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LUMINESCENT TECHNIQUES APPLIED TO BIOANALYSIS

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Content

1. Introduction

The bio- and chemiluminescent reagents, introduced in analytical chemistry since the seventies, allow amplification of the analytical signal and to develop fast and sensitive methods[1]. The bioluminescent enzymatic systems based on firefly or bacterial luciferase, the chemiluminescent emission of the hydrogen peroxide/peroxidase/luminol system or some more recently introduced luminescent substrates (dioxetanes) offer a unique and universal tool to determine the majority of analytes and enzymes of potential interest in bioassays[2].

It has been demonstrated that the best performances were obtained using the immobilized enzymes, because of an increased stability, catalytic activity, the possibility to recover the enzymes and to develop automated systems. Immobilized enzymes are widely used in the biochemical laboratory to assay several analytes and enzyme activities. Metabolite analysis in biological systems requires sensitive and specific methods because analytes are present at low concentrations and further dilution of biological samples is often needed to avoid interference due to matrix effects. In conjunction with bioluminescent enzymes (firefly and bacterial luciferases) and chemiluminescent catalysts (peroxidase and alkaline phosphatase)[3-6], we set up high-sensitive flow sensors based on the use of nylon tube coil or epoxy methacrylate (Eupergit C) column as solid support. When these flow sensors were applied to *in vivo* determination, a suitable microdialysis probe inserted directly into tissues or blood allows continuous measurement of extracellular analyte levels by means of a bioluminescent flow detector system.

In the diagnosis of several microbial diseases, the use of nucleic acid hybridization techniques has led to the use of several radioactive or non isotopic labelled probes which can be immunoenzymatically visualized[7]. It has also been demonstrated that chemiluminescent enzyme substrates are the most sensitive tools available for the detection of enzyme conjugates, including the nucleic acid hybridized probe[8]. The hybridized DNA and RNA probes are generally visualized by Fab fragments labelled with alkaline phosphatase (ALP) or horseradish peroxidase (HRP)[9].

Here we summarize our experience with immobilized luminescent enzymes coupled with the analyte specific enzyme and DNA and RNA chemiluminescent probes applied to viral detection.

2. Luminescent Systems

2.1. Firefly Bioluminescence

Firefly luminescence is the most extensively studied bioluminescent system[4,10]. The light-producing reaction requires the enzyme luciferase, luciferin, Mg^{2+}, ATP, and

molecular oxygen, which give an oxyluciferyl adenylate-enzyme complex in the excited state. After emission (560 nm), the ground-state complex dissociates to form enzyme, AMP, pyrophosphate (PPi), oxyluciferin, and carbon dioxide:

$$\text{ATP} + \text{luciferin} + \text{O2} \xrightarrow{\text{luciferase}} \text{AMP} + \text{PPi} + \text{CO}_2 + \text{oxyluciferin} + \text{LIGHT}$$

The concentration of other metabolites, of cofactors involved in the luminescent reaction like Mg^{2+}, or the activity of enzymes participating in coupled reactions leading to the formation or consumption of ATP can be assayed with the aid of the firefly luciferase-dependent reaction[1,3].

2.2. Bacterial Bioluminescence

The bioluminescent enzyme system from marine bacteria[1-4] consists of a NAD(P)H:FMN oxidoreductase and a luciferase which emits light at 490 nm in the presence of FMN, NAD(P)H, a long chain aliphatic aldehyde and molecular oxygen, according to reactions (7) and (2).

$$\text{NAD(P)H} + \text{FMN} + \text{H}^+ \xrightarrow{\text{NAD(P):FMNoxidoreductase}} \text{NAD(P)}^+ + \text{FMNH}_2 \quad (1)$$

$$\text{FMNH}_2 + \text{RCHO} + \text{O}_2 \xrightarrow{\text{luciferase}} \text{FMN} + \text{RCOOH} + \text{H}_2\text{O} + \text{LIGHT} \quad (2)$$

Various substances of biological interest and enzyme activities can be analysed by coupling the luciferase and the oxidoreductase to a third reaction which either produces or consumes NADH or NADPH.

