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High-Throughput Detection of Thiamine Using Periplasmic Binding Protein-Based Biorecognition

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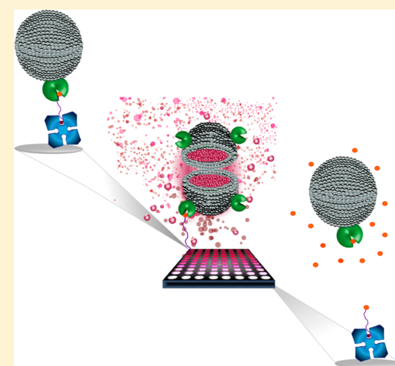
Supporting Information

ABSTRACT: Although antibodies and aptamers are commonly used bioaffinity recognition elements, they are not available for many important analytes. As an alternative, we demonstrate use of a periplasmic binding protein (PBP) to provide high affinity recognition for thiamine (vitamin B1), an analyte of great importance to human and environmental health for which, like so many other small molecules, no suitable biorecognition element is available. We demonstrate that with an appropriate competitive strategy, a highly sensitive (limit of detection of 0.5 nM) and specific bioassay for thiamine and its phosphorylated derivatives can be designed. The high-throughput method relies upon the thiamine periplasmic binding protein (TBP) from *Escherichia coli* for thiamine biorecognition and dye-encapsulating liposomes for signal-enhancement. A thiamine monosuccinate-PEG-biotin derivative was synthesized to serve as an immobilized competitor that overcame constraints imposed by the deep binding cleft and structural recognition requirements of PBPs. The assay was applied to ambient environmental samples with high reproducibility. These findings demonstrate that PBPs can serve as highly specific and sensitive affinity recognition elements in bioanalytical assay formats, thereby opening up the field of affinity sensors to a new range of analytes.



Thiamine (vitamin B1) is essential to human health, serving in its diphosphorylated form as a cofactor to enzymes involved in carbohydrate metabolism. Thiamine deficiencies in people can manifest in diseases known as beriberi and Wernicke-Korsakoff syndrome affecting cardiovascular and neurological systems.[1,2] Thiamine deficiencies may occur due to conditions causing an inability to absorb available dietary thiamine;[3] if dietary intake of thiamine-containing foods is restricted;[4] or if foods containing high levels of enzymes that break down thiamine (thiaminases) are concurrently consumed.[5] Aside from human health, the latter is of great ecological significance, with deficiencies affecting organisms ranging from birds[6] to reptiles[7] and to fish.[8] In predatory fish such as Atlantic salmon, lake trout, and steelhead, thiamine deficiencies have been attributed to consumption of prey fish such as alewives that contain high levels of thiaminases. Such deficiencies have been implicated in muscle weakness, inability to spawn, and poor survival rate of fry known as Early Mortality Syndrome (EMS), Cayuga Syndrome, and the M74 syndrome affecting fish in bodies of water ranging from the Great Lakes to the Baltic Sea.[8−11]

The assessment of thiamine levels typically relies on oxidation of nonfluorescent thiamine to the fluorescent product thiochrome using reagents such as alkaline potassium ferricyanide.[12] The formed thiochrome is then most commonly quantified via reverse-phase HPLC, where thiamine may be differentiated from its mono and diphosphate forms.[12] A variety of separation conditions have been employed yielding detection of as little as 0.002 ng thiamine per injection (5 fmol[13]) by monitoring fluorescence following formation of thiochrome.[14] With detection limits in the ∼200 pM range, HPLC is appropriate for and is widely employed for analysis of thiamine levels in fish tissues.[12] However, this approach is often not cost-effective for analyzing large numbers of samples given the costs of instrumentation, maintenance, and requirements for a skilled analyst. Other assays for thiamine rely on monitoring the growth of auxotrophic microorganisms, including bacteria, algae, yeast, and fungi.[15−17] These assays can achieve low detection limits (low ng/L, pM range) and have had much historical value for the analysis of environmental water samples. However, implementation of these growth-based assays requires access to specific microorganisms, prolonged assay times of several days, and they have the potential for unspecific growth due to thiamine fragments or other constituents in biological samples.[17] Commercially available microbiological kits are available which utilize *Lactobacillus fermentum* and report sensitivities for thiamine of 3 μg/L in serum assessed using turbidity measurements following a 48 h growth period.[18] Enzyme-linked immunosorbent assays (ELISAs) are commonly used for environmental analyses of pesticides, pollutants, and biological toxins, offering high-throughput and relatively low-cost analyses without significant sample preparation requirements.[19] However, the development of traditional immuno-

