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Supramolecular Displacement-Mediated Activation of a Silent Fluorescence Probe for Label-Free Ligand Screening

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Supporting Information

ABSTRACT: We report a new approach for the rapid screening of analyte binding affinities for a target protein. We demonstrate that a molecular probe, with a pro-fluorophore substrate and ligand moieties, can be hindered from enzymatic access when bound to the target protein. When analytes displace the probe from the protein's binding pocket, a fluorescence profile is generated. This profile is used to discriminate analytes based on their relative binding affinities.

The ability of a small molecule to tightly bind to a target protein receptor is arguably the most important criterion in drug development. For this purpose, the development of reliable methods for rapidly screening small molecules against specific proteins is essential. Current methods for small molecule screening include X-ray crystallography,[1] NMR,[2] mass spectrometry,[3] microcalorimetry,[4] and fluorescence based techniques.[5] These techniques are generally complementary to each other; among them, fluorescence stands apart due to its simplicity and speed in analysis. However, most fluorescence-based approaches require labeling the target or the analyte.[6] In this communication, we present a new label-free, fluorescence-based supramolecular platform to rapidly discriminate binding affinities of analytes against a target protein.

In this approach, illustrated in Figure 1a, the probe is designed using a known ligand, complementary to the protein, which is attached to a pro-fluorophore through a linker. The key features of the molecular design are as follows: (*i*) the linker is cleavable by an enzyme; (*ii*) enzymatic cleavage of the linker converts the nonfluorescent precursor to a water-soluble fluorophore; (*iii*) when the substrate is bound to the target protein, the linker is sterically inaccessible for the enzyme. If we were to design a probe molecule that satisfies these requirements, then we envisaged the possibility of using this system to screen small molecule binders for the target protein. Here, the analyte small molecule that has comparable or better binding affinity would be able to displace the probe molecule from the protein, depending on its relative concentration. This displacement would expose the enzyme-active linker, the cleavage of which should result in the formation of the fluorophore. The concentration-dependence of the fluorescence generation can then be used to evaluate the ability of the ligand candidates to bind the target protein.

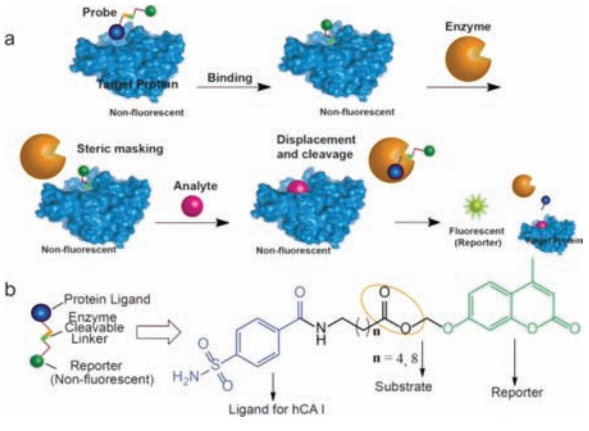

Figure 1. (a) Schematic illustration of the strategy for label-free ligand screening and (b) probe structure.

To test this design hypothesis, we chose human carbonic anhydrase I (hCA1) as the target protein, an interesting model system, since it has been implicated in a variety of pathophysiological processes.[7] The molecular structure of our probe is shown in Figure 1b. Arylsulfonamide is a ligand for hCA1,[8,9] while the coumarin derivative, umbelliferone is an excellent fluorophore. The ligand and the fluorophore are linked through an aliphatic chain with an ester bond. Coumarin is attached to the ester through an acetal moiety, where cleavage of the ester using an esterase (porcine liver esterase (PLE)) would release the fluorophore, umbelliferone.

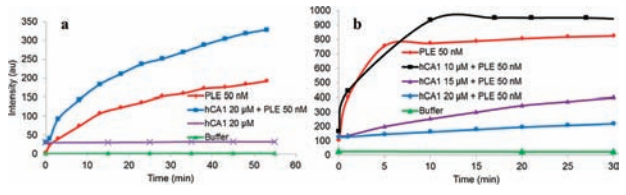

Figure 2. Hindering enzymatic action upon the probe molecule, due to protein binding. (a) hCA1 unable to hinder hydrolysis on the probe with a linker $n = 8$, and (b) hCA1 hinders hydrolysis on the probe with a linker $n = 4$. Probe concentrations in all measurements was 5 μM.

