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CHEMILUMINESCENCE, AN OUTSTANDING PHYSICOCHEMICAL METHOD

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ABSTRACT

Luminescence has been known from ancient times. However, consistent theories about the mechanisms involved in this process have been proposed only during the last few decades. The luminescence phenomenon is the result of the absorption of energy by atoms or molecules followed by their de-excitation, with photon emission in the visible part of the spectrum. The source of energy that leads to the excited species in bioluminescence and chemiluminescence is a chemical reaction that may or may not involve enzymes. This paper presents a short review of the present state of knowledge about chemiluminescence and discusses some of their applications in biology and medicine.

RESUMO

A luminescência é um processo conhecido desde a antiguidade. Teorias consistentes para explicar este fenômeno foram propostas porém somente durante as últimas décadas. A luminescência é o resultado da excitação de átomos ou moléculas seguida por emissão de energia em forma de fótons na região visível do espectro. Na bioluminescência e quimiluminescência a fonte de energia que leva às espécies excitadas é uma reação química que pode ser catalizada ou não por enzimas. O presente trabalho apresenta uma resenha do conhecimento atual sobre quimiluminescência e discute algumas aplicações deste processo na biologia e na medicina.

KEYWORDS: chemiluminescence, bioluminescence, phagocytosis, HPLC-detection, microbiology

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1. A SHORT HISTORY

The luminescence phenomenon of cold light has been known from ancient times and its appearance was often thought to be due to supernatural power. Many old stories refer to strange lights or flames such as glowing hands and luminous corpses, glowing trees or shining animals[1-3].

Many of these stories originate from the observations of natural luminescent phenomena like those enumerated in Table 1.

Table 1- Exhibiting bioluminescence organisms

Noctiluca miliaris	a marine dinoflagelate
Renilla reniformis	sea pensy
Diplocardia longa *Octachaetus multiporus*	earth worms
Latia neretoides	a fresh water limpet
Arachnocampa luminosa	the New Zealand glow-worm
Chaetopterus variopedatus	a marine polichete annelide
Watasenia scintilans	a luminous squid
Photobacterium phosphoreum	a luminous fungus
Photinus Pyralis	the American firefly
Pholas Dactilus	a bivalve

Some of the first references about this phenomenon appeared in the works of Aristotle (384-322 BC) in Ancient Greece, who described the bioluminescence of dead fish and fungi in *De Anima*[4], or three centuries letter when Pliny the Elder during the Roman Empire described some luminescent species known nowadays as *Lucerna piscis* and *Pelagia noctiluca.*

After centuries of apparent silence in this field only in 1669 Robert Boyle (1627-91)[1,2] established some of the properties of bacterial and fungal luminescence and demonstrated the importance of air whose reduction in surrounding medium of 'shining flesh' or 'shining wood' determined a large decrease of the light intensity. In the same year an alchemist of Hamburg, Henning Brand gave the first example of artificial chemiluminescence, by isolating a substance, he called "phosphorus" in distilled urine, and reduction of the remaining material. The product he obtained had a blue light[3] glow and this was thought to be the cause of all cold light phenomena. Only in 1821 this idea was disproved by J. Macaire[5] who concluded that luminous material was composed mainly of 'albumin', and required oxygen. Later, in 1887 Rafael Dubois isolated two extracts from the luminous organ of the click beetle, *Pyrophorus*, extracts he named luciferin and luciferase, a hot-water, respectively a cold-water extract. Separately these two substances did not emit light, though the mixing of the two restored the emission. His conclusion showed clearly that the luminescence was the result of a chemical reaction between the two substances[6].

But it was only the last few decades that the development of some consistent theories about the mechanisms involved in these processes were determined. The

luminescence phenomenon or cold light is the result of the absorption of energy by some atoms or molecules followed by their de-excitation with a photon emission in the visible region of spectrum. The absorption of energy can be the result of different phenomena like: heat (candoluminescence and piroluminescence), irradiation (photoluminescence, radioluminescence), electrical excitation (electro- and piezoluminescence), structural rearrangements in solids (bio- and cristaloluminescence) or chemical reaction (chemi- and bioluminescence).

