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Ionic Topochemical Tuned Biosensor Interface

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Two new hydrophilic, poly(ethylene glycol) (PEG)-based redox copolymers bearing electrochemically active ferrocene (Fc) and thiol/disulfide anchoring functionalities were synthesized. These copolymers are shown to adsorb on gold surfaces causing polymeric self-assembled monolayers (pSAMs) that possess triple functions: "redox-active", "ionic-tunable", and "bio-inert". Both immobilized polymers showed redox potentials at +400 mV (Ag|AgCl), and facilitate the electrocatalytical oxidation of NADH. Additionally, interfacial architecture of the polymers is affected by an increase in Ca^{2+} concentration, which leads to an amplification of the electrochemical response. The electrode current, measured for NADH-oxidation, increased by 80% after addition of 10 mM Ca^{2+} ions. Considering the Ca^{2+} influence on the heterogeneous electron transfer a structural model of the immobilized polymers is proposed based on the strong chelating abilitiy of noncyclic PEG moieties.

Introduction

In natural systems the most widely employed class of oxidoreductases are dehydrogenases dependent on $NAD(P)^+$ as the electron accepting cofactor. These enzymes catalyze the oxidation of various substrates like alcohols, aldehydes, carbohydrates, etc., which are of great interest for analytical applications. The detection of analytes in amperometric systems employing these dehydrogenases is based on the signal generated by the oxidation of NAD(P)H at the electrode surface. The direct electrochemical oxidation of NADH requires high overpotentials at bare electrodes resulting in electrode fouling and principal electrode reactions.[1,2] Therefore, efficient and mild systems with low overpotentials for the cosubstrate regeneration are required for biosensor applications. The construction of chemically modified electrodes (CME) can help to decrease these large overpotentials.[1,3]

Artificial mediators are used instead of natural cosubstrates in the "second generation of biosensors" to mediate electron transfer. Many studies describe various possibilities to utilize enzymes in combination with electrochemical detection for selective analysis by employing various mediator approaches: freely diffusing,[4,5] matrix-embedded,[6] adsorbed,[7,8] or covalently attached to enzyme[9,10] or electrode surfaces.[11−13] It turned out that effective regeneration systems must meet the following requirements: (a) an adequate redox potential for complete substrate conversion, (b) stability in both redox states within the regeneration cycle over a long period of time, (c) fast and selective reaction only with the cofactor, and (d) a reversible reaction at the electrode surface.

The research for NAD-enzymes has focused on various dyes for example phenazine derivatives,[14] Meldola Blue,[15] methylene green,[16] etc. The major body of redox substances studied for electrocatalytic reactions have involved one electron donors/acceptors like Fc, although the redox process of NAD(P)H/$NAD(P)^+$ involves two electrons and one proton.[17−19]

However, CMEs exhibit a slow but continuous desorption due to the solubility of the mediators, which limits the long-term operational stability of these electrodes. A useful approach is covalent coupling of mediator molecules to polymer backbones to hinder their leakage and/or increase the biocompatibility, e.g., inserted in carbon paste electrodes[20−22] or grafted on electrode surfaces.[23,24]

Polymers like polysiloxane, polypyrrole or polyvinylpyridine bearing redox centers like osmium complexes or Fc derivatives were implemented in biosensor applications, unfortunately they are quite hydrophobic.[25−29] Hydrogels, like poly(ethylene glycol)

