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An Abiotic Fluorescent Probe for Cardiac Troponin I

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Supporting Information

ABSTRACT: The first ratiometric fluorescent reporter was designed for the detection of cardiac troponin I (cTnI), a key protein elicited during cardiac muscle cell death. In designing this abiotic fluorescent probe, docking simulation studies were performed to predict the probe/protein interactions along the solvent exposed regions of cTnI. Simple cuvette titration experiments in aqueous buffered solution indicate remarkable selectivity for cardiac troponin in the clinically relevant nM region versus skeletal troponin.

Early clinical detection of molecular biomarkers in serum is the hallmark for point-of-care-testing (POCT) in disease diagnosis. Serum markers can reveal significant information about the onset and progression of many life-threatening diseases ranging from heart attacks to malignancies.[1,2] Methods currently available for detection of disease biomarkers include fluorescence immunoassays,[3] enzyme-linked immunosorbent assays (ELISAs),[4] PCR approaches,[5] biobarcode assays,[6] immunomagnetic diffractometry,[7] and nanoparticle-based biosensors.[8] Despite the diversity of available detection platforms, an underlying clinical bottleneck common to any immunoassay is the requisite incubation time, which in cases such as acute myocardial infarction (AMI) results in the loss of precious time. Currently 85% of patients admitted to an emergency department with possible AMI do not have AMI. Nonetheless, the obligatory "rule-out" process is both costly and time-consuming.[9] On the other hand, approximately 5% of patients with AMI are discharged mistakenly from the ER, often with severe consequences. Following the myocardial damage, the troponin complex is broken up and the individual proteins are released into the bloodstream. Because of its high specificity and its presence in serum within 90 min−3 h upon symptom onset of acute myocardial infarction, cardiac troponin I is considered the "gold standard" marker for AMI.[10] Troponin I levels rise in serum within 3 to 12 h upon the onset of acute myocardial infarction and remain elevated for five to nine days. A positive test for detection of cardiac events occurs when levels of cTnI in serum samples reach levels between 5 and 50 ng/mL (5−50 ug/L).[11]

A paradigm shift in the clinical approach to diagnosis of AMI as well as related problems such as unstable angina would obviate the immunoassay method in favor of an abiotic, highly specific reagent. Toward this end, we disclose the concept and development of an abiotic fluorescent probe specific for cTnI. Utilizing principles recently forged in the area of bioinformatics, an abiotic fluorescent marker is conceived for simple and rapid detection of cTnI. Bioinformatics, in combination with published X-ray or NMR structures, allows for targeting of specific protein regions and incorporating recognition elements onto a unique fluorescent platform.[12] Thus, the development of an "off the shelf" fluorescent probe requiring no incubation time and possessing specific recognition elements for cTnI would hold distinct advantages over existing methods.[13]

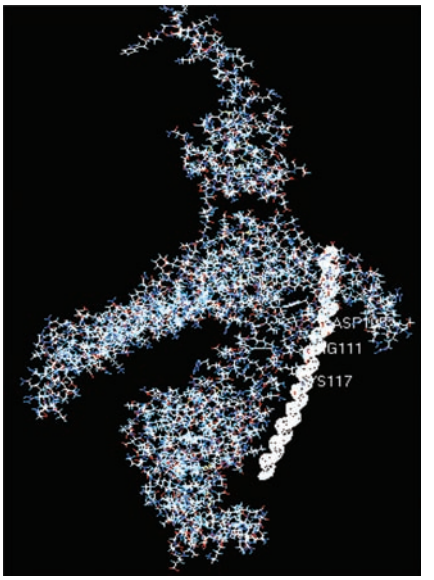

Figure 1. Crystal structure of cardiac troponin I highlighting solvent-exposed α-helical surface.[17]

According to recent reports by us[14] 2,3-naphthalic imide chromophores can be designed to display two emission wavelengths with appropriately placed substituents. In addition, these compounds demonstrate a large change in fluorescence intensity to different solvent polarities. In this regard, their photophysical properties resemble PRODAN, a widely used fluorescent probe for measuring micropolarity. Among the various fluorescent dye platforms from which to assemble our biomarker, 2,3-naphthalic anhydrides feature a high aspect ratio similar to an α-helical secondary structure. Indeed, prior results indicate that *N*-aryl-2,3-naphthalimide (2,3-NI) displays much higher fluorescence sensitivity to helical secondary structures in comparison to other chromophore structures, notably PRODAN.[15]

