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A novel "*pro-sensitizer*" based sensing of enzymes using Tb(III) luminescence in a hydrogel matrix†

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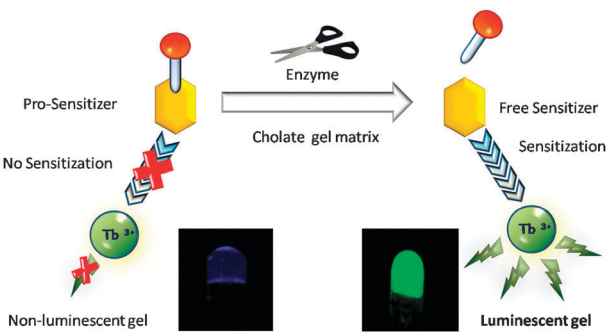
Chemically synthesized "*pro-sensitizers*" release the sensitizer in the presence of lipase or β-glucosidase, triggering a significant luminescence response from a lanthanide based hydrogel.

Optical probes which can provide direct information about enzyme activities are in great demand as enzyme mediated transformations are central to all biological processes. While most enzyme assays are based on colorimetry, recent years have witnessed increasing intent for developing analogous fluorescent systems, mainly due to their higher intrinsic sensitivity.[1] A significant number of fluorescent probes have been reported in the literature which can successfully sense enzymes and other biomolecules, small organic molecules, metal ions *etc*.[2] Fluorescence based sensing of biomolecules such as enzymes or *in vivo* measurements often pose a challenge due to interference from background fluorescence arising either from other fluorogenic species or auto fluorescence from cells.[3] Time-delayed luminescence measurements provide a possible solution to such problems as fluorescence from probes with longer lifetimes can easily be separated from the short-lived background. In this context, luminescent lanthanide complexes are attractive choices because of their long lifetimes, sharp emission bands and negligible interference from the environment.[4] We now report a novel, "*pro-sensitizer*" based sensing of enzymes using a gel-based Tb(III) luminescence assay.

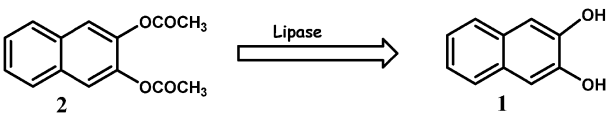
Lanthanide complexes have been extensively used for sensing metal ions, small organic molecules, and also in some cases for enzyme assays. Such examples typically follow the well-known strategy towards lanthanide sensitization by an [enzyme substrate–antenna–ligand] complex of a lanthanide[5,6] or by using a binary system of an appropriate *chelator* and a modified sensitizer.[7] However, *for all these systems, chelation of the lanthanide ion by a suitable multidentate ligand is essential*, usually requiring additional synthetic steps to obtain the chelating ligand suitable for the job.

Recently we discovered a novel way to sensitize trivalent lanthanide ions where the spatial proximity between the sensitizer and the lanthanide ion was achieved in a cholate gel matrix through self-assembly of commercially available ingredients.[8] *We were thus able to eliminate the need for a chelating ligand and use the gel matrix as a general platform to carry out all sensitization and sensing studies*. 2,3-Dihydroxynaphthalene (DHN) was shown to sensitize Tb^{3+} *exclusively in the gel medium* (sensitization by DHN in a fluid medium was negligible). We reasoned that the hydroxyl groups on DHN can in fact be suitably functionalized to generate a *pro-sensitizer* (which would not sensitize the lanthanide ion). The idea was to incorporate the *pro-sensitizer* into the gel media which upon the action of an enzyme would release the sensitizer and hence make the gel luminescent (Scheme 1).