2.3. Luminol Chemiluminescence

The amount of H_2O_2 which derives from the catalysis of a specific oxidase may be assayed by adding excess peroxidase and luminol[2,4] and measuring steady state chemiluminescence intensity according to the scheme:

$$\text{luminol} + 2\ \text{H2O2} + \text{OH}^- \xrightarrow{\text{peroxidase}} \text{aminophthalate} + + 3\ \text{H}_2\text{O} + \text{N}_2 + \text{LIGHT}$$

Since the quantum yield of this reaction is very low (~ 10%), the addition of enhancers, such as *p*-iodophenol or *p*-hydroxycinnamic acid, in the system further increases light emission and transforms the signal from a flash to a steady state output[11]. The enhancers also reduce the background light emission from the luminol-oxidant mixture, further improving the signal/background ratio.

Another field which luminescent detection systems can contribute significantly to the analytical performance of various assays is that of immunoassays, in which the high sensitivity of luminescent substrates can be coupled to the specificity of immunological reagents such as polyclonal or monoclonal antibodies[12].

2.4. Dioxetane Chemiluminescence

New alkaline phosphatase chemiluminescent substrates are the adamantyl-1,2-dioxetane phenyl phosphate (PPD) and its Cl- and Br- derivatives. In the presence of alkaline phosphatase, PPD is dephosphorylated and produces an unstable intermediate which further decomposes, emitting a glow of light in direct proportion to the amount of alkaline phosphatase[6]:

$$\text{PPD} \xrightarrow{\text{alkaline phosphatase}} [\text{PD}]^* + \text{phosphate}$$

$$[\text{PD}]^* + \text{F} \xrightarrow{\text{ENERGY TRANSFER}} \text{PD} + [\text{F}]^*$$

$$[\text{F}]^* \rightarrow \text{F} + \text{LIGHT}$$

where the asterisks represent an excited state and F is the fluorescent acceptor (enhancer). The Cl- and Br-substituted dioxetanes give lower background and lower detection limits when compared with non derivatized ones [13]. The new dioxetanes contain a modified hydrophobic adamantyl group to prevent aggregation, and the background chemiluminescence observed was 50 % lower than that of PPD.

2.5. Acridans Chemiluminescence

Recently another new substrate for horseradish peroxidase (HRP) has been produced: a stable trifluorophenyl-substituted acridan (Lumigen PS-3), which emits fotons at 430 nm. The detection of HRP activity by PS-3 is based on a chemiluminescent process involving the enzymatic generation of an intermediate acridinium ester. In the presence of hydrogen peroxide and *p*-iodophenol as enhancer, the

reaction of the acridan substrate with the HRP label generates thousands of acridinium ester intermediates ($[AE]^+$) per minute, which react with hydrogen peroxide, at alkaline pH, to produce the N-methylacridone in the singlet excited state. This compound comes back to ground state producing a sustained high-intensity chemiluminescence, according to the scheme:

$$\text{PS-3} \xrightarrow{\text{HRP, phenol, } H_2O_2} [AE]^+$$

$$[AE]+ \xrightarrow{\text{Buffer, } H_2O_2} [\text{N-methylacridone}]^* + \text{trifluorophenol}$$

$$[\text{N-methylacridone}]^* \rightarrow \text{N-methylacridone} + \text{LIGHT}$$

3. Immobilized Flow Systems

The continuous flow format of the analytical method offers greater possibilities than a single batch system because it leads to rapid and sensitive assays. The immobilized enzymes, on nylon coils or methacrylate beads, can be used for many analyses and multichannel systems can be developed. The analytical performances in terms of detection limit, linearity and reproducibility for the different methods developed are reported in Table I. As shown, many analytes can be detected at pmol levels, with good precision and a wide range of linearity. Moreover, unless high sensitivity is required, they reduce the analysis time to a few seconds and need smaller serum specimens.