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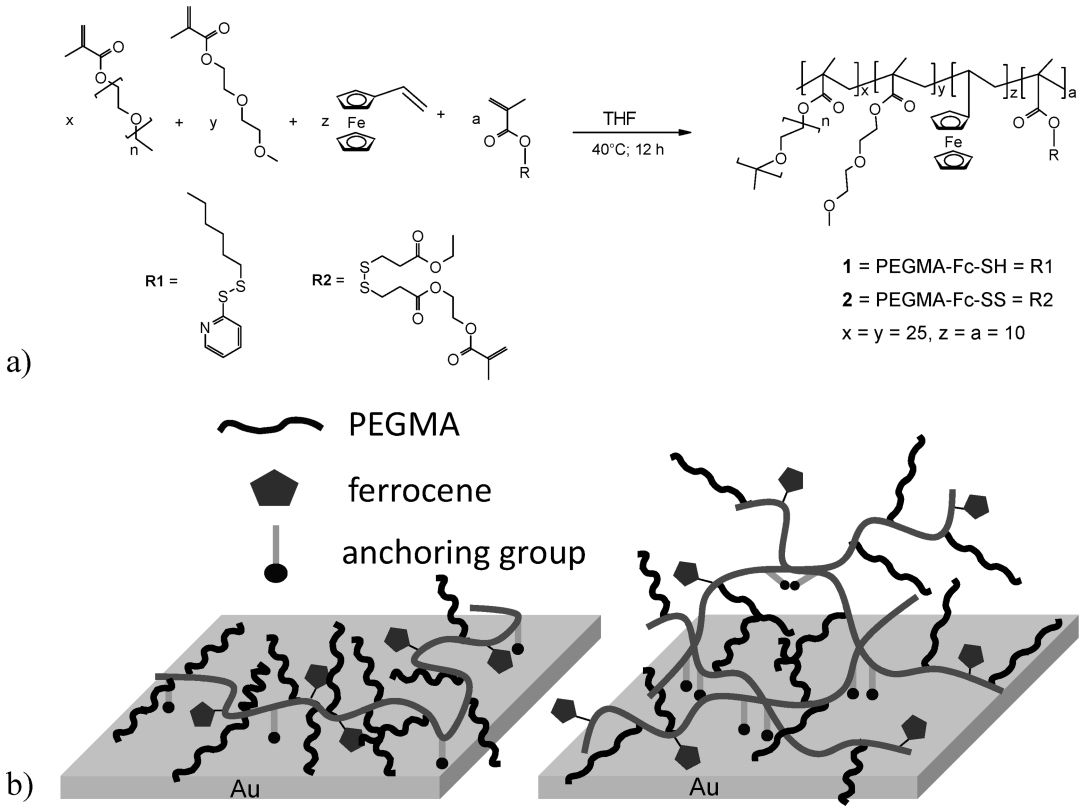
Scheme 1. (a) Synthetic Scheme for the Production of PEGMA-Fc-SH (1)[a] and PEGMA-Fc-SS (2) and (b) Schematic Representation for the Fabrication of pSAMs of 1 (Left) or 2 (Right)



[a] The mercaptopyridine protection group was cleaved prior to surface modification (see Experimental Section)

or poly(*N*-isopropylacrylamide) are three-dimensional networks of hydrophilic polymers, binding a huge amount of water, generally more than 50% of their total weight. Hydrophilic redox polymers mimic the natural surrounding of the enzyme molecule and in this way enhance the electron transfer efficiency.

Poly(ethylene glycol) methacrylate (PEGMA) polymers are polymerizable derivatives of the linear poly(ethylene glycol) (PEG). They are uncharged, hydrophilic, strongly hydrated in aqueous solutions, biocompatible and are generally regarded as an alternative material for preventing fouling behavior.[30]

It has been reported that different conformations of PEG originate in the EG molecule, which can achieve different rotational configurations, that can be described as all-trans and helical.[31−33] The critical length for helical structure is more than four EG units.[32] For instance, in aqueous media the conformation of the EG unit in PEG favors the trans−gauche−trans (TGT) conformational sequence. Furthermore, a structure rearrangement is observed after addition of alkaline and alkaline-earth ions.[31,34] The complexation of alkaline-earth metal ions by poly(ethylene glycol)s was confirmed using NMR, conductivity measurements and ion-selective electrodes.[34−37] In addition, this TGT conformation is convenient due to strong ion-dipole binding affinity toward alkaline and alkaline-earth metal ions. For these kinds of metal-ions there are an optimum number of EG units required, which is five to seven, to form stable five- to seven-membered ring chelates.[34,38]

By taking advantage of the chemical reconfigurability of the PEG with a low number of EG units we demonstrate a facile method to tune the heterogeneous electron transfer using new synthesized poly(ethylene glycol) based, hydrophilic redox polymers with Fc as the key-mediating functionality and thiols/disulfides as anchoring groups with following structures (Scheme1, Figure S2):

These novel designed polymers exhibit quadruple roles: "surface-reactive", "redox-active", "ionic-tunable", and "bio-inert" ones. The polymers are immobilized covalently via a self-assembling step of intrinsic disulfide or thiol groups on gold electrodes. The high number of anchoring groups along the linear polymeric chains are introduced to obtain polymeric self-assembled monolayers (pSAMs), which are reminiscent of similar pSAMs described in previous reports.[39,40] Characteristic for this interfacial architecture is that polymeric chains lay down parallel to the surface plane rather than being orientated toward the surface normal (Scheme 1b).[41,42] The redox active Fc is randomly distributed in a polymeric matrix, which should be beneficial for the ET process. Moreover, this approach provides a protocol for surface modification without the

