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A split ligand for lanthanide binding: facile evaluation of dimerizing proteins†

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Luminescence of lanthanides is attractive for biological applications due to its long lifetime and sharp emission profiles. We describe the split display of a lanthanide binding ligand that allows facile evaluation of dimerizing proteins. The split lanthanide ligand is cysteine reactive, and therefore should be readily applicable to a variety of protein systems.

Protein dimerization is a ubiquitous phenomenon in biology and plays a critical role in transcription regulation and various signaling processes.[1] Methods that allow facile detection and quantification of protein dimers are highly desirable for further elucidating the significance of protein dimerization in physiology and disease. Herein, we describe a novel method that reports on protein dimerization with lanthanide luminescence.

The unique properties of lanthanide luminescence, including long lifetime and sharp emission profiles, have stimulated much interest in developing lanthanide-based imaging agents and biosensors.[2] A key feature of lanthanide luminescence lies in the requirement of light-absorbing ligands as sensitizers owing to the poor absorptivity of lanthanide ions *per se*. Therefore, control of lanthanide–ligand association allows one to switch on/off lanthanide emission, presenting a powerful and versatile strategy for sensing biological transformations.[3] In this contribution, we demonstrate that *split display* of a high-affinity lanthanide ligand on dimerizing proteins allows facile detection and quantification of protein dimers with a luminescence readout.

Our design started with the tetrameric construct of 2-hydroxyisophthalamide (Fig. 1a, dubbed HIP4 for convenience of discussion), a lanthanide sensitizer developed by Raymond and co-workers.[4] HIP4 binds terbium with femtomolar affinity and elicits strong luminescence emission. In sharp contrast, a substructure of HIP4 (HIP2, a dimer of 2-hydroxyisophthalamide) shows no binding to lanthanides with concentrations as high as 10 μM (Fig. S1, ESI†). We hypothesize that proteins labelled with HIP2, upon dimerization, will reconstitute a HIP4-like motif for lanthanide binding and consequently induce luminescence emission (Fig. 1b). In other words, split display of HIP4 should serve as a generally applicable platform

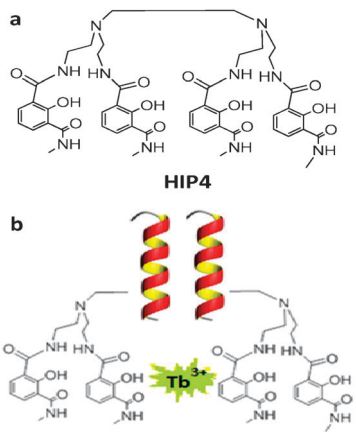
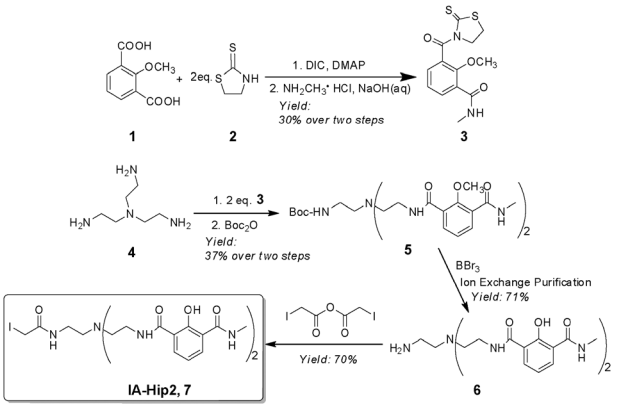


Fig. 1 Structure of the lanthanide ligand HIP4 (a) and schematic illustration of the split display of HIP4 for detecting protein dimers with induced lanthanide luminescence (b).

to report on protein–protein interactions. Importantly, the 2-hydroxyisophthalamide based ligands display maximum absorption at 350 nm.[4] In comparison to other lanthanide sensitizers (*e.g.*, dipicolinic acid[5]), the long-wavelength absorption makes them particularly suitable for biological applications.

Given the broad use of cysteines for protein labeling, we derivatized the HIP2 structure with a thiol-reactive iodoacetamide group (Scheme 1). Our synthesis started with 2-methoxyisophthalic acid **1**. The carboxylic acid groups were activated with 2-mercaptothiazoline and then treated with one equivalent of methylamine to give compound **3**. This singly activated HIP



Scheme 1 Synthesis of the thiol-reactive lanthanide ligand IA-HIP2.

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derivative was mixed with tris(2-aminoethyl)amine in 2 : 1 stoichiometry. Upon completion, the reaction mixture was treated with $(Boc)_2O$ to protect all the remaining amino groups. Boc protection was necessary for purification of the product *via* flash chromatography. The HIP2 derivative **5** was treated with BBr_3 to simultaneously remove the methyl and Boc protecting groups. The resulting compound **6** was purified *via* ion exchange chromatography, and then treated with iodoacetic anhydride to afford the desired final product IA-HIP2 (compound **7**).

