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Electrostatic Immobilization of Glucose Oxidase in a Weak Acid, Polyelectrolyte Hyperbranched Ultrathin Film on Gold: Fabrication, Characterization, and Enzymatic Activity

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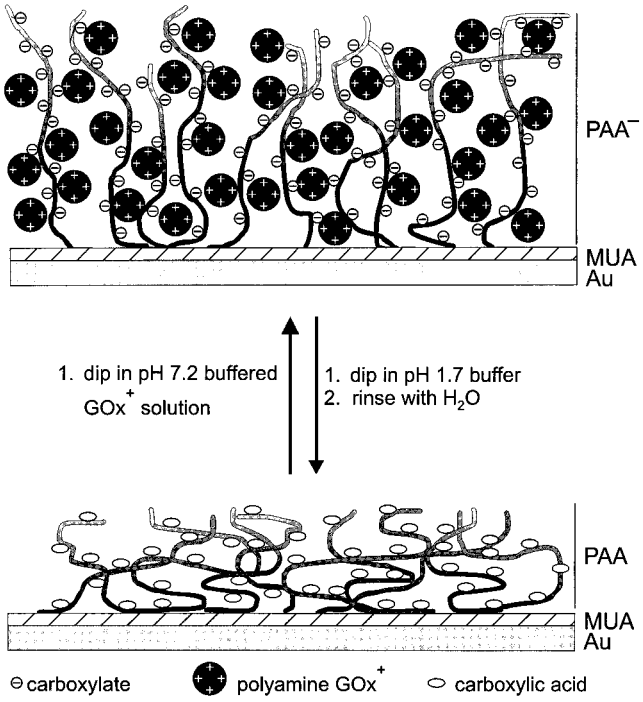
In this paper we show that hyperbranched polymers can be used as a host matrix for electrostatic entrapment of enzymes. Specifically, amine-functionalized glucose oxidase (GOx^+) and horseradish peroxidase, as well as poly(amidoamine) dendrimer-modified horseradish peroxidase, reversibly sorb into polyanionic, hyperbranched poly(sodium acrylate) (PAA^-) films that are on the order of a few hundred angstroms thick. The quantity of GOx^+ entrapped within the PAA^- films depends on the nature of film preparation but is typically on the order of 0.06 unit/cm^2. The extent to which entrapped GOx^+ retains its activity depends on the film history, but for PAA^-/GOx^+ composites not exposed to glucose and stored at 4 °C, the original activity is retained for up to 68 days and perhaps longer.

This paper describes electrostatic immobilization and activity of amine-functionalized glucose oxidase (GOx^+) and horseradish peroxidase (m-HRP^+), as well as poly(amidoamine) (PAMAM) dendrimer-modified horseradish peroxidase (d-HRP^+), within surface-grafted, polyanionic organic thin films (Scheme 1). These composite materials are prepared by electrostatic sorption[1] of GOx^+, m-HRP^+, or d-HRP^+ into solvent-swollen, hyperbranched poly(sodium acrylate) (PAA^-) films confined to Au substrates. Here we report that all three enzymes reversibly sorb into such hyperbranched films and the extent to which their activity is retained therein. Although all three enzymes form composites with the polymer thin film, we focus principally on the properties of the PAA^-/GOx^+ composites because of the technological importance of GOx-based glucose biosensors. The amount of GOx^+ entrapped within the PAA^- films depends on how the composite is prepared but is typically on the order of 0.06 unit/cm^2. The extent to which the entrapped enzyme retains its activity depends on the film history, but for films that are not exposed to glucose and stored in buffer at 4 °C, the original activity is retained for more than 60 days. Although it is not the thrust of these experiments, preliminary studies indicate that this sort of ionic assembly of GOx^+ in highly functionalized ultrathin films is a potentially useful strategy for the development of new amperometric biosensors for the detection of glucose.

Scheme 1



Our previous studies have shown that hyperbranched poly(acrylic acid) (PAA) films are easy to synthesize by sequential grafting steps using simple condensation chemistry (Scheme 2).[2] Such films are stable toward acid, base, extraction, sonication, organic solvents, and extended electrochemical cycling.[2] The

