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A stable three-enzyme creatinine biosensor. 1. Impact of structure, function and environment on PEGylated and immobilized sarcosine oxidase

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Abstract

The determination of creatinine levels in biological fluids is an increasingly important clinical requirement. Amperometric biosensors have been developed based on a three-enzyme system which converts creatinine to amperometrically measurable hydrogen peroxide. The development of the amperometric creatinine biosensor has been slow due the complexity of the three-enzyme system. This paper, the first of three, discusses the chemical modification of sarcosine oxidase and the immobilization and stabilization of this enzyme using polyurethane prepolymers. Sarcosine oxidase was completely inactivated after modification using poly(ethylene glycol) activated with isocyanate. The addition of a competitive inhibitor during enzyme modification was effective in protecting the enzyme from inactivation. Computational analysis of the structure of sarcosine oxidase suggests that there is a lysine in the active site that may be hyper-reactive. The enzyme was irreversibly immobilized using polyurethane prepolymers and retained significant activity. The enzyme's half-life at 37 °C increased from seven days to more than 50 days after immobilization.

1. Introduction

The utility of enzymes in biosensors is limited by their stability. Clinical blood analyzers require enzymes to be used repeatedly while in contact with whole blood [1,2]. Many blood analyzers operate at 37 °C which further limits enzyme stability [3]. A variety of immobilization procedures are described in the literature for use with biosensors. Although most of the procedures described suggest utility for biosensors, they are often not tested under conditions that would be applicable under real-life conditions, such as room temperature or at 37 °C while in contact with fluid. Enzyme immobilization is especially critical for continuous use biosensors where enzyme leaching can be a concern. Enzyme immobilization into a "biopolymer" by multipoint covalent attachment affords a straightforward and convenient method for preparation of immobilized enzymes for biosensors. Not only does multipoint covalent immobilization prevent enzyme leaching, but it also increases enzyme stability to heat, pH, organic solvents, peroxides and proteolytic and microbial degradation [4,5].

We have previously described the immobilization of enzymes into polyurethane materials [6–8]. In this series of papers we extend this work, describing the use of polyurethane prepolymers for the preparation of an enzyme-containing membrane for multiple use

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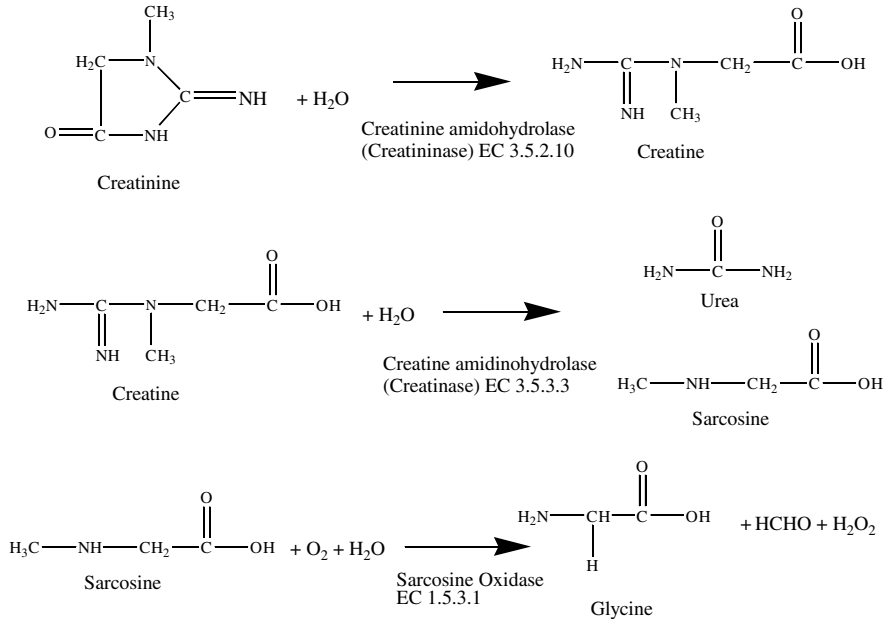

Scheme 1. Three-enzyme creatinine biosensor.

amperometric creatinine biosensors. A critical step in the development of biosensors is effective enzyme immobilization. Incorporation into a polyurethane polymer network through multipoint attachment is a rapid and effective general strategy for enhancing the stability of enzymes, while retaining activity [9]. This strategy involves the production of bioplastics in a single step, employing oligomers capable of chemical reaction with specific functionalities on the enzyme surface. The covalent binding provides protein retention in the matrix so that the advantages of immobilization can be maximized.

