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Toward intrinsically fluorescent proteomimetics: Fluorescent probe response to alpha helix structure of poly-γ-benzyl-L-glutamate

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Abstract—A fluorescent probe (**1**), developed for recognition of alpha helical secondary structure, shows a large fluorescence change upon titration with the synthetic protein PBLG. Compared to fluorophores of similar size and shape, **1** displayed the smallest dissociation constant (K_D = 80 μM) when titrated with PBLG. These preliminary studies are directed toward developing small molecule proteomimetics that have intrinsic fluorescence and are specific for helical-protein binding-sites.

Currently, there is considerable interest in developing molecular scaffolds that can effectively mimic protein secondary structures. Because β-sheets and α-helices mediate a myriad of protein-protein interactions, a number of small-molecule mimetics have been designed to compete as sequence-selective bioactive analogs.[1] As α-helices comprise over 40% of polypeptide amino acids in natural proteins, they represent the most common protein secondary structure.[2] Therefore, the potential clinical applications for fluorescent biomarkers designed to have a specific affinity for helical surfaces are far reaching given the number of protein-protein interactions involving α-helices.[3] The development of such small-molecule biomarkers would hold distinct advantages over current methods involving immunoassays where antibody incubation time, cross-reactions, and mixed epitope regions can hamper clinical findings.[4]

Recent findings from Hamilton's group have highlighted the use of terphenyl derivatives as structural and functional mimics of extended regions of α-helices. Their work demonstrated the similarities in structural motif of tris-functionalized 3,2′,2″-terphenyls to the *i*, *i* + 3, and *i* + 7 side chain residues along one face of the α-helix.[5–7] The critical interactions of α-helical side chains compare well with the 3,2′,2″-substituents when the terphenyl is in a staggered conformation with dihedral angles of 68° between aromatic rings. Because the terphenyl system absorbs at λ_{max} of 260 nm and emits at λ_{max} of 350 nm, its photophysical features lie within the absorption and emission range of the proteins under investigation.[8] To derive binding affinity information between the targeted helical peptide and the proteomimetic, fluorescent labeling of the peptide has provided evidence via (1) fluorescence polarization using a fluorescein-tagged protein to observe displacement of the probe, or (2) titration of a terphenyl proteomimetic with a dansylated heterodimer complexed with the targeted helix.[4,6] Both cases require modification of the native protein.

To allow for direct observation of a proteomimetic's interaction with a protein surface, an intrinsically fluorescent ter-aryl system would obviate the requirement of protein labeling and potentially serve as a fluorescent marker for optical detection of clinically important proteins. An intrinsically fluorescent proteomimetic removes additional fluorophores from the protein/proteomimetic interaction; a current problem in imaging technology.[9] Potential applications for probes with these features are envisioned in the rapid detection of proteins with exposed helical surfaces such as cardiac troponin I released at the onset of cardiac arrest.[4]

N-Arylnaphthalimides represent a diverse class of fluorescent compounds which bear a close structural resemblance to terphenyl compounds. Their photophysical properties, in particular the fluorescence emission, have been found to be very sensitive to substituent groups on the naphthalene ring.[10] As fluorescent biomarkers, such features are especially useful in providing an optical signal beyond the blue emission of autofluorescence.

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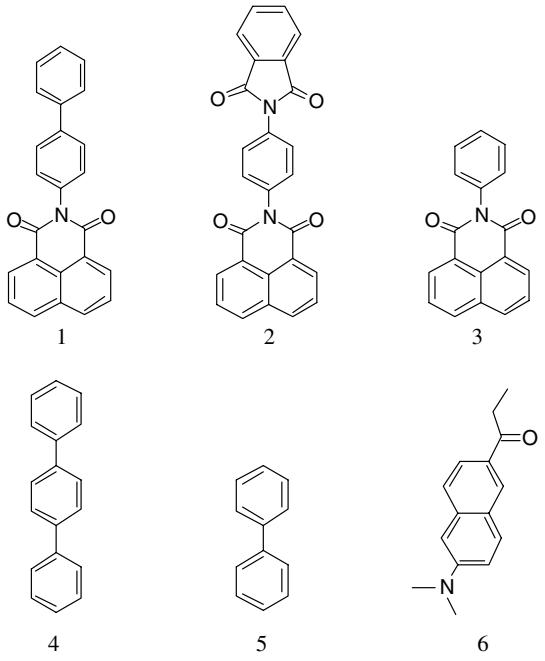

