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A Platform for the Detection of Trypanosomes via Selective Small Molecule Recognition

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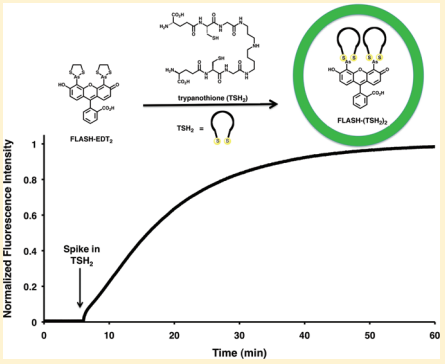
Supporting Information

ABSTRACT: Trypanothione (TSH_2), a metabolite unique to trypanosomal parasites, was evaluated as a potential biomarker for trypanosomal infection using fluorescence as the means of detection. Fluoroescein arsenical helix binder (FLASH) was prepared and used to detect TSH_2. Since it has low background fluorescence and forms a highly emissive complex with TSH_2, it can be used to detect low micromolar concentrations of TSH_2 in serum. The large dynamic range of FLASH and its selectivity for detection of the dithiol metabolite indicate that arsenical probes may offer a promising new platform for the diagnosis of trypanosomal infection.

KEYWORDS: Trypanosomes, trypanothione, Chagas' disease, human African trypanosomiasis, African sleeping sickness, leishmaniasis, diagnostic, fluorescein arsenical helix binder (FLASH)



Three devastating human diseases are caused by parasitic protozoa of the order Kinestoplastida and the family Trypanosomatidae: Chagas' disease (*Trypanosoma cruzi*), Leishmaniasis (*Leishmania* spp.), and human African trypanosomiasis (HAT; *Trypanosoma brucei*). The World Health Organization estimates that there are 40000 new cases of Chagas' disease, 2 million new cases of leishmaniasis, and 70 000 new cases of HAT each year.[1−7] Collectively, estimates indicate that these three diseases cause tens of thousands of deaths annually.[4,5,7,8] These diseases are all termed neglected tropical diseases (NTDs) since they primarily affect people in rural areas or urban slums where resources are limited. Considering their impact on child health, pregnancy outcomes, and worker productivity, they are a major reason that many in these regions cannot escape poverty.

Current diagnostic techniques fall into two classes: parasitological (those that detect the parasite directly) and immunological (those that detect markers from a patient's immune response).[2,7,9] The former are highly specific but are only sensitive enough to provide a diagnosis in instances of high parasitemia (typically during early or acute infection).[2,6,8,9] Immunological methods are highly sensitive for diagnosing disease when levels of parasitemia are low (typically during an indeterminate or chronic infection), but they are not always predictors of clinical status due to false positives from nonpathogenic parasites, persisting antibodies from prior infections, or subclinical infections.[7,9,10] Diagnosis for trypanosomal infections is further complicated by the dramatic variations in their clinical presentation and, in the case of leishmaniasis, the number of pathogenic species that can cause the human disease.[9] Since clinical symptoms for these diseases may not manifest until months after exposure, the need to develop diagnostics that function long before chronic symptoms emerge is urgent.

We are developing a new parasitological screening method that detects a parasite-specific metabolite trypanothione (TSH_2, **1**) as a biomarker for infection. Trypanosomal parasites maintain an intracellular reducing environment using a redox pair that is unique to this family: TSH_2 and trypanothione reductase (TyR).[11,12] This pair is analogous to that of glutathione and glutathione reductase in the mammalian host. The putative mechanism of action for heavy metal therapeutics is either the sequestration of TSH_2 or the inhibition of TyR by the TSH_2−metal conjugate.[13,14] Since TSH_2 is a dithiol metabolite with bidentate binding capacity, heavy metals preferentially bind the metabolite rather than endogenous monothiols such as glutathione and *N*-acetyl cysteine. We aim to harness the specificity of this pharmacologically relevant target in the development of a parasitological diagnostic. Since this metabolite is required for redox homeostasis in this family of parasites, it is present at high levels in all three human pathogens.[15] Therefore, our new parasitological screening method should function as a platform diagnostic for all three types of human trypanosomal infections.