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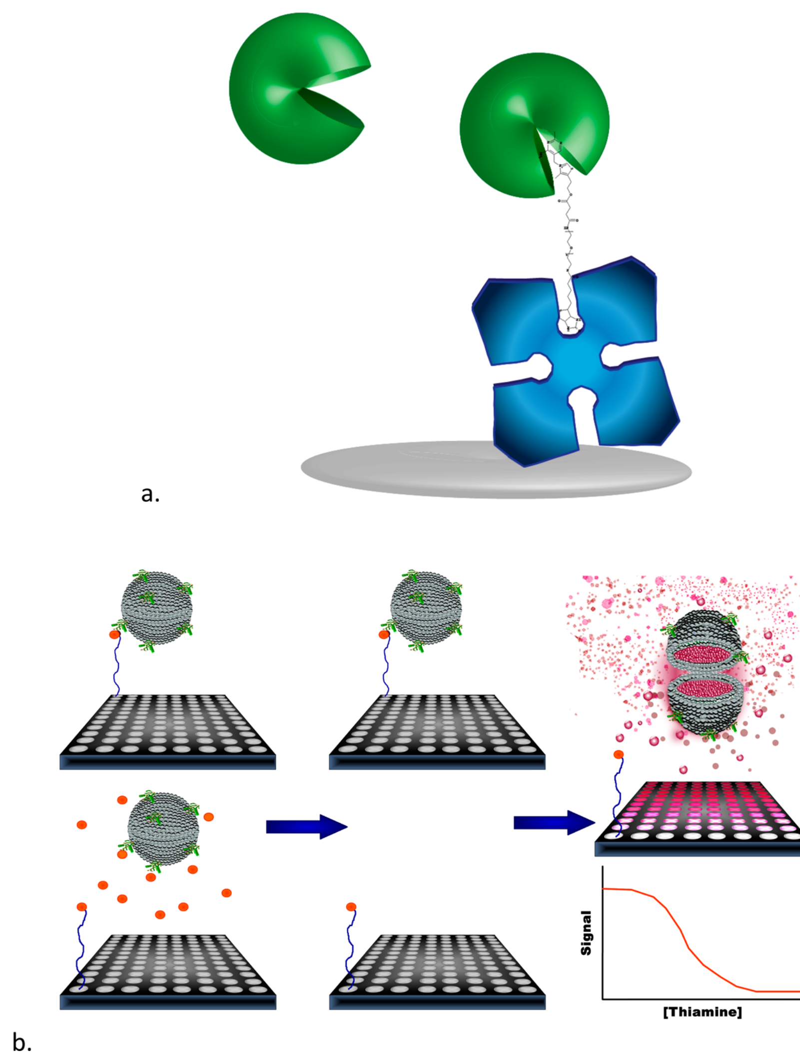

Figure 1. Competitive assay for thiamine using fluorescent dye-encapsulating liposomes for signal amplification and the thiamine binding protein for specific biorecognition. (a) Thiamine derivative was designed with a long PEG spacer between thiamine monosuccinate and biotin to accommodate the binding sites of both TBP used for thiamine recognition and tetrameric streptavidin used for immobilization. (b) Competitive assay with thiamine monosuccinate-PEG11-biotin immobilized via streptavidin in microtiter plates and detected via TBP conjugated to the lipid bilayer of sulforhodamine B encapsulating liposomes (left). After competition with sample thiamine, unbound materials are removed (middle) and liposomes remaining bound are lysed to release dye yielding a signal inversely proportional to thiamine concentration (right).

assays for thiamine is not possible due to the lack of availability of antibodies specific to thiamine.

One potential option obviating the current assay limitations for thiamine is to rely on periplasmic binding proteins (PBPs) rather than antibodies in the development of high-throughput assays. PBPs are located at high concentrations (10^{-4} M) between the inner and the outer membranes of Gram-negative bacteria such as *Escherichia coli* and *Salmonella typhimurium*.[20] They are involved in the uptake of solutes such as inorganic ions, amino acids, peptides, sugars, and vitamins[21−24] through coupling to transporters within the inner membrane that mediate their transport or chemotaxis toward nutrient sources.[25] These protein structures are characterized by two globular domains connected by a short hinge region which undergo a significant conformational change between the protein's closed form when the ligand is bound deep within the cleft between the two domains and its open form when the ligand is unbound.[26] This has formed the basis of numerous fluorescence resonance energy transfer (FRET)-based sensors[27−29] which have demonstrated minimal cross-reactivity of PBPs toward molecules other than their target analytes.[30] However, challenges with FRET include the need for site-specific chemical modification or genetic engineering of the protein, which can add complexity and expense in bioanalytical sensor development, high background signal, limited dynamic range, and often poor sensitivity.[31,32]

We have recently demonstrated that the utility of such PBPs is not limited to FRET-based detection and that these molecules can serve as substitutes for antibody-based recognition in a standard heterogeneous binding assay platform.[33] In the previous assay for D-(+)-maltose, competition between the sample analyte and amylose magnetic beads for maltose binding protein (MBP) conjugated fluorescent dye-encapsulating liposomes took place resulting in a limit of detection of 78 nM. This assay yielded exceptional specificity toward maltose versus numerous other monosaccharides and disaccharides, which was remarkable given the simple structure of these molecules. Further, given that the binding targets of PBPs are generally not suitable for antibody generation, this advance was anticipated to expand the repertoire of solid-phase

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binding assays to targets otherwise challenging to bioanalytical assay development.

The thiamine binding protein from *E. coli* (hereon referred to as TBP) is a monomeric 34.2 kDa periplasmic binding protein with high affinity for thiamine (K_D = 3.8 nM) and 1:1 stoichiometry for its ligand.[34,35] It has also been shown to recognize thiamine monophosphate and thiamine diphosphate. Techniques for its cloning and overexpression produce this plasmid-derived protein in high yield.[34] This PBP, when modified by site-directed mutagenesis and conjugation with thiol-selective fluorescent dyes, has been used previously to construct a homogeneous assay for thiamine based on the reduction in fluorescence upon binding.[36]

In this work, TBP was investigated as a biorecognition element in a competitive solid-phase binding assay for thiamine. Here, a PEGylated thiamine analogue was developed to serve as an immobilized competitor for sample thiamine and fluorescent dye-encapsulating liposomes conjugated to TBP served to provide signal enhancement and biorecognition, respectively (Figure 1). Such liposomes that encapsulate hundreds of thousands of fluorescent dye molecules within their interior cavity have advantages over enzymatic amplification, which include improved assay sensitivity, instantaneous signaling, and long-term stability of encapsulated materials.[37,38] The specificity of TBP alone and TBP conjugated liposomes in the full assay format was evaluated and function was demonstrated in an environmental water sample. The success of the approach described within provides further evidence that the PBP liposome-based approach demonstrated previously[33] could serve as a platform technology for further heterogeneous assay development.

