TECHNICAL NOTES

PEGylation of a Maltose Biosensor Promotes Enhanced Signal Response When Immobilized in a Silica Sol−Gel

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A robust method to immobilize a maltose biosensor is described using an engineered maltose periplasmic binding protein (PBP) covalently coupled to NBDamide, an environmentally sensitive fluorophore. A mesoporous silica sol−gel derived from diglycerylsilane (DGS) was constructed to embed the maltose biosensor, and the ligand reporting fluorescence properties were measured. When sequestered in the DGS-derived silica matrix, the biosensor retained maltose-dependent fluorescence sensing capability with micromolar affinity, which is consistent with the protein free in solution. The MBP-NBD conjugate was further modified by covalent conjugation with poly(ethylene glycol)-5000 (PEG) to promote the retention of water molecules around the protein and to reduce possible steric effects between the silica matrix and protein. Bioconjugation with PEG molecules does not significantly affect the signaling response of the protein in solution. When immobilized in the DGS polymer, a consistent increase in fluorescence intensity was observed as compared to the protein not functionalized with PEG. To our knowledge, this report presents the first successful method to embed a PBP biosensor in a polymerized matrix and retain signaling response using an environmentally sensitive probe. The immobilization method presented here should be easily adaptable to all conformation-dependent biosensors.

INTRODUCTION

The construction of protein biosensors for the detection of analytical targets requires high ligand specificity and selectivity, combined with a robust delivery strategy (e.g., *refs* 1, 2). The first two criteria are determined by the polypeptide utilized in the biosensing scheme, whereas a reliable way to mobilize the sensor out of the lab requires a deliberate, empirical approach that ideally leads to operational and storage stability (*3*). One protein superfamily studied for the design of fluorescent protein biosensors is the periplasmic binding proteins [1](PBPs), which facilitate the collection of nutrients from the surrounding environment for use in bacterial metabolism (*4*). A diverse set of PBPs has been characterized with binding capabilities to a variety of ligands including metals, sugars, amino acids, and anions (*5*−*7*). PBPs share a conserved structural organization whereby a single polypeptide chain folds into two easily identifiable domains separated by a hinge region where binding to cognate ligand may occur (*8*). Upon ligand binding, the protein domains undergo large bending or rotational motions around the hinge region. As a result of these well-documented conformational changes, many spectroscopic and electrochemical techniques provide a measurable signal output to report on ligand binding.

Two factors have inhibited the advancement of promoting the design of PBP biosensors. First, because large conformational changes are required for signal transduction, it has been difficult to construct a system using these potential biosensors embedded in a polymeric matrix which typically prevents conformational flexibility. Second, the majority of PBP biosensors are designed using environmentally sensitive probes covalently attached to single cysteine residues (*5*). Because all measurements and associated calculations are optimized in an aqueous solution, transfer of the system to an altered environment (e.g., different polarity medium) may have a significant impact on signal output, response, and stability. In this report, we tested a silica gel polymer derived from diglycerylsilane (DGS) as a carrier for a maltose biosensor. Sugar alcohol- and polyol-modified silanes, such as DGS, have been extensively used for the gentle encapsulation of biomolecules because these materials condense at neutral pH while releasing benign glycerol molecules (*9*−*11*). We also examined the effect of covalent attachment of poly(ethylene glycol) (PEG) on maltose-dependent fluorescence intensity changes upon encapsulation in the sol−gel; this polymer conjugation is intended to increase the hydration of the protein, as well as prevent interactions between the protein and silica matrix walls. To our knowledge, this report presents the first successful method to embed a PBP biosensor in a polymerized matrix and retain signaling response using an

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[1]Abbreviations: PBP, bacterial periplasmic binding proteins; MBP, *Escherichia coli* maltose binding protein; NBD, *N*,*N*′-dimethyl-*N*-(iodoacetyl)-*N*′-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine; DGS, diglycerylsilane; PEG, poly(ethylene glycol) 5000-NHS ester; MALDI-TOF, matrix assisted laser desorption/ionization−time-of-flight; MS, mass spectrometry.

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environmentally sensitive probe. The immobilization method presented here is general and should be easily adaptable to other conformation-dependent biosensors.