We first tested our hypothesis with the probe shown in Figure 1b, where the linker length was $n = 8$. The results of these experiments are shown in Figure 2a. First, the probe itself does not hydrolyze in the buffer without the enzyme. Second, we were surprised to find that the presence of hCA1 enhanced the enzymatic cleavage of the probe. This could be because the

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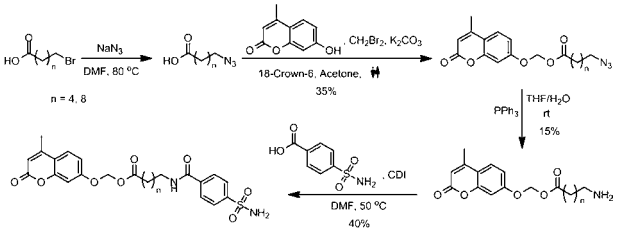
protein does not sufficiently mask the ester moiety upon binding but helps in improving the probe's solubility.

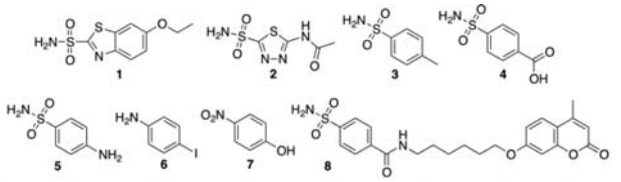
To test this possibility, we modified the linker length, a critical component of our molecular design, as this would impact the enzyme's accessibility to the ester moiety. We synthesized the probe molecule with a shorter linker length (n = 4, Scheme 1). While a 5 μM concentration of the probe (n = 4) with 50 nM PLE generated significant fluorescence within a few seconds, this combination did not generate any fluorescence in the presence of 20 μM hCA1. At smaller concentrations of hCA1, there was intermediate fluorescence (Figure 2b). Also, we found the optimal preincubation time to be ~15 min (Figure S1).

Scheme 1. Synthesis of the Probe



With the identification of a combination of the probe and the optimal relative probe/protein concentrations, we were interested in testing the possibility of competitive displacement of the probe from the hCA1 binding pocket as the assay for the relative affinity of an analyte molecule. The ligand molecules, used for this purpose, are shown in Chart 1. Ligands **1**–**5** are known to be good inhibitors for hCA1, while molecules **6** and **7** are randomly chosen and are presumably not good ligands for hCA1. Analyte **8**, structurally analogous to the probe, should exhibit similar binding affinity as the probe.

Chart 1. Structure of the Analyte Molecules Used in the Assay and Their Literature-Reported Binding Affinities[9]



Comp.	1	2	3	4	5	6	7
K_D(M)	9×10^{-9}	1.15×10^{-6}	1.67×10^{-6}	3.4×10^{-6}	90×10^{-6}	N/A	N/A

When different amounts of these molecules were introduced into a solution containing a mixture of the probe/hCA1 (5:20 μM) and 50 nM PLE, molecules **1**–**5** showed an increase in fluorescence with increasing concentration (Figure 3a). However, molecules **6** and **7** did not exhibit any appreciable change in fluorescence with concentration. Three key features are evident from these plots: (*i*) analytes with strong to moderate binding affinities can displace the probe, and the displacement profile can be traced by the fluorescence evolution; (*ii*) since the relative concentration of probe vs hCA1 dictates that there are some free proteins in the system, the fluorescence response remains flat in the initial part of the plot. This is taken to indicate that the ligands are first binding to the excess free proteins;[10] and (*iii*) the slope of the fluorescence change correlates with the analyte binding affinities, thus providing an opportunity to assess the relative binding affinities of the analyte molecules tested (Figure 3b).

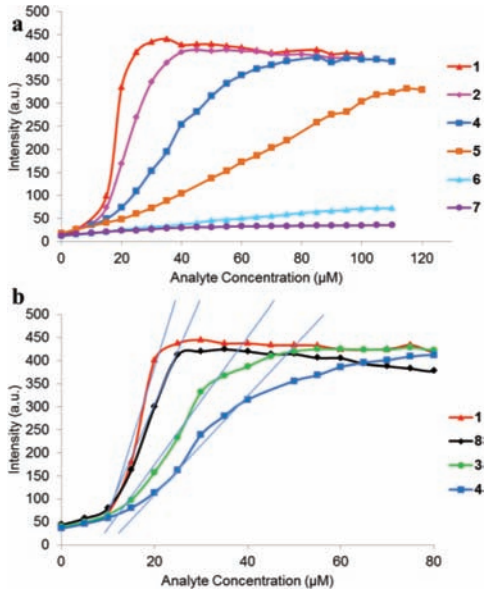


Figure 3. Displacement-mediated activity profiles generated by (a) the analytes **1**, **2**, **4**–**7**; and (b) **1**, **8**, **3**, **4** with the slopes of the curve corresponding to the effective probe displacement by the analytes (experimental parameters on p S4).

