A chemiluminescent catalytic antibody†

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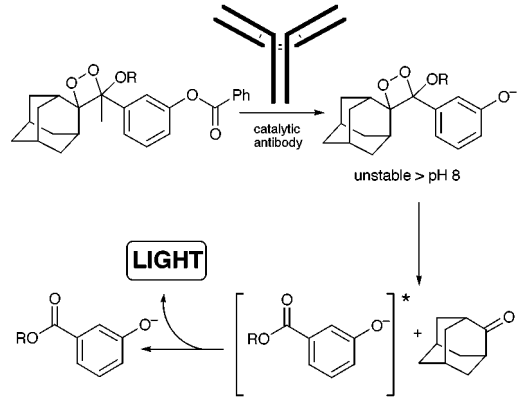
A monoclonal antibody elicited against a phosphonate transition state analogue was shown to initiate chemiluminescence from luminogenic spiro[adamantane-2,3′-(1,2-dioxetane)] substrates by selectively cleaving a benzoate ester triggering group.

Serendipity has played a large part in the identification of many of the catalytic antibodies reported in the literature to date.[1] Typically, 5–50 monoclonal antibodies selected on the basis of their affinity for a transition-state analogue as judged by Enzyme-Linked ImmunoSorbent Assay (ELISA)[2] have been produced in sufficient quantity to screen for catalytic activity. Such an approach has led to the identification of, at best, a handful of catalysts. Given that the humoral response generates at least 10^8 unique antibody binding sites, only a tiny fraction are examined for their catalytic activity. The probability of identifying the most efficient antibody catalyst for any given reaction from the library available using this approach is therefore very low, as screening for catalysis only occurs at a late stage. As a result it is currently difficult to optimise either the design of the antigen to elicit antibodies for a specific reaction, or the immunisation and immortalisation protocol (*in vivo* or *in vitro*, hybridoma or bacteriophage display) used in their creation. In order to overcome this limitation we present here an example of the use of a chemiluminescent reporter assay in the identification of an antibody catalyst.[3] Chemiluminescent assays benefit from low background interference and are finding increasing use in the analytical laboratory. The assay system described here offers the opportunity for catalytic activity to be detected at an early stage in antibody production when only small quantities of antibody are available, and hence should allow large libraries of antibodies or other materials to be screened for hydrolytic activities.

Recently, a number of papers have appeared which use either *screening* or *selection* methods for the identification of new catalysts: the complementation of auxotrophic bacteria,[4] the use of fluorogenic substrates,[5] immobilised mechanism based inhibitors,[6] catELISA,[7] proximity coupling,[8] or pH indicators.[9] Compared to many of these approaches the highly sensitive chemiluminescent assay system based around a spiroadamantyl-substituted dioxetane substrate offers a simple one-step approach for which automated equipment is already commercially available.

Thermally stable spiroadamantyl-substituted dioxetanes, which undergo enzyme triggered chemiluminescence, have been developed for use in chemiluminescent reporter assays.[10] These luminogenic substrates undergo Chemically Initiated Electron-Exchange Luminescence (CIEEL) following cleavage of a triggering group which can be tailored to the desired enzyme activity. The dioxetanes offer the advantages of an efficient chemiluminescent reaction (ϕ_{CL} = 10^{-3}–10^{-6} in aqueous solution) which has both a long half-life and low background interference. To determine the utility of CIEEL for identifying new catalytic activities we chose the simple example



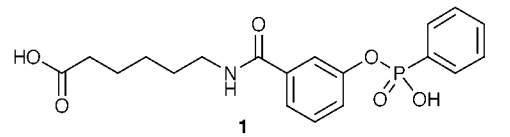
Scheme 1

of ester hydrolysis (Scheme 1), and designed a transition-state analogue (hapten **1**)† based around a phosphonate core accordingly. In order to direct most of the antibody–antigen recognition towards the phosphonate portion of the transition-state analogue, the reactive and potentially immunogenic adamantyl dioxetane moiety was omitted from hapten **1**. The position of the linker arm in the hapten for attachment to an appropriate carrier protein was chosen to orientate the dioxetane group away from the antigen binding site. This ensures that the excited ester generated following dioxetane decomposition will be released quickly from the antigen binding site, minimising the possibility of quenching of the excited state through molecular collision with the antibody, rather than through light emission.

Hapten **1** was conjugated to both Bovine Serum Albumin (BSA) and Keyhole Limpet Hemocyanin (KLH) *via* its *N*-hydroxysuccinimide ester, using standard carbodiimide chemistry.[11] The degree of conjugation to the relevant carrier protein was established using the trinitrobenzenesulfonic acid (TNBS) assay.[12]

Three Balb/c mice were hyperimmunised with the KLH conjugate (4 × 100 μg per mouse) over a period of 15 weeks, and hybridomas were generated from the spleens of these mice by fusion with P3X63Ag8 myeloma cells using standard protocols.[13] Twenty-six hybridomas producing antibodies specific for hapten **1** were identified by ELISA, and 10 of these were selected for cloning.