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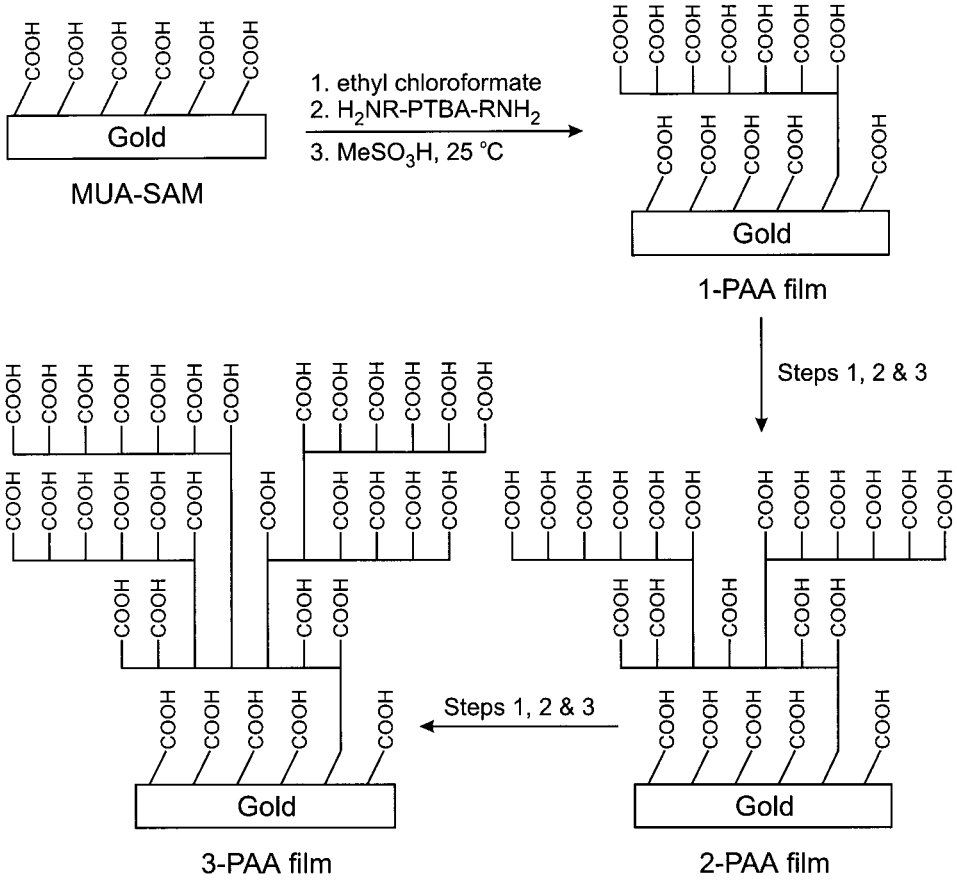
Scheme 2



highly functionalized PAA films are also chemically versatile intermediates for the synthesis of more sophisticated materials having applications to a variety of technologies.[2,3] For example, we have previously shown that PAA films can be covalently modified with dyes, fluorophores, perfluorinated amines, and electroactive groups.[2] More recently we demonstrated that these hyperbranched films can also be modified ionically by electrostatic sorption of polycations such as poly(D-lysine), PAMAM dendrimers, and poly(allylamine).[1] The work described here is an extension of this ionic entrapment approach, focusing specifically on the entrapment of biologically active species.

Several techniques have been used to immobilize biologically active molecules within or on polymer-based devices. These include retention by adsorption at solid interfaces,[4−7] cross-linking of the enzyme to a support or to other enzymes with bifunctional agents,[8−12] physical retention within polymeric matrixes,[13−16] and covalent[17−22] or ionic[23−26] binding to functionalized supports.[27−30] While these methods have been used successfully, significant

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difficulties may be encountered during implementation. For example, harsh synthetic conditions required for immobilization may lead to enzyme degradation, the enzyme immobilization matrix may lead to slow substrate mass transfer, and leaching of the enzyme results in sensor drift.[27−29] Another difficulty involves low capacity of the host for the enzyme. For example, a terpolymer of poly(*N*-methyl-4-vinylpyridinium chloride), 4-aminostyrene, and the poly(4-vinylpyridine) complex of $[Os(bpy)_2Cl_2]$ immobilized on graphite ionically binds native, unmodified glucose oxidase (GOx) very strongly under mild conditions, but the capacity of such systems is limited.[23] We reasoned that the hyperbranched polyelectrolyte thin films described here would be a useful alternative matrix for enzyme immobilization, because they can entrap large quantities of cationic polyelectrolytes under mild conditions[1] and because mass transfer of substrates in to and out of the films should be fairly rapid due to the unique hyperbranched structure of the film. Indeed, such films do entrap enzymes under very mild conditions while retaining a practical level of enzyme activity and without loss of the enzyme from the polymeric host. Additionally, the highly functionalized hyperbranched grafts offer the potential for straightforward incorporation of electron-transfer mediators, which could be useful for shuttling electrons to or from the catalytic sites within the film. Because hyperbranched grafts are synthesized using a layer-by-layer approach, it is easy to control film thickness and introduce thin biocompatible terminal layers.[31−33] Finally, these grafts are covalently linked to the substrate as a lightly cross-linked polymer film and are thus not prone to delamination.