■ EXPERIMENTAL SECTION

Thiamine binding protein (TBP) was purchased from the Cornell Protein Purification and Production Facility (Ithaca, NY) and was provided as a solution in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.4). EZ-Link Amine-PEG$_n$-biotin and fluorescence biotin quantification kit were purchased from Pierce (Ipswich, MA). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)], sodium salt (DPPG), N-glutaryl 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine (N-glutaryl-DPPE), cholesterol, and the extrusion membranes were purchased from Avanti Polar Lipids (Alabaster, AL). Sulforhodamine B (SRB) dye and streptavidin were purchased from Thermo Fisher Scientific (Waltham, MA). The Nanosep 10K MWCO centrifugal filter devices were purchased from Pall (Port Washington, NY). MicroFloat-A-Lyzer dialysis devices with 0.1−0.5 kDa membranes were purchased from Spectrum Laboratories (Rancho Dominguez, CA). Medium binding polystyrene plates were manufactured by Greiner Bio-One (Monroe, NC). Black Maxisorp high-binding polystyrene microtiter plates were purchased from Nunc (Roskilde, Denmark.) The Costar clear medium binding 96-well microtiter plates were purchased from Corning, Inc. (Corning, NY). The potassium phosphate monobasic standard was purchased from Ricca Chemical Company (Arlington, TX). All buffers were prepared with HPLC grade water, manufactured by JT Baker (Phillipsburg, NJ). All other reagents were molecular biology grade and purchased from VWR Scientific (West Chester, PA) or Sigma-Aldrich Corp. (St. Louis, MO). Fluorescence and UV/visible measurements were made using FLX800 and PowerWave XS microtiter plate readers, respectively (Bio-Tek Instruments, Winooski, VT). Zeta potential measurements were made using a Zetasizer Nano (Malvern, Worcestershire, U.K.). The procedures for liposome preparation and protein conjugation were carried out as reported previously, using a lipid composition of 36.2 mol % DPPC, 17.8 mol % DPPG, 45.9 mol % cholesterol, and 6 mol % N-glutaryl DPPE and 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide (EDC)-mediated conjugation of TBP amine groups to liposomal carboxylic acid groups.[33,39] The MS, ^{1}H NMR, and ^{13}C NMR analyses were carried out by NuMega Resonance Lab (San Diego, CA). The thiamine ELISA kit (catalog #LS-F10664) was purchased from LifeSpan BioSciences, Inc. (Seattle, WA).

Equilibrium Filtration Assays. To optimize binding and assess the specificity of unconjugated TBP, equilibrium filtration assays were developed and carried out. Thiamine was diluted to 0.6 μM in 20 mM MES, 20 mM NaCl pH 6.5 containing 0 or 2 μM TBP. The mixture was incubated at 21 °C for 2 h, then was centrifuged for 5 min at 4000 rpm using a centrifugal filter device. A total of 60 μL of the filtrate was diluted with 120 μL of HPLC grade water, then 50 μL of this mixture was oxidized using 100 μL of 0.015% (w/v) potassium ferricyanide in 15% (w/v) sodium hydroxide to form thiochrome prior to fluorescence measurement at λ_{ex} = 360/40 nm and λ_{em} = 450/50 nm.

Various parameters were studied using this approach, including pH, ionic strength, buffer type, and presence of additives, such as BSA, casein, Tween-20, and PVP. For competitive binding studies, the same procedure was utilized, except including additionally 6 μM of thiamine analogues or fragments as competitors in the initial solution. The lack of thiochrome fluorescence from possible competitors was confirmed in advance.

Synthesis of Thiamine PEG Biotin. To develop a competitive binding assay using TBP, a thiamine derivative that could be immobilized and recognized by TBP was required. Thiamine monosuccinate was formed following modifications of the procedure of Jayamani and Low.[40] Thiamine (3.37 g, 10 mmol) was suspended in 50 mL of pyridine containing succinic anhydride (5.0 g, 50 mmol) and 4-dimethylaminopyridine (DMAP; 1.22 g, 10 mmol). The reaction was heated to reflux at 115 °C under N_2 for 1 h, then the thick slurry was cooled to 21 °C and filtered. The solids were washed with acetone and then were resuspended in 25 mL of anhydrous denatured ethyl alcohol with rotation at 50 °C. The slurry was cooled to 21 °C, then the solids were filtered and washed thoroughly with 200 proof ethanol under vacuum, yielding 1.42 g of a white powder, which was used without further purification. NMR and mass spectra are provided in the Supporting Information. ^{1}H NMR (500 MHz, D_2O) δ 2.50 (t, 2H), 2.58 (m, 8H), 3.39 (t, 2H), 4.41 (t, 2H), 5.54 (s, 2H), 8.03 (s, 1H). ^{13}C NMR (125.7 MHz, D_2O) δ 11.2, 22.2, 26.0, 30.2, 31.1, 50.6, 63.7, 105.6, 135.3, 143.6, 149.0, 162.8, 165.3, 175.3, 179.5. ESI MS positive mode: (m/z, M + H) 365.3, 244.2.

The carboxylic acid group of the formed thiamine monosuccinate (ThSu) was then reacted with the amine group of EZ-link amine-PEG3-biotin or amine-PEG11-biotin using EDC. A total of 25 μL of EZ-Link amine-PEG11-biotin at 100 mM in PBS was added to 125 μL of ThSu at 1.0 M in 100 mM MES, pH 5.0. EDC was diluted to 500 mM in 100 mM MES, pH 5.0, and 125 μL was added to the reaction mixture. The reaction was incubated on a vortex for 1 h prior to size-

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exclusion chromatography using Sephadex G10 equilibrated with 20 mM MES, 20 mM NaCl, pH 6.5. Elution of the PEG conjugate was monitored by UV at 242 nm and by fluorescence after oxidation to thiochrome as above (Supporting Information, Figure S-6).

Fractions containing the ThSu derivatized PEG3 or PEG11 biotin were then dialyzed extensively using microFloat-A-Lyzer devices with several buffer changes of 20 mM MES, 20 mM MES, pH 6.5 until the dialysate contained undetectable levels of thiochrome. The concentration of the ThSu and biotin content of the product was assessed using a thiochrome calibration curve described as above and a fluorescence biotin quantification kit, respectively. The latter was carried out as per the manufacturer's instructions, except the samples and standards were diluted with 20 mM MES, 20 mM NaCl, pH 6.5.