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use of bifunctional molecules where steric constraints preclude the formation of ordered structure (e.g., clustering of Fc-alkanthiols on gold surfaces).[43,44] These polymers were studied for the electrocatalytic enzyme-coupled oxidation of glucose with the respective NAD-dependent dehydrogenase. Therefore, the "bio-inert" (antibiofouling) surface property was implemented to minimize adsorption of proteins that results in a poor signal-to-noise ratio (S/N ratio) in electrochemical experiments.[45−47] The "ionic-tunable" property is shown by the effect of complexation of Ca^{2+} by the PEG moieties of the pSAM, connected with its electron transfer behavior. A model of the structural change of the polymers can be derived on the basis of the effective complexation with alkaline-earth cations accompanied by a partial dehydration and a reduced radius of gyration.[32,33]

Experimental Section

Chemicals. 3,3′-Dithiodipropionic acid di(*N*-hydroxysuccinimide ester), 2-hydroxyethyl methacrylate, and NAD-dependent glucose dehydrogenase (NAD-GDH, *Pseudomonas sp*.) were purchased from Fluka, triethylene glycol monoethyl ether methacrylate and diethylene glycol methyl ether methacrylate from Aldrich, vinylferrocene from Alfa Aesar and 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) from Wako. All substances were used as obtained.

Synthesis of 6-(Pyridine-2-yl disulfanyl)hexyl Methacrylate (3). A 5 g (22,7 mmol) sample of 2,2-dithiopyridine was dissolved in 50 mL of MeOH containing 333 μL of acetic acid, and a mixture of 1.5 mL (1.47 g, 11 mmol) of 6-mercapto-1-hexanol in 4 mL of MeOH was added dropwise. The mixture was stirred for 3 h at room temperature. The reaction was monitored by TLC (SiO_2, DCM). After completion the crude product was purified by column chromatography on silica with $CHCl_3$ as mobile phase. The main fraction was evaporated to dryness to give a colorless crystalline solid, which was dissolved in 50 mL of DCM containing 8 mL of triethylamine. The mixture was cooled down to 0 °C and a solution of 1.12 mL (1.2 g, 11.5 mmol) of methacrylic acid chloride in 20 mL of DCM was slowly added. The mixture was allowed to warm to room temperature and stirred for further 24 h. The reaction mixture was quenched by saline solution and the organic layer was extracted with water. The product was purified by column chromatography on silica with petrolether/ethyl acetate 1:1 as mobile phase to yield after evaporation colorless oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.45 ppm (d, CH-1); 7.7 - 7.05 ppm (m, Ar CH-2 to CH-4); 6.2 ppm (d, CH_2-14a); 6.05 ppm (d, CH_2−14b); 4.1 ppm (m, CH_2-11); 2.56 ppm (m, CH_2-6); 1.9 ppm (s, CH_3-15); 1.65−1.2 ppm (m, CH_3-7 to CH-10). Numeration is related to Figure S2.

Synthesis of PEGMA-Fc-SH (1). A 319 mg (1.3 mmol) sample of triethylene glycol monoethyl ether methacrylate, 244 mg (1,3 mmol) of diethylene glycol methyl ether methacrylate, 162 mg (520 μmol) of 6-(pyridine-2-yl disulfanyl)hexyl methacrylate (**3**), and 110 mg (520 μmol) of vinylferrocene were dissolved in 3 mL of degassed THF. The polymerization was initiated by addition of 33 mg 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) and proceeded 24 h at 40 °C. After purification an orange-yellow oil with a yield of 87% was obtained.

UV/vis (λ_{max}/nm) in ethanol: 450

Synthesis of Dithiodi((2-propanoyloxy)ethyl methacrylate) (4). A 1 g (2.8 mmol) sample of 3,3′-dithiodipropionic acid di(*N*-hydroxysuccinimide ester) and 0.77 g (5.9 mmol) of 2-hydroxyethyl methacrylate were dissolved in 50 mL of DCM, purged with argon, and stirred for 48 h at room temperature. The new substance was characterized by ^{1}H NMR and mass spectrometry.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.15 ppm (d, CH-3a); 5.1 ppm (d, CH-3b); 4.35−4.25 ppm (m, CH_2-5 to CH_2-6); 3.85 ppm (m, CH_2-8); 3.05 ppm (m, CH_2-7); 2.83 ppm (s, CH_3-1). Numeration is related to Figure S2.