EXPERIMENTAL SECTION

Buffer Solutions. To provide a nearly constant refractive index and ionic strength, buffers were prepared in an aqueous 0.1 M NaCl solution using 18 MΩ·cm deionized (DI) water (Milli-Q, Millipore). Buffers were prepared as described previously.[1]

Reagents. Glucose oxidase (EC 1.1.3.4 type X-S from *Aspergillus niger*, 208 800 units/g), horseradish peroxidase (HRP, EC 1.11.1.7, type VI, 290 000 units/g), and sodium *m*-periodate (Sigma Chemical Co., St. Louis, MO) were used as received. 4,4-Difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid succinimdyl ester (BODIPY 530/550, SE, Molecular Probes, Eugene, OR) was used as received. Pentaethylenehexamine (3,6,9,12-tetraazatetradecane-1,14-diamine), mercaptoundecanoic acid (MUA), third-generation (G3) Starburst PAMAM dendrimers (Dendritech, Inc., Midland, MI), sodium cyanoborohydride, and *o*-dianisidine (3,3′-dimethoxybenzidine) (Aldrich Chemical Co., Milwaukee, WI) were used as received. The protein assay dye (Catalog No. 500-0006, Bio-Rad, Cambridge, MA) was used as received.

Substrate Preparation. Au-coated substrates were prepared by electron beam deposition of 10 nm of Ti followed by 200 nm of Au onto Si (100) wafers. Before each experiment, all wafers were cleaned in a low-energy ozone cleaner for 10 min (Boekel Industries, Inc., model 135500).

Preparation of α,ω-Diaminopoly(*tert*-butyl acrylate) (NH_2R-PTBA-RNH_2). The polymer NH_2R-PTBA-RNH_2 (R = CH_2CH_2-$NHCOCH_2CH_2C(CN)(CH_3)$) was synthesized as previously described with only minor modifications.[3a] At the diacid polymer step, the product was twice precipitated from methanol/distilled water (1:1) yielding a polymer dispersity of 1.9 (M_n = 15 000, GPC). The final diamino-terminated polymer had a polymer dispersity of 2.0 (M_n = 18 000, GPC).

Preparation and Derivatization of Poly(acrylic acid) Films. Hyperbranched PAA films on Au-coated Si wafers were prepared by grafting NH_2R-PTBA-RNH_2 onto an ethyl chloroformate-activated MUA self-assembled monolayer (SAM).[3] Hydrolysis of the *tert*-butyl ester groups occurs in 15 min in a solution of $MeSO_3H/CH_2Cl_2$ (0.5 mL/15 mL) at 25 °C. Hydrolysis is followed by two additional stages of activation, grafting, and hydrolysis to yield a grafted hyperbranched 3-PAA film (Scheme 2). The 3-PAA films prepared in this way were 23 ± 3 nm thick.

Amine Functionalization of GOx and Purification of GOx^+. GOx is a dimer glycoprotein that has a molecular mass of 186 000 Da and an isoelectric point of ∼3. The majority of the predominantly hydrophilic exterior consists of mannose-rich oligosaccharide chains, which comprise 10−15% of the weight of the native enzyme.[34] Modification of GOx was necessary, because the native enzyme could not be intercalated within the anionic hyperbranched film. Amine-functionalized glucose oxidase has several amine groups on the periphery which are protonated in pH 7.2 buffer solution. The modification of GOx was accomplished using a three-step procedure based upon a literature method.[34−38] First, 100 μL of 20 mg/mL GOx in 100 mM aqueous bicarbonate solution was oxidized with 50 μL of a 12 mg/mL sodium *m*-periodate solution at 25 °C for 1 h in the dark. This results in formation of aldehyde functional groups in the oligosaccharide chains. Next, 100 μL of pentaethylenehexamine (20 mg/mL in water, pH 8) was added to the solution and allowed to react for 2 h to yield a Schiff base. Finally, 100 μL of a 20 mg/mL sodium cyanoborohydride solution was added and allowed to react overnight to reduce the Schiff base. After incubation, the solution was dialyzed against 0.1 M phosphate-buffered saline (PBS) for 4 h. Dialysis was performed using Slide-A-Lyzer dialysis cassettes (Pierce Co., Rockford, IL), with a sample volume ranging from 0.1 to 0.5 mL and a molecular weight cutoff of 3500.