Competitive Assay for Thiamine. High binding polystyrene microtiter plates were washed with PBS (200 μL/well), then streptavidin at 10 μg/mL in PBS was added (100 μL) to all wells and the plates were stored overnight at 4 °C. The wells were then washed with 3 × 200 μL PBS. ThSu-PEG11-biotin was then added (1.25 μM) in 20 mM MES, 20 mM sodium chloride, pH 6.5 and the sealed plate incubated at 21 °C for 2 h. The wells were washed with 200 μL 20 mM MES, 20 mM sodium chloride, then blocked with 0.1% (w/v) casein, 0.05% (w/v) Tween-20 in 20 mM MES, and 20 mM sodium chloride sealed at 22 °C for 1 h (200 μL/well). The solution was then removed and the wells were washed with 3 × 200 μL MESS (20 mM MES, 200 mM sodium chloride) buffer, then tapped dry onto several layers of Kimwipes. Dilutions of thiamine (10 pM to 100 μM) in MESS were added (50 μL/well). TBP-tagged SRB-encapsulating liposomes diluted to 6.25 μM phospholipid in MESS (50 μL/well) were then mixed with the well contents, plate sealed, and incubated for 1 h at 22 °C on a vortex set at low speed and equipped with a plate adapter. The plate was washed with 3 × 200 μL MESS, then liposomes remaining bound were lysed with 50 μL of 30 mM OG. The fluorescence of the lysed liposomes previously remaining bound to the beads was then read at λ_{ex} = 540/35 nm and λ_{em} = 590/20 nm.

The fluorescence intensities were correlated to thiamine concentrations using a five-parameter logistic (5-PL, eq 1) using XLFit software (IDBS, Bridgewater, NJ):

$$y = b + \frac{a - b}{\left(1 + \left(\frac{x}{c}\right)^{d}\right)^{e}} \quad (1)$$

where a is the response at zero concentration, b is the response at maximum concentration, x is the thiamine concentration, c is the concentration yielding 50% response, d is a slope factor, and e is an asymmetry factor.[41,42] The limit of detection was defined as the concentration equivalent to the background signal minus 3× standard deviation[43] and was determined based on the 5-PL fit of the acquired data. The background signal was the signal resulting from the above steps using MESS without thiamine and was used in all signal-to-noise calculations.

To determine the above conditions, the following optimizations were carried out: ThSu-PEG3-biotin and ThSu-PEG11-biotin concentration varied from 10 nM to 10 μM; TBP-labeled liposome incubation carried out for 15, 30, 60, or 90 min; TBP conjugated using 2.5−15 mol equiv of EDC, and TBP-labeled liposome concentration varied from 0.625 to 50 μM phospholipid. Blocking reagents containing BSA, casein, and Tween-20 were tested and the blocking times were also varied from 15 min at 21 °C to overnight at 4 °C.

To assess specificity, the following compounds were tested at concentrations ranging from 2 nM to 2 mM: thiamine monophosphate, thiamine diphosphate, oxythiamine, fursultiamine, pyrithiamine HBr, thiamine impurity E, thiamine disulfide, 3-benzyl-5-(2-hydroxyethyl)-4-methylthiazolium chloride, toxopyrimidine, thiazole, 4-methylthiazole, 4-methyl-5-thiazoethanol, 4-aminopyrimidine, and biotin.

The thiamine ELISA was carried out as per the manufacturer's instructions, using thiamine and thiamine derivatives as samples diluted in the sample diluent supplied with the kit. The same compounds were tested with the TBP-liposome assay, except with dilution in MESS buffer.

To assess function in environmental water, a sample of natural pond water was filtered first through grade 1 filter paper, followed by 0.2 μm pore size, 13 mm polypropylene syringe filters (both Whatman, Florham Park, NJ). A total of 4 mL of the filtered water was adjusted to a final composition of 20 mM MES, 200 mM NaCl, pH 6.5, then spiked with thiamine concentrations ranging from 0.78 to 50 nM. The same dilutions were made using HPLC grade water prior to analysis, as described above.

■ RESULTS AND DISCUSSION

In this work, we sought to explore the use of periplasmic binding proteins as affinity recognition elements in high-throughput assays for small analytes such as thiamine. Antibodies are usually the first line choice in such affinity-based assay development due to their widespread commercial availability and convenience of established protocols, but can be limited by their expense and restricted utility toward target molecules that are endogenous, toxic, or structurally simple. For small molecule targets, a conjugate of the target with a carrier protein is required to produce an immune response, which places consideration to covalent linkages and carrier protein cross-reactivity. Of the few antibodies that are marketed commercially for thiamine, the manufacturers often report recognition of only the thiamine-BSA conjugate used as an immunogen and not the free vitamin. Even this critical specificity information is often not available from the manufacturers and cannot be derived experimentally without a costly trial and error process. Additionally, such antibodies are prohibitively expensive (as of the date of this publication, $845/100 μg[44]) for use in high-throughput assays. Hence, thiamine is a prime example of an analyte where an alternative to immunological detection is highly desirable.

