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# Enhanced chemiluminescence enzyme immunoassay

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Abstract - The characteristics and applications of enhanced chemiluminescent reactions for the determination of peroxidase labels in ligand-binder assays are reviewed.

## INTRODUCTION

Exploitation of light-emitting reactions (chemiluminescent or bioluminescent) in immunoassay falls into two main areas. Components of a chemiluminescent (e.g., luminol) or bioluminescent reaction (e.g., a luciferase, aequorin) can be used as a label, or a chemiluminescent or bioluminescent reaction can be used to monitor an enzyme label or its products. Table 1 lists some representative examples of immunoassays which have end-points based on light emission. Recently, considerable attention has focussed on the chemiluminescent assay of horseradish peroxidase labels following the discovery of a new enhanced chemiluminescent end-point for peroxidase labels (ref. 1).

## ENHANCED CHEMILUMINESCENT ASSAY OF HORSERADISH PEROXIDASE

Peroxidase catalyses the chemiluminescent oxidation of cyclic diacyl hydrazides by hydrogen peroxide (Fig. 1). This assay procedure has been significantly improved by the discovery of a series of compounds which, when added to the reaction, increase the light emission by several orders of magnitude. These compounds are known as enhancers and Fig. 2 illustrates some typical examples of molecules with enhancement properties.

TABLE 1 Immunoassays based on a light-emitting end-point

| Analyte | Label | Detection system | Reference |
|---|---|---|---|
| Digoxin | Horseradish peroxidase | Luminol-$H_2O_2$-enhancer | 1 |
| Trinitrotoluene | Firefly luciferase | Luciferin-ATP | 2 |
| Rubella specific IgG | Bacterial luciferase | $FMNH_2$-decanal | 3 |
| Estriol | NAD | Bacterial luciferase NAD:FMN oxidoreductase | 4 |
| Estradiol | Aminobutylethyl-isoluminol | $H_2O_2$-microperoxidase | 5 |
| Thyroxine | Acridinium ester | $H_2O_2$-NaOH | 6 |
| IgG | Phenanthridinium ester | $H_2O_2$ | 7 |
| Thyroxine | Glucose oxidase | TCPO-ANS | 8 |
| Progesterone | Glucose-6-phosphate dehydrogenase | Bacterial luciferase-NAD:FMN oxidoreductase | 9 |
| Kallikrein | Alkaline phosphatase | D-luciferin-O-phosphate-ATP-firefly luciferase | 10 |
| Human chorionic gonadotrophin | Horseradish peroxidase | *Pholas dactylus* luciferin | 11 |

Abbreviations: ANS - 8-anilino-naphthalenesulphonic acid, ATP - adenosine 5'-triphosphate, FMN - flavine mononucleotide, NAD - nicotinamide adenine dinucleotide, TCPO - bis(2,4,6-trichlorophenyl)oxalate

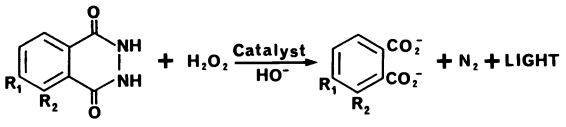
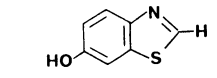


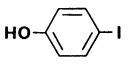

Fig. 1. The chemiluminescent oxidation of cyclic diacyl hydrazides.

luminol $R_1$-H $R_2$-$NH_2$

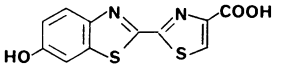
isoluminol $R_1$-$NH_2$ $R_2$-H



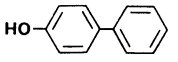

6-Hydroxybenzothiazole



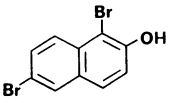

p-Iodophenol



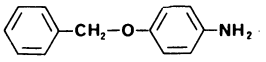

Dehydroluciferin




p-Phenylphenol




1,6-Dibromo-2-naphthol




4-Benzyloxyaniline

Fig. 2. Molecules capable of enhancing light emission from the HRP-catalysed oxidation of luminol.

Enhancers act synergistically in the peroxidase-catalysed luminol-peroxide reaction. The degree of enhancement varies depending upon the conditions and the identity of the enhancer. For example, with p-iodophenol as the enhancer light intensity can be increased by up to approximately 2500-fold, compared to an unenhanced reaction. Enhancement is also pH- and concentration-dependent. Significant enhancement occurs between pH 7 and 9.5 and maximum enhancement is seen close to pH 8.6. Enhancers show an optimal concentration for enhancement and above this further addition of enhancer to a peroxidase-luminol-peroxide reaction decreases the degree of enhancement. Cyclic hydrazides other than luminol can be used in an enhanced reaction (e.g., isoluminol, N-(6-aminobutyl)-N-ethyl isoluminol, 7-dimethylaminonaphthalene-1,2-dicarbonic acid hydrazide) and peroxide can be replaced by oxidants such as perborate. An important feature of enhanced chemiluminescent reactions is that the light is emitted as a prolonged glow. This simplifies the analytical application of the reaction because it can be initiated in isolation and then a light-intensity measurement made, for example, up to 20 min later. The assay is very sensitive. For example <2.5 fmol of horseradish peroxidase can be detected using a p-iodophenol enhanced reaction and a luminometer operating in photocurrent mode.