Prior to enzyme modification, assays indicated the amount of active enzyme was 4.9 mg/mL. After enzyme modification, the amount of active enzyme was reduced by 35%, which compares favorably with a ∼50% decrease reported previously.[35] However, total protein assays indicated no change in the amount of enzyme present before and after modification (5.7 mg/mL), confirming that loss in activity is due to enzyme deactivation.

Preparation and Purification of Amine and Dendrimer-Functionalized HRP. HRP is a monomer glycoprotein with a

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molecular mass of ~44 000 Da and an isoelectric point of ~9; ~20% of the enzyme is glycosylated.[39] Using the same procedure as above, HRP was modified using pentaethylenehexamine and third generation PAMAM dendrimers to obtain m-HRP$^+$ and d-HRP$^+$, respectively.[34−38] The dialysis weight cutoff used to purify d-HRP$^+$ was 10 000 to remove the unreacted high-molecular-weight G-3 PAMAM dendrimers (7500).

Preparation of 3-PAA$^-$/GOx$^+$ and 3-PAA$^-$/d-HRP$^+$ Salt Films. The 3-PAA$^-$ composite salt films were prepared by soaking 3-PAA films for 20 min in a 5-mL solution of pH 7.2 buffer containing ~2.7 nmol of GOx$^+$ or 1.3 nmol of d-HRP$^+$, to form their respective film composites. The enzyme is not stable in the presence of organic solvents, and therefore, only water was used for washing after enzyme incorporation. After being blown dry with N_2, all films were stored dry in a refrigerator at ~4 °C prior to use in the assay protocol.

Preparation of 3-PAA$^-$/m-HRP$^+$ Salt Films. The 3-PAA$^-$ composite salt films were prepared by soaking 3-PAA films for 20 min in a 5-mL solution of pH 6.6 buffer containing ~2.0 nmol of m-HRP$^+$. The enzyme is not stable in the presence of organic solvents, and therefore, only water was used for washing after enzyme incorporation. After being blown dry with N_2, all films were stored dry in a refrigerator at ~4 °C prior to use in the assay protocol.

Infrared and UV−Vis Spectroscopic Analyses. Fourier transform infrared-external reflectance spectroscopy (FT-IR-ERS) measurements were made using a Bio-Rad FTS-40 spectrometer equipped with a Harrick Scientific Seagull reflection accessory (Harrick Instruments Inc., Ossining, NY) and a liquid N_2-cooled, narrow-band MCT detector. Spectra were obtained using *p*-polarized light at an 85° angle of incidence with respect to the substrate normal. Spectra were measured at 4-cm^{-1} resolution using between 100 and 256 scans.[40] The FT-IR-ERS spectra of deprotonated PAA films were obtained by immersing the films in a pH 9.9 buffer solution (0.01 M Na_2CO_3, 0.01 M $NaHCO_3$) for 20 min, rinsing with ethanol, and then drying under flowing N_2. The PAA$^-$ films were reprotonated by immersion of the film in a pH 1.7 buffer solution (0.01 M Na_2SO_4 + 0.02 M HCl) for 20 min, rinsing with ethanol, and drying under flowing N_2. A UV−vis spectrophotometer (model 420, Spectral Instruments, Inc., Tucson, AZ) was used to analyze the enzymatic activity and total protein assay results.

Ellipsometry. Ellipsometric measurements were performed using a Gaertner model L2W26D ellipsometer (Gaertner Scientific Corp., Chicago, IL) employing either a 488.0-nm Ar$^+$ laser or a 633.0-nm He/Ne laser and a 70.00 ± 0.02° angle of incidence. Refractive indexes (n_f) and film thicknesses were calculated by assuming a homogeneous film model using Gaertner software. To calculate the thickness of dry PAA films, a refractive index of 1.54, determined experimentally by fitting ellipsometric data obtained from thicker (>30 nm) PAA films, was used.[2] The optical properties of the 3-PAA$^-$/GOx$^+$ salt films were comparable to previously described 3-PAA$^-$/PAMAM$^+$−dendrimer composite salt films.[1] For example, the refractive indexes of these films were 1.56 and 1.58, respectively, and the thicknesses were both ~65 nm (both GOx and the G-4 PAMAM dendrimers are ~4.5 nm in diameter).[41,42] Ex situ thickness measurements of all dry polyelectrolyte-intercalated 3-PAA$^-$ salt films were compared with those of a 3-PAA$^-$ film dipped in a pH 9.9 buffer solution for 20 min, rinsed with water, and then dried. In situ ellipsometry was performed using a trapezoidal prism-shaped cell, having glass windows oriented perpendicular to the incident laser beam. The refractive index of the 0.1 M buffer solutions was measured to be 1.338 and the refractive index of pure water at 488.0 nm is 1.337.[43,44] For the in situ ellipsometric measurements, it was possible to measure both the refractive indexes and thicknesses of the films.[1]