Synthesis of PEGMA-Fc-SS (2). A 319 mg (1.3 mmol) sapmle of triethylene glycol monoethyl ether methacrylate, 244 mg (1.3 mmol) of diethylene glycol methyl ether methacrylate, 225 mg (520 μmol) of dithiodi((2-propanoyloxy)ethyl methacrylate) (**4**), and 110 mg (520 μmol) of vinylferrocene were dissolved in 2 mL of THF. The polymerization was initiated by addition of 36 mg of 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) and proceeded at 40 °C for 24 h. The orange-colored resin-like polymer was obtained with a yield of 89%.

UV/vis (λ_{max}/nm) in ethanol: 450

Mounting the Polymer to Gold Surfaces. Clean gold electrodes were incubated in a solution of 1 mg/mL of polymer in ethanol overnight at room temperature. Before immobilization of PEGMA-Fc-SH the protecting group is cleaved by 5 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP HCl) in H_2O as a reducing reagent. After 12 h, they were rinsed with copious amount of water, and then the electrodes were directly used for electrochemical measurements.

Electrochemical Measurements. A three electrode system was used consisting of a polymer modified gold working electrode, Ag|AgCl|3 M KCl reference electrode (CH Instruments, Inc., USA) and a platinum counter electrode. Cyclic voltammograms (CVs) were recorded in HBS buffer (20 mM HEPES, 20 mM KCl, 1 mM $CaCl_2$, pH 8) with an Autolab System (Metrohm, Germany) connected to a personal computer. For amperometric measurements at different Ca^{2+} concentrations the electrode system was set to +400 mV vs Ag|AgCl|3 M KCl using a potentiostat (Biometra GmbH, Germany), measurements were recorded in 20 mM HEPES/KCl buffer pH 8 in the presence of 2.5 mM NAD^+, 100 mM glucose, and 50 μg/mL NAD-GDH.

Gel Permeation Chromatography (GPC). GPC was carried out using a setup from Thermo Separation Products together with a GRAL-LIN (7 μ, 100 + 1000) column (PSS, Mainz). The flow rate of the eluent DMSO + 0.5 g/L LiBr was 1.0 mL/min at 25 °C. Poly(methyl methacrylate) was used as a standard compound.

Ca^{2+} Effects. Turbidimetric measurements of the polymers were carried out using a V-570 spectrophotometer from JASCO and PMMA cuvettes (Roth). The changes in absorbance of the aqueous polymer solutions through different Ca^{2+} concentrations were detected at 600 nm. Different concentrations of $CaCl_2$ were added to different cuvettes with aqueous polymer solutions. Ca^{2+} effects to the electrochemical measurements were recorded as described above.

Results and Discussion

The new redox polymers were synthesized as described in the Experimental Section. PEGMA-Fc-SH (**1**) is a linear polymer compared to the PEGMA-Fc-SS (**2**), which is cross-linked via disulfide functionalities. Both polymers contain ferrocene as the redox mediating moiety, the polymer side chains consist of poly(ethylene glycol) (PEG). The hydrophilic chains are hydrated in aqueous solutions under hydrogel formation.

The apparent molecular weight of the polymers was determined by gel permeation chromatography (GPC) in polymethylmethacrylic acid equivalents in DMSO. The apparent molecular weight of PEGMA-Fc-SH (**1**) was about 4 kDa (M_n) and a polydispersity (M_w/M_n) of 1.7 was determined. These values are