2. ELECTRONIC EXCITATION

Chemiluminescence (CL) is a complex phenomenon in which a product of an exo-energetic reaction is endowed with the energy released in one crucial step. This excited product follows the universal laws of photophysics and returns to its ground state with photon emission to give direct chemiluminescence or transfers its excitation energy to another fluorescer giving rise to sensitised chemiluminescence The basic differences between chemiluminescence and the better known fluorescence and phosphorescence involves the excitation mechanisms and the final structure of atom/molecule processed.

i) in fluorescence and phosphorescence the excitation mechanism is the absorption of a photon that forms the excited state of the molecule in chemiluminescence the excited state is formed as a result of a chemical reaction,

ii) in fluorescence and phosphorescence the de-excitation with photon emission will result in the return of the atom or molecule to the ground state. In chemiluminescence the chemical reaction will lead to a new product formation, in an excited state and this will return to the ground state with the emission of light in the visible region of the spectrum. In fluorescence and phosphorescence the absorption-emission processes may be repeated over and over while in chemiluminescence it occurs only once time for each molecule.

No matter how the luminescence arise, the main step involved is the electronic excitation.

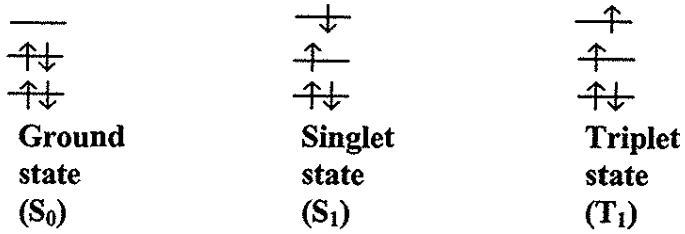


Fig. 1 - Electronic configuration of a ground state (S_0) and electronically excited singlet (S_1) and triplet (T_1) state

This is illustrated in Fig.1. where the electronic configuration of a molecule in the ground state is depicted by S_0, the first excited singlet state by S_1, which represents the fluorescent state, and the first triplet state T_1 defines the phosphorescent state. The crucial orbitals that show the electron population are the highest occupied and lowest

unoccupied molecular orbitals. In the S_0 and S_1 states, the electrons possess antiparallel spin configurations (hence, singlet states) and in T_1, a parallel arrangement (hence, triplet state)

While in the ground state S_0 the electrons to be excited are accommodated in the same molecular orbital, i.e., the highest occupied molecular orbitals, in the excited states S_1 and T_1 the highest occupied and lowest unoccupied molecular orbitals are singly occupied.

While singlet excited states with lifetimes shorter than 10^{-8} seconds are less important in most bimolecular processes of biological systems the triplet states with longer lifetimes are prone to participate in bimolecular events such as chemical reactions or physical processes (energy transfer) with biomolecules, even when the latter ones are present only in low concentrations. The energy transfer processes may lead to sensitized emission like in sensitised chemiluminescence .

3. THE EMISSION EFFICIENCY

As was already mentioned chemiluminescence is observed when light is emitted from a chemical reaction. If the reaction occurs in a living system or is derived from one, the process is called bioluminescence (BL). The intensity of light is generally observed to increase initially and later decrease with time as the reactants are consumed. This fact can be described by the chemiluminescence quantum yield or efficiency which is photon emitted per mole of added or reacted chemiluminescence species and is expressed in Einstein mol^{-1} (1 Einstein = 6.023×10^{23} photons). An empirical equation relates the chemiluminescence quantum yield $\mathbf{Q_{CL}}$ with the yield of the chemical reaction $\mathbf{Y_R}$, the fluorescence quantum yield of the primary excited product, $\mathbf{Q_F}$ and the chemiexcitation efficiency, $\mathbf{Q_{CE}}$

$$\mathbf{Q_{CL} = Y_R \times Q_F \times Q_{CE}} \qquad (1)$$

$\mathbf{Q_{CE}}$ is the percentage of molecules crossing from the transition state of the reactant to an electronically excited state of the product. This process is not well understood, Q_{CE} can only be calculated from E_q (1) and is the measure of the chemiexcitation step of the entire process.

