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ENZYME-ENCAPSULATED SILICA MONOLAYERS FOR RAPID FUNCTIONALIZATION OF A GOLD SURFACE

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We report a simple and rapid method for the deposition of amorphous silica onto a gold surface. The method is based on the ability of lysozyme to mediate the formation of silica nanoparticles. A monolayer of lysozyme is deposited via non-specific binding to gold. The lysozyme then mediates the self-assembled formation of a silica monolayer. The silica formation described herein occurs on a surface plasmon resonance (SPR) gold surface and is characterized by SPR spectroscopy. The silica layer significantly increases the surface area compared to the gold substrate and is directly compatible with a detection system. The maximum surface concentration of lysozyme was found to be a monolayer of 2.6 ng/nm^2 which allowed the deposition of a silica layer of a further 2 ng/mm^2. For additional surface functionalization, the silica was also demonstrated to be a suitable matrix for immobilization of biomolecules. The encapsulation of organophosphate hydrolase (OPH) was demonstrated as a model system. The silica forms at ambient conditions in a reaction that allows the encapsulation of enzymes directly during silica formation. OPH was successfully encapsulated within the silica particl3es and a detection limit for the substrate, paraoxon, using the surface-encapsulated enzyme was found to be 20 μM.

15. SUBJECT TERMS

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Enzyme-encapsulated silica monolayers for rapid functionalization of a gold surface

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Abstract

We report a simple and rapid method for the deposition of amorphous silica onto a gold surface. The method is based on the ability of lysozyme to mediate the formation of silica nanoparticles. A monolayer of lysozyme is deposited via non-specific binding to gold. The lysozyme then mediates the self-assembled formation of a silica monolayer. The silica formation described herein occurs on a surface plasmon resonance (SPR) gold surface and is characterized by SPR spectroscopy. The silica layer significantly increases the surface area compared to the gold substrate and is directly compatible with a detection system. The maximum surface concentration of lysozyme was found to be a monolayer of 2.6 ng/mm^2 which allowed the deposition of a silica layer of a further 2 ng/mm^2. For additional surface functionalization, the silica was also demonstrated to be a suitable matrix for immobilization of biomolecules. The encapsulation of organophosphate hydrolase (OPH) was demonstrated as a model system. The silica forms at ambient conditions in a reaction that allows the encapsulation of enzymes directly during silica formation. OPH was successfully encapsulated within the silica particles and a detection limit for the substrate, paraoxon, using the surface-encapsulated enzyme was found to be 20 μM.

Keywords: Surface plasmon resonance; Silica; Organophosphate hydrolase; Enzyme immobilization; Paraoxon

1. Introduction

Immobilization of enzymes on solid substrates, such as silicon [1,2], polymers [3] and glass [4] is of great interest for a variety of applications including biocatalysis, biosensors and formation of protein arrays for biological screening. Often, the platform is merely an inactive support for the biomolecule. Recent interest however, has advanced to attaching biomolecules directly to a transducer surface to allow *in situ* and real-time detection of enzymatic activity [5,6].

Surface plasmon resonance (SPR) is a versatile analytical method for real-time monitoring of interactions at a solid/liquid transducer surface. SPR uses the principle of total internal reflectance occurring at the interface between materials with differing refractive indices. An evanescent wave penetrates the interface (modified with a thin layer of gold) and couples with surface plasmons (oscillating free electrons). The interaction causes a change in reflectivity and a concurrent change in resonance angle, which correlates to the refractive index (RI) of the adjacent medium. The RI is therefore directly related to changes in surface concentration of interacting ligands. The change in RI is continuously monitored to produce a sensorgram of refractive index unit (RIU) as a function of time [7–9]. SPR has proven to be particularly useful for the analysis of biological systems and can be used for example, to determine kinetic parameters and reaction characteristics [9,10]. SPR has been recently used to study enzymatic reactions on various surfaces and microarrays. Kim et al. [11] for example, performed enzymatic reactions on surface bound substrates and measured adsorbed enzyme concentrations and substrate cleavage rates by the use of combined SPR and surface-plasmon enhanced fluorescence techniques. SPR has also been demonstrated as a method for determining the kinetics of surface enzyme reaction based on Langmuir adsorption and Michaelis–Menten kinetics [12,13]. SPR is adaptable to a wide range of biomolecular reactions as labelling of ligands or receptor molecules are not required. The use of SPR for biological

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systems however, generally requires the development of specific methods to attach biomolecules on the sensor surface and orient the molecules for optimal biological activity. Maintaining an interaction between biomolecules and the SPR waveguide surface generally requires covalent modification, which can change biological function and lower the catalytic activity as the orientation of the enzyme active site is hindered by attachment [14].