Here, we chose to pursue the thiamine periplasmic binding protein from *E. coli* based on our recent success demonstrating the exceptional specificity of such PBPs toward simple sugar molecules.[33] Initial experiments intended to build a profile of conditions compatible with TBP were carried out using equilibrium filtration assays. In this approach, binding takes place in solution and unbound thiamine is quantified after separation following filtration through a MWCO membrane chosen to retain TBP. These experiments indicated that in the solution phase, TBP bound thiamine over a wide range of pH values, salt concentrations, and protein, polymer, and surfactant additives (Supporting Information, Figures S-1 and S-2). Thus, thiamine binding by this PBP was highly promising and

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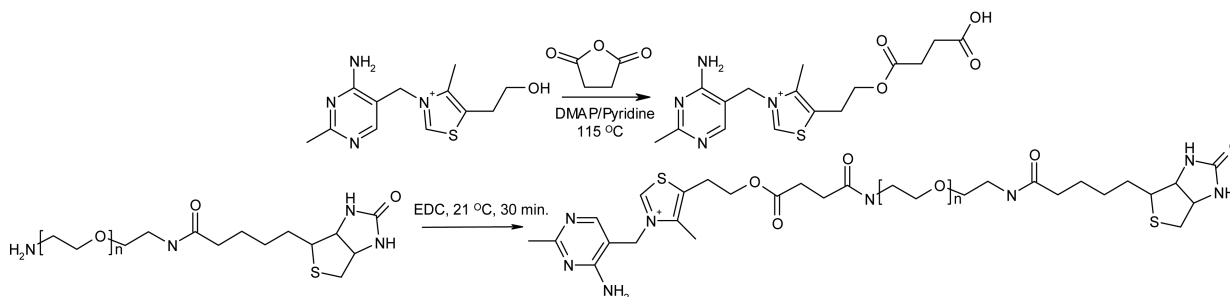
Scheme 1



expected to be compatible with varying conditions in a heterogeneous assay.

Design of a Thiamine Competitor. The aim of this work was to develop a heterogeneous competitive assay format for thiamine analogous to an ELISA, except using a PBP rather than an antibody and liposomes rather than an enzyme for signal generation. As such, either an immobilized or a labeled solution phase thiamine derivative needed to be developed to serve as a competitor. The previously reported crystal structure for ligand-bound TBP indicated that the pyrimidine ring of thiamine monophosphate was situated near the interior of the protein, while its phosphate group resided toward the edge of the binding cleft.[35] Hydrogen bonds reportedly form between various TBP amino acids and the pyrimidine ring nitrogens and amino group as well as phosphate groups on TMP and TDP. Water-mediated interactions with TBP residues occur with the pyrimidine amino group and TMP phosphate group. Here, to assess specificity, both direct binding and competitive binding experiments were carried out with TBP using equilibrium filtration assays. These experiments allowed the critical features of thiamine-related structures for protein recognition to be elucidated so an appropriate thiamine conjugate could be developed for the purposes of a competitive assay.

In direct binding experiments, it was determined that TBP bound thiamine phosphate derivatives in the order of TDP > TMP > thiamine (Supporting Information, Figure S-3). This is consistent with the findings of previous investigators[45] and indicated that modifications to the hydroxyethyl group of thiamine could be tolerated. In competitive binding experiments, structural analogues and fragments of thiamine (Supporting Information, Table S-1) were tested in competition with free thiamine for TBP binding. As the thiamine concentration in the filtrate was assayed via thiochrome fluorescence, it was confirmed in advance that these structures did not form or interfere with the formation of a fluorescent product. At a 10-fold greater concentration than thiamine, there were no competitive effects for thiamine binding by any thiamine fragments, including thiazole and pyrimidine ring derivatives. This confirmed the importance of both the pyrimidine ring and thiazole ring structures in combination for protein recognition. The intact 4-methyl-5-thiazoethanol structure linked to a benzene ring, rather than 4-aminopyrimidine, also yielded no competitive effects. This confirmed the importance of the pyrimidine ring nitrogens and amino group in protein recognition. Further, no competitive effects were observed with oxythiamine which has the same structure as thiamine, but with a carbonyl group rather than the amino group on the pyrimidine ring. Fursultiamine, which contains the 4-aminopyrimidine ring, but an extended chain structure, rather than 4-methyl-5-thiazoethanol, was not recognized, again suggesting the need for both the pyrimidine and the thiazole ring structures. Only thiamine impurity E yielded any competitive binding for TBP, yielding a reduction in thiamine binding from 28% to 36% under the conditions employed in this experiment (Supporting Information, Figure S-4). Thiamine impurity E (otherwise known as thiothiamine) has a thione group linked to the thiazole carbon between its ring nitrogen and sulfur atoms, suggesting that substituents to this ring structure may be more tolerated for protein recognition.

With these findings in mind, a thiamine derivative that ideally maintained both the 4-aminopyrimidine ring and the methylthiazole ring, with modifications to the hydroxyethyl substituent was designed. For covalent modification to surfaces, a functional group amenable to conjugation such as an amine, carboxyl, or sulfhydryl group was required. Several previously published strategies toward synthesizing an appropriate molecule toward this end were investigated, including linkage of the distal phosphate of thiamine diphosphate to amine groups using EDC;[46] targeting the thiamine hydroxyl via NHS under aqueous conditions[47] or via 1,1′-carbonyldiimidazole (CDI) in methanol.[48] Consistent with prior reports,[46] the TDP-amine linkage had poor stability, evidenced here by thiochrome fluorescence of the supernatants from previously washed aminated surfaces increasing over time. This approach is suitable for protein purification where the support can be used soon after preparation, but is not ideal for a solid-phase assay where surface stability is mandatory. Attempting to link the thiamine hydroxyl to NHS-PEG-fluorescein in PBS or to an amine dendrimer in methanol using CDI under previously reported conditions[47,48] did not yield a successful conjugate, perhaps because the hydroxyl groups from the respective solvents in these reactions outcompeted that available on thiamine. However, if the hydroxyl group is instead targeted under anhydrous conditions as reported by Jayamani and Low, it may be derivatized in high yield.[40] By treating thiamine with succinic anhydride under reflux with pyridine in the presence of DMAP, the desired thiamine derivative with an extended carboxylic acid functionality (thiamine monosuccinate) was obtained (Scheme 1) and structure confirmed by MS and NMR analyses (Supporting Information, Figure S-5).