Figure 1. Structures **1**–**6**, respectively, *N*-4,4′-biphenyl-1,8-naphthalenedicarboximide, *N*-1-(phthalimido)-*N*′-4-(1′,8′-naphthalimido)benzene, *N*-phenyl-1,8-naphthalene dicarboximide, *p*-terphenyl, biphenyl, and Prodan.

In addition, these compounds display large changes in fluorescence intensity with solvents of different polarity. In this regard, their photophysical properties resemble Prodan, a widely used fluorescent probe for measuring micropolarity.[11] By extending the *N*-arylnaphthalimide core to an *N*-biarylnaphthalimide platform, a prototype for a terphenyl analog is conceived that is intrinsically fluorescent and highly sensitive to solvent polarity. Fluorescent probe **1** was readily prepared with 1,8-naphthalenedicarboxylic anhydride and 4-aminobiphenyl.[†] Figure 1 indicates the structural similarities between *p*-terphenyl and compound **1** along with structurally related probes such as Prodan, *N*-phenyl-1,8-naphthalic carboximide, and biphenyl used in our initial study with a helical peptide.

To assess the fluorescence response of these probes with an α-helical secondary structure, the well-studied synthetic polypeptide, poly-γ-benzyl-L-glutamate, was selected for its rigid coil morphology and co-solubility with probes **1**–**6** in ordinary organic solvents.[12] PBLG has attracted considerable attention as a building block for the development of self-assembled materials.[13] More recently, such synthetic peptides have found use as biofoul-resistant polymers for medical devices.[14] Terphenyl, which when decorated with recognition groups represents the current aromatic scaffold for peptide recognition, displayed erratic change in its 300 nm fluorescence (see Supplementary data) when titrated with PBLG. Of the six systems used in our investigation, Figure 2 shows dramatic quenching of the probe's 420 nm emission upon titration of fluorescent probe **1** with PBLG (MW 20,000) solution in dichloromethane. The emission spectrum of **1** is more highly structured than the absorption spectrum indicating that the rings become more coplanar in the excited state.[8] In this case as both synthetic protein and probe absorbed at 260 nm, increased emission at 300 nm indicates higher concentrations of the PBLG.

Following Benesi–Hildebrand analysis of this data set, a K_D value of 80 μM was obtained based on a 1:1 binding isotherm (Table 1).[15] In comparison, the other isomorphous probes **2** and **3** displayed smaller changes in their fluorescence response. In case the biphenyl component of **1** was responsible for the peptide recognition, **5** was examined for its fluorescence response. Although **5** displayed more significant fluorescence quenching than probes **2**–**4**, its affinity for the synthetic protein was less than **1** (K_D = 130 μM) and its bluer emission at 300 nm was less desirable. To assess the possibility that probe **1** is displaying changes solely due to environmental polarity, Prodan, a well-known probe for micropolarity measurements, was selected for titration with PBLG. Here, only modest changes were observed with increasing amounts of the synthetic peptide and indicate that probe **1**'s ter-aryl morphology plays a role in protein recognition.