## APPLICATIONS

Enhanced chemiluminescent detection of horseradish peroxidase labels in enzyme immunoassays has been extensively tested. Data are now available for competitive and immunoextraction (sandwich) type assays, a wide range of analytes (e.g., drugs, antibodies, cancer markers, steroids, peptide hormones, thyroid hormones) and for assays based on the spectrum of commonly used solid supports (e.g., tubes, beads, microtitre wells, membranes, micropins, magnetic particles). In many cases the detection limit of the enhanced chemiluminescent enzyme immunoassay is superior to conventional immunoassays. Some representative data are collected in Table 2.

TABLE 2 Enhanced chemiluminescent enzyme immunoassays

| Analyte | Solid support | Detection limit | Ref |
|---|---|---|---|
| Estradiol | Microtitre plate | 1.8 fmol | 12 |
| Follicle-stimulating hormone | Microtitre plate | 0.01 mU | 12 |
| Thyroid-stimulating hormone | Microtitre well | <0.04 μIU $ml^{-1}$ | 13 |
| Human chorionic gonadotropin | Microtitre well | <0.6 mIU $ml^{-1}$ | 14 |

TABLE 3. Commercially available enzyme immunoassays modified to an enhanced chemiluminescent end-point

| Company | Analyte | Ref |
|---|---|---|
| Abbott | Carcinoembryonic antigen | 21 |
| Boehringer Mannheim | Digoxin | 22 |
| Walker Laboratories | Beta-2-microglobulin | 23 |
| Sibar | Testosterone | 23 |
| Hoechst | IgE | 24 |

DNA probe-based assays are assuming increasing importance but they are only likely to enter routine use if a suitable non-radioactive label can be developed to replace the conventional $^{32}P$ label. Several groups have investigated peroxidase labels detected using enhanced chemiluminescence. Various enhancers have been tested including firefly luciferin (ref. 15), p-iodophenol and p-hydroxycinnamic acid (ref. 16). Preliminary results have been very encouraging and a detection limit of 1 pg has been achieved for pBR 322, a genetically engineered DNA fragment.

## COMMERCIAL REAGENTS AND SYSTEMS

The feasibility of adapting commercially available enzyme immunoassays to an enhanced chemiluminescent end-point has been demonstrated with products from various companies (Table 3). Replacement of a colorimetric end-point by a rapid enhanced chemiluminescent end-point reduces overall assay time significantly. It had no detrimental effect on assay performance (e.g., precision) despite the fact that the enzyme immunoassay reagent formats (tubes, beads, etc.) were not ideally matched to the available light-measuring equipment. Latterly, an enhanced chemiluminescent enzyme immunoassay system under the trade name "Amerlite" has become available from Amersham International PLC. In this system the reagent format and light-measuring instrumentation are matched to ensure efficient light measurement. The "Amerlite" system uses coated opaque white microtitre wells which are read using an automatic luminescence microtitre plate reader. After stabilisation of the light emission (approximately 2 min after initiation) the light emission from individual wells is measured for 0.2 s . A complete plate of 96 wells can be read and the results processed in less than 2 min (ref. 17).

A range of assays has been developed for this system, including carcinoembryonic antigen, thyroid-stimulating hormone, alpha-fetoprotein, luteinising hormone, thyroxine and tri-iodothyronine, and several evaluations of the system have been reported (refs. 18-20).

## EXTRA-LABORATORY TESTING

The combination of enhanced chemiluminescence and either photographic film or luminometers based on silicon photodiode detectors has considerable potential as a testing system for use outside conventional laboratories. The intense glow of an enhanced reaction permits simplification of luminometer design in two ways. Firstly, reactions can be initiated and then transferred to the luminometer, thus obviating the need for injectors to initiate individual reactions in front of the photodetector. Secondly, the intensity of the light emission is such that a photomultiplier tube can be replaced with a simple detector such as photographic film or a photodiode (ref. 25).

Simple luminometers based on instant photographic film (camera luminometers) have been developed which can simultaneously monitor up to 63 individual reactions contained in the wells of a modified microtitre plate (ref. 26). The camera is small, requires no power source, is inexpensive to produce and is portable. Examples of chemiluminescent enzyme immunoassays performed photographically are listed in Table 4. The photographic

TABLE 4. Photographically monitored chemiluminescent enzyme immunoassays

| Analyte | Detection System | Type of film | Reference |
|---|---|---|---|
| Cytomegalovirus specific IgG | Luminol-peroxide-6-hydroxybenzothiazole (or p-iodophenol) | Polaroid Type 612 | 27,28 |
| Factor VIII related antigen | Luminol-peroxide-p-iodophenol | Polaroid Type 612 | 29 |
| Ferritin | Luminol-peroxide-firefly luciferin | Polaroid Type 612 | 30 |
| IgE | Cyclic diacyl-hydrazide-peroxide | Polaroid Type 57 | 31 |
| Malaria specific IgG | Luminol-peroxide-p-iodophenol | Polaroid Type 612 | 32 |
| Rubella specific IgG | Luminol-peroxide-p-iodophenol | Polaroid Type 612 | 33 |
| Progesterone | Luminol-peroxide-p-iodophenol | Polaroid Type 612 | 34 |

results are qualitative but a degree of semi-quantitation can be achieved by interposing a stepped neutral density filter between the instant film and the glowing reaction vessel.

## CONCLUSION

Enhanced chemiluminescent reactions have many advantages in the detection and quantitation of horseradish peroxidase and horseradish peroxidase conjugates. The reactions are sensitive, rapid, employ inexpensive reagents and mild reaction conditions. Light emission is intense and prolonged and this simplifies both the initiation of the light-emitting reaction and the measurement of the light. The intense nature of the light emission also allows the use of simple detectors such as photographic film and silicon photodiodes thus reducing the cost of instrumentation.

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