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A Green Fluorescent Chemosensor for Amino Acids Provides a Versatile High-Throughput Screening (HTS) Assay for Proteases

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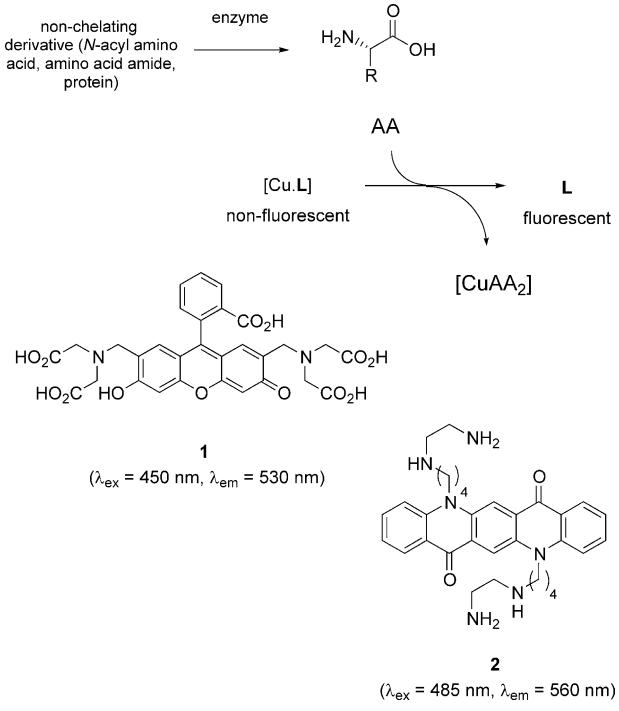
Abstract—The water soluble fluorescein-based ligand **1** forms a non-fluorescent complex with Cu^{2+}. This complex serves as a fluorescent sensor for amino acids in the 10^{-3} M concentration range. Since the signal response is very fast, the sensor can be used to detect the hydrolytic activity of various proteases (trypsin, chymotrypsin, subtilisin) on bovine serum albumin as a whole protein substrate, and more generally to follow reactions releasing or removing free amino acids, in real time.

Chemosensors are molecules capable of binding a given analyte selectively such that the binding event induces a measurable signal, most often a change in the spectral properties of a chromophore or fluorophore.[1] In one particular design, a reporter molecule occupies the binding site for the target analyte within the receptor and signalling occurs upon competitive displacement of this reporter molecule from the binding site by the analyte. This concept is well known in competitive ELISA using antibodies[2,3] and has been more recently applied in small molecule chemosensors.[4] We recently reported that the copper complex of the quinacridone ligand **2** operates as a fluorescent sensor for amino acids following this principle, whereby the metal center serves as the analyte binding site and the metal coordinating fluorophore as the reporter molecule.[5] The rapid ligand exchange kinetics of this sensor allowed us to follow the reaction of acylase and aminopeptidase, two enzymes which release free amino acids from non-coordinating precursors. However, the orange fluorescent quinacridone ligand **2** had to be prepared by a tedious synthesis and showed limited solubility in aqueous buffer, which severely restricted its use. We therefore set out to find a more practical alternative to this ligand. Herein, we report that calcein **1**, a commercially available and inexpensive bisiminodiacetate derivative of fluorescein, can advantageously replace **2** for sensing applications. Calcein **1** forms a non-fluorescent complex with Cu^{2+} that is completely soluble in aqueous buffers. This complex serves as a versatile green fluorescent chemosensor for amino acids that can be used to follow the proteolytic activity of proteases on whole protein substrates in real time (Scheme 1).



Scheme 1. Principle of fluorescence assay for amino acids using copper–ligand complexes.

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The fluorescent detection of amino acids by [**2**.Cu] relied in principle solely on the coordinating ability of amino acids for copper and there was no direct contact between the amino acids and the quinacridone fluorescent reporter of ligand **2**. This implied that ligand **2** should be replaceable by other ligands with similar coordination and fluorescence quenching properties. Calcein **1**, a bisiminodiacetate derivative of fluorescein, showed the required properties. The green fluorescence of **1** at $\lambda_{em} = 530$ nm ($\lambda_{ex} = 450$ nm) was quenched by approximately 90% upon addition of Cu^{2+}, Ni^{2+} and Co^{2+}, probably by an energy transfer quenching mechanism (Fig. 1).