Determination of the amount of creatinine in biological fluids is an increasingly important clinical measurement used in the evaluation of renal function and muscle damage. There has been considerable interest in the design of biosensors specific for creatinine. Amperometric biosensors have been developed based on a three-enzyme system which converts creatinine to amperometrically measurable hydrogen peroxide (H_2O_2) as described in Scheme 1 [10]. Due to the complexity of the three-enzyme system, development of these biosensors has been slow [11].

In this report, we discuss the chemical modification and immobilization of sarcosine oxidase into polyurethane polymers. Sarcosine oxidase (EC 1.5.3.1) catalyzes the oxidative demethylation of sarcosine (*N*-methylglycine) and forms equimolar amounts of formaldehyde, glycine, and hydrogen peroxide. Sarcosine oxidase from the *Arthrobacter* sp. is a monomer with a molecular weight of 43 kDa [12]. The monomeric sarcosine oxidases (MSOX) are flavine proteins that contain 1 mol of flavine adenine dinucleotide (FAD) that is covalently linked to the enzyme by a cysteine residue [13]. Herein we describe the structure–function–environment relationships that are required to maintain sarcosine oxidase in a polymeric sensor environment. The reaction catalyzed by sarcosine oxidase is shown below.

2. Materials and methods

2.1. Materials

Sarcosine oxidase (from *Arthrobacter* sp., SAO-341) was purchased from Toyobo Co., Ltd. Horseradish peroxidase was purchase from Sigma–Aldrich (St. Louis, MO). All enzymes were used without further purification. Poly(ethylene glycol) activated with isocyanate (mPEG–NCO) (M_w 5000) and succinimidyl propionic acid (mPEG–SPA) (M_w 5000) were obtained from Shearwater Polymers Inc. (Huntsville, AL). Hypol 2060G prepolymer, a toluene diisocyanate based prepolymer [14], was purchased from Hampshire Chemical (Lexington, MA). All other reagents were purchased from Sigma–Aldrich Chemicals (St. Louis, MO) and were of the highest purity available.

2.2. PEGylation of sarcosine oxidase

Sarcosine oxidase was dissolved in an aqueous buffer (50 mM phosphate buffer, pH 7.5 or 50 mM borate buffer, pH 8.5) at a concentration of 1 mg/mL. PEG–NCO or PEG–SPA was added in excess to the enzyme at a molar ratio of 1:100 to ensure complete enzyme modification. In some experiments sarcosine oxidase inhibitors

(50 mM methylthioacetic acid or 50 mM pyrrole-2-carboxylic acid) were added to help prevent inactivation of the enzyme. The reaction mixture was mixed for 30 min followed by dialysis (12,000 M_w cutoff) against 50 mM phosphate buffer [15].

2.3. Synthesis of sarcosine oxidase-containing polyurethane

Hypol prepolymer 2060G (0.4 g) was added to a buffered solution (3.6 g of 50 mM phosphate buffer, 50 mM inhibitor, pH 7.5) containing sarcosine oxidase (0–200 units enzyme per gram of prepolymer). The aqueous polymer solution was vigorously mixed for 30 s in a weigh boat until the onset of gelation. Polymerization was very rapid and gelation usually took place within one minute.

2.4. Characterization of enzyme modification

MALDI-MS analyses were performed using a Perspective Biosystems Voyager Elite MALDI-TOF. The acceleration voltage was set to 20 kV in a linear mode. One microliter of PEGylated enzyme solution (0.1 mg/mL) was mixed with 1 μL of matrix solution (0.5 mL water, 0.5 mL of acetonitrile, 1 μL of trifluoroacetic acid, and 10 mg of sinapinic acid) and then spotted on the target plate. Spectra were recorded after evaporation of the solvent mixture and were calibrated externally with equine cytochrome *C* (12,361.96 Da (ave.)), rabbit muscle aldolase (39,212.28 Da (ave.)) and bovine serum albumin (66,430.09 Da (ave.)).