Recent studies have shown that silica formation can be catalyzed by simple peptides or proteins, such as lysozyme, in a silicification reaction analogous to the formation of silica in biological systems [15–18]. The lysozyme-precipitated silica nanoparticles proved suitable for immobilization of other enzymes. The silicification reaction yields a network of fused silica nanospheres, providing a high surface area for encapsulation and permitting high enzyme loading capacities [19]. We now report herein, a versatile method for immobilization of biomolecules directly onto a SPR transducer surface by encapsulating biomolecules within a lysozyme-mediated self-assembled layer of silica particles. The immobilization of lysozyme is based on non-specific physical adsorption of the protein to the gold SPR surface through a combination of electrostatic and surface interactions [20]. Non-specific binding will therefore result in the formation of a film of lysozyme upon the gold surface, which is then available to participate in the silicification reaction and direct the assembly of a layer of silica at the surface. Physical adsorption generally causes little conformational change of the enzyme and no reagents or pretreatment and activation of the surface is required. A disadvantage is enzyme leaching during continuous use, as the binding is primarily due to weak hydrogen bonding and Van der Waals forces [21]. Previous literature reports however, indicate that lysozyme retains its tertiary structure when adsorbed to a hydrophilic interface, no significant denaturation occurs, and in addition, the binding is irreversible [22].

The fabrication of SPR chips, consisting of gold films coated with thin silicon dioxide layers has been recently reported [23]. The method however, involves vapor-deposited silica layers that showed a lack of stability in aqueous buffer solutions and is unsuitable for enzyme immobilization. A sol–gel technique has been successfully applied to generate stable gold/silica interfaces, which allowed further functionalization but preparation required multi-step attachment using biotin and streptavidin binding chemistries [24]. The lysozyme-mediated silica formation described herein provides a method for coating a gold surface with a thin layer of silica particles, greatly increasing the surface area of the transducer. In addition, the silica provides a matrix for the encapsulation of additional biomolecules, significantly enhancing the functionality of the resulting silica layers by directing the attachment of immobilized biomolecules directly at the gold surface.

2. Experimental

2.1. Enzymes and reagents

Potassium phosphate buffer (0.1N NaOH, 0.1 M KH_2PO_4, pH 8) was used throughout unless otherwise stated. Paraoxon was obtained from ChemService, West Chester, PA. All other reagents and chemicals were of analytical grade and obtained from Sigma–Aldrich (St. Louis, MO). Silicic acid was prepared as described previously [19]. Organophosphorus hydrolase (OPH) was generously provided by James Wild and his research group (Texas A&M University). The enzyme purification method has been described previously [25].

2.2. Formation of silica nanoparticles on the gold surface

The formation of silica particles was characterized by SPR using SPREETA^{TM} sensors (Texas Instruments) with two analysis channels. A gold surfaced SPR sensor module, and its supporting hardware and software (SPREETA, Texas Instruments) were coupled to a continuous-flow cell to allow contact with reaction solutions. Experimental setup and cleaning steps were performed as previously described [26]. The sensor was docked with the fluidics block and reference measurements were obtained with air and water as baseline measurements. An *in situ* washing step (0.12N NaOH, 1% Triton-X) was performed to ensure that the surface remained hydrophilic. A further baseline with phosphate buffer was taken as a reference measurement. Initially, lysozyme (1 mg/ml) was non-specifically adsorbed to the gold surface and any excess was removed by washing with phosphate buffer. Silicification was carried out *in situ* by introducing TMOS (100 mM tetramethyl orthosilicate in 1 mM HCl) to the lysozyme-modified surface. This process was repeated with different lysozyme concentrations (5, 25 and 50 mg/ml) to determine the optimum enzyme concentration. All immobilization procedures were performed at room temperature (∼22 °C). Immobilization steps were monitored by measuring the change in refractive index (RI) as a function of time followed by integration using SPREETA software. Net responses were calculated by comparison of 'working' and 'control' channels. Calculations and statistical analysis were performed with OriginPro 7.5 software (OriginLab Corporation, Massachusetts, USA).