Given that arsenical therapeutics have affinity and selectivity for the intracellular dithiol TSH_2, we hypothesized that arsenical probes could be used for the detection of trypanosomal parasites (Scheme 1). We selected the fluorescein arsenical helix-ethane dithiol (FLASH-EDT$_2$) probe (**2**) to test this hypothesis. FLASH-EDT$_2$ originally was developed for the in vivo imaging of peptides engineered to contain a tetracysteine motif.[16] When unbound, FLASH-EDT$_2$ (**2**) is virtually nonfluorescent. When it binds the tetracysteine peptide, free rotation about the As−C bond is

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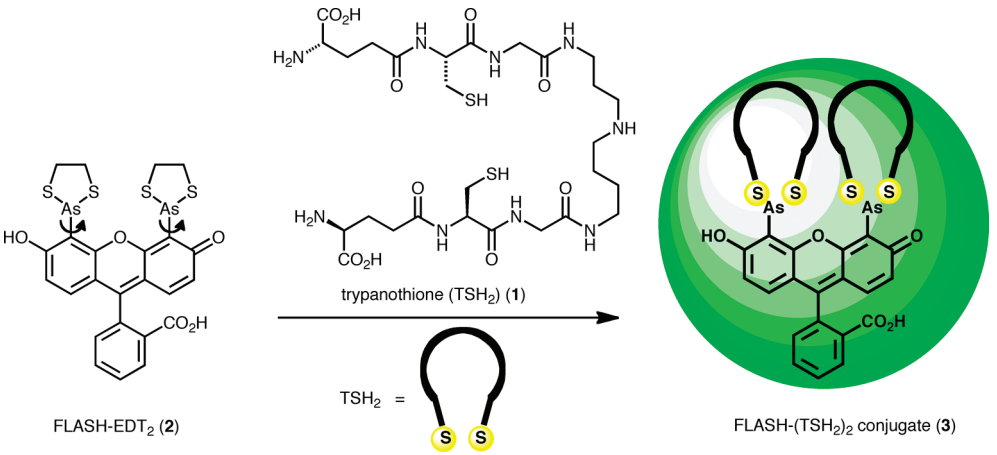
Scheme 1. Fluorimetric Response on Binding of TSH_2 to FLASH-EDT_2



restricted, and the dye becomes brightly fluorescent. Since this visualization method works to label proteins inside cells with little background fluorescence, we hypothesized that this sensor could be used for diagnostic purposes without interference from nontrypanosomal thiols. The binding of two molecules of TSH_2 should provide sufficient bulk to prevent the free rotation about the As−C bond in FLASH, causing a fluorimetric response. This strategy exploits the thiophilic nature of the arsenic nucleus in the detection of the biomarker TSH_2 for the diagnosis of trypanosomal infection.

FLASH-EDT_2 was synthesized according to literature protocols.[17,18] To synthesize TSH_2, we treated commercially available trypanothione disulfide (TS_2) with tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl). This reagent is attractive for this application because it is a water-soluble reagent and reduces disulfides rapidly over a wide pH range.[19]

FLASH-EDT_2 was treated with an excess of TSH_2 under the conditions used for the visualization of the engineered peptides (see the Supporting Information). FLASH-EDT_2 was dissolved in 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer. FLASH-EDT_2 was virtually nonfluorescent, but the fluorescence intensity immediately increased after introduction of TSH_2, and the reaction reached saturation within 1 h. Using the normalized fluorescence measurements (Figure 1), we observed that the FLASH-EDT_2 reagent had 0.45% of the fluorescence of the FLASH-$(TSH_2)_2$ conjugate. The identity of the fluorescent species was determined to be FLASH-$(TSH_2)_2$ using HPLC with fluorescence detection in conjunction with LCMS data (see the Supporting Information). The binding of TSH_2 was completely reversible by the addition of a large excess of EDT.

The excitation and emission maxima for the conjugate were 505 and 527 nm, respectively, which are 20 nm longer than fluorescein itself. The quantum yield for our complex was determined using fluorescein in 0.1 N NaOH (ϕ = 0.95) as the standard.[20] The quantum yield of the FLASH-$(TSH_2)_2$ complex was 0.24. The quantum yield for the FLASH-$(TSH_2)_2$ was lower than that reported for the complex of FLASH with tetracysteine peptides.[16] This could be due to the difference in restricting As−C bond rotation by the steric hindrance of two molecules with bidentate binding in the former versus unimolecular tetradentate binding in the latter.