2.5. Measurement of sarcosine oxidase activity using an end point assay

The production of hydrogen peroxide was measured by using the 4-aminoantipyrene–peroxidase system [16]. Typically, an enzyme solution (0.05 mL) was incubated with a mixture (1.0 mL total) of 95 mM sarcosine, 0.47 mM 4-aminoantipyrene, 2.0 mM phenol, 0.045% Triton X-100, 50 mM sodium phosphate (pH 8.0) and 5 units/mL of horseradish peroxidase at 37 °C for 10 min. The reaction was terminated by addition of 2.0 mL of 0.25% sodium dodecyl sulfate (SDS) solution and the absorbance at 500 nm was measured. One unit was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol of substrate per minute.

2.6. Measurement of sarcosine oxidase activity using an oxygen monitor assay

The initial rate of oxygen consumption was also measured at 37 °C with a Clark oxygen electrode from Yellow Springs Instruments (Yellow Springs, OH). The reaction was initiated by adding an enzyme solution (1 μL) or an enzyme-containing polymer (10–100 mg cut into small pieces) to 5.0 mL of substrate (50 mM sarcosine in 50 mM phosphate buffer, pH 7.5). Before measurement, the assay solution was allowed to equilibrate to 37 °C with air. Oxygen consumption was measured for 5–10 min.

2.7. Thermostability of sarcosine oxidase

Sarcosine oxidase was added to a buffered medium (50 mM sodium phosphate, 2 mM EDTA, pH 7.5). The native enzyme concentration used was 0.06 mg/mL. The activity of sarcosine oxidase was followed over time at room temperature (22 °C), and at 4 °C and 37 °C using the end point assay described above.

Thermoinactivation of PEG–sarcosine oxidase was monitored at 37 °C in buffer (50 mM sodium phosphate, 2 mM EDTA, pH 7.5) as described for the native enzyme. The enzyme concentration in all samples was adjusted to 0.05 mg/mL.

Enzyme polymer samples were cut into small pieces and added to buffer (50 mM phosphate, pH 7.5) and incubated at 37 °C. Samples were removed over time and assayed for enzymatic activity using the oxygen electrode.

2.8. Inhibition of sarcosine oxidase by silver ions

To determine the effect of silver ions on sarcosine oxidase, 0.07 mg/mL of sarcosine oxidase were incubated in 20 mM Tris–HCl (pH 7.5) with silver nitrate (0–1 mM) at room temperature. Samples were removed periodically and assayed using the end point assay for sarcosine oxidase activity.

2.9. Computational analysis of sarcosine oxidase structure to predict lysine reactivity

2.9.1. Homology modeling

An energy-minimized structural model for the monomeric sarcosine oxidase (MSOX) of the strain TE1826 from *Arthrobacter* sp. [12] was obtained from a comparative modeling approach [17]. The template was chosen from the closest homologous enzyme of known structure, MSOX from *Bacillus* sp. (Protein data bank (PDB) code:1EL5), which has a sequence identity of 83% [18]. The Molecular Operating Environment package (MOE) was used for this modeling work. It utilizes a segment-matching procedure [19] together with modeling of insertions and deletions [20]. The insertions were modeled from available structural motifs for generating the possible conformations of groups of residues followed by a standard energy minimization approach. Amber 94 force field was used in this process [21,22].

2.9.2. Prediction of solvent accessibility

The solvent accessibility of individual residues was calculated in Swiss-PdbViewer [23]. The relative value is shown in percentage scale. One hundred percent solvent accessibility for a single residue X is achieved when the residue X has the same exposed surface area as that when it is presented in a pentapeptide form, flanked by two glycines on each side (GGXGG) in the solution. When residue X is totally surrounded by its neighboring residues, its relative solvent accessibility is 0%.

2.9.3. Prediction of lysine pK_a values

Some amino acids in a protein are titratable residues which exchange protons with their environment. These residues (His, Lys, Arg, Asp, Glu, Tyr and Ser) possess pK_as that can be influenced by their environments. The charged states of these residues have a direct impact on protein function. UHBD (University of Houston Brownian Dynamics), is a software package capable of solving the linearized and non-linear Poisson–Boltzmann equation using a finite-difference method [24,25]. The program calculates the electrostatic free energy differences for ionization of a given site in the model amino acid and in the protein for a given residue. For N ionizable residues, there will be $2N$ possible ionization states. The average pK_a is obtained over all states with probabilities based on Boltzmann weighting factor [26].

3. Results and discussion

3.1. Effect of PEGylation on enzyme activity and stability

Since the immobilization of enzymes into polyurethane polymers involves isocyanate reacting with nucleophilic residues on the enzyme surface it is convenient to model the process of chemical modification using soluble polymers. Modification using this technique allows us to mimic the effect of covalent immobilization on enzyme activity while still working with a soluble enzyme. Once the enzyme is incorporated into a polymer, mass transfer effects can complicate the analysis.