The uncertainty of the in situ film thickness measurements is ±3 nm while the uncertainty in the film refractive index is ±0.01.[1] These errors are larger than those for typical measurements made in air because of the similarity of the refractive indexes of the buffer solutions and the swollen films. For all ellipsometric measurements, it was assumed that refractive indexes were uniform throughout the films.[1]

Enzyme and Total Protein Assays. Total protein assays of the GOx$^+$ and HRP$^+$ solutions were conducted according to the Bradford method.[45] Microcentrifuge tubes were filled with 785 μL of water, 195 μL of dye reagent, and 19.5 μL of sample. These solutions were mixed and allowed to incubate for 20 min, and then their absorbance was measured at a wavelength of 595 nm. The absorbance of the solution containing GOx$^+$ was compared to a calibration curve prepared by measuring the absorbance of GOx$^+$ solutions of known concentrations.

Enzymatic activity determinations were made using assays based on published methods.[46] Typical aqueous assay solutions consisted of 24 mL of 0.02 mg/mL *o*-dianisidine, 80 μL of a 2 M glucose solution, and 240 μg of HRP$^+$. To generate a calibration curve, 1.0 μL of a GOx$^+$ solution (of known protein concentration and activity, ranging from 0.05 to 2.0 M) was added to the assay solution, and the evolution of colored assay product was monitored over the course of 3 min at a wavelength of 460 nm. Activity levels of the enzyme-modified wafers were determined by plotting the absorbance values for solutions containing the wafers against the calibration curve. Those wafers that were stored under dry conditions were immersed in a PBS solution for 5 min before the assay. Wafers that were to be stored under dry conditions were washed with PBS, allowed to air-dry, and then refrigerated after assaying. Wafers stored under wet conditions were washed with PBS after the assay and then returned to their original PBS solution in the refrigerator. Enzyme activity assays for m-HRP$^+$ and d-HRP$^+$were conducted using a published protocol, with slight modifications.[47]

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Quartz Crystal-Based Microgravimetry. Quartz crystal-based microgravimetry (QCM) was performed using 10-MHz, Au-coated, AT-cut quartz crystals (International Crystals, Oklahoma City, OK) operated as thickness shear mode resonators (TSMRs). The frequency response was measured with a frequency counter (Hewlett-Packard HP8204A, Corvallis, OR), and data were recorded using LabView software (National Instruments, Austin, TX). The Au electrode surfaces of the quartz oscillators were modified with 3-PAA films as described earlier. The oscillator frequency was allowed to stabilize and was then measured. The films were then exposed to a buffered solution containing GOx^+ for 20 min, removed and rinsed with water, and finally dried with N_2. The oscillators were again allowed to stabilize and the frequency was measured. The difference in frequency was then converted to a mass/area change in the film using the Sauerbrey equation.[48] We assumed that the entire frequency change was attributable to mass change and that viscoelastic effects were negligible.[49] Control experiments performed with a $3\text{-}PAA^-$ film and a solution of unmodified GOx resulted in no measurable mass change. All of the TSMR experiments were conducted on deprotonated 3-PAA films to account for the mass of Na^+ ions, which would be present in all of the intercalated films but not in the protonated films.

Labeling of GOx^+ with BODIPY 530/550 SE. GOx^+ was labeled with BODIPY 530/550 SE using an established protocol.[50] Immediately before labeling, 1 mg of BODIPY 530/550 SE was dissolved in 100 μL of dimethyl sulfoxide (DMSO). This solution was added to a 400-μL solution of GOx^+, and the mixture was mixed vigorously for 1 h at 25 °C. Finally, dialysis was carried out for 24 h using a Slide-A-Lyzer dialysis cassette suspended in 500 mL of 0.1 M PBS (pH 7.2). A Bradford assay, described earlier, was used to determine the concentration of the BODIPY 530/550 SE-labeled GOx^+ (4.0 μg/μL).