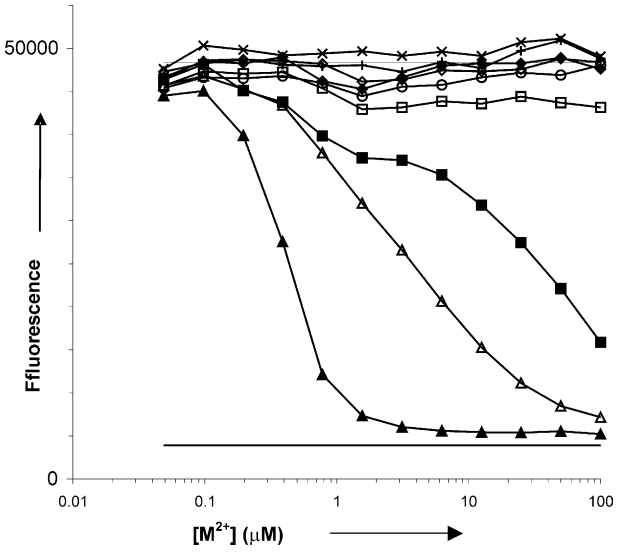

Figure 1. Fluorescence quenching of calcein **1** by addition of divalent metal ions. Conditions: 1 μM **1** in 5 mM aq phosphate pH 7.0, 25 °C, $\lambda_{em} = 530 \pm 25$ nm ($\lambda_{ex} = 450 \pm 50$ nm). All measurements were done by serial dilutions in V-bottom polypropylene 96-well-plates (Costar) and recorded using a Cytofluor II Fluorescence Plate Reader (Perseptive Biosystems). (▲) Cu^{2+}, (◆) Mg^{2+}, (x) Ca^{2+}, (■) Co^{2+}, (○) Sr^{2+}, (△) Ni^{2+}, (◇) Zn^{2+}, (+) Ba^{2+}, (□) Fe^{2+}. Upper and lower horizontal lines are set at the average fluorescence reading for 1 μM **1** (upper line) and 1 μM **1** + 10 μM Cu^{2+} (lower line). Each data point represents the average value obtained from at least two independent serial dilution experiments.

While the cobalt and nickel complexes showed no effect upon addition of amino acids, the copper–calcein complex showed an increase in fluorescence, showing that calcein coordination to copper was weak enough to be displaced by the bidentate amino acid ligands and thus release the metal-free, green fluorescent ligand **1** into solution. Similar response curves were obtained for 0.1 μM < [**1**] < 1 μM and one to five equivalents of Cu^{2+}, with the most sensitive response occurring at the lower concentration of sensor. The response of the copper–calcein complex occurred in the 10^{-3} M concentration range with most amino acids. Cysteine resulted in full return of fluorescence upon addition of one equivalent of amino acid relative to Cu^{2+}. However the signal was unstable and equilibrated to a displacement curve reflecting the equilibrium for the oxidized cystine (Table 1).

The copper–calcein system was investigated as a fluorescent sensor for enzyme-catalyzed reactions releasing free amino acids from non-coordinating precursors. The activities of acylase I, which deacetylates *N*-acetyl L-methionine, and aminopeptidase, which hydrolyses L-leucinamide to L-leucine, were readily followed by fluorescence in aqueous buffer under their respective pH optima of pH 7.2 and pH 9.0 for acylase and aminopeptidase, respectively, showing the flexibility of the system to different operating conditions (Fig. 2A and B).

The most important class of enzymes releasing free amino acids from non-coordinating precursors are proteases. Protease assays are particularly important in relation with high-throughput screening for specific inhibitors. These enzymes can be assayed by chromogenic substrates such as peptide nitroanilides or by fluorogenic substrates such as peptide aminocoumarine amides or FRET-type peptides.[6] However, all of these assays involve expensive synthetic reagents. Our copper–calcein complex [**1**.Cu] operated as a fluorescent sensor for protease activity using bovine serum albumin (BSA) as a whole protein substrate (Fig. 3). While there was no response of the sensor in the presence of BSA alone, incubation with various proteases induced a time

Table 1. Fluorescence sensing by copper-calceine [**1**.Cu]

Ligand	Sensor composition, pH	EC_{50}[a] (mM)
Alanine	0.1 μM **1**, 0.1 μM Cu^{2+}, pH 7.0	~0.8
β-Alanine	0.1 μM **1**, 0.1 μM Cu^{2+}, pH 7.0	20% at 10
Serine	0.1 μM **1**, 0.1 μM Cu^{2+}, pH 7.0	~0.7
Glycine	0.1 μM **1**, 0.1 μM Cu^{2+}, pH 7.0	~0.5
Valine	0.1 μM **1**, 0.1 μM Cu^{2+}, pH 7.0	~1.5
Histidine	0.1 μM **1**, 0.1 μM Cu^{2+}, pH 7.0	~0.01
Cysteine[b]	0.1 μM **1**, 0.1 μM Cu^{2+}, pH 7.0	~0.02
Methionine	1.0 μM **1**, 5 μM Cu^{2+}, pH 7.2	~0.8
N-Acetylmethionine	1.0 μM **1**, 5 μM Cu^{2+}, pH 7.2	<5% at 10
Leucine	1.0 μM **1**, 10 μM Cu^{2+}, pH 9.0	~5
Leucinamide	1.0 μM **1**, 10 μM Cu^{2+}, pH 9.0	~12
EDTA	0.1 μM **1**, 0.1 μM Cu^{2+}, pH 7.0	~0.010