Monomeric sarcosine oxidase was modified using PEG–NCO (5000 M_w) at pH 7.5 (50 mM phosphate buffer) and pH 8.5 (50 mM borate buffer) using different isocyanate to enzyme ratios. The amount of native enzyme remaining was quantified using MALDI-TOF analysis and plotted versus the activity retention of sarcosine oxidase following modification (Fig. 1). When the enzyme was modified at pH 7.5, the percentage of activity retained was proportional to the percentage of unmodified enzyme. This suggests that a critical residue from the perspective of the enzyme mechanism (or group of residues) dominates the reaction with the isocyanate. However, modification at pH 8.5 led to increased activity retention probably due to the increased nucleophilicity of the ε-amines of non-critical lysines with increased pH.

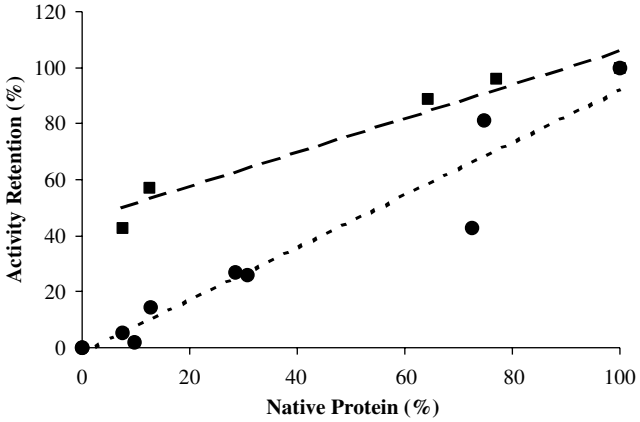

Fig. 1. Activity retention of PEGylated MSOX modified using PEG–NCO at pH 7.5 (closed circles) and pH 8.5 (closed squares).

Since a single modification by isocyanate appeared to deactivate sarcosine oxidase, a method will be required to protect the enzyme during immobilization within polyurethane polymers. If modification is occurring at or near an active site residue, an inhibitor may be added to solution to help increase activity retention by blocking the active site cleft. Although this method of protection is often suggested, the literature contains precious few examples of where inhibitor protection strategies work effectively [27,28]. A number of inhibitors have been identified for sarcosine oxidase [29]. Pyrrole-2-carboxylic acid (K_i = 1.37 mM) and (methylthio)acetic acid (K_i = 2.60 mM) were selected since they had low K_i values and do not contain functional groups that are highly reactive with isocyanate. The native substrate, sarcosine (K_M = 0.6), was not used as a protecting agent since the catalytic oxidation product, hydrogen peroxide, can inactivate the enzyme and may also oxidize the PEG backbone. Modification of sarcosine oxidase at pH 7.5 showed considerably improved activity retention compared to modification without inhibitors (Fig. 2). MALDI-TOF analysis confirmed that the majority of the enzyme was modified with PEG and that the presence of the inhibitor had minimal effect on the modification process itself.

The stabilities of native and PEG-modified sarcosine oxidases were measured at 37 °C in 50 mM phosphate buffer (Fig. 3). Enzyme with an average of one PEG chain attached per molecule of enzyme had a stability similar to native enzyme (half-life of 7 days). Enzyme with an average of three PEG chains attached per molecule of enzyme had an improved half-life (17 days).

3.2. Understanding the nature of chemical modification

Isocyanates are capable of reacting with amino, sulfhydryl, carboxyl, phenolic hydroxyl, imidazole, and

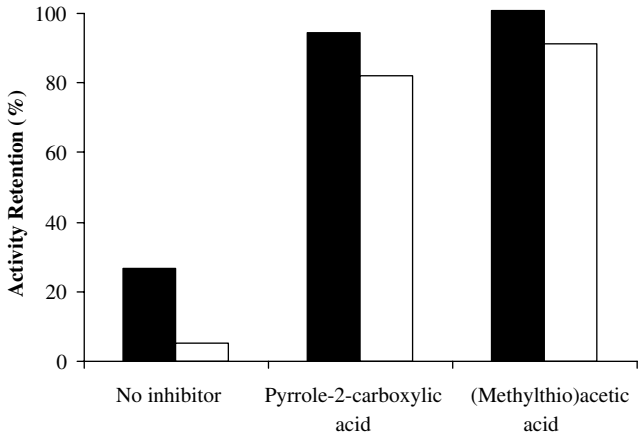

Fig. 2. Activity retention of MSOX after PEGylation in the presence of inhibitors (10:1 (solid) and 20:1 (white) isocyanate to amine ratio). The inhibitor concentration was 50 mM.