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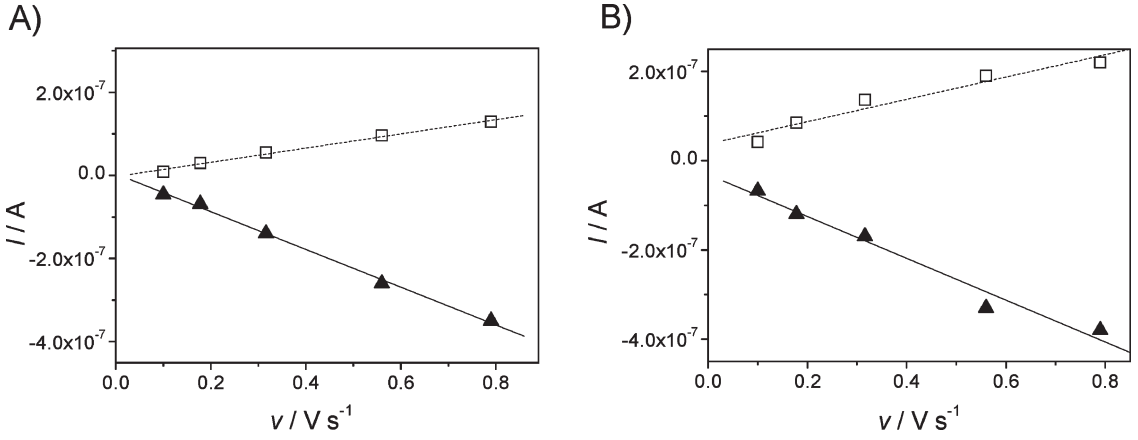

Figure 1. Anodic and cathodic peak currents as a function of the scan rate for PEGMA-Fc-SH (**1**) (A) and PEGMA-Fc-SS (**2**) (B) pSAM electrodes in HBS pH 8. RE: Ag|AgCl. CE: platinum.

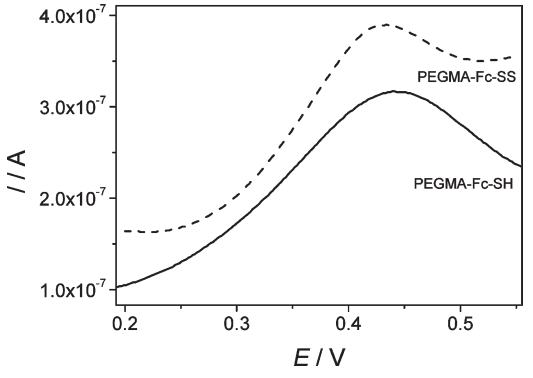

Figure 2. Square wave voltammogram of PEGMA-Fc-SH-modified (solid) and PEGMA-Fc-SS-modified (dashed) gold electrodes in HBS pH 8: frequency, 10 Hz; step potential, 2 mV; RE, Ag|AgCl; CE, platinum.

in a good agreement with polydispersities of comparable polymers.[48] The apparent molecular weight of the second new polymer, PEGMA-Fc-SS (**2**), was about 8 kDa and a polydispersity of 1.4 was found. The integration of the mediator is confirmed by spectrophotometry (see Supporting Information, Figure S1). UV/vis spectra of the polymers PEGMA-Fc-SH (**1**) and PEGMA-Fc-SS (**2**) show similar absorption at 450 nm in ethanol, which is consistent with the absorption of the vinylferrocene in DMSO, which confims the integration of ferrocene.

The polymers were immobilized via intramolecular disulfide or prior deprotected thiol functionalities to gold surfaces under formation of flexible, polymeric self-assembled monolayers (pSAM), enabling short electron transfer ways.

The covalent coupling of the redox mediator to the electrode surface was confirmed by recording cyclic voltammograms at different scan rates (SR). Both covalently attached polymers showed a linear dependence between the peak current (I_p) and the SR (Figure 1) indicating surface bound mediators.

Square wave voltammetry (SWV) was used to determine the redox potentials of PEGMA-Fc-SH (**1**)/SS (**2**) mounted on gold electrodes. Values of +400 mV vs. Ag|AgCl in HBS at pH 8 for both polymers (Figure 2) comparable to monolayers of ferrocene derivatives and ferrocene-modified poly(*N*-isopropylacrylamide) polymers were measured.[13,43,44,49] Integration of the current peaks leads to surface coverages of 1.5×10^{-11} mol cm^{-2} a value, which is in good agreement with thin polymer films for both polymers.[50] Although, the coverage of the pSAM is ten times less than a respective SAM of 9-mercaptononyl-5′-ferrocenylpentanoate, the heterogeneous electron transfer constants (k_s) were found to be 70 and 50 s^{-1} for PEGMA-Fc-SH (**1**) and PEGMA-Fc-SS **2**, respectively, which are comparable to k_s to Fc-containing SAMs.[49]

NAD^+-dependent enzymes like glucose dehydrogenase (GDH), alcohol DH or sorbitol DH oxidize their analytes and NAD^+ is reduced during this process. The recycling of NADH at the polymer modified electrodes via electrocatalytic oxidation could be observed. The mediator can be regenerated at the electrode and a current proportional to the analyte amount can be determined.