With the small molecule in hand, we investigated its potential as a dimerization indicator by using a well-characterized model protein α_2D. This thirty-five-residue polypeptide folds into dimeric helix bundles (Fig. 2a) following a simple two-state equilibrium: the unfolded monomer and folded dimer.[6] Prior work from our group has shown that the stability of α_2D can be tuned by fluorinating its core residues.[7] For example, mutating the phenylalanine residues in the hydrophobic core to perfluorophenylalanines (Z) gives the mutant α_2D-ZZ that is more stable than α_2D-WT by over 6 kcal mol^{-1}. Consequently, at low concentrations (*e.g.*, 2 μM) α_2D-ZZ folds completely into dimers while α_2D-WT is largely unfolded (Fig. S2, ESI†). These two α_2D variants were synthesized through solid phase peptide synthesis, through which we also introduced the H30C mutation for labelling with IA-HIP2. Due to the C_2 symmetry of the α_2D dimer structure, residue 30 of one monomer is positioned in close proximity of the other. Thus dimerization of the HIP2-labelled α_2D is anticipated to reconstitute a HIP4-like motif for lanthanide binding (Fig. 2a).

The cysteine mutants of α_2D reacted readily with IA-HIP2 under mildly basic conditions. HIP2 labelling minimally affected the structure and folding of the α_2D variants as revealed by circular dichroism spectroscopy (Fig. S2, ESI†). The dimerizing behaviour of α_2D was examined by monitoring lanthanide luminescence. With an excess amount of terbium,[8] α_2D-WT at 2 μM gives slightly higher emission than the HIP2 control (compound **6**), consistent with its predominantly monomeric state under the experimental conditions. In sharp contrast, α_2D-ZZ elicits a strong terbium luminescence (Fig. 2b), presumably due to the formation of α_2D-ZZ dimers. At equal concentrations, the luminescence emission afforded by the α_2D-ZZ dimer is ~50% as strong as that of HIP4, suggesting that the lanthanide binding motif reconstituted by the peptide dimers gives good, albeit less than ideal, efficiency for sensitizing terbium emission. A titration experiment yields a linear relationship between the terbium luminescence intensity and the concentration of α_2D-ZZ (Fig. 2c), showcasing the potential of using lanthanide luminescence to quantify the amount of protein dimers. Importantly, the strong lanthanide emission enables analysis of α_2D dimerization even at sub-micromolar concentrations (Fig. 2c), which is often challenging for other techniques including CD and FT-IR. Furthermore, the dynamic (un)folding behaviour of α_2D can be readily monitored by terbium luminescence. As shown in Fig. 2d, similar structural transition profiles are displayed by the folding (cooling down) and unfolding (heating up) curves. The slightly reduced luminescence of the unfolding curve is presumably due to photobleaching over time.

To test the general applicability of IA-HIP2, we evaluated another dimerizing model protein GCN4, the coiled coil