The quantum yields found in nature are surprisingly high and often approach unity, in contrast to those of nonbiological origin, which with few exceptions (like oxalate chemiluminescence) are the best of the order of 10^{-2} Einstein mol^{-1}.

4. METHODOLOGY IN CHEMI AND BIOLUMINESCENCE (CL and BL)

For the generalised chemiluminescence reaction, E_q (2) any of the components, including the catalyst if one is used, can be measured as the analyte.

$$\mathbf{A + B \xrightarrow{(catalyst)} C^* \rightarrow C + light} \qquad (2)$$

An efficient chemiluminescence reaction may be dependent on five factors:

1. - The chemiluminescence substrate whose reaction determines the formation of the molecule in excited state, molecule that is responsible for luminescent emission (direct chemiluminescence) or acts as a ground state for the de-excitation by photon emission (sensitized chemiluminescence).

2. - An electron acceptor, like oxygen in the case of a oxidising reaction - all chemiluminescence reactions

3. - A catalyst - could be an enzyme or a metal ion. It can have one of three functions:

- reduce the activation energy by accelerating the reaction
- provide a high efficiency medium for the chemiluminescence reaction
- develop an oxidant.

4. - Cofactors - usually convert one or more substrates in a form able to react or interact with the catalyst.

5. - An energy acceptor, if the chemiluminescence process is of the sensitising type.

Not always all these components are necessary. The simplest case is that in which only components 1 and 2 are involved. The reaction conditions are adjusted so that the light measured is a function of the level of analyse to be determined.

Because the signal is transient, measurement of the emission intensity is time dependent. The signal is often recorded at a specific time after mixing or by integration of light during the entire time or during a specific fraction of time when light is emitted. The instrumentation usually involves some means of mixing the reactants and a detector to measure light. The reactants can be mixed directly in front of the detector, or an optical fiber can be used to transmit the emitted light. Sometimes other sources of light are removed from the chemiluminescence signal with a filter that is placed in front of the detector.

The measurements reported for the applications described in this paper are performed in one of three ways. Static measurements in solution, involve mixing reagents in front of the detector. Often, mixing is achieved by the forced injection of a final reagent into a tube containing the other reagents. The last reagent has to be chosen so that it triggers the chemical reaction. Flow systems can be used to mix the reagents when the analyte is injected into one, or more streams of chemiluminescence reagents. Analyte on solid surfaces such as filter papers can be measured by saturating the surface with chemiluminescence reagents and recording the light emitted with a microtiter plate reader or by contact printing with photographic detection.

Investigation of chemiluminescence systems has proved difficult due to the often complex, multistep chemical reactions involved, and the variety of parameters which influence light emission. Early reaction steps and competing luminescent reaction may obscure the identity of the reaction step which produces the emitting species.

Reactants and solvents require careful purification to remove trace amounts of contaminants. This involves repeated treatment until constant light output is achieved. Even low amounts of impurities may markedly affect the intensity-time decay curve or result in as much as a tenfold variation of light intensity.

Mechanistic studies follow three approaches: (1) the reaction pathway for the chemical process must be established in terms of the intermediates (stable and transitory) and reaction products, (2) the key reaction step involving conversion of chemical energy into electronic excitation energy is identified, and (3) the mechanism whereby excitation energy appears as the excited state of the emitter is characterised. Kinetic studies may indicate the portion of the overall reaction directly related to the light emission process. The light produced in these reactions must be characterised in terms of spectral distribution and total quanta of emitted light. A conventional spectrograph may be used to determine the spectral distribution. Some other techniques for the same purpose involve the use of a rapid scan monochromators using photographic films or a phototube-recorder to display intensity[6-7].

The central unit of the detecting system is the photomultiplier tube. Recent developments afford routine measurement of light intensities of less 1000 photons/sec incident to the photocathode. Thus systems with extremely low light output (quantum yield less then 10^{-12}) can be studied. A number of factors affect photomultiplier accuracy in making absolute or even relative measurements of light intensity. These effects may be minimised by using calibrations methods using standard radioactive sources or simple chemical light sources.