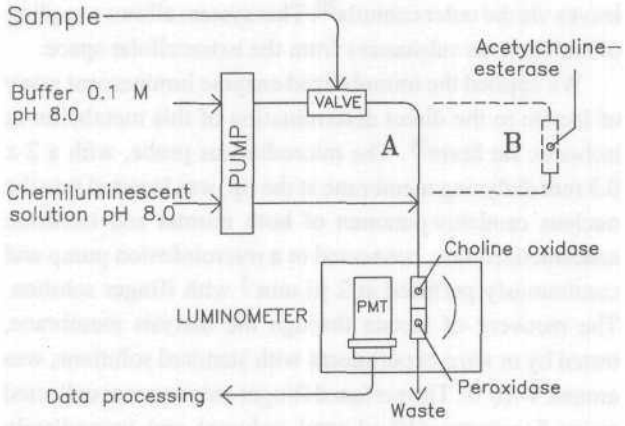

Fig. 1. **Example of typical luminescent flow sensing device** (manifold for pesticide chemiluminescent flow assay with one(A) or two (B) columns using immobilized enzymes)

Table I
Analytical performance of the immobilized luminescent enzymes

Analyte[a]	Range (pmoles in sample)	Detection limit [$\mu mol.l^{-1}$]	Precision Content [pmol]	Precision RSD [%]
NADH (B)	1–2500	0.1	2.5	3.7
NADPH (B)	5–1500	1	10	5.3
α-Bile acid (B)	1–1000	0.5	2.7	5.9
3α-Bile acid (B)	1–1000	0.5	2.8	7.1
12α-Bile acid (B)	10–1000	1	5.2	6.3
Ethanol (B)	50–2500	1	100	8.3
Glycerol (B)	50–500	2	70	7.2
Acetaldehyde (B)	50–1000	2	100	6.8
L-Alanine (B)	5–500	0.5	75	7.4
Branched-chain (B) aminoacids	20–2000	0.5	75	7.8
ATP (L)	0.05–100	0.02	1.5	8.0
ADP (L)	0.6–100	0.1	15	8.2
H_2O_2 (C)	50–8000	1	150	7.2
Oxalate (C)	50–8000	1	150	9.1
ATP (M, r-LM)	1–500	0.3	10	9.3
ATP (r-LN)	0.3–100	0.06	10	5.2
L-Lactic acid (B)	1–500	0.1	7	5.2
D-Lactic acid (B)	10–500	5	50	7.8
L-Glutamic acid (B)	50–1000	10	500	3.7
L-Phenyl-alanine (B)	1–100	0.5	50	4.3
Choline (C)	20–1000	1.2	75	8.4
Magnesium (L)	0.05–6.7	50	0.67	4.5
Magnesium (r-LN,M)	0.01–6.7	10	0.99	2
Lactate dehydrogenase (B)	3–2000[b]	1[b]	151[b]	8.8
Creatine kinase (L)	0.1–100[b]	0.1[b]	17[b]	8.2
Phospholipase D (C)	0.2–100[b]	0.17[b]	40[b]	9.2

[a]Abbreviations: (B) bioluminescent bacterial system on nylon, (L) bioluminescent firefly system on nylon, (C) chemiluminescent luminol system on nylon, (M) bioluminescent firefly system on methacrylate beads, (r-LM) recombinant firefly luciferase on methacrylate beads, (r-LN) recombinant firefly luciferase on nylon, [b]$\mu mol.min^{-1}.l^{-1}$

3.1. Apparatus

The general scheme of the system is designed to be as simple and versatile as possible (Fig. 1). Using enzyme

immobilized on nylon coils, the system is schematically composed of a sampler which permits insertion of the sample within two bubbles of air or directly after a suitable time interval by a three-way valve, another two or three streams for buffer or substrates supply, specific immobilized enzyme, and finally the luminescent enzyme(s) reactor inserted directly into the luminometer. In order to achieve reproducible and accurate measurements, the sample must be separated from the working solution stream by two air bubbles to prevent axial diffusion caused by the concentration gradient which can reduce reproducibility.

The beads (usually Eupergit) with immobilized enzymes are generally packed into a small Plexiglas column (3 cm x 2 mm i.d.) and the column is located inside the luminometer in front of the photomultiplier window. The same system as used for coils can be employed for the Eupergit column, except that the air is replaced with buffer in the second stream and the presence of air bubbles inside the column is carefully avoided. The Eupergit column can be also coupled with a nylon coil containing immobilized enzymes (not luminescent), that can be placed before the column, outside the luminometer.

3.2. Applications

Table I shows a list of analytes, which have been determined using immobilized luminescent enzymes.