[a]EC_{50} is the concentration of ligand necessary for obtaining 50% of the fluorescence intensity of copper-free calcein **1** under the assay conditions. Fluorescence at $\lambda_{em} = 530 \pm 25$ nm ($\lambda_{ex} = 450 \pm 50$ nm) was recorded at 25 °C after 30 min equilibration in 5 mM phosphate (pH 7.0), Bis-tris (pH 7.2) or Tris (pH 9.0). EC_{50} were estimated from serial dilutions in 96-well microtiter plates.
[b]Cysteine initially induced full return of fluorescence at one equivalent relative to Cu^{2+}. The signal then decreased to a lower fluorescence reflecting the displacement equilibrium for the oxidized cystine.

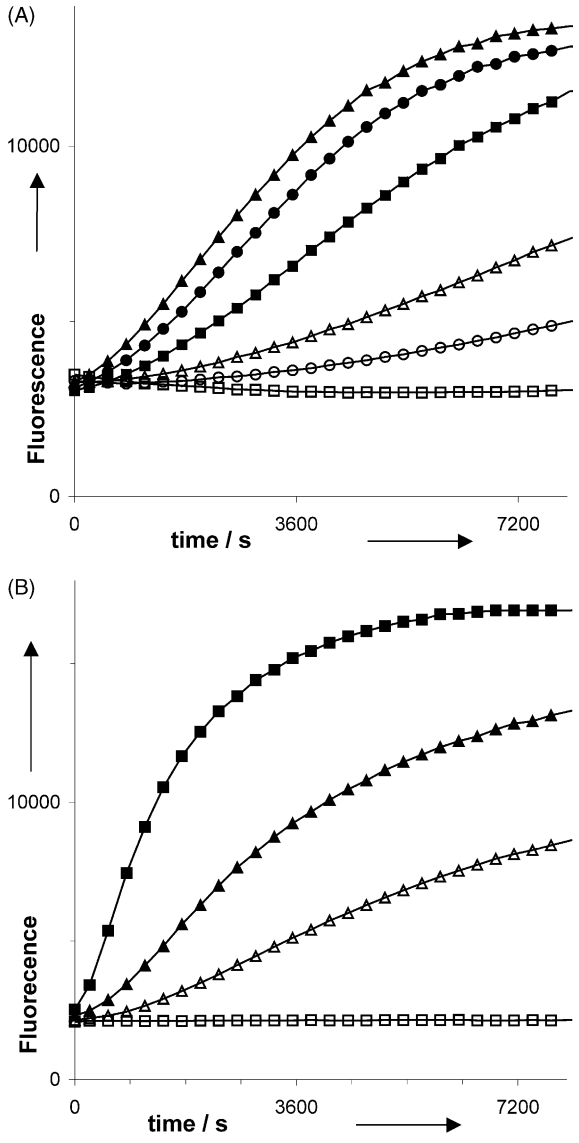

Figure 2. (A) Fluorescence assay for the hydrolysis of L-leucinamide to L-leucine by aminopeptidase. Conditions: 5 mM leucinamide in aq 5 mM Tris buffer pH 9.0, 25 °C, 1 μM **1**, 10 μM $CuCl_2$, 0.1 mM $MgSO_4$, $\lambda_{em} = 530 \pm 25$ nm ($\lambda_{ex} = 450 \pm 50$ nm), with no enzyme (□) and with aminopeptidase at (○) 0.5 μg mL^{-1}; (△) 1.0 μg mL^{-1} (■); 2.0 μg mL^{-1} (●); 2.5 μg mL^{-1} (▲). The maximum fluorescence observed corresponds to that of copper-free calcein **1** under the same assay conditions. (B) Fluorescence assay for the hydrolysis of *N*-acetyl L-methionine to L-methionine by acylase I. Conditions: 10 mM *N*-acetyl-L-methionine in aq 5 mM Bis–tris buffer pH 7.2, 25 °C, 1 μM **1**, 5 μM $CuCl_2$, $\lambda_{em} = 530 \pm 25$ nm ($\lambda_{ex} = 450 \pm 50$ nm), with no enzyme (□) and with acylase I at (△) 2.5 μg mL^{-1}; 5.0 μg mL^{-1} (▲); 10 μg mL^{-1} (■).The maximum fluorescence observed corresponds to that of copper-free calcein **1** under the same assay conditions.