The development of "image intensifiers" offered photon gains of up to 10^6. This kind of device is similar to a photomultiplier in its mode of operation. Incident photons are converted to electrons as they strike a photoactive cathode and are then accelerated to the first of several plates at which point several secondary electrons are released. These secondary electrons are in their turn accelerated to another plate when the releasing electron process is repeated. But the essential difference is that the photomultiplier tube provides an output electrical signal which is independent of the point at which the photons strike the cathode surface while the image intensifier tube converts the amplified electrons back into photons at a phosphor anode exit screen and with focusing methods provides an exit image identical with that formed by the original photons, image that is recorded photographically.

5. BIOLUMINESCENCE ASSAYS

The luminescence associated with biological systems that achieve such high quantum yields as mentioned before is known as bioluminescence. Some other differences between chemi- and bioluminescence are summarised in Table 2.

Table 2. Differences between bioluminescence and chemiluminescence

Bioluminescence	**Chemiluminescence**
enzymatic process	usually non-enzymatic process
biological function in: protection reproduction nurturing	apparently non-biological function
developed organs and special structures for guiding amplifying and changing the colour of the emitted light.	is usually spontaneous and does not develop special organs.

The components involved in the light generating reaction in bioluminescence include a luciferin in reduced form as substrate, together with a luciferase as specific enzyme. An energy-supplying substrate or cofactor is present, often in the form of NAD(P)H or ATP.

Firefly luciferase for example in the presence of luceferin (LH_2), ATP, Mg^{2+} and molecular oxygen catalyses the production of light according to the net following reaction (Eq. 3).

$$\mathbf{ATP + LH_2 + O_2 \rightarrow AMP + oxyluciferine + PP_i + CO_2 + h\nu} \qquad \mathbf{(3)}$$

where **hν** will have a λ_{max} of 560 nm.

Under appropriate conditions the intensity of the light produced is proportional to the ATP concentration.

In the luminescent marine bacteria systems used for analytical purposes, the light is produced by two consecutive enzymatic reactions. In the first one, catalysed by NAD(P)H: FMN oxidoreductase, $FMNH_2$ is produced (reaction 4) and there utilised in the second reaction catalysed by a luciferase to produce the luminescent signal (reaction 5) in the presence of molecular oxygen and an aldehyde (R-CHO)

$$\mathbf{NAD(P)H + H^+ + FMN \rightarrow NAD(P)^+ + FMNH_2} \qquad \mathbf{(4)}$$

$$\mathbf{FMNH_2 + R\text{-}CHO + O_2 \rightarrow FMN + R\text{-}COOH + H_2O + h\upsilon} \qquad \mathbf{(5)}$$

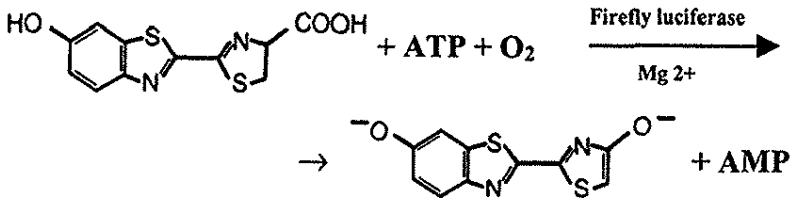
where **hυ** has a λ_{max} of about 490 nm.

When NAD(P)H is the limiting substrate of this bi-enzymatic system, the light intensity is proportional to the NAD(P)H concentration. The two most useful light - emitting enzyme systems were isolated from *Beneckea harvey* and *Photobacterium fischeri.*

In addition of the direct measurement of either NAD(P)H or ATP by the bioluminescence reactions described above, the analysis of some NAD(P)H or ATP dependent enzymes and their substrates can be performed via the bacterial or the firefly luminescent system.