Fluorescence Assay. The BODIPY 530/550 SE-labeled GOx^+-entrapped $3\text{-}PAA^-$ films were prepared as described earlier for $3\text{-}PAA/GOx^+$ films, but using fluorescently labeled GOx^+. A standard fluorescence curve for BODIPY 530/550 SE-labeled GOx^+ was constructed using concentrations ranging from 0.5 to 10 μg/mL at pH 1.7 (QuantaMaster Fluorescence System, QM-1, Photon Technology International). A 250-W xenon arc lamp was used to excite the samples at 530 nm. Fluorescence emission was measured at 90° between 540 and 600 nm using a photomultiplier tube (PMT) equipped with a TE chiller (R928, Products for Research, Inc., Danvers, MA). The data were analyzed using FeliX software (Photon Technology International, South Brunswick, NJ). The BODIPY 530/550 SE-labeled GOx^+ was removed from the $3\text{-}PAA^-$ film by soaking the film in a pH 1.7 buffer for 30 min. The 3-PAA film was then removed from the solution and the fluorescence emission of the solution was compared with the calibration curve.

RESULTS AND DISCUSSION

Entrapment of GOx^+ (Scheme 1) was confirmed using FT-IR-ERS, ellipsometry, and microgravimetry. Figure 1 compares the spectra of "dry" protonated, deprotonated, and enzyme-entrapped 3-PAA films. Part a of Figure 1 is the FT-IR-ERS spectrum of a protonated 3-PAA film. The dominant features are an acid carbonyl band at 1740 cm^{-1}, an $\alpha\text{-}CH_2$ band at 1450 cm^{-1}, and a C−O stretching band at 1265 cm^{-1}. Deprotonation of the 3-PAA film in a pH 9.9 buffer solution, followed by washing with ethanol and drying under N_2, results in the disappearance of the acid carbonyl band and the appearance of asymmetric and symmetric carboxylate peaks at 1600 and 1415 cm^{-1}, respectively (part b of Figure 1). Enzyme modification of the 3-PAA film was accomplished by immersion of a 3-PAA film in 5 mL of a pH 7.2 buffer solution containing 2.7 nmol of GOx^+. After intercalation of GOx^+ into the $3\text{-}PAA^-$ film, two new bands are evident in the IR spectrum at 1660 and 1565 cm^{-1}. The 1660-cm^{-1} band arises from amide bonds within the enzyme, and the band at 1565 cm^{-1} is a composite of the amide II and the asymmetric carboxylate bands, as well as asymmetric and symmetric N−H stretching modes associated with the protonated primary and secondary amine groups of pentaethylenehexamine-modified GOx (part c of Figure 1).[51] The IR evidence for intercalation of $m\text{-}HRP^+$ and $d\text{-}HRP^+$ into PAA^- is very similar to the data shown in part c of Figure 1.

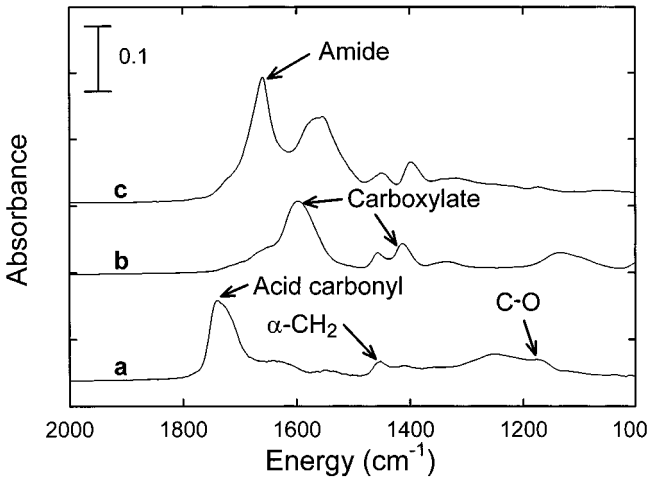

Figure 1. FT-IR-ERS spectra of (a) a protonated 3-PAA film, (b) a $3\text{-}PAA^-$ film, and (c) a $3\text{-}PAA^-/GOx^+$ composite film.

Immersion of a 21-nm-thick, protonated 3-PAA film into a pH 9.9 buffer solution for 20 min followed by rinsing with ethanol and drying under N_2 results in a thickness increase of 10 nm due to the formation of carboxylate groups within the deprotonated film.[1] In contrast, when a 3-PAA film is exposed to a pH 7.2 buffer solution containing GOx^+, rinsed with water, and then dried under N_2, a thickness increases of ~36 nm results. Similar thickness increases occur when $m\text{-}HRP^+$ and $d\text{-}HRP^+$ are intercalated into PAA^- films (Table 1). The 32−41 nm thickness difference between the $3\text{-}PAA^-$/enzyme and $3\text{-}PAA^-/Na^+$ composite films is a consequence of the difference in size of Na^+ compared to the bulky sorbed polycationic enzymes. We have observed thickness increases of similar magnitude when polycationic G4 PAMAM dendrimers are used as counterions in $3\text{-}PAA^-$ salt films.[1] Since both G4 PAMAM dendrimers and GOx^+ are globular macromolecules with approximate diameters of 4.5 and 5.1 nm, respectively, it is not surprising that they yield composite $3\text{-}PAA^-$ films of similar thickness.