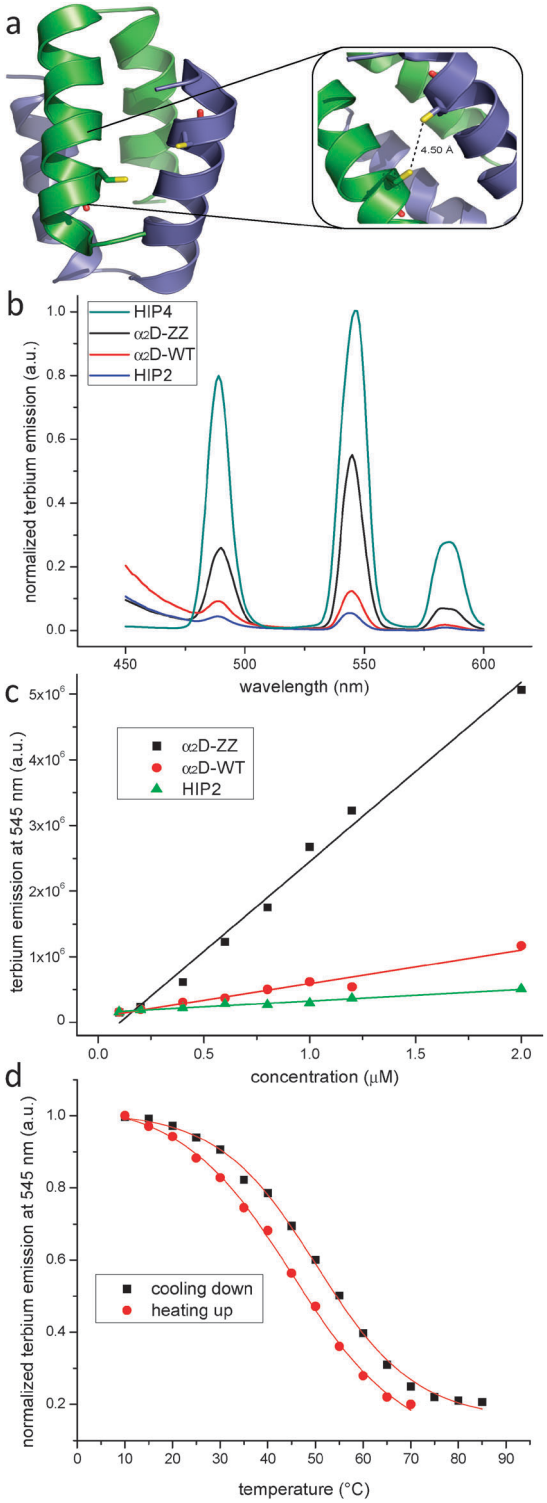

Fig. 2 Analyzing α_2D dimerization with terbium luminescence. (a) Cartoon representation of the α_2D dimer (PDB: 1QP6); the monomers are colored green and purple, respectively, and the cysteine residues for HIP2 labeling are shown in sticks (sulfur atoms are depicted in yellow). (b) Luminescence spectra of the α_2D variants and small molecule controls. The emission intensities are normalized against that of HIP4 at 545 nm. (c) Concentration profiles of terbium emission showing that luminescence correlates linearly with peptide concentration. (d) Thermal folding/unfolding curves monitored by terbium luminescence. The luminescence intensities were normalized against that at 10 °C.

GCN4-WT : CGGQLEDKVEELLSKNYHLENEVARLKKLVG
GCN4-L19P : CGGQLEDKVEELLSKNYHPENEVARLKKLVG

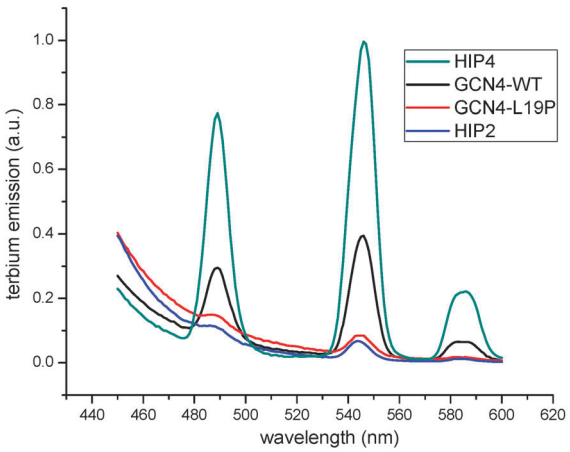

Fig. 3 Analysis of GCN4 dimerization in the presence of bovine serum. The luminescence data are normalized against the maximum emission intensity of HIP4 (at 545 nm). Terbium luminescence effectively differentiates the GCN4 monomer and dimer even in the presence of bovine serum.

domain from the bZip repressor.[9] There has been long-standing interest in deciphering the dimerization specificities of this family of transcription factors.[10] Truncated versions of GCN4 (~30 residues) were shown to fold to parallel dimers following a two-state mechanism.[11] Through solid phase peptide synthesis, we have prepared the wild type GCN4, as well as the L19P mutant for comparison (Fig. 3). The incorporation of proline completely abolishes the dimerization behaviour of GCN4 as revealed by circular dichroism spectroscopy (Fig. S3, ESI†). Instead of labelling at the dimer interface, a tripeptide (Cys-Gly-Gly) fragment is added to the N-terminus of the GCN4 peptides for HIP2 conjugation. Similar to the α_2D system, the dimerizing peptide GCN4-WT gives a strong luminescence emission, while the monomeric mutant (GCN4-L19P) yields a comparable readout as the HIP2 control (Fig. S5, ESI†). Again the terbium luminescence increases linearly with the GCN4-WT concentration (Fig. S5, ESI†), suggesting the promise of using HIP2 to quantify protein dimers. As a preliminary test of this assay in complex biological systems, we examined the GCN4 peptides in the presence of bovine serum. Although the addition of 10% bovine serum causes significant quenching for all samples (Fig. S6, ESI†), the normalized luminescence profiles of the peptides with serum (Fig. 3) are essentially identical to those without (Fig. S5, ESI†), suggesting that terbium luminescence reliably reports on GCN4 dimerization even in the presence of high concentrations of random proteins.

In summary, we have successfully developed a novel method that robustly detects protein dimerization with a luminescence readout. Our method builds on the split display of a high-affinity lanthanide ligand on target proteins, which upon dimerization induces lanthanide binding and luminescence emission. An analogous strategy was recently reported that utilized the split display of a tetracysteine motif to probe protein folding and assembly processes.[12] In comparison, the current lanthanide-based method does not involve covalent bond formation as does the tetracysteine–FlAsH system; the fast lanthanide–ligand (dis)association allows one to monitor the dynamic behaviour of target proteins. In addition, the high sensitivity of lanthanide emission and the possibility of time-gated measurement make this assay particularly attractive for applications in complex biological systems. Although the current report focuses on protein dimerization, the split lanthanide ligand display should be extendable to the study of protein–protein interactions in general, as well as protein aggregation processes.

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