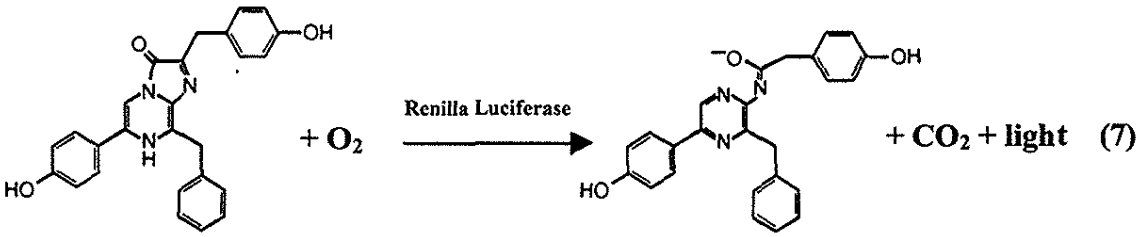
A new development of the bioluminescence technique is refereed as dual luciferase reporter assay used in the quantification of gene expression. Changes in the activity of one reports correlate to the effects of the specific experimental conditions of gene expression, while the constitutive activity of the second reporter provides an internal control by which experimental values can be normalised.

The system can use both firefly and Renilla luciferases in a doubly emitting light system produced by the reactions 6 and 7.



$$\rightarrow \qquad \mathbf{+ AMP + PPi + CO_2 + light} \qquad \mathbf{(6)}$$

Beatle luciferin



(7)

Coelenterazine

Some other bioluminescence assays involve dehydrogenase and kinase and components of reactions catalysed by these enzymes. Bioluminescence can be measured using NAD(P)H - dependent BL marine bacterial reaction and ATP - dependent firefly luciferase reaction.

This type of reaction is very sensitive and offers possibilities to be used it in analysis of microsamples of ATP and phosphocreatinine in simple muscle fibres[7], pyrophosphate in mucosal biopsies as a marker of malignancy in the large intestine[8], intracellular inorganic pyrophosphate in lymphocytes and nucleotide triphosphate pyrophosphatydialase in fibroblast[9]. Bioluminescence assays, unlike spectrophotometric assays, are less effected by turbidity and this can be exploited in coupled enzyme assays for bile acids in hyperlipemic sera[10].

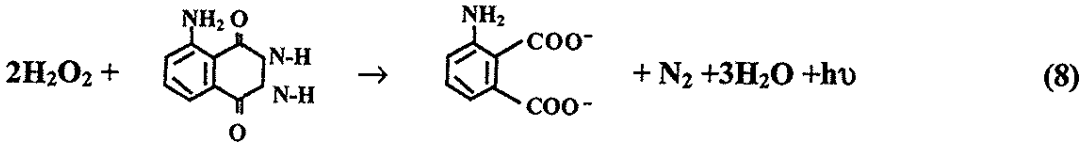
Recombinant auxotrophic microorganisms containing the bacteria luciferase gene provide novel means of assaying vitamins and aminoacids; e.g., in the presence of thiamine and a long chain aldehyde, the microorganism produces bacterial luciferase, which oxidises the aldehyde in a bioluminescence reaction[11].

Photoproteins, such as aequorin, form the basis of very sensitive assays for ionised calcium[12,13] Aequorin can also be used in an immobilised form (covalent or physical adsorption) [14,15].

6. CHEMILUMINESCENT ASSAYS

Any component of a reaction that involves hydrogen peroxide is amenable to chemiluminescence analysis. Usually peroxide production is detected using the luminol or a peroxyoxalate reaction. New assays based on this principle continue to appear e.g., platelet - activating factors[16] oxalate[17,18] phosphatidylcholine hydroperoxides in blood plasma[19], choline containing phospholipids[20] and acetylcholine[21], cephalosporine antibiotics intensify and prolong the light emission from Co^{11} - luminol-peroxide reaction and form the basis of a simple assay for compounds such as cephalothin[22].

The best known chemiluminescent synthetic molecule is luminol (5 - amino - 2,3 - dihydro - 1,4 -phthalasinedione), which can produce light in the presence of H_2O_2 and a catalyst (reaction 8).



(8)

Luminol **3-aminophthalate**

where hυ has a max of 430 nm.