We also performed binding experiments using the monothiol sodium mercaptoethanesulfonate (MES). Earlier work with

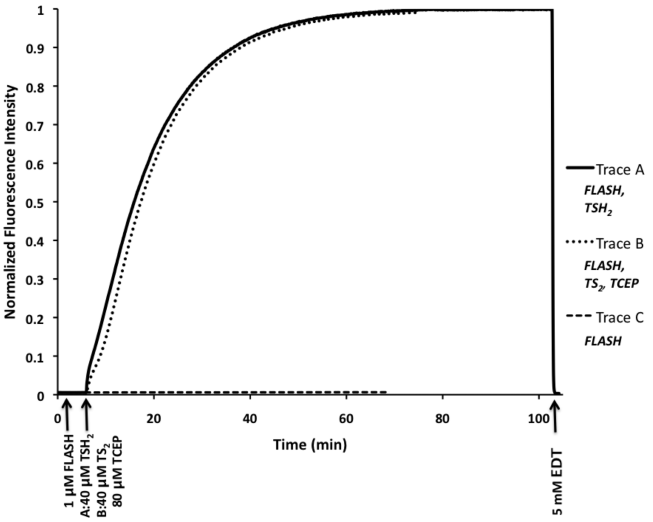


Figure 1. Fluorimetric response on TSH_2 binding. A comparison of the rate for conjugation with in situ reduction of TS_2 vs addition of the reduced substrate TSH_2 illustrates that the reduction by the phosphine is facile and does not impact the time for detection. (Trace A) To a 1.0 μM solution of FLASH-EDT_2 in MOPS buffer at pH 6.94 was added 40.0 μM TSH_2. Full conjugation was achieved within 1 h. Binding was reversed within 30 s by the addition of 5.0 mM of free EDT. (Trace B) To a 1.0 μM solution of FLASH-EDT_2 and 40.0 μM TS_2 in MOPS buffer at pH 6.94 was added 80 μM TCEP·HCl. Full conjugation was achieved within 1 h of development. (Trace C) The background fluorescence of a 1.0 μM solution of FLASH-EDT_2 in MOPS buffer at pH 6.94.

FLASH-EDT_2 indicated that the efficient labeling of tetracysteine peptides typically required the presence of monothiols in low millimolar concentrations.[21] We observed that the presence of low millimolar concentrations of MES decreased the rate of conjugation for TSH_2 to FLASH-EDT_2 and reduced the observable fluorescence intensity by over 20% (see the Supporting Information). These experiments illustrated the selectivity of FLASH-EDT_2 for the dithiol metabolite TSH_2.

In a field setting, a clinical sample could contain both the dithiol metabolite TSH_2 and the oxidized metabolite TS_2. To mimic field detection, we monitored reaction progress for the reduction of TS_2 and concomitant binding to the arsenical

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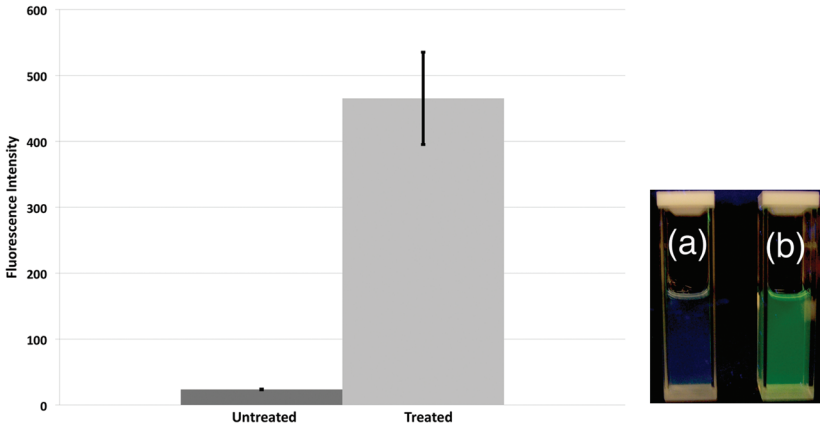

Figure 2. Conjugation of TSH_2 from serum extracts. The fluorescence of untreated serum sample is less than 10% that of the treated serum sample. This difference is visible with a hand-held UV light source (λ_{ex} = 365 nm). The left sample (a) contains the serum extracts from the vial that did not receive TS_2. The sample on the right (b) contains the serum extracts from the treated vial and exhibits the characteristic yellow-green fluorescence.

probe (Figure 1, trace B). In this reaction, FLASH-EDT_2 was preincubated with TS_2, and no increase in fluorescence was observed. When TCEP·HCl was added, the fluorescence intensity immediately began to rise. While the initial rate was slower than that of TSH_2 alone, the reaction reached saturation within 45 min. This indicated that the reduction with the phosphine is rapid and does not limit the rate of development of the fluorimetric response. Neither the phosphine nor TS_2 alone generated a fluorimetric response in the presence of FLASH-EDT_2 (see the Supporting Information).