Figure 3 shows cyclic voltammograms for PEGMA-Fc-SH (**1**) and PEGMA-Fc-SS (**2**) modified gold electrodes in absence (dotted lines) and presence (solid lines) of NAD^+-dependent GDH (NAD-GDH) and substrates. In the presence of enzyme and substrates an increase of the anodic peak current demonstrating an electrocatalytic effect of the polymers was observed. Table 1 summerizes the main characteristics of the two polymers.

The PEG side chains were used to create a more biocompatible redox electrode and moreover an external tunable redox activity to influence the heterogeneous ET. This effect can be reached by a change of the polymeric, interfacial structure. Since it is known, that PEG exhibit effective complexation of alkali and alkaline-earth ions with relative high association constants,[34,35,37] which leads to a conformational change of the PEG unit and subsequently to interfacial order. The employed short PEG chains can not form helical structures, so that we assume a stable ring chelate is an apparently convenient conformation. This assumption agrees very well with those obtained by Okahara et al. using NMR-studies.[34]

Inter- and intramolecular complexes of the PEG-oxygen atoms with Ca^{2+} can be formed.[35] For this reason the influence of Ca^{2+} on the interfacial structure was investigated using turbidimetry (polymer in solution) and amperometry (with polymer modified gold electrodes).

In UV/vis titration experiments the cross-linked polymer showed increasing absorption signal until 10 mM Ca^{2+} while the linear polymer does not show any visible effect (Figure 4).

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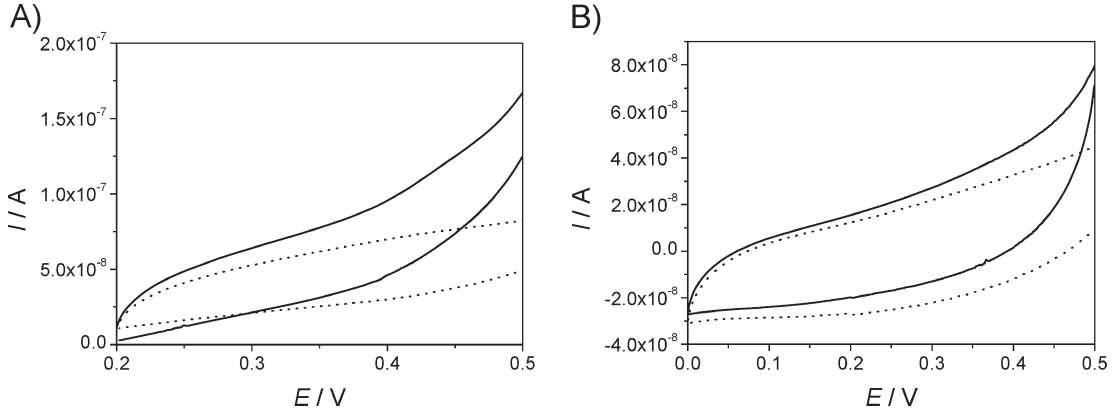

Figure 3. Cyclic voltammograms of PEGMA-Fc-SS (**2**) (A) and PEGMA-Fc-SH (**1**) (B) in HBS buffer (dotted line) and HBS buffer +2.5 mM NAD^+, 50 μg mL^{-1} NAD-GDH, and 100 mM glucose (solid line): scan rate, 2 mV s^{-1}; RE, Ag|AgCl; CE, platinum.

Table 1. Summarized Main Features of the Polymers

polymer	apparent molecular weight (M_n)[a] (kDa)	polydispersity M_w/M_n	$E_{1/2}$ (mV)	electrocatalytic oxidation of NADH
PEGMA-Fc-SH (**1**)	4	1.7	+400	+
PEGMA-Fc-SS (**2**)	8	1.4	+400	+

[a] M_n was measured by GPC using DMSO + 0.5 g/L LiBr, standard PMMA.

