) and 3-(*p*-carboxybenzyl)-7,8,10-trimethylisoalloxazine (**7**) (see Figure 5), relative to the activity of **6**. First, no activity was found for the polyanion without flavin (**4a**; $\alpha = 0$). Flavin-containing polyacids, however, showed very high reaction rates. The rate constant appeared to be dependent on the amount of the incorporated flavin. For low flavin content (**4b–d**) the highest reaction rates were found, all about 70 times higher than that for the low molar mass analogue. If the flavin content was higher (**4e,f**), the reaction was much slower. The polymers with the highest α's (**4g,h**) were no longer soluble in the aqueous medium. Increasing α of the polyacid may cause the polymer coils to collapse due to intramolecular aggregation of the hydrophobic flavin moieties, which are known to form aggregates in aqueous solution very easily.[29,30] Furthermore, the increasing concentration of flavin units in the polymer coils as α becomes higher will eventually lead to a lower mean activity of the flavin units due to diffusional problems.

The enhanced reactivity after binding flavin to a polyelectrolyte has been described earlier[11,12] for a polycationic system (under the same reaction conditions), where a 17-fold increase was found. This phenomenon was ascribed mainly to the accumulation of the substrate in the polymer domains due to apolar interactions and to the increased hydride-anion transfer rate due to the favorable polarity of the flavin microenvironment. Here the increase is even stronger. In both the polycationic and the polyanionic system described here, a styrene-based monomer was used. Since the level of substrate enrichment in the polymer coils depends mainly upon the lowered polarity of the polymer domain, caused by apolar segments of the chain, one may assume that this effect is similar in both cases. This means that the further increase of reaction rates with the flavin-containing polyanions should be caused by a faster hydride transfer, which is the rate-limiting step in the reaction.

It has been reported that an acidic group near to the (polymer-bound) flavin has a positive effect upon its activity,[12,25,26] due to the higher stability of the intermediate flavin anion, and it is tempting to assume that this is also the case here. In the polyacids described here, an *intra*molecular hydrogen bridge can be formed between the pendent flavin and the carboxylic acid groups, with a relatively small loss of entropy. Covalent binding of a carboxylic acid group to a low molar mass flavin (**7**) did not lead to a positive effect upon the catalytic activity (see Table I). This acid group, however, is not available for intramolecular hydrogen bonding due to its steric positioning with respect to the isoalloxazine ring system, so that only the entropically unfavorable *inter*molecular interaction is possible. This means that only the negative electrostatic effect of a dissociated acid group upon the hydride-anion transfer reaction remains, so that the catalytic activity (at pH = 8) is even lower than that for **6**.

An interesting property of the flavin-containing polyacids is the pH dependence of their catalytic activity.

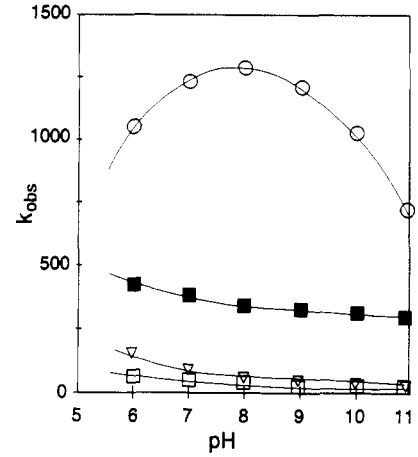

Figure 7. Activity of flavins in the oxidation of BNAH (standard medium): (□) unbound flavin (**6**); (▽) unbound flavin (**6**) with 10 equiv of COOH (**4a**) per flavin; (■) flavin-containing polycation ($\beta = 0.97$, see ref 12); (○) flavin-containing polyanion (**4d**).

Figure 7 shows the (apparent) second-order rate constants for the reaction catalyzed by polyanion-bound (**4d**) and low molar mass flavin with BNAH as a function of the reaction medium pH. For comparison the activity of a flavin-containing polycation[11,12] has also been plotted. Both the polycationic and the low molar mass flavins show only a small increase with lower pH, whereas the polyanionic flavin shows a distinct pH dependency. This probably is related to the degree of protonation of the polyacid. At high pH a large fraction of the carboxylic acid groups is ionized, so that the proposed hydrogen-bonding effect becomes small and the activity of the flavin is relatively low. Furthermore, the anionic charge of the polymer coil will induce an energetically unfavorable electrostatic interaction during the reaction, when the flavin anion is formed. If the pH is decreased, two effects are supposed to influence the activity of the flavin units. First, the increasing protonation will allow more of the favorable hydrogen bonding with flavin to take place. Second, protonation of the polymer will diminish the ionic character of the polymer, leading to contraction of the polymer coils and eventually (at pH < 6) to precipitation. With the reduced ionization of the polymer, the dielectric constant of the microenvironment of the polymer-bound flavin will also decrease, and this is known to decrease its activity.[31] With this in mind it is not surprising that a maximum in the catalytic activity is found at intermediate pH.

The large enhancement in reactivity of the flavin by the carboxylic acid group is definitely connected to the fact that both are bound to the same polymer, so that *intra*molecular hydrogen bonding is possible. If a polyacid **4a** is added to a low molar mass flavin, only a small increase in the activity is found at low pH (see Figure 7). This increase is probably due to enrichment of the hydrophobic (protonated) polymer coils with both the flavin and the substrate molecules, since both of them are quite hydrophobic. The effect of hydrogen bonding of flavin with the polymer, if at all present, is of minor importance here since only the entropically unfavorable *inter*molecular interaction can take place.