Next, we were interested in evaluating the predictive capability of the assay developed here. Figure 4 shows a correlation between the literature-reported K_D values and the slopes obtained from the linear regime of the plot (Figure 3a), where there is significant fluorescence change. It is clear that the trend in the slopes obtained in our assays correlates well with the literature-reported K_D values. Considering the empirical nature of the correlation, we consider this correlation to be relatively qualitative. We provide an example of the type of qualitative comparison and numeric correlation that one could obtain with this method. Molecules **3** and **4** have identical binding moieties (benzene sulfonamide) but are different in their hydrophilicities. This difference can be attributed to the higher affinity of **3**. Molecule **8** has the carboxamide functional group at the para-position (similar to the carboxylic acid in **4**) but is more hydrophobic than **3**. To test whether the hydrophobicity indeed influences the binding affinity, we tested the relative binding affinity of **8**.[11] Our results suggest that **8** is indeed better than **3** but is not as good as **1** (Figure 3b). We indeed found the IC50 of **8** to be lower at 0.10 μM (the IC50 of the n = 4 probe was 0.04 μM; see Supporting Information for details). It is also possible that the coumaryl

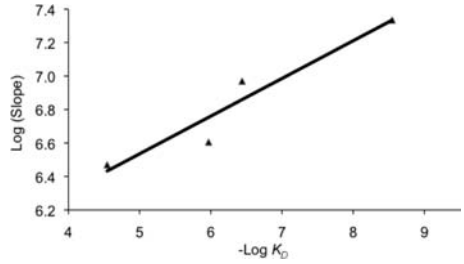


Figure 4. Correlation between literature-reported binding constants and slopes.[9] Data from Figure 3a (see Table S1).

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moiety contributes to the affinity of **8** and, consequently, the affinity of the probe.[12]

An important control experiment, for this approach, is to ensure that the analyte molecules themselves do not alter the activity of the enzyme (PLE) in any way. Therefore, it is essential that we routinely carry out a control experiment, where we assay the activity of the enzyme against its substrate in the presence of the ligand candidates. At high concentrations of the analyte molecules, we found that the activity of PLE was indeed unaffected (Figure S3).

The approach outlined here works well in providing an evaluation of the relative affinities but does not provide direct quantitative binding affinity measurements. Therefore, for such an approach to be useful, it is necessary that we can adapt this strategy for the rapid screening of analytes. For this purpose, we further tested the versatility of our approach in a 96-well plate reader setup. In such a setting, the volume of the components needed to generate a data point would be low and the screening can be done in a highly parallel fashion. The data generated from this experiment are shown in Figure 5 (compare with Figure 3a). The results indeed are consistent and are reliably reproducible.

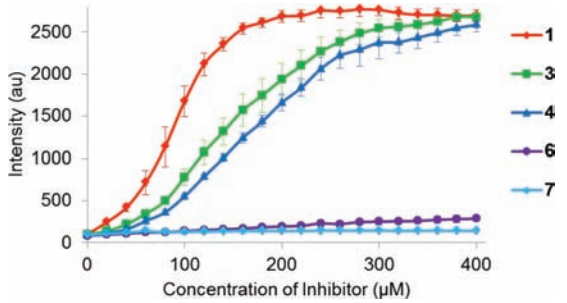

Figure 5. Probe (n = 4) displacement-mediated activity profiles generated by the analytes **1**, **3**, **4**, **6**, **7** with 96-well plate reader.

In summary, we have introduced a new approach to screen the binding of small molecules to proteins using a supramolecular displacement approach. Following are the noteworthy features of our approach: (*i*) a protein-specific ligand is attached to a fluorophore, via an enzyme cleavable linker. This functionality is chosen such that the cleavage results in the generation of a water-soluble, high quantum yield fluorophore, umbelliferone. (*ii*) The linker length is chosen such that the enzyme-cleavable functional group is sterically masked from the enzyme, when bound to the protein. (*iii*) Molecules with different binding affinities for the protein show different probe displacement profiles to expose the enzyme cleavable functionality and thus exhibit an affinity-dependent fluorescence response. (*iv*) This approach can be rendered high throughput, as this is easily translated to a multiwell plate reader based fluorescence measurement. (*v*) A limitation of the approach is that one initial molecule with a reasonable binding affinity for the target protein must be known to successfully design the fluorescent probe and execute further ligand optimization. We envision that the design principles, outlined here, have the potential to be broadly adapted to rapidly screen small molecules against a protein target.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures, characterizations, and control experiments are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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