In the present work, these fluorescent studies were limited to PBLG due to the solubility properties of the probes under investigation. Attempts to include water-soluble α-helical proteins such as polyleucine as well as the non-helical polyisoleucine met with precipitation of the fluorescent probe. Synthesis of water-soluble versions of the probe is currently underway. Nevertheless, these in vitro studies on **1** should not preclude its potential value in biological systems as hydrophobic pockets are commonly found features in protein-protein interactions. The red-shifted emission (120 nm) of **1** relative to **5** indicates participation of the naphthalimide ring in the photophysics as well as the fluorescence quenching upon recognition of the helical secondary structure.

In short, this new fluorescent platform sets the stage for additional luminescent proteomimetics of α-helical structure. Despite dissociation constants in the micromolar region compared to Hamilton's terphenyl systems where nanomolar binding is observed, a preliminary analysis of the data suggests that the shape and surface area of **1** correlates well with the π–π stacking arrays presented by the poly-γ-benzyl-L-glutamate. Future versions of this probe are planned that include recognition components for the naphthalimide system along the 3′ and 2′ positions of the biphenyl for other biologically relevant proteins. Such functionality should significantly improve the K_D values relative to the parent hydrocarbon system observed with compound **1**. Finally, the naphthalimide chromophore can be readily attenuated

[†] Mp 237 °C (dec); UV–vis λ_{max} = 257 nm Emission = 415 nm, ^{1}H NMR (300 MHz, $CDCl_3$) 8.66 (d, *J* = 7.93 Hz, 2H) 8.63 (d, *J* = 4.12 Hz, 2H), 8.29 (t, *J* = 10.53 Hz, 2H) 7.92 (m, 4H), 7.64 (d, *J* = 8.23 Hz, 2H), 7.43 (m, 3H). ^{13}C (75 MHz, DMSO-d_6 164.5, 161.1, 135.9, 135.3, 135.4, 133.1, 131.9, 131.4, 130.3, 128.1, 127.7, 125.9, 124.0, 123.1, 119.5. IR ν/cm^{-1}: 1733, 1364, 1229, 1216, 1204. Anal. Calcd for $C_{24}H_{15}NO_2$: C, 82.52%, H, 4.29%, N 4.01%. Found: C, 82.41%, H, 4.28%, N, 3.83%.

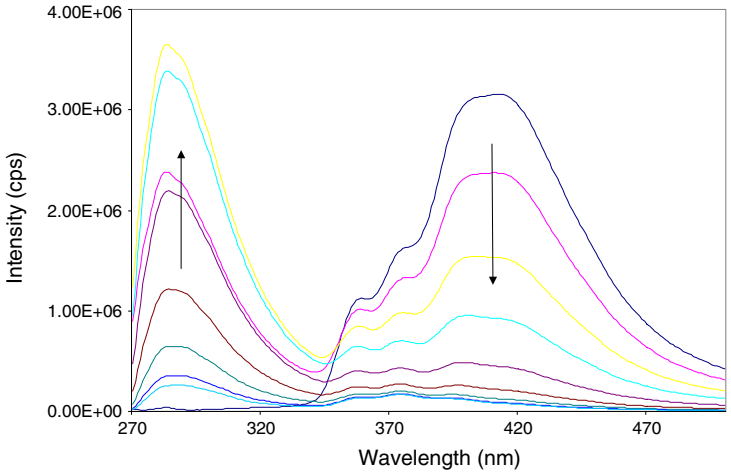

Figure 2. Stacked fluorescence spectra of *N*-4,4′-biphenyl-1,8-naphthalenedicarboximide (**1**) at constant concentration of 10 μM with PBLG in aliquots of 10 μM using λ_{exc} at 260 nm.

Table 1. Comparison of dissociation constants between PBLG and fluorescent probes

Probe	K_D (μM)
1	80
2	230
5	130

Percent error for K_D values is ±10%. Probes **3**, **4**, and **6** did not display significant changes in fluorescence suitable for binding isotherm analysis.

(with substituents) to absorb at longer wavelengths thereby removing protein autofluorescence from the spectral window.

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Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bmcl.2006.07.050.

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