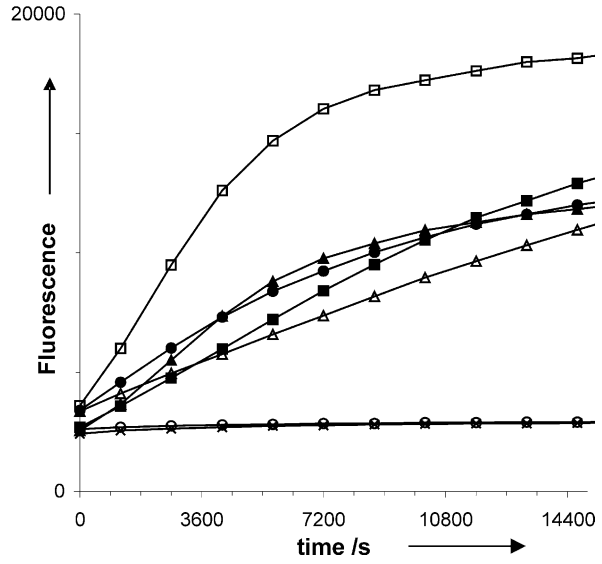

Figure 3. Fluorescence assay for proteases using calcein copper. Proteases (10 μg mL^{-1}) were added to BSA (0.5 mg mL^{-1}) in 5 mM aq. Bis-tris buffer pH 7.2, 25 °C, with 1 μM **1** and 5 μM $CuCl_2$. Fluorescence was recorded at $\lambda_{ex} = 450 \pm 50$ nm ($\lambda_{em} = 530 \pm 25$ nm). All measurements were done in round-bottom polypropylene 96-well-plates (Costar) and recorded using a Cytofluor II Fluorescence Plate Reader (Perseptive Biosystems). (□) subtilisin, (▲) elastase, (●) trypsin (■) thermolysin, (△) α-chymotrypsin, (○) pepsin, (×) no enzyme. The maximum fluorescence observed corresponds to that of copper-free calcein **1** under the same assay conditions.

dependent increase in fluorescence. SDS–PAGE analysis of the reaction showed that the protein substrate was indeed partially degraded in assays with a positive fluorescence signal, while negative assays showed no degradation. There was no response of the sensor in the presence of BSA upon addition of only guanidinium hydrochloride or urea up to denaturating concentrations (8 M), indicating that the sensor did not simply respond to a denaturation induced by proteolysis. The sensor probably responded to free amino acids or metal-coordinating short peptide fragments liberated by proteolysis. Interestingly, the cysteine proteases papain and pepsin showed no signal with 1 μM **1** and 5 μM Cu^{2+}, but showed a clear proteolysis signal above background when using 1 μM **1** and 1 μM Cu^{2+}. This suggests that the excess free Cu^{2+} in the first set-up acted as an inhibitor of these proteases, possibly by inducing an oxidation of the thiol group, under the assay conditions.

The ability to operate under a wide variety of conditions is essential if a chemosensor is to be generally applicable. The calcein–copper complex [**1**.Cu] shown here is quite generally applicable as long as the copper coordination by the ligand **1** is not perturbed. Thus, strongly coordinating buffers, such as Bis–tris–propane, which competitively bind the Cu^{2+}, and low pH values, which protonates the carboxyl groups of calcein **1** and lower its coordinating ability, must be avoided. Nevertheless, at pH 5.5 the 50% quenching of fluorescence induced by one equivalent of Cu^{2+} is already sufficient for sensitive chemosensor work, and from pH 6 to pH 10, with Bis–tris, phosphate, Tris and borate buffers at a range of buffer concentrations, quenching was typically 85–90%. Further, it should be noted that the fluorescence levels of 1 μM calcein, with and without 1 μM Cu^{2+}, remained essentially constant for solutions containing 0–50% of the most commonly used cosolvents; dioxane, acetonitrile, DMSO, DMF, methanol and ethanol, implying that the sensor can also be used under these various mixed solvent systems.

In summary, calcein–copper [**1**.Cu] operates as a fast switching fluorescent sensor for amino acids and other metal coordinating ligands in aqueous buffer. There is no response from whole proteins such as BSA. The calcein fluorophore **1** is commercially available at reasonable price (ca. 20 € per gram), which is negligible if one considers that only 1 μM concentration of the fluorophore is used in the assay (1.0 g calcein suffices for 1.6 million assays at 0.1 mL each). In principle, the fluorescence modulation of calcein copper responds to variations in the concentration of coordinating ligands in solution. It should thus also be possible to follow the decrease of this concentration with time. Indeed, a fluorescence decrease is observed for the reaction of free methionine with acylating agents, which shows that the kinetic properties of the sensor are as rapid for the formation of the quenched calcein–copper complex as for its break-up. Thus, calcein–copper is a versatile system to follow reactions producing or consuming free amino acids or other metal-chelating compounds, and should be applicable for hydrolase-type processes as well as for redox-type reactions.

Acknowledgements

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