EXPERIMENTAL SECTION

Construction of the Maltose Biosensor. Purification of *E. coli* MBP D95C was performed as previously described using Ni-NTA affinity chromatography (*12*). Covalent attachment of a thiol-reactive NBDamide (Anaspec, Inc., San Jose, CA) was performed either at room temperature for 4 h or overnight at 4 °C in the dark with a 3-fold molar excess of dye. Unbound probe was removed using PD-10 gel filtration chromatography followed by dialysis against 5 mM phosphate buffer, pH 7.0. Typically, PEGylation was then performed by combining an 8-fold molar excess of NHS-PEG-5000 (Nanocs, Inc.) to MBP D95C for 12 h at room temperature. The mixture was then filtered through a 10 kDa microcentrifuge column (Microcon) to remove unreacted PEG; no further attempt was made to separate PEGylated protein from the unmodified protein. Dynamic light scattering (Zetasizer Nano, Malvern Instruments) was used to measure the hydrodynamic radii of the protein before and after PEGylation. Mass spectrometry was performed on a 4800 Plus (ABI, Framingham, MA) MALDI-TOF mass spectrometer.

DGS was synthesized following previously reported procedures (*13, 14*). In a typical silica gel preparation, ~150 mg of DGS was partially dissolved in deionized water (>18.0 MΩ cm) and sonicated for 30 min at 10−15 °C. The resulting solution was filtered through a 0.45 μm polycarbonate filter to remove any undissolved particulates of DGS and then mixed in a 96 well plate with an equal volume of labeled protein in 5 mM phosphate buffer (pH 7). The resulting monoliths measure 7 mm × 6 mm (height × width). The plate was covered with parafilm, and samples were allowed to gel at 4 °C for at least 5 days before performing maltose binding assays. Sensors were stable and operationally viable for at least 2 months stored at 4 °C.

Steady-State Fluorescence. Fluorescence measurements were performed at room temperature using a Varian Cary Eclipse spectrofluorometer equipped with a plate reader and thin film polarizers set to the magic angle (0° excitation and 54.7° emission). Protein conjugate samples were excited at 480 nm and were titrated with increasing amounts of maltose to achieve signal saturation. Fluorescence intensity at the emission maximum was plotted against maltose concentration to determine the apparent binding constant of the protein conjugate as previously described (*15*). Data analysis was performed using the nonlinear curve fitting procedures in the *Origin 7.0* software.

RESULTS AND DISCUSSION

The maltose-dependent fluorescence response of a MBP-D95C mutant labeled with IANBDamide (NBD) has been characterized previously (*12*). This bioconjugate sensing system is one of the few PBP biosensors where the mechanism of ligand-dependent fluorescence change is understood to result from a specific hydrogen bond interaction between the NBD and the hydroxyl group on Tyr171. Upon binding maltose, the hinge-bending motion of MBP removes this contact resulting in an increase in NBD emission. For device applications, immobilization of these types of proteins in a solid matrix is preferred. However, many encapsulating systems, like silica, dehydrate over time causing proteins to denature. Functionalization of MBP with a poly(ethylene glycol) (PEG) polymer may improve the protein's ability to maintain proper conformation in a solid matrix, as PEG is known to be heavily hydrated by water (*16*). In addition, the steric bulk of the PEG moieties should reduce protein−matrix sidewall interactions, which can disrupt protein function (*17*).

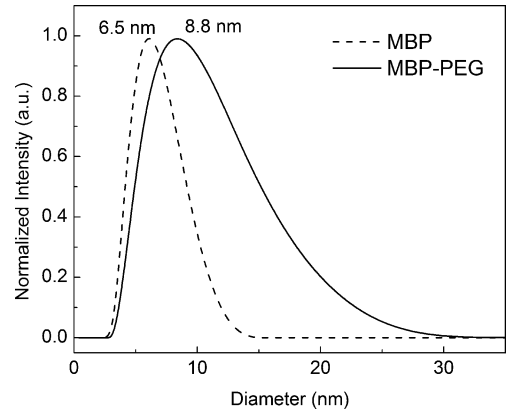

Figure 1. Representative dynamic light scattering data for MBP D95C before (6.5 nm) and after (8.8 nm) the reaction with an 8-fold molar excess of amine-reactive PEG-5000.