Diaryl oxalates such as TCPO (bis [2, 4, 6 - trichlorophenyl] oxalate) also undergoes a chemiluminescence oxidation reaction with hydrogen peroxide. The diaryl oxalate reaction, sensitized with fluorphore can be then used for H_2O_2 determination.

Enzyme - catalysed system producing hydrogen peroxide can be coupled to chemiluminescence detection. Some examples, are given below:

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow[\text{oxidase}]{\text{Glucose}} \text{Glucuronic acid} + H_2O_2 \qquad (9)$$

$$\text{Cholesterol} + O_2 + H_2O \xrightarrow[\text{oxidase}]{\text{Cholesterol}} \Delta\text{-4-cholestenone} + H_2O_2 \qquad (10)$$

$$\text{L-lactate} + O_2 + H_2O \xrightarrow[\text{oxidase}]{\text{Lactate}} \text{Pyruvate} + H_2O_2 \qquad (11)$$

Working with a luminol H_2O_2 system under standard conditions the antioxidant or pro-oxidant capacity of a third molecule can be tested. In this way the antioxidant capacities of some vitamins like ascorbic acid (vitamin C)[23,30], vitamins K_3[24] B_2 and A[25] the pineal gland hormone, melatonin[26,27] or some purinic and pirimidinic bases[28] were tested. It was also possible the testing of the influence of some iron containing ions[29].

7. MICROBIOLOGY AND COMPETITIVE BINDING ASSAYS

ATP is present in all living cells and thus cells can be detected and enumerated via assay for ATP using bioluminescence firefly luciferase-luciferin reaction[31-33]. This bioluminescence assay has brand applications e.g. antimicrobial susceptibility testing[34-36] determination of bactericidal activity[37] evaluation of microbial growth[38], studying cell chemotoxis[39], sperm viability[40] and antitumor chemosensitivity testing[41]. This type of assay has been automated using a microtiter plate luminometer[42] where both somatic and microbial cell ATP can be determined in a sample using a sequential analytical process involving a detergent, lipid to protect the bioluminescence reagents, a hydrolytic enzyme, and a bactericide[43]. Cell counts can also be determined with a chemiluminescence method using a menadione-Fe-EDTA-luminol reagent (detection limit for yeast cells 8.8×10^4 [44].

Since the development of radioimmunoassays, many assays that rely on the specificity of the antigen-antibody binding reaction, have been based on this principle. Scheme (12) represent a typical competitive binding assay for the determination of an antigen (Ag) where unlabelled and labelled antigen (Ag) compete for the sites on the antibody (Ab)

$$Ag + Ag^* + Ab \rightarrow AgAb + Ag^*Ab \qquad (12)$$

After equilibration, the amount of bound and free labelled antigen can be measured, and a calibration curve can be used to determine the analyte.

The earliest immunoassays used radioactive labels. Difficulties with these labels, including waste disposal and unstable reagents, have prompted the development of nonradioactive tags, including fluorescent derivatives. However, even methods using fluorescent labels have not provided the low levels of detectability required for most immunoassay. For this reason chemiluminescence togs are attractive alternatives because chemiluminescence are among the few with the required sensitivity and detectability.

The chemiluminescence immunoassay is carried out similarly to radioimmunoassays using an immunogen labelled with a chemiluminescence reagent, and the final measurement is made by adding the required reagents and recording the light emitted. Unfortunately, unlike radioactive and even many fluorescent labels, which are relatively unaffected by binding to another species, the chemical binding of a chemiluminescence reagent often significantly affects its ability to chemiluminescence efficiently. A series of chemiluminescence immunoassays are presented in Table 3.