2.3. Calculation of adlayer thickness and surface coverage

The adlayer thickness and surface coverage of each monolayer was calculated using the formula described by Jung et al [27,28]; $d_a = (l_d/2) \times [(n_{eff} - n_b)/(n_a - n_b)]$, where d_a is the thickness of the adlayer, l_d the characteristic decay length of an evanescent wave at 307 nm, n_{eff} the effective RI of the adlayer (from the SPR signal), n_b the RI of the buffer (from reference reading), and n_a is the RI of the adlayer material assuming an RI of 1.57 for protein and an RI of 1.43 for biosilica [29].

2.4. Enzyme assay for immobilized organophosphate hydrolase activity

OPH was encapsulated within the silica matrix by adapting the method described above. The initial protein monolayer was established using a solution of 25 mg/ml lysozyme to coat the SPR surface. A solution of 100 mM TMOS containing OPH was then passed over the surface for approximately 45 min to yield the silica layer and co-encapsulate OPH during the silici-

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fication reaction. The SPR surface was rinsed thoroughly with buffer to remove any loosely associated enzyme and silica prior to further analysis. Enzyme activity was determined by measuring the hydrolysis of paraoxon as described previously [30]. Paraoxon (1–500 μM) was circulated across the surface at a flow rate of 100 μl/min for 2 min. Enzyme activity was determined by collecting 200 μl of the paraoxon hydrolysis product (*p*-nitrophenol). The absorbance of the hydrolysis product was measured at 405 nm using a UV–vis fiber optic spectrophotometer (Ocean Optics Inc., Dunedin, FL).

2.5. Scanning electron microscopy

For scanning electron microscopy imaging, glass slides were coated with a chromium adhesion layer (~2 nm) followed by ~50 nm gold films. The film was cleaned with freshly prepared piranha solution (3:1, H_2SO_4 and H_2O_2. *Caution*: Piranha solution is dangerous and should be handled with care) followed by thorough rinsing with DI water. The slides were then sonicated in acetone (5 min), rinsed with DI water, and sonicated in ethanol (5 min) before plasma cleaning in air (5 min). The slides were prepared as described above with a range of lysozyme concentrations, followed by silica formation in the presence of 100 mM TMOS. The samples were then coated with a thin layer of gold (~10 nm) and imaged using a JEOL JSM 7000F field emission scanning electron microscope (JEOL USA, Inc., Peabody, MA).

3. Results and discussion

3.1. SPR analysis of lysozyme and silica nanocomposite films

SPR spectroscopy revealed rapid adsorption of lysozyme to the gold waveguide surface (Fig. 1a). The change in surface density results in small changes in RI at the interface and a corresponding shift in the resonance angle. Upon introduction of lysozyme, an initial rapid signal increase is observed and is attributed to the change in the bulk refractive index of the circulating solution. The change in RI then increased gradually, corresponding to the adsorption of lysozyme to the gold surface. Surface saturation was indicated by a plateau in the RI signal. The decrease in the RI during the wash step was due to removal of unbound lysozyme. The RI signal change increased linearly with higher protein concentrations (Fig. 1b). The lysozyme adlayer also thickened with increasing lysozyme concentration but showed a plateau at 25 and 50 mg/ml (Table 1),

Table 1
Effect of lysozyme concentration on thickness of lysozyme and silica adlayers

[Lysozyme] (mg/ml)	Thickness of protein adlayer, *d* (nm)	Surface coverage (molecules/mm^2)	Thickness of silica adlayer, *d* (nm)
1	0.934	5.23E+10	3.74
5	0.978	5.47E+10	3.75
25	1.963	1.10E+11	6.56
50	2.029	1.14E+11	6.60