Prior to encapsulation, we investigated the addition of an amine-reactive PEG-5000 moiety to lysine residues of the maltose biosensor. Because PEG is spectroscopically inactive, we used dynamic light scattering to measure the increase in molecular volume upon reaction with PEG and MALDI-TOF MS to further characterize the PEGylated biomolecules. Before the PEGylation reaction, the ensemble of proteins in the MBP solution showed a narrow light scattering curve with an average diameter of 6.5 nm, as seen in Figure 1, which is consistent with previous determinations of the hydrodynamic radius for similar MBP monomeric proteins in solution (*18*). Following the labeling of amino groups with 8 mol equiv of PEG, the mean distribution broadened significantly and shifted to an average diameter near 8.8 nm (Figure 1). While there is a clear increase in the hydrodynamic diameter for the PEGylated-MBP which represents a correlation to the increase in size of the protein−PEG conjugate, it is difficult to quantitatively determine the average number of PEG molecules attached to each protein (*19*). A distribution of the number of PEG units covalently attached to the protein is observed, and additional experimentation with changes to the PEG and protein concentration had little effect on the resulting scattering data providing consistent evidence for the addition of PEG molecules to the protein. To further investigate what PEGylated species were present following conjugation, MALDI-TOF MS was used. Prior to PEGylation, a singly charged MBP parent ion was observed around 42 kDa. After PEGylation, three new peaks were observed at ~46 kDa, 52 kDa, and 56 kDa as expected for MBP plus one, two, or three PEG molecules, respectively (see Supporting Information), along with the parent MBP ion at ~42 kDa. While MALDI of intact proteins is not accurately quantitative, these spectra provide a reasonable estimate of the level of PEGylation in our samples. Taken together, the DLS and MALDI-TOF data demonstrate that MBP was labeled with a distribution of PEG groups.

Following PEGylation, we tested the protein samples for any quantitative differences in maltose sensing ability in solution compared to the non-PEGylated protein sensor. The fluorescence response of NBD-labeled MBP with and without PEG showed no significant differences in phosphate buffered solution with respect to reporting on maltose concentration (Figure 2). A 4.5-fold increase in fluorescence was measured for both systems, and the dissociation constant for maltose of the PEGylated protein conjugate was determined to be 5 μM (Figure 2, inset). PEGylation of the protein does not produce a noticeable lag in response time in solution, where signal equilibrium is reached within the mixing time. Also, the dissociation constant measured

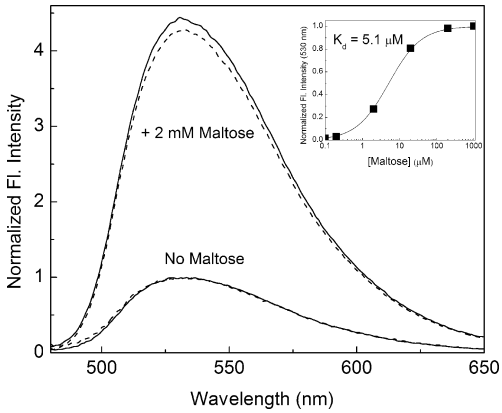

Figure 2. Fluorescence emission spectra of MBP D95C-NBD with (solid lines) and without (dashed lines) the attachment of PEG in the presence and absence of maltose. (inset) Titration of PEGylated protein with maltose in solution yields a dissociation constant of 5 μM.

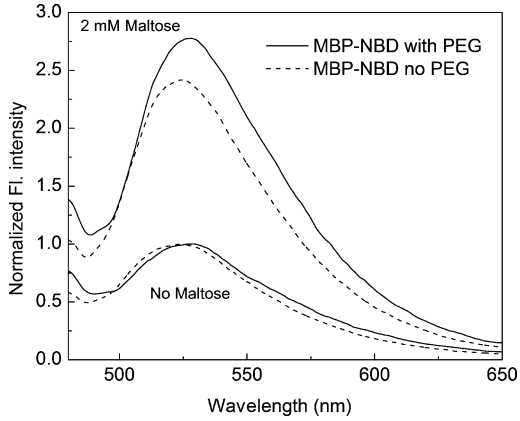

Figure 3. Fluorescense spectra of a maltose biosensor embedded in DGS before (dashed) and after (solid) conjugation with PEG. Spectra were normalized to the signal measured for the unbound protein immobilized in DGS. Samples were prehydrated with phosphate buffer, pD 7.0, and measurement was performed in a 96 well plate.

for the PEGylated protein is consistent with the value found previously for the maltose sensor in the absence of PEG, providing further support that the PEG molecules conjugated to the MBP sensor do not produce a significant effect on the binding characteristics of the protein (*12*).