The carboxylic acid group of this derivative was subsequently linked to the amine group at the distal end of a long chain PEG biotin via an amide bond using EDC. The conjugate was purified by size-exclusion chromatography (SEC) and the fractions from the column were monitored using thiochrome fluorescence (Supporting Information, Figure S-6). This was followed by dialysis to remove any residual unreacted thiamine to ensure isolation of thiamine-PEG-biotin. The material was analyzed for its thiamine monosuccinate content by thiochrome

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fluorescence and its biotin content by a commercially available biotin assay, which yielded a 1:1 molar ratio when fully purified.

Development of a Competitive Surface. To minimize steric effects, in the design of the thiamine conjugate, two PEG chain lengths, PEG3 and PEG11, were utilized between thiamine monosuccinate and biotin corresponding to spacer lengths of 22.9 and 53.2 Å, respectively. These conjugates were immobilized onto a streptavidin-functionalized surface at concentrations ranging from 10 nM to 10 μM, then binding by TBP-functionalized liposomes was assessed in the presence and absence of 5 nM thiamine. The results indicate that strong binding occurred to the conjugate with the PEG11 spacer, but that to the PEG3 conjugate was markedly lower (Figure 2).

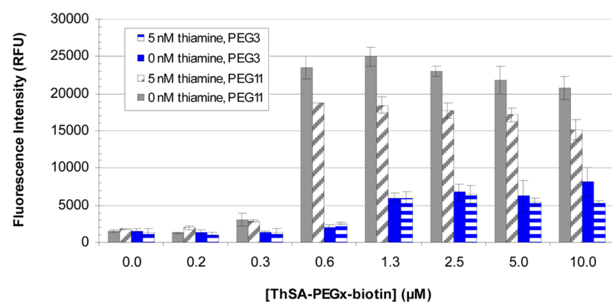

Figure 2. Effect of thiamine monosuccinate-biotin spacer chain length (PEG3 or PEG11) and concentration (0–10 μM) on fluorescence response to 0 and 5 nM thiamine. Each bar represents the average of triplicate determinations with error bars representing the standard deviation of these measurements.

The crystal structure for TBP indicates that its binding cleft for thiamine is ~6 Å wide, 17 Å long, and 12 Å deep.[35] Based on the reported dimensions of the binding cleft, it was anticipated that either of these spacers would allow the PBP to bind to the thiamine moiety while leaving the biotin group on the distal end extending outside of the cleft. However, in the solid-phase assay format utilized here, this biotin group was not free in solution, but rather immobilized to the underlying plate surface via streptavidin. In its interaction with streptavidin, biotin has been shown to be buried deeply within the protein,[49,50] with an average binding site depth of 8.5 Å.[51] As the distance required for streptavidin–biotin binding and the distance required for thiamine–TBP binding is cumulative in this format, it is assumed that the spacer length of 22.9 Å is insufficient to allow for access to both binding sites. Being classified as a group II periplasmic binding protein, TBP undergoes a significant conformational shift upon binding, which also likely places constraints on the accessibility of any immobilized target. However, a spacer length of 53.2 Å was more than adequate to overcome any potential steric or repulsive effects.

Assay Optimization. Of the optimizations carried out (Supporting Information, Figures S-7 and S-8), pH yielded the most significant impact. Binding to the immobilized thiamine-PEG-biotin remained constant over a pH range from 5.0 to 6.5, but the assay was more responsive to solution phase thiamine at pH 6.5 (Figure 3). Higher pH values with this buffer were not tested as the buffering range for MES is between 5.0 and 6.5; however, a marked loss of binding to the immobilized thiamine-PEG-biotin was observed at higher pH values with other buffer types (HEPES and Tris). As the stability of thiamine is reduced at alkaline pH values, the buffer pH was maintained at 6.5 from

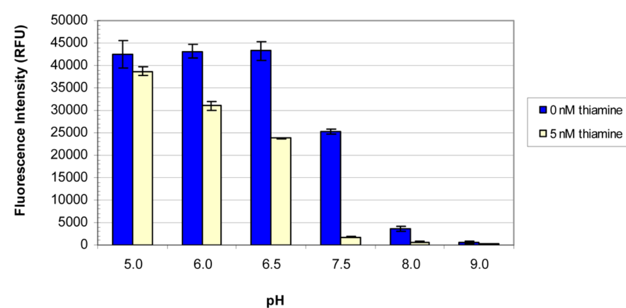

Figure 3. Effect of pH: 20 mM MES at pH 5.0, 6.0, and 6.5; HEPES at pH 7.5; and Tris at pH 8.0 and 9.0 containing 200 mM NaCl were used as both the liposome diluent and the thiamine diluent. Each point represents the average of triplicate determinations with error bars representing the standard deviation of these measurements.

the thiamine monosuccinate-PEG11-biotin immobilization step forward. However, there may be room for improvement in the limit of detection if the pH is increased slightly as the trend indicates more competition with solution phase thiamine at higher pH values.

The fluorescent dye-encapsulating liposomes used for signal amplification here were prepared and conjugated to TBP as described in our previous PBP-based assay for D-(+)-maltose.[33] This particular high cholesterol content, negatively charged lipid formulation has been demonstrated to yield liposomes with exceptional long-term stability.[52] Despite the negative zeta potential (Supporting Information, Figure S-9), these liposomes interacted with the cationic ThSu-PEG-biotin at detectable levels only when conjugated to TBP (Supporting Information, Figure S-8). This indicated a specific binding event between the immobilized thiamine derivative and the TBP liposomes, rather than an electrostatic interaction with the lipids. Incubation of solution-phase thiamine in the μM range with unconjugated liposomes did not yield detectable losses using equilibrium filtration assays. This also suggested that thiamine did not electrostatically interact with the liposomal lipids under the high salt (0.2 M) conditions employed. The competitive assay for thiamine using this formulation functioned with high specificity and sensitivity, but reformulation of the liposomes using PEG to shield the negative charge[53] may be considered if lower limits of detection are needed.