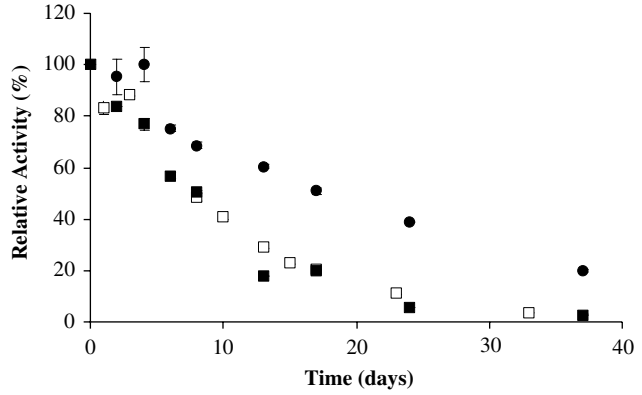

Fig. 3. Stability of sarcosine oxidase as a function of number of PEGs attached at 37 °C. Native enzyme (open squares); one PEG attached (closed squares); three PEGs attached (closed circles).

phosphate groups in proteins [30,31]. Reactions with sulfhydryl, imidazole, tyrosyl, and carboxyl groups give relatively unstable adducts that may decompose upon dilution or change in pH [32]. Due to the high nucleophilicity of amino groups, these are the most easily modified groups that form a stable adduct. Since hydroxyl and phenolic groups generally have nucleophilicities very similar to that of water, they are not typically easily modified.

Inactivation by cyanate and isocyanate has been reported for a number of enzymes including pepsin [33], papain [34], trypsin and chymotrypsin [35,36], and glutathione reductase [37]. The proteolytic activity of pepsin was inhibited when tyrosine residues were carbamylated by potassium cyanate; however treatment with hydroxylamine was effective in reversing the inactivation by decarbamylating the residues [33]. Papain is also inactivated by cyanate; in fact, the active site thiol of papain is about 3000 times more reactive with cyanate than the thiol group of free cysteine [34]. The inactivation is reversible upon dilution, due to the lability of the carbamylated sulfhydryl group. Chymotrypsin was also shown to be inactivated by cyanate [35]. Modification of the active site serine is the cause of the inactivation and similar effects have been reported for trypsin and subtilisin [35].

In order to determine if sarcosine oxidase modified with isocyanate was modified at a residue giving an unstable adduct (a modified sulfhydryl or tyrosyl residue for example), the enzyme was "re-activated" in a number of ways. Neither dilution nor dialysis overnight at pH 7.5 was successful in reactivating the enzyme. Treatment with hydroxylamine is a method frequently used to remove weakly bound conjugates from enzymes [38]. Treatment of isocyanate-modified sarcosine oxidase with hydroxylamine (500 mM, pH 7) for up to 24 h was ineffective in reviving the enzyme. Thus, we can reasonably presume that the PEG–isocyanate has reacted with an amine on the surface of the protein and thereby inactivated the enzyme.

In order to determine if inactivation was specific for isocyanate, or whether other amine-modifying PEGs would have a similar effect, sarcosine oxidase was modified using PEG–SPA. PEG–SPA is an NHS-modified PEG that forms stable amide linkages with amines [32]. Interestingly, low degrees of modification (average of 1 to 3 PEGs per enzyme) of sarcosine oxidase with PEG–SPA caused a loss of less than 5% activity. At higher degrees of modification (>5 PEG chains attached) more significant loss of enzyme activity was apparent which could be prevented using the inhibitors pyrrole-2-carboxylic and (methylthio)acetic acid (data not shown). This suggests that the reactivity of PEG–SPA with residues on the surface of sarcosine oxidase is different than the reactivity of PEG–NCO with sarcosine oxidase.