Entrapped GOx^+ is not released from the PAA^- films even after several water or ethanol rinses because of the multiple electrostatic

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Table 1. Ellipsometrically Measured Thicknesses of 3-PAA and 3-PAA$^-$/Enzyme Composite Films

	thickness (nm)			average thickness (nm)
	film 1	film 2	film 3	
3-PAA (dry, pH 1.7)	21	22	20	21
3-PAA$^-$ (dry, pH 9.9)	30	31	31	31
3-PAA$^-$ (wet, pH 7.2)[a]	123	122	123	123
3-PAA$^-$/GOx$^+$ (wet, pH 7.2)[a]	135	136	139	137
3-PAA$^-$/GOx$^+$ (dry, pH 7.2)	68	67	66	67
3-PAA/GOx$^+$ (dry, pH 1.7)	23	26	21	24
3-PAA$^-$/m-HRP$^+$ (dry, pH 6.6) (av 3-PAA thickness 26 nm)	63	63	64	64
3-PAA$^-$/d-HRP$^+$ (dry, pH 7.2) (av 3-PAA thickness 27 nm)	72	71	73	72

[a] This thickness measurement was made in situ. See Experimental Section for details.

interactions between its protonated amines and the carboxylate groups of the PAA$^-$ film. However, ellipsometry and FT-IR-ERS confirm that the polycationic enzyme can be removed by soaking the 3-PAA$^-$/GOx$^+$ composite salt film in pH 1.7 buffer for 30 min (Scheme 1 and Table 1).

If the 36-nm difference in thickness between the 3-PAA$^-$/GOx$^+$ and 3-PAA$^-$/Na$^+$ films results solely from enzyme entrapment, then the amount of GOx$^+$ within the film is estimated to be 0.75 unit/cm^2 (1.2×10^{-11} mol/cm^2). This value is calculated using the molecular weight (186 000) and the approximate number density of the enzyme, 1.7×10^{-12} mol/cm^2 (which corresponds to a monolayer of enzyme, determined using a cross-sectional area of 100 nm^2/enzyme), and the assumption that the enzyme is hexagonal close-packed within the film.[42,52] Of course the nature of enzyme packing is unknown, so this assumption simply sets an upper limit on the magnitude of the entrapped mass of enzyme permeated throughout the film.

When the quantity of enzyme in the films is determined on the basis of TSMR-based microgravimetry, the sorbed mass is found to be somewhat smaller than the ellipsometrically determined value (0.41 and 0.75 unit/cm^2, respectively). A standard UV−vis spectroscopic assay of enzyme activity indicates that ~0.06 unit/cm^2 is present within the 3-PAA film. It was also possible to estimate the amount of GOx$^+$ immobilized within the 3-PAA$^-$ films by labeling the enzyme with the fluorescent dye BODIPY 530/550. In this experiment, the dye-labeled GOx$^+$ was entrapped within a 3-PAA$^-$ film and released from the film within a fluorescence cuvette, and then the fluorescence was determined and correlated to the enzyme concentration using a calibration curve. The amount of GOx$^+$ detected in this case was 0.27 unit/cm^2, which is slightly lower than the TMSR data but higher than the assay data. These four independent estimates for the amount of entrapped enzyme are in reasonable agreement given the possibility of enzyme activity loss upon immobilization and the assumptions and limitations inherent to the TSMR and ellipsometric thickness measurements, which are discussed in the Experimental Section. In any case, comparison of the assay data with the TMSR or ellipsometric thickness estimates indicate some loss of enzymatic activity after GOx$^+$ is immobilized in the 3-PAA$^-$ film. The important point is that electrostatic immobilization of