The fluorescently tagged MBP with and without PEG conjugation was encapsulated into DGS-derived sol−gel slabs or cast as thin films in 96 well plates. Several spectral differences were observed between unmodified and PEGylated protein upon encapsulation into the DGS polymer and titration with maltose. The fluorescence intensity of the starting material was checked prior to addition of ligand to ensure consistency of the experimental data collected. Following immobilization in the DGS-derived sol−gels, the PEGylated sensor demonstrated a 2.8-fold increase in fluorescence intensity upon saturation with maltose, while the protein without PEG showed a smaller 2.4-fold change in emission intensity (Figure 3). In general, we observed a ∼10% increase in fluorescence from PEG-functionalized MBP compared to unfunctionalized MBP upon encapsulation and exposure to maltose. Additionally, we observed a 5 nm red shift of the NBD emission maximum in the PEGylated MBP, which suggests that the probe molecule may be experiencing an increased level of polarity in its microenvironment compared to the protein free in solution (*20*). While a difference in signal intensity is observed between the two protein conjugates trapped in DGS-derived sol−gel films, titration with

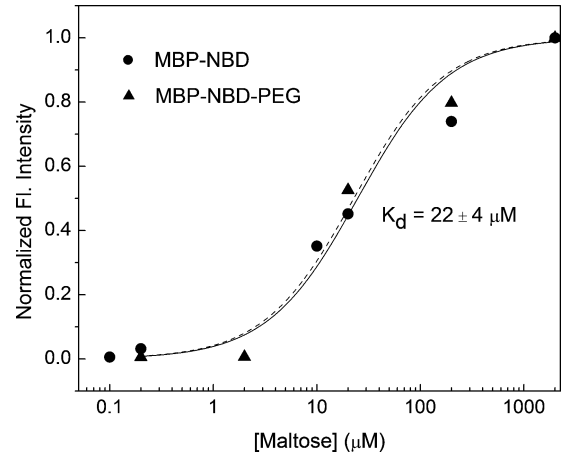

Figure 4. Representative maltose titration data for the constructed biosensor in DGS with (triangle) and without (circle) the covalent attachment of PRG-5000. The data were fit to a single binding isotherm and the K_d was calculated to be approximately 22 μM for maltose for both sensors.

increasing concentrations of maltose (0 to 2 mM) yielded similar dissociation constants (∼22 μM) as shown in Figure 4.

There have been few reports in the literature describing the successful interface of PBP-based fluorescent biosensors on surfaces for the construction of immobilized biosensors. Cass and colleagues reported the first successful surface immobilization of a PBP biosensor using a glutamine binding protein translational fusion with a C-terminal hydrophobic polypeptide (*21*). Using thermophilic PBPs, deLormier et al. were able to covalently attach glucose and ribose sensing proteins to the bottom of well plates via masked cysteine residues (*22*). However, both of these methods required further engineering of the protein biosensor, and because emission is provided from a single layer of protein molecules, there is a reduced level of signal intensity which may lower the sensitivity of the sensor. There are examples in the literature of protein immobilization entrapment in silica-based polymers with retention of ligand binding and fluorescence signaling (*20, 23*); however, these systems typically do not require the magnitude of the conformational flexibility needed for ligand binding in PBPs.

We tested the ability of an MBP sensor to respond to maltose when encapsulated in a DGS-derived silica matrix. The ideal matrix needs to allow easy diffusion of ligand to the protein, as well as allowing for the conformational transition between open and closed states of MBP, which is required for proper signal transduction of the binding event. Typical matrices used to embed proteins include polyvinyl alcohol, carboxydextran, and polyethylenimine. All of these polymers have been tried and found to greatly reduce or eliminate the ligand-dependent fluorescence response of PBP-based biosensors (unpublished observations). DGS, however, forms a silica gel that has not previously been used with PBP sensing systems and has several benefits including condensation at neutral pH and optical transparency. The DGS matrix was shown to be effective at allowing an observable signaling response to maltose. Although there was a considerable lag in response time compared to solution (∼20 min for 50% signal intensity), there is no difference in time response between PEGylated and non-PEG protein in DGS, suggesting that the time required for signaling is most likely due to diffusion of maltose into the DGS matrix. Retention of protein flexibility upon entrapment is clearly adequate for maintenance of equilibrium binding affinity, despite the slowed dynamics upon entrapment within the biogels. This trend is consistent with earlier observations showing arrested nanosecond rotational reorientation dynamics for intact poly-

clonal antidansyl antibodies sequestered within sol−gel-derived biogels accompanied by minimal losses in the equilibrium binding constant describing antibody association with its target hapten (*24*). Covalent conjugation with PEG may have increased the stability of MBP by keeping it better hydrated even after immobilization within the silica gel, as well as minimizing denaturing steric effects. We believe that the beneficial effects of PEGylation will be even more significant for PBP biosensors where the signaling response is not as well-characterized as the MBP system studied here. In summary, the covalent attachment of PEG chains to residues on protein surfaces, followed by mild immobilization procedures based upon bioconjugate entrapment within sol−gels formed from biofriendly precursors such as DGS, may prove to be a generic approach to designing robust, portable fluorescent protein biosensor platforms and forms the subject of future research in our group.

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Supporting Information Available: Additional figure as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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