Since modification of sarcosine oxidase at pH 7.5 caused a greater inactivation than at pH 8.5, it seems likely that the modification of the terminal amine may be responsible for the loss of activity (the terminal amine has a lower pK_a than the ε-amine of lysine). In fact, modification of sarcosine oxidase by PEG–NCO caused no noticable shift in the visible absorption spectrum of sarcosine oxidase (data not shown) suggesting that modification did not occur in the core of the protein. The absorption spectrum of the enzyme-bound FAD of *Corynebacterium* sarcosine oxidase has been reported to change (red shift of absorption peak from 455 to 462 nm) upon modification of a histidine residue with diethylpyrocarbonate (DEP), probably in the vicinity of the flavin moiety [39]. DEP-modified sarcosine oxidase is completely inactive; however, activity can be recovered by treatment with hydroxylamine. Although the absorption spectrum of *Arthrobacter* sarcosine oxidase did not shift upon modification with PEG–NCO, this is not absolute evidence that modification did not

occur in the active site. Since the terminal amine is located 44 Å from the nearest catalytic residue (Gly 344), it seems unlikely that the modification of this residue is responsible for the complete inactivation of the enzyme.

3.3. Prediction of chemical modification site

In order to better understand the effect of chemical modification on sarcosine oxidase activity, computational studies were performed to predict the most important residues for enzyme activity/stability and the most reactive residues for modification.

Glocker et al. have measured the reactivity of lysine residues in RNAse A as a function of the solvent accessibility (SA) of a residue and the pK_a value of lysines [40]. Similar data have been collected for deoxy-hemoglobin [41] and horseradish peroxidase [42]. Specifically, reactivity increases with increasing SA. However, even with low SA, the reactivity increases dramatically when the pK_a of a given lysine residue is especially low (signifying a very good nucleophile). Hence, lysine residues located on the surface of the enzyme will have a reactivity that is proportional to SA. However, residues located in an environment which may have a significant effect on pK_a, like the active site lysine of bovine ribonuclease A, will have a significantly different pK_a and altered reactivity.

The predicted pK_as for all lysine residues in sarcosine oxidase (FAD free-MSOX) are shown in Table 1. The SA was calculated from SWISS PDB viewer and estimated pK_a was obtained from the UHBD algorithm. Based on these results, we believe it is likely that lysine 351, an active site residue, may be the most reactive lysine since the pK_a of this residue is 9.18. Lysine 268 and 322 are within 6 Å of the active site. Lysine 268 has the second lowest pK_a (9.68) and is probably the second most reactive residue. Lysine 322 has a significantly increased pK_a (13.67) and is not likely to be easily modified.

Table 1
Solvent accessibility and pK_a predicted for the terminal amine and the lysine residues in sarcosine oxidase

Residue number	Solvent accessibility	pK_a
MET 1	83.40	7.39
LYS 4	43.22	10.66
LYS 5	29.67	11.30
LYS 27	30.40	12.22
LYS 31	31.87	11.51
LYS 80	58.97	11.38
LYS 85	30.04	11.79
LYS 89	34.07	10.72
LYS 98	37.36	11.34
LYS 112	54.95	11.22
LYS 128	56.04	10.80
LYS 145	36.63	11.07
LYS 169	31.14	11.36
LYS 187	20.88	10.69
LYS 199	19.05	11.88
LYS 210	29.30	13.04
LYS 214	35.53	12.09
LYS 236	57.51	10.61
LYS 237	26.74	11.86
LYS 268	**2.20**	**9.68**
LYS 277	48.72	11.38
LYS 299	46.52	11.23
LYS 313	38.10	12.12
LYS 322	**24.18**	**13.67**
LYS 351	**4.76**	**9.18**
LYS 368	45.42	10.90
LYS 384	45.42	10.86
LYS 386	43.96	11.34

Rows in bold show the residues located in or within 6 Å of the active site of the enzyme.

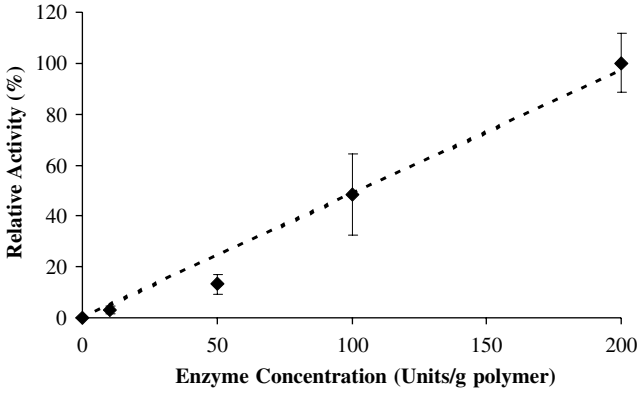

Fig. 4. Relative activity of MSOX containing polyurethanes as a function of enzyme content.