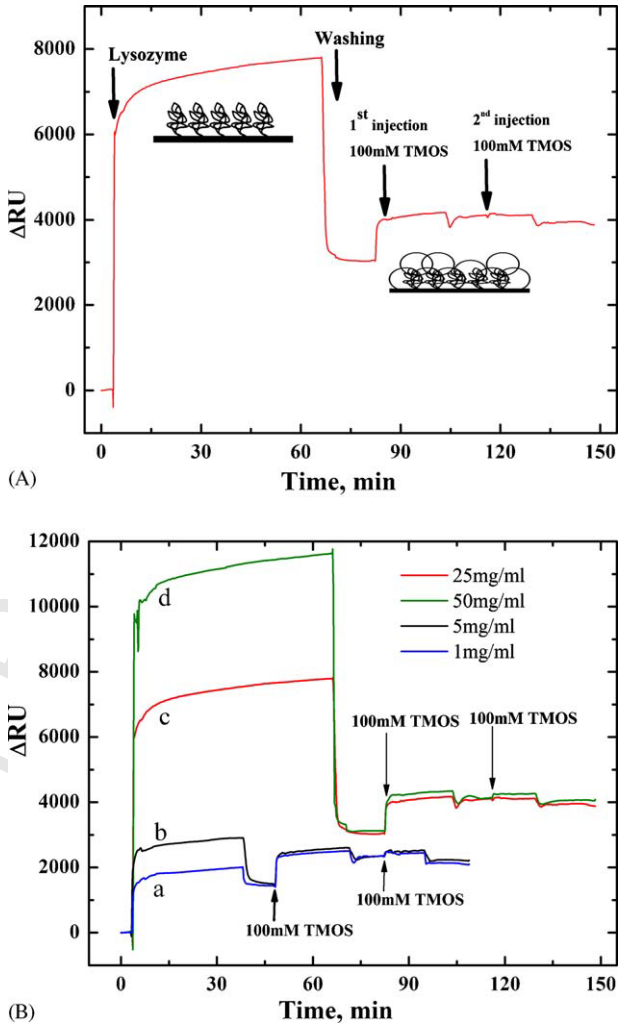

Fig. 1. (A) SPR spectroscopy response showing the binding of lysozyme and formation of silica at the SPR surface. Sensorgram shows addition of 25 mg/ml lysozyme, followed by washing. An 100 mM TMOS was added as a precursor for silica formation. (B) Formation of lysozyme-mediated silica coating on gold. SPR response of lysozyme deposited to the gold surface at a range of concentrations: 1 mg/ml (curve a), 5 mg/ml (curve b), 25 mg/ml (curve c) and 50 mg/ml (curve d). Addition of TMOS (100 mM) is indicated by an arrow.

indicating that the gold surface was saturated at high protein concentrations. The surface coverage of lysozyme was calculated and revealed a maximum surface concentration of ~2.6 ng/mm^2 (~1.10×10^{11} molecules/mm^2) and a maximum film thickness of ~2 nm (±0.047) (Table 1). The measured maximum coverage of lysozyme at saturation is in agreement with the theoretical surface density for a monolayer of lysozyme (1.8–2.7 ng/mm^2), based on a protein with a molecular mass of 14 kDa and dimensions of 4.5 nm × 3.0 nm × 3.0 nm [20,21]. SEM images of the monolayer showed a glass-like film of lysozyme across the surface of the waveguide (Fig. 2a).

The bound lysozyme molecules mediated the formation of a silica adlayer *in situ*. Introduction of a solution of TMOS caused a rapid increase in RI, indicating changes in surface refraction consistent with the formation of a second distinct adlayer of silica (Fig. 1B). The reaction was rapid and approximately 90% of silica formation occurred within the first minutes of contact.

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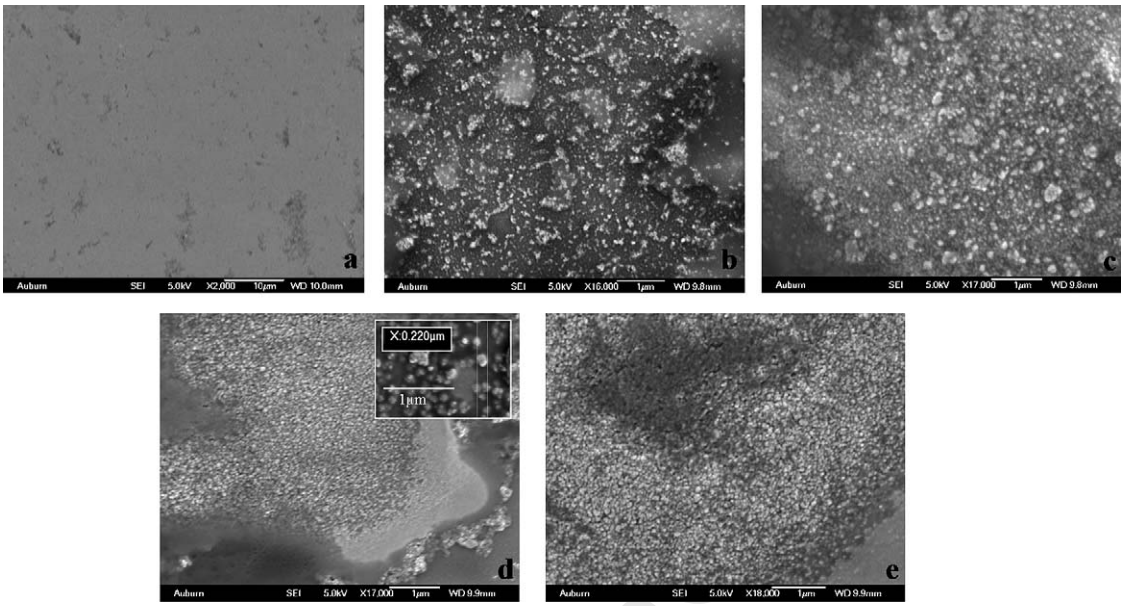