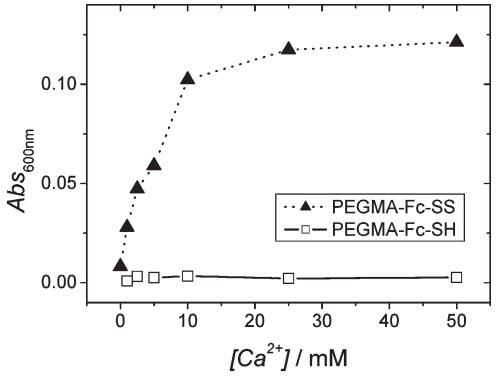

Figure 4. Influence of Ca^{2+} on polymer structure measured by turbidimetry in H_2O: PEGMA-Fc-SH (**1**) (squares) and PEGMA-Fc-SS (**2**) (triangles).

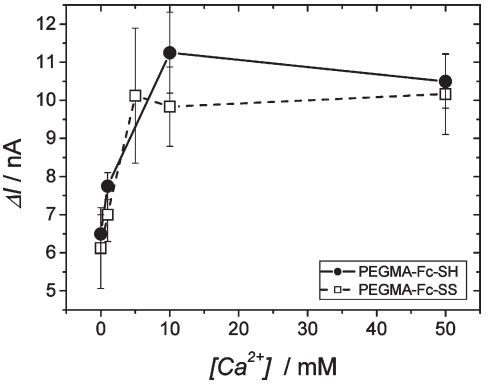

Figure 5. Influence of Ca^{2+} on polymer structure measured by amperometry, with electrode current for 2.5 mM NAD^+, 50 μg/mL NAD-GDH, and 100 mM glucose, E = +400 mV: PEGMA-Fc-SS (**2**) (triangles) and PEGMA-Fc-SH (**1**) (squares) modified gold electrodes in 20 mM HEPES/KCl pH 8. RE: Ag|AgCl. CE: platinum, n = 3.

Both solutions have the same polymer concentrations, but the molecular weight of PEGMA-Fc-SH is half as great as for PEGMA-Fc-SS. The cross-linked polymer forms huge aggregates throughout inter- and intramolecular interactions, which finally precipitate in solution. The shorter linear chains of (**1**) possibly change their structure without detectable effect in the surrounding solution. Maybe the formation of intermolecular bridges is prevented and only intramolecular complexes are formed, which do not lead to precipitation. Hence, the solution remained transparent.

The influence of these structural changes to the electrochemical response was investigated amperometrically at different Ca^{2+} concentrations reflected in terms of enzyme-converted NAD^+. The current maximum for 100 mM glucose and 2.5 mM NAD^+ was reached at 10 mM Ca^{2+} for both polymers, the same concentration as in turbidimetric measurements (Figure 5). In the presence of 10 mM Ca^{2+}, a $\sim$ 80% higher current signal as compared to a Ca^{2+}-free solution, was detected for PEGMA-Fc-SS (**2**) and PEGMA-Fc-SH (**1**).

The enzyme activity was not affected by the Ca^{2+}-concentration confirmed in a control experiment. Thus, the obtained increase in electrode response is related to changes in polymer structure. The structural change of PEGMA-Fc-SS (**2**), suggested already in spectroscopic measurements, is confirmed by the electrochemical measurements. In contrast to the spectroscopic studies, the PEGMA-Fc-SH (**1**) showed an increase in current signal with increasing Ca^{2+} concentration (Figure 5). This effect can be ascribed to the PEG chains binding Ca^{2+} via the coordinative use of the lone pair electrons on the PEG units, which result in an dehydration process and both phenomena lead to a collapsed state. The surface-bound polymers form predominantly intramolecular Ca^{2+} complexes caused by reduced flexibility of the polymers chains due to the anchoring. An expansion of the polymer chains after complexation of cations via repulsion forces is, opposite to literature,[37] prevented by alternating functional groups through Fc, thiol, or disulfide monomers in the copolymer. While the polymeric microenvironment around the mediator moieties withers, cavities are generated allowing an efficient interaction between coenzyme and mediator. Short electron