3.4. Immobilization of sarcosine oxidase in polyurethane polymers

After finding the optimal conditions for modification of sarcosine oxidase to preserve enzyme activity during modification with isocyanate, we looked to immobilize the enzyme in polyurethane hydrogels. Enzyme-containing polymers that were prepared with enzyme concentrations of 0–200 U/g of polymer had a specific activity that was directly proportional to enzyme concentration (Fig. 4). Since the reaction rate is proportional to enzyme concentration, this ensures that the rates measured under these conditions are not diffusion limited and represent only the rate from the enzyme-catalyzed reaction [43]. Enzyme–polymers prepared without inhibitor retained no activity verifying that the effects seen with the soluble modifiers translated directly to the polyurethane biopolymer. Enzyme immobilized in polymers prepared with inhibitor retained approximately 10% of their initial activity after rinsing to remove the inhibitor.

The enzyme–polymers were tested for reusability by repeatedly assaying a single gel sample for eight cycles with no loss of apparent activity (Fig. 5). As seen with other polyurethane chemistries, the enzyme was well

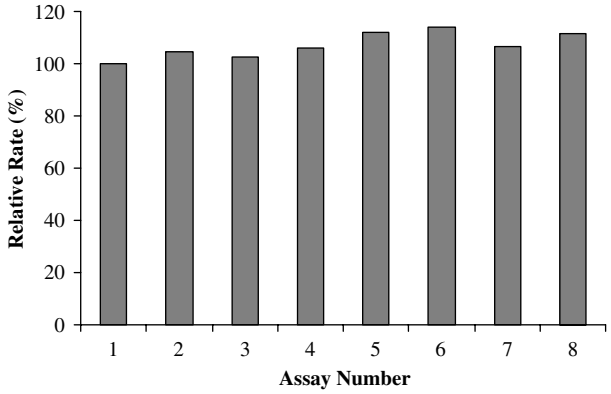

Fig. 5. Reusability of MSOX containing polyurethane hydrogels.

immobilized within the gel and no leaching of enzyme from the polymer to the surrounding solvent was observed [6–8]. Polymers were also prepared and stored in buffer at 4 °C under gentle agitation. Samples were removed from the liquid phase to determine the degree of enzyme leaching from the polymer. No activity was detected in the liquid phase over a nearly 50-day period (Fig. 6). Since the enzyme is irreversibly immobilized in the polyurethane hydrogel material, this immobilization technique will be ideal for use in clinical biosensors.

The thermostability of the enzyme in the polymer was determined by incubating enzyme–polymer samples in buffer at 37 °C and removing samples for assay periodically (Fig. 7). The half-life of native sarcosine oxidase at 37 °C is 7 days whereas immobilized sarcosine oxidase retained >50% activity after incubation at 37 °C in buffer for more than 50 days. Obviously, inhibitor-protected immobilization of sarcosine oxidase in polyurethane polymers is effective in stabilizing the enzyme. High enzymatic stability, especially at elevated temperatures, will be essential for successful application in continuous use clinical blood analyzers.

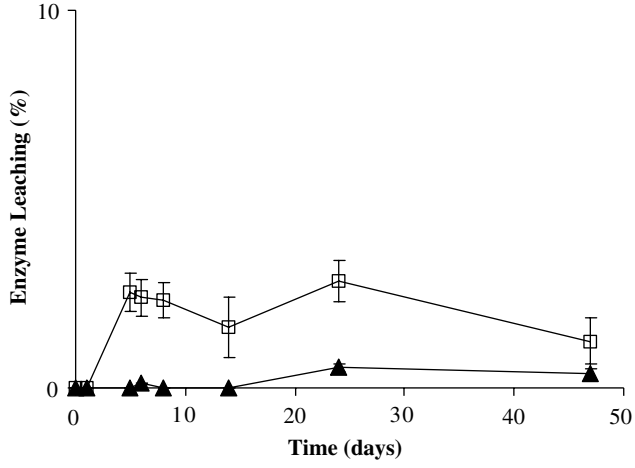

Fig. 6. Leaching (cumulative) of sarcosine oxidase from polyurethane hydrogels. 1 mg MSOX/g polymer (closed triangles); 2 mg MSOX/g polymer (open squares).