Fig. 2. SEM images of silica-encapsulated OPH at the SPR surface. Gold surface modified with (a) lysozyme (25 mg/ml); (b) lysozyme (1 mg/ml) with 100 mM TMOS; (c) lysozyme (5 mg/ml) with 100 mM TMOS; (d) lysozyme (25 mg/ml) with 100 mM TMOS; (e) lysozyme (25 mg/ml) with 100 mM TMOS and OPH (0.1 mg/ml).

Washing the silica layer with buffer did not decrease the signal significantly, indicating that the silica was firmly attached to the surface. The change in the RI was used to calculate the deposition characteristics of the silica particles. The maximum thickness of the layer was calculated to be ~6.6 nm (Table 1). The thickness of the silica layer did not increase following a second injection of TMOS suggesting that the surface was saturated with silica and conditions were not substrate limited. SEM analysis confirmed the formation of an interconnected, dense coating of silica nanospheres formed upon the gold surface. At low concentrations of lysozyme, a scattered deposition of silica was observed with silica particles having an average size ~10 nm (Fig. 2). When lysozyme was present in excess, however, dense coatings of interconnected aggregates of much larger silica particles (~230 nm) formed in addition to the initial monolayer of silica nanospheres (Fig. 2c and d). In aqueous static suspensions, lysozyme forms silica spheres of approximately 570 nm diameter [15]. The reduction in size of the silica particles observed here is attributed the formation of the silica particles under continuous flow conditions. Silica spheres are the lowest free energy structure formed in a static environment, but application of a dynamic flow will affect the formation and aggregation of silica.

Even though the SEM shows the size of the nanoparticle as 230 nm the thickness measured by SPR for the silica layer is significantly less (~7 nm). The results are consistent with the immediate formation of a thin film of silica directly at the surface which provides a template for subsequent formation of larger silica particles, as observed for many silicification reactions [31]. The surface plasmon resonance phenomenon occurs at the metal–liquid interface and is highly sensitive to specific interactions at the interface which may be on the order of only a few nanometers. Although a generated evanescent wave can travel up to ~300 nm in the z direction [27], the medium beyond the interface will affect the observed RI. The inability to see the depth of the whole silica structure using SPR in the present work is in agreement with previous literature reports where silica layers of greater than 44 nm did not show significant SPR response [23].

The lysozyme is presumably attached at the surface in an orientation which does not diminish its ability to mediate silica formation. Variations in TMOS concentration may theoretically affect the thickness of the silica layer. Preliminary control experiments in static suspensions however, revealed that silica formation does not occur if the TMOS concentration is below 25 mM (data not shown), accordingly, silica adlayer formation was not investigated at lower precursor concentrations. In control experiments, no formation of silica particles was observed in the absence of lysozyme. Bovine serum albumin (BSA) adsorbed to the gold surface but did not precipitate silica in the presence of TMOS, confirming that lysozyme is integral to the silica formation at the surface (data not shown).

3.2. Encapsulation of organophosphate hydrolase

The further biofunctionalization of the silica particles at the surface was investigated using organophosphate hydrolase (OPH) as a model system. The gold surface was saturated with lysozyme as defined above and used to mediate the formation of silica particles containing various concentrations of organophosphate hydrolase (OPH). The silica particles formed on the gold surface as described above and, examination using SEM clearly showed that the surface was coated with a film of evenly distributed spheres (Fig. 2e). The addition of OPH to the hydrolyzed TMOS solution did not result in any significant changes in the morphology of the silica surface (Fig. 2d and e). OPH encapsulated within the silica coating maintains activity, confirmed by the hydrolysis of paraoxon and the activity of OPH correlates with protein concentrations used in the encapsulation step