Starting with a BLAST search of human toponin I cardiac muscle, protein sequences with similarities to slow-twitch and fast-twitch skeletal muscle (sTnI) isoforms were obtained in our search for unique regions of cTnI (Supporting Information).[16] Following skeletal muscle damage, both sTnI proteins become an interfering analyte and can exist in human sera at concentrations equivalent to cTnI. Sera proteins such as serum albumin

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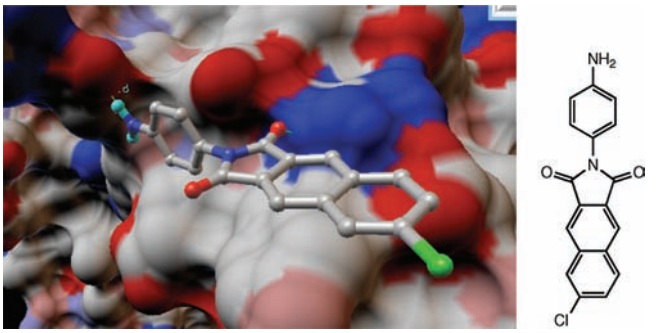

Figure 2. (a) Autodock 4.0 depiction of crystal structure of human cTnI with dual fluorescent dye **1**. (b) Structure of **1**.

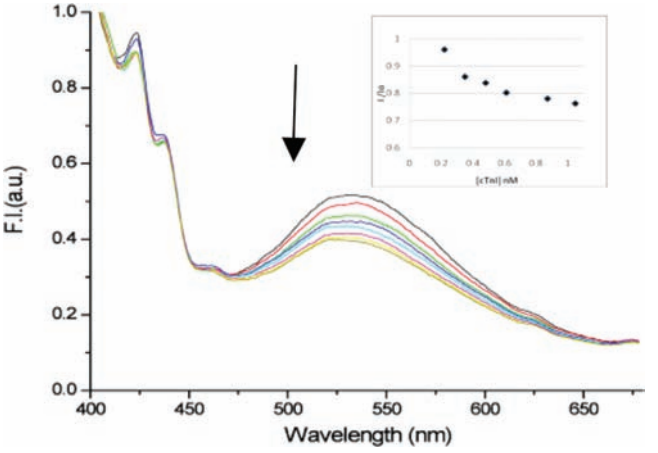

Figure 3. (a) Dual fluorescent dye **1** (2.6 μM) titrated with 5−20 μL aliquots of cardiac troponin I (cTnI, 20 μg/mL; aqueous buffered solution).

and cytochrome C were not included as these components are routinely separated in troponin immunoassays.[13] Utilizing the crystallographic data[17] and the aid of Deep View,[18] Figure 1 depicts a 3-D structure of cTnI as well as the accessibility of a protein sequence to solvent. The section that is marked as a white helical ribbon represents the greatest surface region exposed to solvent. Based on the fluorescence response of 2,3-NI to helical surfaces,[15] computational docking experiments on suitably substituted versions were conducted with Autodock 4.0. Figure 2 shows an overlay of the top ranked docking result. A key correlation was observed, specifically that the designed probe docked in the region that our bioinformatical method (graphically illustrated using DeepView) had indicated to be solvent exposed. Energy minimization calculations for cTnI, probe **1**, and docking studies using AutoDock 4.0 with Lamarckian Genetic Algorithm generated a binding energy value of −4.58 Kcal/mol. Docking interactions, though fully depicted in the Supporting Information, indicate multipoint interactions. Specifically, these interactions consist of the reporter **1** amino group and aspartic acid-105 of cTnI, imide carbonyl of **1** with arginine-111 amide hydrogen, and chlorine of **1** with the guanidinium group of arginine-111. Secondary lipophilic/π-interactions with reporter **1** involve lysine-117 and tyrosine-112, respectively.