A typical example of these analytical systems is a manifold using bacterial luciferase for L-phenylalanine assay[14] developed with two separate nylon coils: the first one contains the specific L-phenylalanine dehydrogenase (L-PheDH) enzyme:

$$\text{L-phenylalanine} + NAD^+ \xrightarrow{\text{L-PheDH}} \text{L-phenylpyruvate} + NADH + H^+ + NH_4^+$$

and the second the bacterial bioluminescent enzymes. This system allows reaching a detection limit of 0.5 $\mu mol.l^{-1}$.

A continuous-flow system was developed for the assay of magnesium (II) in serum, drugs and beverages, making use of firefly luciferase (LUC) or recombinant luciferase (r-LUC) from *Escherichia coli* immobilized both on nylon coil and on epoxy methacrylate[15]. The sample assay rate was 20 $samples.h^{-1}$ with no carry-over; 10 µl samples were analysed. The detection limit of the assay was 0.05 $mmol.l^{-1}$ for LUC-nylon coil and 0.01 $mmol.l^{-1}$ for LUC-Eupergit column and r-LUC-nylon coil. No interference from ions present in the samples (Ca^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}) was found. The nylon-immobilized enzymes give a relatively high stablility (1-4 months), despite of a low recovery in terms of activity with respect to the soluble forms; Eupergit C gives better sensitivity and activity recovery but lower enzyme stability (3 days–1 month).

The manifold showed on Fig. 1A has been used for choline and/or phospholipase D assay[16]. Two columns manifold (Fig. 1B) successfully allowed determination of pesticides, like paraoxon and aldicarb[17]. These flow systems do not require thermostating, light emission being constant in the range 18-28 °C;however, this can be done if an accurate control of temperature is needed, particularly when enzymatic activity is measured[16], e.g. phospholipase D. On the other hand, flow-rate variations do not influence light emission over a sufficiently wide range. Variations in sample volume obviously have greater effects when a short coil is used (50 cm) since saturation values are soon reached. Using a longer coil (e.g. 1 m or more), the light signal remains constant over a larger range of sample volumes. If highly sensitive analytical performance is needed, the low-light imaging luminograph can be used[18]. This instrument can be also applied for batch enzyme activity assay[19].

3.3. Microdialysis Technique

In vivo metabolite determinations can be performed by inserting „an artificial capillary" into a target area (brain area, muscle, adipose tissue, blood, etc). The probe consists of two concentric steel cannulas covered at the tip by a dialysing membrane (molecular mass cut-off around 20,000); a physiological fluid is introduced through the inner cannula, flushes the inside of the membrane, and leaves via the outer cannula[20]. This system allows sampling of endogenous substances from the extracellular space.

We applied the immobilized enzyme luminescent assay of lactate to the direct determination of this metabolite in ischemic rat brain[21]. The microdialysis probe, with a 2 x 0.5 mm dialysing membrane at the tip, was inserted into the nucleus caudatus-putamen of both normal and ischemic anaesthetized rats, connected to a microinfusion pump and continuously perfused at 2 $\mu l.min^{-1}$ with Ringer solution. The recovery of lactate through the dialysis membrane, tested by *in vitro* experiments with standard solutions, was around 4–10 %. The perfused Ringer solution was collected every 5 minutes (10 µl total volume) and immediately analyzed for lactate content. Results were in agreement with the values obtained at the same conditions by other measurement techniques, such as HPLC, which, however, require larger injection volumes.

4. DNA and RNA Probes

We have developed dot blot hybridization immunoenzymatic assays with chemiluminescent substrates for the sensitive and quantitative detection of cytomegalovirus, B19 Parvovirus and plasmid pBR328 DNAs[7,22]. Digoxigenin-labelled probes were used and then visualized by anti-digoxigenin Fab fragments labelled with alkaline phosphatase or horseradish peroxidase[8,9]. Digoxigenin was preferred as a nucleotide marker in order to avoid problems that may arise from the use of biotin-labelled probes detected with avidin systems in sera and because of its high specificity and sensitivity similar to that of radiolabelled probes, but without the potential health hazards, disposal problems and instability associated with radioisotopes.