Since the parent dye FLASH-EDT_2 has 0.45% the fluorescence of the conjugate, we observed a clear "on" response that could be used to indicate the presence of blood-borne trypanosomal parasites. To test the feasibility of detection of this metabolite in biological samples, we spiked rat serum with TS_2. Since proteins present in the blood produce a large background fluorescent signal, we precipitated the protein matter and used the extracts for detection in serum. This also eliminated the known thiol-independent fluorogenic response of FLASH-EDT_2 with albumin.[21] Following precipitation with acetonitrile (MeCN) and centrifugation, serum extracts were treated with TCEP·HCl and stirred for 0.5 h, and the fluorescent dye was added. While the rate of conjugation was slower in serum extracts, the "on" response was detectable within 20 min. The increase in fluorescence was readily detectable without a fluorescence spectrometer using a simple hand-held UV lamp (λ_{ex} = 365 nm, Figure 2b). Only the vial to which TS_2 was added exhibited the yellow-green fluorescence. The ability to use a simple hand-held lamp provides a low-technology detection method that could be implemented in a resource-poor setting.

We examined the limits of detection for TSH_2 with FLASH-EDT_2 (Figure 3). In our liquid−liquid method, we found that a 10-fold excess of the metabolite was sufficient to generate an observable fluorimetric response within 30 min. Although lower concentrations were detectable using a fluorescence spectrometer, a 10% increase in fluorescence was not perceptible using the hand-held UV lamp, and the conjugation was too slow to be practical. The lower limit of detection for this solution-based assay using FLASH-EDT_2 in 1 μM concentration is 10 μM TSH_2.

FLASH-EDT_2 provides a visible fluorimetric response capable of detecting low concentrations of the parasite metabolite TSH_2

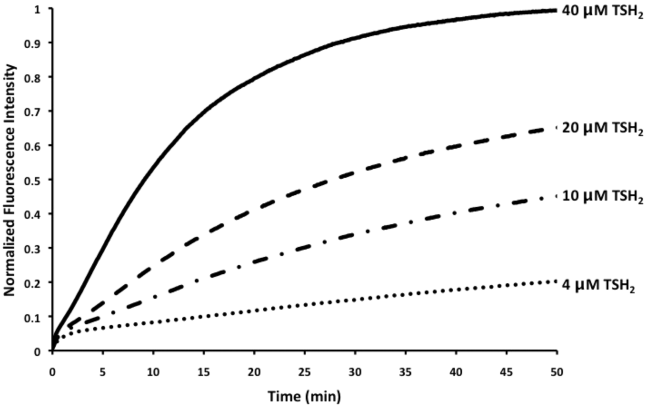

Figure 3. Limit of detection of TSH_2 with FLASH. A 1.0 μM concentration of FLASH-EDT_2 was dissolved in MOPS buffer, pH 6.94. Different amounts of TSH_2 were added, and binding was monitored. The 10-fold increase in fluorescence in the presence of 10.0 μM TSH_2 was visible both with a fluorimeter and a hand-held UV lamp (see Figure 2b). The addition of 4.0 μM TSH_2 was not perceptible with the hand-held device.

using a simple hand-held UV light source. However, this probe is not optimized for this purpose. We are developing next generation probes that have better optical properties and faster development times for binding TSH_2. We are also examining the use of solid-supported arsenical probes for the detection of TSH_2 to obviate the need for a protein precipitation step that increases the instrumentation required for field applications. We are confident that exploiting the molecular recognition of arsenic for TSH_2 can lead to a new, rapid field diagnostic for the early detection of these devastating parasitic diseases and, thus, could have a significant positive impact in global health.

■ ASSOCIATED CONTENT

Supporting Information. Experimental details of binding experiments, quantum yield determination, and limit of detection experiments. Contour plot used to determine maxima for λ_{ex} and λ_{em} (Figure S1), results of additional competitive binding experiments with FLASH (Figure S2), competitive

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binding experiments in the presence of the monothiol MES (Figure S3), HPLC chromatograms of FLASH-EDT_2 with UV and fluorescence detection (Figures S4 and S5), LCMS chromatogram with mass spectrum for FLASH-EDT_2 (Figure S6), HPLC chromatograms from the reaction mixture of FLASH-TSH_2 with a 10-fold excess of TSH_2 with both UV and fluorescence detection (Figures S7 and S8), LCMS chromatograms and mass spectra for the three UV active peaks (Figures S9−S11). This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

TSH_2, trypanothione; FLASH, fluorescein arsenical helix binder; HAT, human African trypanosomaisis; NTDs, neglected tropical diseases; TyR, trypanothione reductase; EDT, ethane dithiol; TS_2, trypanothione disulfide; TCEP·HCl, tris(2-carboxyethyl)-phosphine hydrochloride; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MES, sodium mercaptoethanesulfonate; MeCN, acetonitrile

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