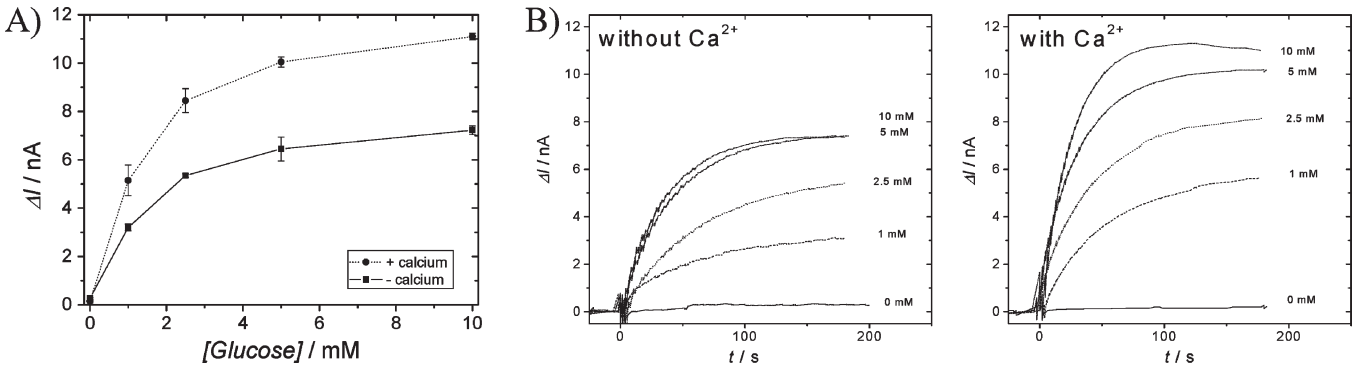

Figure 6. Ca^{2+} effect to different glucose concentrations with PEGMA-Fc-SS electrodes in the absence and presence of 10 mM Ca^{2+}. (B) Original data. 50 μg mL^{-1} NAD-GDH + 2.5 mM NAD^+, E = +400 mV. RE: Ag|AgCl. CE: platinum.

transfer pathways between mediator and enzyme and between mediator and electrode are ensured and ET is facilitated. In the absence of Ca^{2+} the oxygen atoms of PEG form hydrogen bonds to water molecules, hence, a relaxation of the PEG chains is obseverd and the polymer swells. Because of the relaxation the mediator is surrounded by PEG chains and the interaction with the coenzyme is hindered and thus, less efficient. The complexation of the metal ions has been found to be reversible by using EDTA in the washing step between the electrochemical measurements. These results agree with the measured association constants of noncyclic PEG.[34]

The effect of Ca^{2+} to the electrocatalytical response was shown for different glucose concentrations with the PEGMA-Fc-SS-modified electrode and NAD-GDH in absence and presence of 10 mM Ca^{2+}. The sensitivity for the glucose concentrations could be increased by up to 80% by using Ca^{2+} (Figure 6A). The original curves reach a plateau after ~100 s. The curves for 100 and 20 mM glucose concentrations show decreasing signals in the presence of calcium originated in diffusion limitations via high substrate concentrations.

Conclusion

To overcome the high overpotential of the direct oxidation of NADH at different electrode materials (~1 V)[51] and the mediator leakage two different redox polymers with quadruple functionalities based on bioinert, ionic-tunable poly(ethylene glycol) methacrylates and redox-active ferrocene were synthesized, with surface-reactive thiol and disulfide functionality, respectively, to form linear and cross-linked polymeric self-assembled monolayers (pSAM) on gold surfaces.

These thin layers enable short electron transfer pathways, leading to fast and efficient electron transfer between enzyme and electrode. Both polymers were useful for the electrocatalytic oxidation of NADH. The electron transfer efficiency is increased by using Ca^{2+}. Thus, the sensitivity could be increased by ~80% after addition of 10 mM Ca^{2+}. The effects of Ca^{2+} to structural changes of the PEG chains were studied. On the basis of previous studies Ca^{2+} complexation of noncyclic PEGs results in a metal-ion chelate similar to the initial state in template synthesis of crown ethers. Because of the complexation a structural change of the whole ensemble is observed, which can be attributed to a collapsed structure. This leads to a better electrode response to NAD-GDH catalyzed glucose conversion. These results give an impression how to control catalytic redox response via external stimuli. This could be implemented in fabrication of parallel electrode arrays with single addressable electrodes. The irreversible immobilization of both polymers and mediators allows easy handling for large scale production of electrodes.

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Supporting Information Available: Figures showing absorption spectra of the polymers **1** and **2** and the chemical structures of **3** and **4** including ^1H NMR numeration. This material is available free of charge via the Internet at http://pubs.acs.org.

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