Having previously prepared fluorescent probe **1** and reported its photophysical features,[14b] we briefly point out that its optical

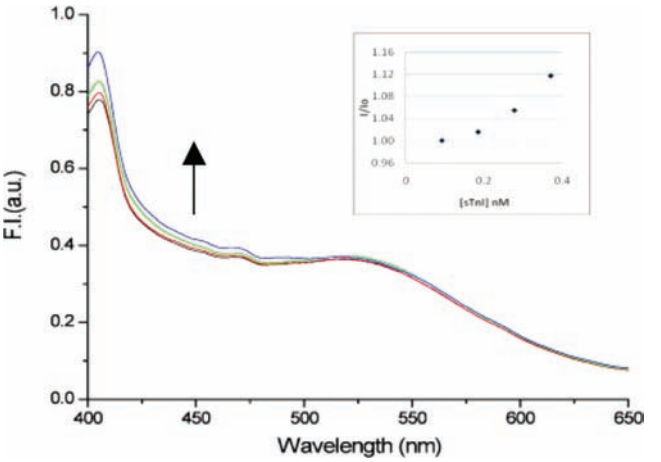

Figure 4. Dual fluorescent dye **1** (2.6 μM) titrated with 5−20 μL aliquots of skeletal troponin I protein (sTnI, 10 μg/mL; aqueous buffered solution).

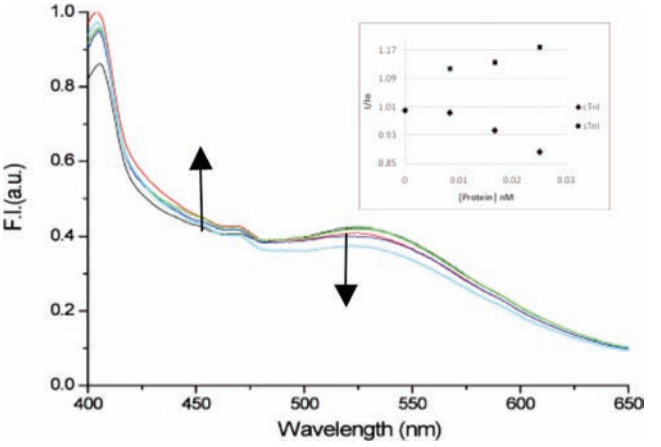

Figure 5. Dual fluorescent dye **1** (2.6 μM) titrated with 10 μL amounts of both cardiac and skeletal troponin I (cTnI 20 μg/mL, 10 μg/mL respectively) mixed aqueous buffered solution.

absorbance spectrum exhibits λ_{max} in the violet region of the visible spectrum (~390 nm), well removed from the optical absorption range of cardiac troponin I (λ_{max} ~280 nm). Moreover, reporter **1** possesses the unusual yet advantageous feature of dual fluorescence (two well-separated emission bands), an optical feature that provides ratiometric detection. Following a simple cuvette assay, 2.6 μM of compound **1** was prepared from an aqueous solution of 0.10% DMSO. The photophysical properties of **1** were obtained in phosphate buffer solutions (100 mM, pH = 7.1). In Figure 3, the addition of cardiac troponin I (cTnI, 5−20 μL aliquots) to the buffered solutions of **1** (2.4 μM) causes strong fluorescence intensity quenching in the presence of cTnI. Benesi−Hildebrand analysis of these data leads to a K_d value of 0.92 nM. In the presence of skeletal toponin I (sTnI, 10 μg/mL), **1** displayed the opposite response with increasing fluorescence intensity (Figure 4) and a K_d value of 1.10 nM. Finally, mixed solutions using cTnI and sTnI in various concentrations were titrated with reporter **1**. The addition of 10 μL aliquots each of sTnI and cTnI to the buffered solutions of reporter **1** caused a ratiometric response in the fluorescence spectrum (Figure 5). Thus, according to these titrations involving mixed troponins,

the ratiometric fluorescence response of **1** clearly indicates a difference between cTnI and sTnI. Though untested in human sera, reporter **1** appears to be highly selective between these two isoforms and presents a promising abiotic probe with ratiometric spectral features.[11]

Because α-helices and β-sheets mediate a myriad of protein−protein interactions, α-helices on protein surfaces represent one of the most common structural motifs and recognition sites. Therefore, small molecule fluorescent biomarkers have potential clinical applications since they have a specific affinity for helical surfaces. This study provides the first successful demonstration of how an initial bioinformatical analysis led to a key solvent-exposed protein region suitable for design and synthesis of a small fluorescent probe. Unlike conventional chemosensors, where the recognition component is designed to coordinate or complex an ion or small molecule, here the reporting system is orders of magnitude smaller than the analyte of interest. The preliminary nature of these findings opens up tremendous possibilities for nonimmnunological probe design, not only for cardiac troponins but other proteins possessing α-helical regions.[19]

■ ASSOCIATED CONTENT

Supporting Information. This includes bioinformatical analysis, Autodock data, and graphical analysis of titration data. This material is available free of charge via the Internet at http://pubs.acs.org.

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