An obvious starting point was to test a diester of DHN and cleave it with an esterase to release DHN (Scheme 2). A lipase was chosen for this purpose, since lipases belong to a very well known class of enzymes. A malfunctioning lipase metabolic pathway is reflected in high blood cholesterol and triglyceride levels and can lead to several cardiac diseases and diseases related to liver such as hepatomegaly, liver fibrosis, splenomegaly *etc*.[9] A simple and efficient lipase assay system is thus of



Scheme 1 A strategy towards the design of enzyme-induced luminescent gel.



Scheme 2 Free sensitizer generation due to lipase activity.

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† Electronic supplementary information (ESI) available: Synthetic procedures, details of gel preparation, microscopy. See DOI: 10.1039/c2cc30904a

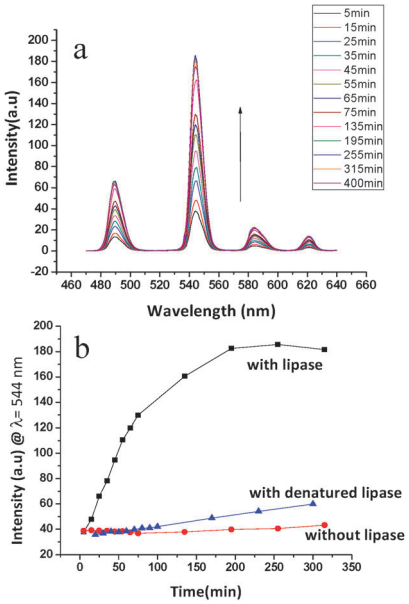


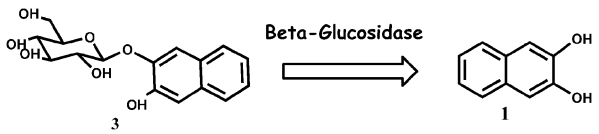
Fig. 1 Lipase (0.9 mg mL^{-1}) assay with **2** (33 μM) in a Tb : cholate gel system (5 mM : 15 mM) at 298 K; (a) evolution of luminescence spectra with time; (b) increase in intensity (λ 544 nm) as a function of time for native enzyme and comparison with denatured enzyme or without any enzyme.

considerable importance. The substrates for lipases are generally hydrophobic in nature, which makes designing of an assay system based on a fluorogenic probe in aqueous media non-trivial. Interestingly, bile salts are critical for the activity and efficiency of most classes of lipases.[10] The hydrogel system enjoys an advantage as the gel network primarily consists of bile salt aggregates thus providing a near-natural environment for the enzyme. Lipase from *Candida rugosa* sp. was used for all the assays. The enzyme was dissolved in aqueous terbium acetate, while the pro-sensitizer (**2**) was dissolved in sodium cholate stock solution.

The gel formed upon mild sonication of the mixture, whose (time-delayed) luminescence was recorded as a function of time (Fig. 1).

Compound **2** did not appreciably sensitize Tb^{3+} and *only in the presence of native lipase* the luminescence intensity started to increase. A remarkable enhancement in lanthanide emission was observed that was clearly visible even with a hand-held long-wave UV lamp (photo in Scheme 1). A control was done using the denatured enzyme which did not show any enhanced luminescence from the lanthanide ion, thereby proving that the luminescence response was indeed a result of the enzyme activity.‡ Higher analogues of DHN diesters (with propionate, butyrate and hexanoate) were also studied with similar results.

In order to explore the generality of the sensing system with a completely different type of enzyme, β-glucosidase, which cleaves water-soluble β-glucosides to release sugars and plays



Scheme 3 Free sensitizer generation due to β-glucosidase activity.