Light emission can be revealed both by instant-development photographic film as a qualitative test and by instruments like luminometer or luminograph as a reliable quantitative assay in clinical samples. In recent years, the research and synthesis of improved chemiluminescent substrates has been matched by new developments in photon imaging instrumentation such as ultrasensitive luminographs based on an intensified charge-coupled device (CCD) video camera. This instrument allows the quantification of the light signal at the level of a single photon and localization of the chemiluminescent emissions on a target surface, such as tissue or membranes[22]. Spots of hybridized DNA, cut out from nylon membrane, were placed into cuvettes containing the chemiluminescent mixture and directly analysed in a luminometer, and were revealed by ALP-conjugated and by HRP-conjugated reagents.

Of the two labelling systems, the ALP system, using PPD as the chemiluminescent substrate, was more reliable and appliable. In fact, the samples can be stored dry at 4 °C for at least 1 week and it is not necessary to measure them immediately like for HRP conjugates; the measurements can be performed all together, not one by one because the light emission is not an unstable flash but glows for several days. The results obtained with ALP detection were more reproducible: the relative standard deviation was about 6 %, whereas that obtained with the HRP detection system was 13 %.

The sensitivity of the chemiluminescent assay is compared with that of the colorimetric one in Table II. Moreover, for membrane-based applications (Southern blotting and DNA sequencing) the Cl-derivatives give, in comparison with PPD, faster kinetics, permitting faster exposures, and sharper images of the bands obtained for DNA sequencing blots. For both systems, a key step was the use of the same kind and batch of nylon membrane, because some types are very highly charged and can cause a high background; to avoid this, each batch must be pre-tested.

Table II
Sensitivity of colorimetric and dioxetane ALP chemiluminescent photographic test detection of viral and plasmid DNAs using different concentrations of digoxigenin labelled probes

Probe concentrations	Detection limit	
	colorimetric	chemiluminescent
10 ng.ml^{-1}	5 pg	1 pg – 500 fg
40 ng.ml^{-1}	1 pg – 500 fg	200 fg – 100 fg
160 ng.ml^{-1}	500 fg – 100 fg	50 fg – 10 fg

5. Conclusion

The variety of the assays reported here displays the great versatility of the luminescent detection systems. The main advantages are the high sensitivity and specificity, that reduce to the minimum the sample treatment. Immobilized systems allow to reduce greatly the cost per assay, on the other hand their preparation requires a good expertise, especially in the surface activation step on nylon tubes.

The bioluminescent flow sensors based on enzymes immobilized on nylon tubes and Eupergit columns specifically assay several analytes and enzymes activities (Table I) and are simple, require only minor modifications of a commercial detector and allow the analysis of about 20-30 samples per hour. Therefore the flow bioluminescent methods appear highly competitive with other methods such as radioimmunoassay, enzyme immunoassay, high performance liquid chromatography, and fluorimetry which require radioactive materials, separation steps, sample manipulation or expensive equipment.

When the flow sensors are used coupled with microdialysis probe there are several advantages:

- the reaction takes place in few minutes allowing continuous analysis,
- sensitivity is in the order of pmoles,
- the microdialysis probe allows biological specimens to be drawn without proteins or macromolecules.

This approach can be extended to the analysis of other analytes which need to be detected continuously, such as during therapy monitoring or in emergency care units.

The use of chemiluminescent dot blot DNA and RNA hybridization techniques suggest that chemiluminescent substrates can be considered useful tools for the detection of nucleic acid hybridization reactions because of their high sensitivity and specificity. In the case of *in situ* hybridization assays the best performance can be obtained using new instruments, such as videocamera luminograph with computer collecting data[19].

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S. Girotti[a], E. N. Ferri[a], S. Ghini[a], F. Fini[a], M. Musiani[b], G. Carrea[c], A. Roda[d], P. Rauch[e] *("Institute of Chemical Sciences, University of Bologna, [b]Institute of Microbiology, University of Bologna, [c]Institute of Hormone Chemistry, CNR, Milano, [d]Department of Pharmaceutical Sciences, University of Bologna, Italy, [e]Institute of Chemical Technology, Prague):* **Luminescent Techniques Applied to Bioanalysis**

The principle of luminescent systems (firefly and bacterial bioluminescence, luminol, dioxetane and acridans chemiluminescence) is described. The bio- and chemiluminescent reagents can be employed as highly sensitive and, together with immobilized enzymes, also specific detection systems in bioanalytical assays both by using batch determination and continuous flow systems. An example of versatile manifold (flow sensing device) is shown. A list of possible bioanalytical applications is given.