Specificity. The equilibrium filtration experiments assessed the specificity of the protein itself in solution. However, it was necessary to assess the specificity of the complete assay which includes the effect of immobilization on the protein, the relative affinity for the immobilized thiamine derivative, presence of liposomes, and buffer conditions. It was found that in the absence of thiamine monosuccinate-PEG11-biotin, no significant binding to the underlying streptavidin surface occurred (Figure 2). If liposomes that were not conjugated to TBP were employed, no binding to the thiamine monosuccinate-PEG11-biotin/streptavidin surface occurred (Supporting Information, Figure S-8). This indicated that the binding was specific to the thiamine monosuccinate-PEG11 conjugate/TBP interaction, however, the relative specificity of this interaction required investigation.

Consistent with the equilibrium filtration experiments for TBP, the assay yielded a slightly greater response to thiamine monophosphate (TMP) and thiamine diphosphate (TDP; Figure 4a). However, the assay was found to be unresponsive to the constituent fragments of thiamine, including toxopyrimi-

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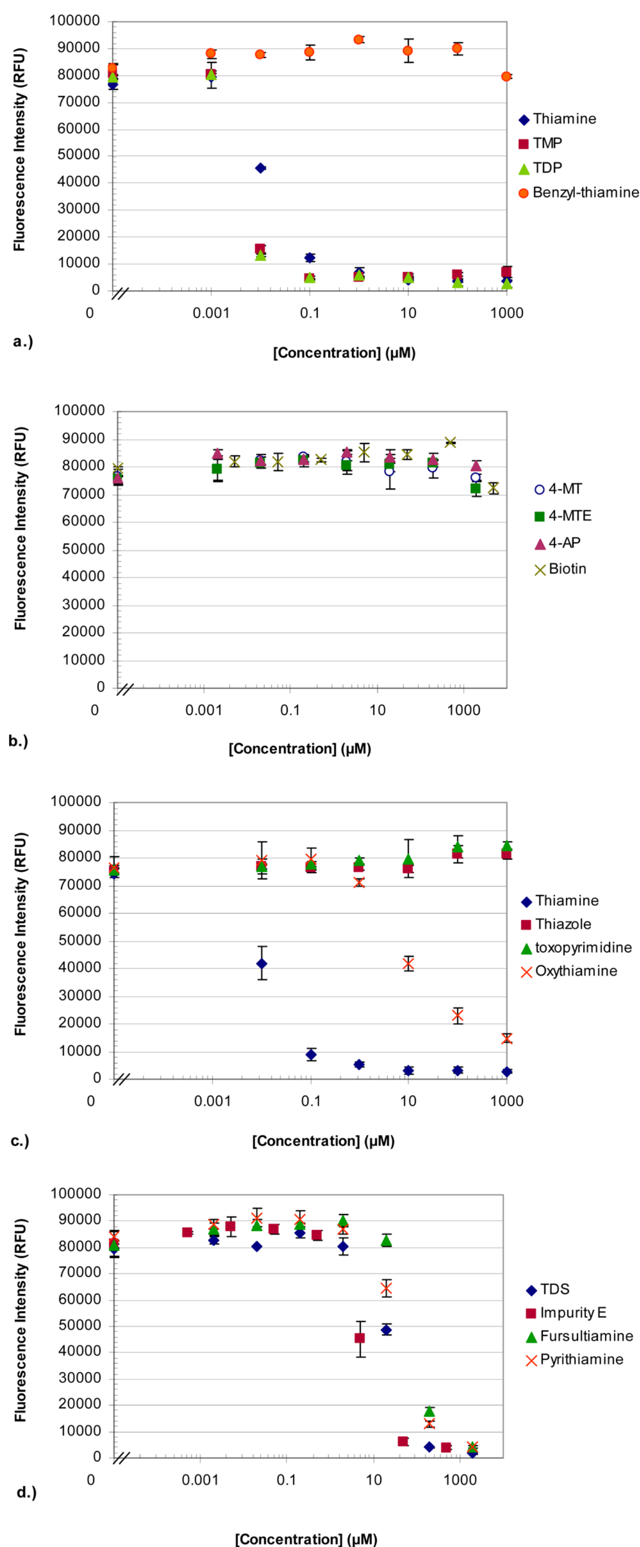

Figure 4. Specificity of the competitive assay to thiamine versus thiamine fragments and derivatives. (a) TMP, TDP, and 3-benzyl-5-(2-hydroxyethyl)-4-methylthiazolium chloride; (b) 4-methylthiazole (4-MT), 4-methyl-5-thiazoethanol (4-MTE), 4-aminopyridine, and biotin; (c) thiazole, toxopyrimidine, and oxythiamine; and (d) thiamine disulfide, thiamine impurity E, fursultiamine, and pyrithiamine. Each point represents the average of triplicate determinations with error bars representing the standard deviation of these measurements.

dine, 4-aminopyrimidine, 4-methythiazole, thiazole, and 4-(β-hydroxyethyl)-5-methylthiazole, which is consistent with the protein's binding specificity as reported previously (Figure 4b,c).[35] This lack of cross-reactivity is very important as it will allow for specific detection of thiamine among thiamine fragments which may be present in biological samples. Similarly, no response was observed to 3-benzyl-5-(2-hydroxyethyl)-4-methylthiazolium chloride at concentrations as high as 1 mM (Figure 4a). Intact structures related to thiamine that are of pharmaceutical interest, including pyrithiamine, fursultiamine, oxythiamine, thiamine disulfide, and thiamine impurity E, showed responses only at markedly higher concentrations (Figure 4d). Of these compounds, consistent with the equilibrium filtration results, the derivative which yielded the response at the lowest concentration was thiamine impurity E, but still only at a 1000-fold greater concentration than thiamine (Figure 4d). As these compounds would not likely be present in environmental or vitamin preparations, their cross-reactivity at such elevated concentrations is not a concern.