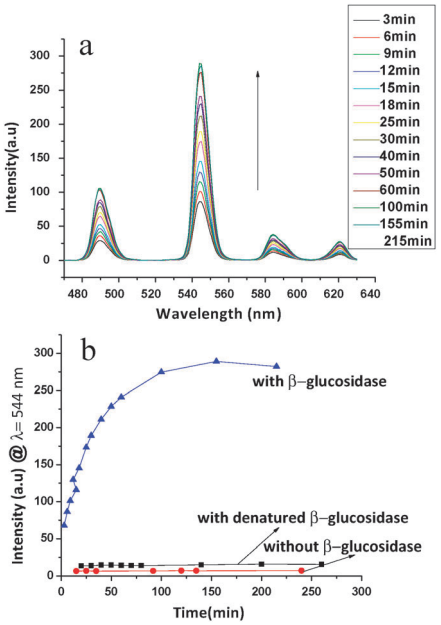


Fig. 2 β-Glucosidase (0.7 mg mL^{-1}) assay with **3** (0.37 mM) in a Tb : cholate gel system (5 mM : 15 mM) at 298 K; (a) evolution of luminescence spectra with time; (b) increase in intensity (λ 544 nm) as a function of time for native β-glucosidase and comparison with denatured enzyme or without any enzyme.

a key role in the carbohydrate metabolism, was chosen.[11] β-(Mono)glucoside derivative of DHN (**3**) was synthesized (see ESI†) to act as a pro-sensitizer (Scheme 3).

The luminescence studies showed similar enhancements in intensity for the assay with the native enzyme as observed with lipase (Fig. 2).

In order to unambiguously establish that it is indeed the generation of free DHN that is responsible for the luminescence

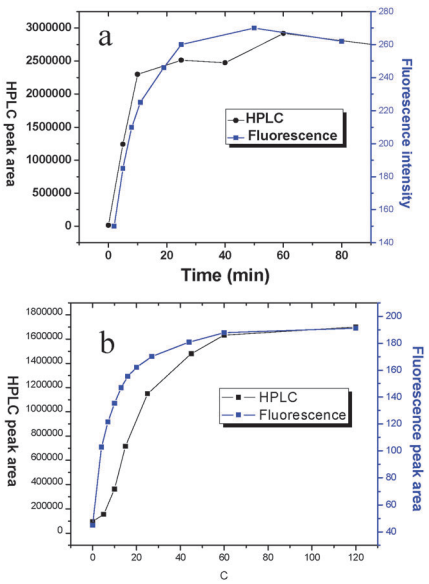


Fig. 3 Comparison of enzyme activity measured by luminescence and HPLC studies for (a) lipase (33 μM of **2** and 0.9 mg mL^{-1} enzyme were used for both luminescence and HPLC measurements) and (b) β-glucosidase (0.37 mM of **3** and 0.7 mg mL^{-1} enzyme were used for both luminescence and HPLC measurements).

enhancement, HPLC analysis for both the gel systems was carried out to monitor the evolution of DHN with time (Fig. 3).

When the relative peak areas obtained from the HPLC analysis were plotted against time, the trends matched quite well with the luminescence enhancement, confirming that both were indeed the result of the same process.

In conclusion, we have developed a novel luminogenic lanthanide based gel probe for inexpensive and rapid detection of two completely different types of enzymes, lipase and β-glucosidase. The sensing technique does not involve any specialized multidentate chelating ligand and instead uses the gel matrix as the general platform. To the best of our knowledge this is the first report describing a luminescent gel system used for enzyme sensing. The synthesis of our pro-sensitizers is straightforward and has a unique advantage of being adaptable to assay different enzymes. We believe that this technique would provide an exciting alternate approach towards the sensing of various enzymes and should enjoy significant advantages over the other existing sensing techniques because of its simplicity and generality. This strategy should also allow one to rapidly test enzyme inhibition. We are currently working on the design of other pro-sensitizers for clinically important enzymes and fine tuning the assay system to make it more sensitive.

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Notes and references

‡ (a) Moderate concentrations of enzymes were used for all the studies as the intrinsic activities were less for both enzymes, lipase (2.9 U mg^{-1}) and β-glucosidase (6.1 U mg^{-1}). (b) Both lipase and β-glucosidase were denatured by heating a sealed container of enzyme stock solution at 85 °C for 10 min.

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