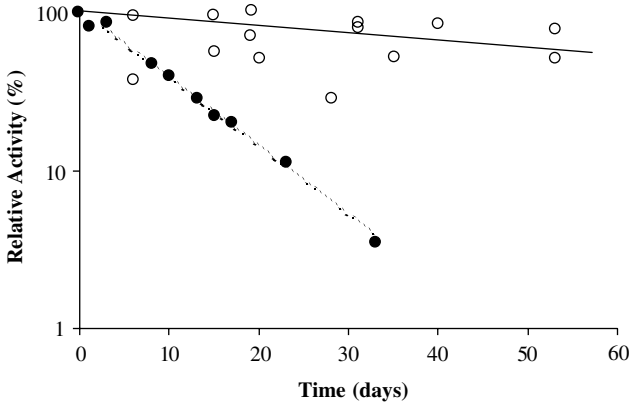

Fig. 7. Stability of MSOX containing polyurethane hydrogels stored in buffer at 37 °C. Rates were normalized to the rate of oxygen consumption after the first day. Native enzyme (closed circles); immobilized enzyme (open circles).

3.5. Effect of silver ions on enzyme activity

Since our goal is to use sarcosine oxidase in conjunction with an amperometric electrode that contains a silver/AgCl reference electrode, the effect of silver ions on enzyme activity must be explored. Silver ions that leach from reference electrodes can bind tightly to an enzyme and lead to inactivation [3,44]. Although no data is available describing the concentration of silver ions generated by silver electrodes, the concentration of silver ions in equilibrium with a saturated solution of silver chloride is approximated 10 μM [45]. The impact of silver ions on sarcosine oxidase activity was determined by adding 1 nM to 1 mM silver nitrate to a solution of enzyme and then assaying in the absence of silver (Fig. 8). Obviously, silver is an effective irreversible inhibitor of sarcosine oxidase. The enzyme inhibition appears to be slow requiring long incubation times to see an effect at low silver concentrations. Although the inhibition is

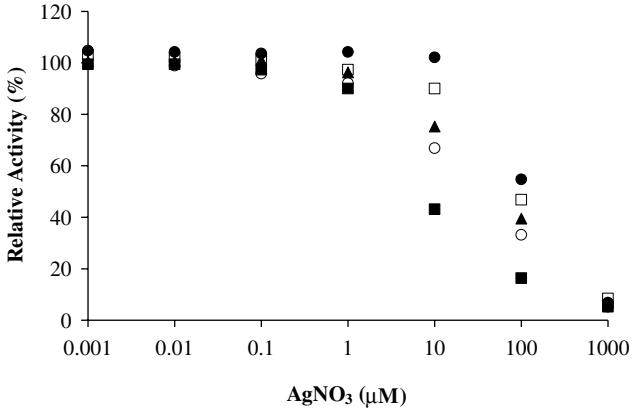

Fig. 8. Silver ion induced irreversible inhibition of sarcosine oxidase activity. Incubation time: 5 min (closed circles); 1 h (open squares); 3 h (closed triangles); 5 h (open circles); 21 h (closed squares).

not instantaneous, since these sensors may be used over a period of weeks, inhibition by silver may drastically shorten sensor half-lives and we will report on strategies to diminish the impact of silver ions on biocatalyst-based sensors in a separate paper.

4. Conclusions

We modeled the immobilization of sarcosine oxidase in polyurethane polymers using PEG–NCO. Sarcosine oxidase was completely inactivated when modified by a single molecule of PEG–NCO. Using a pK_a prediction program, we were able to show that two residues in the active site of sarcosine oxidase are predicted to have decreased pK_a values making them hyper-reactive. Two irreversible inhibitors, (methylthio)acetic acid and pyrrole-2-carboxylic acid, were effective in preventing complete enzyme inactivation during modification. Sarcosine oxidase was successfully and irreversibly immobilized during polymerization by chemical crosslinking into polyurethane hydrogel-forming polymers, retaining approximately 10% activity. Polymers containing sarcosine oxidase were more than sufficiently stable to be used for 30 days in a buffered solution at 37 °C. Sarcosine oxidase is susceptible to inhibition by silver and incubation with micromolar quantities of silver caused enzyme inactivation. Susceptibility to silver ions may be important when using the enzyme in amperometric creatinine biosensors with silver-containing electrodes.

Acknowledgement

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