Commercially available ELISA kits for thiamine report detection of thiamine at concentrations as low as 1.5 μg/L (5.7 nM).[44] For comparison purposes, an assessment of the specificity of a commercially available ELISA to the same thiamine derivatives used above with the TBP-based assay was attempted. However, while this ELISA kit was marketed to detect thiamine in cell culture supernatants, plasma, tissue homogenates, and serum samples, the results of this experiment indicated that only the proprietary standard supplied with the kit could be detected, not thiamine from a commercial chemical vendor (Supporting Information, Figure S-10). Unfortunately, the identity of the proprietary standard was not provided from the manufacturer upon request. By contrast, the TBP-liposome based assay yielded excellent specificity toward thiamine and thiamine monophosphate, with little cross reactivity toward other thiamine derivatives even at markedly higher concentrations (Supporting Information, Figure S-11).

Application. The optimized assay yielded a limit of detection of 0.5 nM for thiamine and quantifiable range from 1 to 370 nM (Figure 5).

The response of the assay in an environmental matrix was tested by spiking HPLC grade water and filtered natural pond water with known amounts of thiamine. The response was nearly identical in the pure HPLC grade water matrix versus in pond water, the latter of which would be expected to contain both inorganic and organic species (Figure 6).

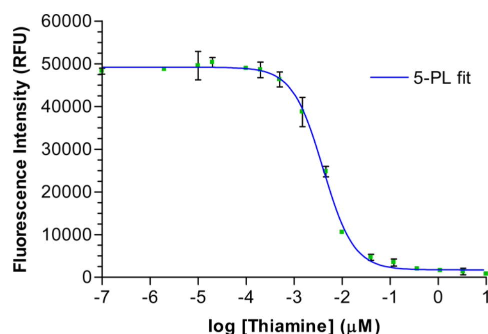

Figure 5. Response to thiamine (0.1 nM to 10 μM). Each point represents the average of triplicate determinations with error bars representing the standard deviation of these measurements. The five-parameter logistic fit of the data is shown as the curve.

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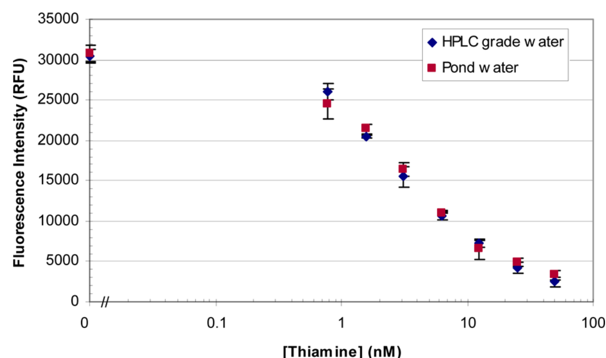

Figure 6. Response of the assay to thiamine spiked into HPLC grade water and filtered pond water, both adjusted to a final composition of 20 mM MES, 200 mM NaCl, pH 6.5. Each point represents the average of triplicate determinations with error bars representing the standard deviation of these measurements.

CONCLUSIONS

We have demonstrated the successful development of a high-throughput competitive microtiter plate assay for thiamine relying on periplasmic binding proteins and liposomal signal amplification. The assay described within is the second successful application of fluorescent dye-encapsulating liposomes as signal enhancement reagents in conjunction with PBP-based biorecognition, giving strong evidence that this is a useful approach for analytes refractory to traditional antibody-based analyses. Thiamine is but one example of an endogenous small molecule to which immunological detection is not possible, yet its concentration is critical to both human and environmental health.

We anticipate that the sensitivity and high-throughput nature of this assay will have significant utility for analyses especially in the environmental realm. The competitive assay itself could be completed in as little as 30 min including washing and lysis steps and, as there are no time-based substrate conversions or bead-based immobilizations involved, it could readily be expanded to a 384-well plate format for higher throughput. In preliminary testing, no matrix interference was observed in natural pond water versus standard HPLC grade water. The range of detection is suitable for the analysis of thiamine levels in fish eggs, which is of particular interest due to increased morbidity and mortality of fry in bodies of water ranging from the Great Lakes to the Baltic Sea.[8−11] Thiamine is also an important growth factor for algae, including those responsible for harmful algal blooms.[54,55] A preconcentration step or further assay optimization will be needed for direct measurement of thiamine levels in environmental water samples, as these are typically less than 0.2 nM. We have previously demonstrated the use of a similar liposome formulation to that employed here for the quantification of CD4+ T-cells isolated from whole blood using a sandwich immunoassay.[53] With a competitive assay, the liposomes are in direct contact with the sample matrix rather than being introduced after multiple wash steps. Should matrix constituents of complex environmental or clinical samples cause liposome lysis, one strategy to employ would be to separate the protein and liposome binding steps by relying on a biotin−streptavidin interaction rather than the currently used covalent linkage and instead use a covalently immobilized thiamine derivative for the plate surface. The present negatively charged liposome formulation has been demonstrated previously to have excellent long-term stability.[52] Although not evidenced in the work within, with the negatively charged formulation, it remains conceivable that some of the thiamine that is present at low levels may be lost due to electrostatic interaction with the lipids themselves, rather than via specific interaction with the attached TBP. For future work, the limit of detection may be improved through inclusion of PEGylated lipids in the liposome formulation which would serve to shield the negative charge.

Our future work will focus on application of this assay and determination of its benefits and limitations in various environmental, clinical, and food matrices.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b02092.

Additional information including liposome preparation details, specificity results, characterization, assay optimization, and zeta potential data (PDF).

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS:

BSA: bovine serum albumin; DPPC: 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPG: 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)], sodium salt; EDC: 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide; LOQ: limit of quantitation; PBP: periplasmic binding protein; TBP: thiamine binding protein; MES: 4-morpholineethanesulfonic acid sodium salt; OG: *n*-octyl-*β*-D-glucopyranoside; PBS: phosphate buffered saline; SRB: sulforhodamine B

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