Substrate **2** was synthesised by modifying a route previously described.[14] Introduction of the benzoate triggering group could



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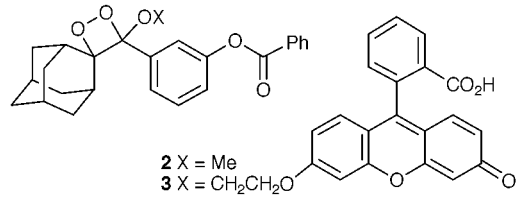

† Experimental details for the preparation and characterisation of hapten **1** and substrates **2** and **3,** and the monoclonal antibodies together with kinetic data are provided as electronic supplementary information. See http://www.rsc.org/suppdata/cc/1999/2105/

only be accomplished on the methyl enol ether intermediate, and not on the 3-hydroxyaryl spiroadamantyl dioxetane itself. The conditions for the chemiluminescent assay were as follows: the antibody (2 μM final concentration) was taken up in 190 μl of Tris HCl (50 mM, pH 9.0) in individual microtitre plate wells and was preincubated at 30 °C. The dioxetane **2** in MeCN (10 μl) (at the appropriate stock concentration to give a final substrate concentration of 1–100 μM in the incubation) was then added, and the light generated over a period of 2 h was measured using a Dynex MLX Microtitre® plate luminometer. The chemiluminescent decomposition of **2** occurs in a reasonable quantum yield ($\phi_{CL} = 1.4 \times 10^{-5}$), however, this was significantly improved by addition of fluorescein (2 mM) to the incubation medium ($\phi_{CL} = 1.0 \times 10^{-4}$). A further improvement in signal was obtained by covalently tethering the fluorescein to the dioxetane substrate **3** ($\phi_{CL} = 1.1 \times 10^{-3}$).[10] This increases the quantum yield from 0.0012% for the phenolate generated from **2** to 0.39% for the phenolate from **3** due to the efficient intramolecular energy transfer to the fluorescer. In turn this extends the lower detection limit for dioxetane decomposition from 10^{-13} moles for **2** to 10^{-16} moles for **3**. The synthetic route for the preparation of substrate **3** is described in the supporting material.† The increase in sensitivity it provides is clearly seen in Fig. 1, which also demonstrates that the antibodies are capable of at least five substrate turnovers per antigen-binding site.

Of the eight monoclonal antibodies which survived to this stage, antibody 7F11 was identified as an active catalyst and produced a chemiluminescent signal which was well above background levels with both dioxetane substrates. Larger quantities of this antibody were produced for detailed kinetic studies. The antibody-catalysed hydrolysis obeyed saturation kinetics with $k_{cat} = 0.020\ s^{-1}$, $K_m = 2.5$ μM for substrate **2** and $k_{cat} = 0.0028\ s^{-1}$, $K_m = 3.1$ μM for substrate **3** respectively. The lower k_{cat} value for substrate **3** can be explained by an increased amount of non-productive binding of this substrate (the reaction is inhibited by fluorescein which bears some structural resemblance to the hapten). No light was detected in the absence of the antibody, or in the presence of non-catalytic mAb's with either substrate. Therefore, assuming an upper limit for k_{uncat} of $1 \times 10^{-8}\ s^{-1}$ based on the detection limit of the chemiluminescent assay, the rate enhancement (k_{cat}/k_{uncat}) observed is 2.0×10^6 for substrate **2**, and 2.8×10^5 for substrate **3**, respectively. This suggests that 7F11 is one of the most efficient antibodies at catalysing benzoate hydrolysis that has yet been obtained,[1] although it is still several orders of magnitude slower than comparable esterases. The antibody is inhibited competitively by hapten **1** with Dixon analysis of the kinetic data affording a K_i value of 97 nM.† This result demonstrates that the catalysis is due to an antibody, rather than a contaminating enzyme. We are currently producing a single-chain antigen-binding fragment of 7F11 to further confirm this fact.

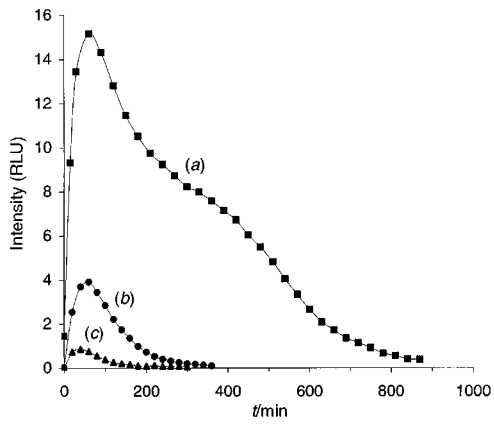

Fig. 1 Total light emission for 20 μM dioxetane (**2** or **3**) in 200 μl of 50 mM Tris buffer at pH 9.0, 37 °C in the presence of antibody 7F11 (2 μM): (*a*) **3**, (*b*) **2** + fluorescein (2 mM) and (*c*) **2**.

In conclusion we have shown that it is possible to identify antibody catalysts using a chemiluminescent substrate system. The absolute sensitivity of the assay using substrate **3** is sufficient for detection of catalysts in hybridoma supernatants or phage-displayed antibody libraries[5] and these are currently being screened. Modification of the spiroadamantyl dioxetane to allow screening of other hydrolytic activities, and for 'remote' release is currently underway. The use of the chemiluminescent catalytic antibody in a chemiluminescent imaging system is being investigated as a possible replacement for enzyme–secondary antibody conjugates, or for enzyme–antibody/protein A fusion proteins as required by ELISA.[15] These self-marking antibodies could be useful in a wide range of immunochemical assays including microtitre plate immunoassays, immunoblotting and immunohistochemistry.

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