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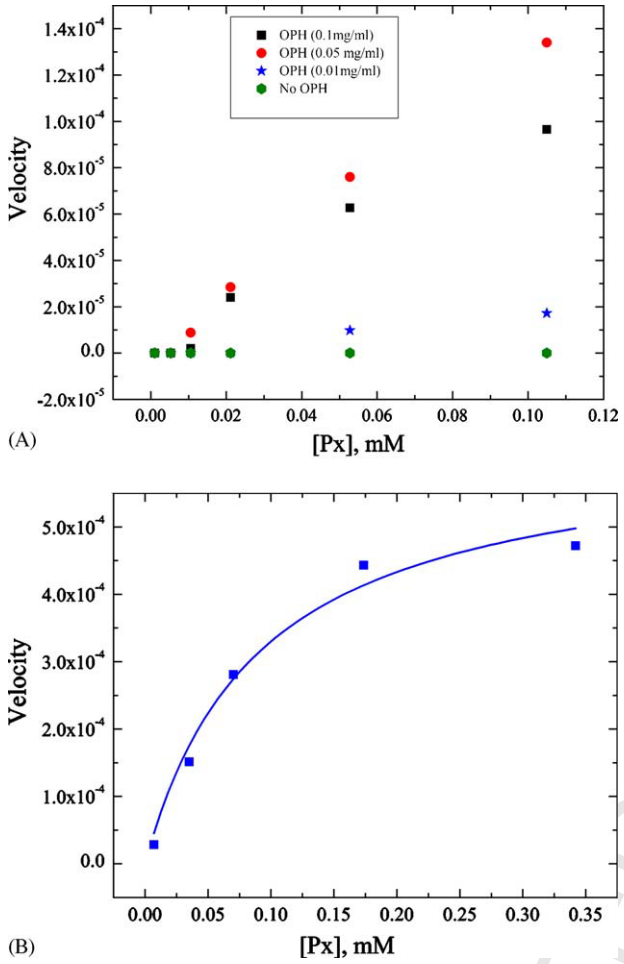

Fig. 3. Paraoxon hydrolysis by silica-encapsulated organophosphate hydrolase immobilized to the SPR surface. (A) Hydrolase activity in relation to OPH concentrations present during silicification step at a range of substrate concentrations. (B) Michaelis–Menten plot of immobilized OPH (0.5 mg/ml during silicification) to reveal maximal velocity of reaction.

(Fig. 3a). The kinetic parameters of the encapsulated enzyme were determined by contacting the encapsulated enzyme at the surface with paraoxon at a range of concentrations. At low concentrations of paraoxon (20–100 μM), the silica-encapsulated OPH shows a linear response (Fig. 3a) but enzyme activity becomes saturated at paraoxon concentrations above 300 μM (Fig. 3b). A reproducible detection limit of 20 μM paraoxon was achieved with OPH concentrations greater than 0.05 mg/ml. A decrease in the concentration of encapsulated OPH resulted in a proportional reduction in detection sensitivity. The kinetic parameters of the encapsulated OPH ($K_m = 0.09(\pm 0.022)$) were determined (Fig. 3b) are in good agreement with the kinetics of OPH in solution [32] indicating that immobilization of OPH in silica does not significantly hinder the mass transport of substrate.

4. Conclusion

The formation of silica using lysozyme precipitation provides a simple and rapid method for the deposition of silica films directly to a gold surface. The silica layer proved sufficiently stable under continuous flow conditions to allow measurement of enzyme kinetic parameters. The silica deposition and surface immobilization of OPH demonstrated in this study provides a model system with potential application to a range of formats. The surface encapsulated OPH could be reused continually for over 2 days, but lost activity gradually over the time period, concurrent with a loss of silica film thickness (data not shown). The immobilization efficiency and stability achieved were sufficient for demonstrating the concept, but further analysis of the silica coating is required to optimize the approach. The formation of a silica layer on the gold surface significantly increases the surface area at the transducer interface and potentially enhances the sensitivity of SPR spectroscopy applications [33]. The silica layer also proved suitable for encapsulation of OPH and the immobilized enzyme retained activity over a period of several hours, providing accurate and reproducible measurements of immobilized enzyme kinetics. The immobilization technique described provides a versatile method for enzyme encapsulation that selectively immobilizes proteins directly on a transducer surface with no requirement for surface modification before immobilization. OPH is not directly tethered to the SPR surface, which may limit any restriction in the orientation of the active site, as often observed when enzymes are covalent attached to a surface.

The approach may lead to development of a versatile method for the immobilization of enzymes on an SPR transducer surface that might be applied to biosensors or protein microarrays [34–36]. In addition, the methodology developed for OPH immobilization on the gold surface may be applied to other electrochemical detection platforms.

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