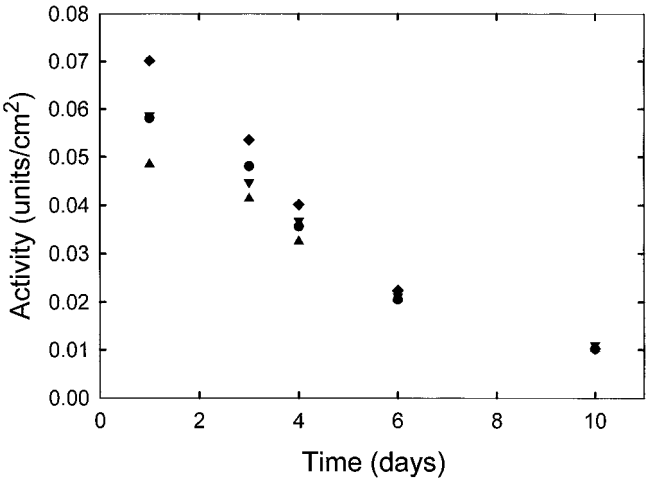

Figure 2. Plot of GOx$^+$ activity (units/cm^2) in PAA$^-$/GOx$^+$ composite films vs time. Data shown are illustrative of four separate 3-PAA$^-$/GOx$^+$ films (◆, ▼, ▲, ●) that were stored at 4 °C in PBS between assays. These enzyme-intercalated films lose enzyme activity as a consequence of enzyme turnover during the assays. Unassayed films do not lose activity when stored in buffer for up to 68 days.

GOx$^+$ within the hyperbranched film results in a significant loading of active enzyme within the ultrathin 3-PAA$^-$ film. The activities of the 3-PAA$^-$/m-HRP$^+$ and 3-PAA$^-$/d-HRP$^+$ composite films are lower than 3-PAA$^-$/GOx$^+$ films: 1.6×10^{-3} and 1.7×10^{-3} unit/cm^2, respectively.

We showed previously that while polyamines are entrapped and retained within hyperbranched PAA$^-$ films, mono- and diamines are not retained because they lack the necessary polypodal interactions.[1] Similarly, we find that unmodified GOx is not sorbed into the 3-PAA$^-$ films. This result is supported by the absence of measurable enzymatic activity, mass or thickness increase, or change in the FT-IR-ERS spectra of 3-PAA$^-$ films before and after exposure to unmodified GOx. These important control experiments support our contention that GOx$^+$ intercalates into the films and is retained therein through multiple electrostatic interactions.

The activity of the 3-PAA$^-$-entrapped GOx$^+$ as a function of time was tested under two sets of conditions: the wafers were stored at 4 °C either dry or in 0.1 M PBS (pH ~7.4). Since the PAA films are pH responsive and undergo extremes of swelling and contraction, storage conditions play a key role in determining long-term activity. Activity data (Figure 2) show that the initial average enzyme activity of ~0.06 unit/cm^2 decreases gradually over 10 days. This loss in activity is apparently due to enzyme deactivation since TMSR and ellipsometric data (vide supra) indicate no mass or thickness loss corresponding to the activity decrease. Moreover, at the end of the time-dependent assay (Figure 2) the 3-PAA$^-$/GOx$^+$ films were analyzed by FT-IR-ERS, and the data revealed a shift in both the amide I ($\Delta\nu = 8$ cm^{-1}) and II ($\Delta\nu = 13$ cm^{-1}) bands. These shifts may be the result of a conformational change within the enzyme, which could be associated with the loss of enzymatic activity.[53,54] The time-dependent decrease in enzyme activity may also be accelerated

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by the gradual accumulation of assay-associated byproducts in the films. Evidence for this assertion comes from an experiment in which a 3-PAA^-/GOx^+ film was stored in PBS buffer for 68 days at 4 °C and retained its original enzymatic activity of 0.06 unit/cm^2 when assayed on the 69th day. Such long-term shelf life is important for biomedical applications.

SUMMARY AND CONCLUSIONS

In summary, we used in situ and ex situ ellipsometry, FT-IRERS, fluorescence spectroscopy, and TSMR mass measurements to confirm the ionic assembly of GOx^+ within composite hyperbranched 3-PAA^- organic thin films (Scheme 1). The intercalation of m-HRP^+ and d-HRP^+ was also confirmed, which suggests this is a general approach for enzyme immobilization. The key result is that enzymes are present at technologically relevant loadings within the films and that they retain their biological activity after loading. The PAA^-/enzyme composites present key advantages compared to alternative enzyme immobilization strategies. These include the mild intercalation conditions and the controllable thickness and synthetic flexibility of the PAA films. Moreover, removal of GOx^+ from the films only occurs at very low pH. Finally, hyperbranched grafting can be performed on many different substrate materials, including metals and plastics of various geometries, so the immobilization strategy described here should be broadly useful. Though it was not the thrust of these experiments, preliminary results show that these enzyme intercalated-hyperbranched thin films may, in the future, be useful in designing amperometric biosensors.

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