Complexation with a Polycation. In earlier publications[12,32] we have shown the advantages of immobilization of catalytically active (flavin-containing) polycations in so-called polyelectrolyte complexes[33,34] (PEC). It appeared that the activity of the immobilized flavin units in the reaction with BNAH was influenced by the complexing polyanion. Complexation with a strong polyanion lowered the reaction rates,[32] while a weak polyacid

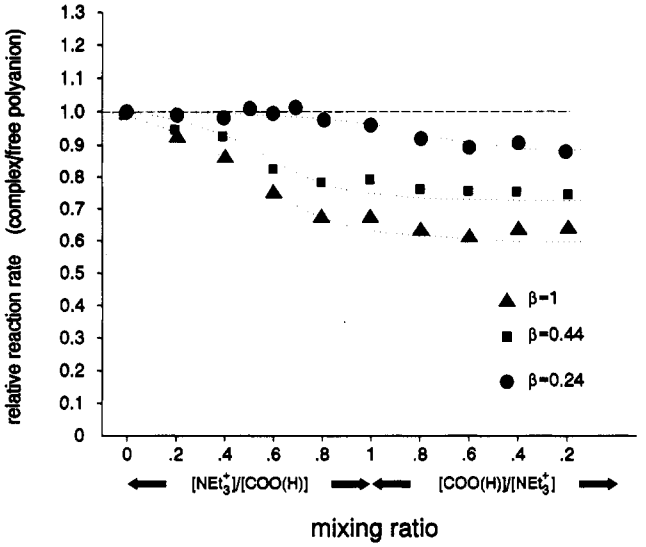

Figure 8. Initial reaction rates of flavin-containing polyanion (**4d**) after complexation with polycations (**5a–c**), relative to the activity of the free polyanion (in a standard medium).

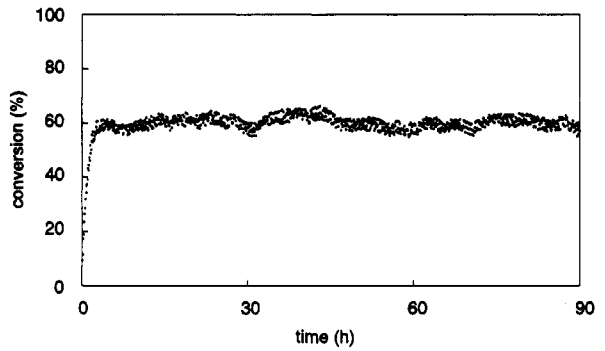

Figure 9. Conversion of BNAH in a continuous reaction catalyzed by a stoichiometric flavin-containing PEC (**4d** and **5a**) in a sandwich membrane.

enhanced the flavin catalytic activity.[12] Here we have formed a PEC of the flavin-containing polyacid (**4d**) with several polycations (**5**), having varying degrees of quaternization (β). Figure 8 shows the initial reaction rates after complexation, relative to the rate with the free polyanion in solution. Complexation with polycations appears to have a negative influence upon the activity of the polyanion-bound flavin. This effect is stronger when the charge density of the polycation (β) is higher, which is in agreement with earlier observations.[12,32] There are two reasons for the reduced reaction rates. First, the complexation induces an increased ionization of the polyacid,[35] and, second, the complexed polyanion will be less mobile due to the (ionic) cross-linking. Both these effects will reduce the degree of hydrogen bonding and thus the reactivity of the flavin.

Stability. Continuous oxidation experiments with these complexes give the possibility of determining the stability of such systems, especially when sandwich membranes[12] (see the Experimental Section) are applied. The complexes prepared here were tested in a continuous reaction for almost 4 days (Figure 9). During this period no significant change in the catalytic activity of the polyelectrolyte complex membrane was observed. This demonstrates the high stability of the flavin-containing polyacid and the practical applicability of polyelectrolyte complex formation for the immobilization of such catalytically active polymers. In addition to the high stability of the PEC under a wide range of reaction conditions, the ionic type of cross-linking allows *reversible* immobilization of the catalytic center. Under specific medium conditions (pH, cosolvent, ionic strength, etc.) the polyelectrolyte complexes can be dissociated, as was shown earlier for PECs consisting of two linear polyelectrolytes[36,37] and for polycations complexed to the surface of a cross-linked polyanionic material.[38]

Conclusions

We have presented the synthesis of the first flavin-containing polyanion, having pendent flavin and carboxylic acid groups, and of a "flavin homopolymer". The flavin units, bound covalently to the polyacid, show a large increase in the catalytic activity in the aerobic oxidation of BNAH as compared to the unbound, low molar mass flavin. This increase is attributed to the increased dielectric constant of the flavin microenvironment and to intramolecular hydrogen bonding of the acid groups to the flavin units prior to or during the reaction with BNAH. The activity of the flavin polyanions was shown to be pH-dependent, with a maximum at pH = 8. After complexation of the polyanion with a polycation the activity of the flavin units decreased (by 10–30%), especially when a polycation with a high charge density was applied. These polyelectrolyte complexes appeared to be quite stable, as was found in a continuous oxidation reaction of the substrate, showing once again the practical applicability of polymer complexation in the immobilization of catalytically active polymers.

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Registry No. 1, 136951-08-1; 1 (homopolymer), 138835-40-2; (1)(2) (copolymer), 138835-39-9; 2, 84740-98-7; 2 (homopolymer), 91380-16-4; 6, 14684-43-6; 7, 138835-38-8; BNAH, 952-92-1; KOBu-*t*, 865-47-4; $ClCH_2C_6H_4$-*p*-$CH{=}CH_2$, 1592-20-7; lumiflavin, 1088-56-8; *p*-vinylbenzoic acid chloride, 1565-41-9.