Table 3. Examples of Chemiluminescence Immunoassays

Analyte	**Label**	**Chemiluminescence system**	**Detection limit**
Methotrexate	Firefly luciferase	luciferin/ATP	2.5 pmol
Dinitrophenol	Bacterial luciferase	luciferin/ATP	15 pmol
Estriol	Bacterial luciferase	FMNH/oxidoreductase/ NADH/decanol	50 pg
Dinitrophenol	ATP	luciferin/luciferase	10 mM
Dihidroepian-drosterone	HRP	luminol/hydrogen peroxide	25 pg
Albumin	Fe (III) complex	luminol/hydrogen peroxide	2 μg
Estriol-16α-glu-curonide	Aminobutyl-ethyl isoluminol	hydrogen peroxide/microperoxidase	10 pg
Thyroxine	Fluorescamine	trichlorophenyloxalate/hydrogen peroxide	2ng/ml

8. PHAGOCYTIC CHEMILUMINESCENCE

The production of reactive oxygen species by phagocytic cells of the immune system, such as polymorphonuclear leukocytes and macrophages is recognised to be a key event in the function of these cells during infections and inflammations[47, 48, 49].

The release of reactive oxygen species by activated polymorphonuclear leukocytes and macrophages is also associated with an emission of chemiluminescence of low intensity. Depending on the particular requirements of an assay systems whole[50] purified polymorphonuclear leukocytes[45-47, 51-53] or alveolar macrophages[53] natural kills cells[50] when are used as a source of chemiluminescence The in vitro activation of the phagocytic cells was triggered by various stimuli such as: opsonized zymosan[45-47, 51-52]

or bacteria[54] and phorbol myrimethionyl-lucyl-phenilalanine[55]. In several studies[45, 46] was reported that chemiluminescence emission of human activated polymorphonuclear leukocytes in healthy individuals depends on age and the presence of physical or psychical stress, but also on different chemicals and is induced by the ingestion vitamins and certain drugs. In this way ascorbic acid and natural antioxidants like, superoxide dismutase and melatonine were tested, but also antiinflammatory drugs like; indomethacyn, diclofenac, phenylbutazone, sodium salicylate and acetyl salicylate. The results evidenced an inhibitory effect of the chemiluminescence emission of polymorphonuclear leukocytes of the ascorbic acid and natural antioxidants while some cytostatics were reported to enhance leukocyte phagocytosis[56].

9. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY DETECTION

One area of great interest to researchers is the development of detectors for high performance liquid chromatography and the use of postcolumn reactions to active selectivity and sensitivity. Chemiluminescence is particularly attractive in this mode, especially when sensitivity is needed. A variety of reactors an presented in Table 4.

Table 4. Examples of chemiluminescence detection with high performance liquid chromatography

ANALYTE	LABEL	CHEMILUMINESCENCE SYSTEM	DETECTION LIMIT
Creatinine kinase isoenzymms		Firefly luciferin/luciferase	350 u/l
Bile acids		Bacterio-luminescence	
Aminoacids	Dansyl chloride	Trichlorophenyl oxalate/hydrogen peroxide	low fmol
Polycyclic aromatic amines		Trichlorophenyl oxalate/hydrogen peroxide	sub pg
Co(II), Cu(II)		Luminol/hydrogen peroxide	sub pg
Aliphatic alcohols aldehydes ethers sacharides		Luminol/Co(II)	low µg
Cholic acid	Aminobuthyl ethyl isoluminol	Hydrogen peroxide/Fe(III)	20 fmol

The luminol reaction was the first chemiluminescence reaction demonstrated in a postcolumn reactor. Luminol and hydrogen peroxide were added postcolumn, but researchers had to ensure the compatibility of separation conditions with postcolumn reaction conditions. Metals are usually separated under acidic conditions using hydrogen

chloride, but as previously mentioned, the luminol reaction is most efficient in basic conditions. Therefore lithium chloride was used in the mobile phase.

Coupling a photochemical reaction that produces hydrogen peroxide to be detected with luminol, chemiluminescence has been used to determine aliphatic alcohols, aldehydes, ethers, and saccharides.

The isoluminol label used in immunoassay applications has been adapted as the high performance liquid chromatography derivatizing agent for amines and carboxylic acids. Addition of hydrogen peroxide and metal catalyst postcolumn allows the detection of femtomole amounts of these compounds. Metamphetamine and its metabolite amphetamine can be detected in urine at 5 and 10 pmol, respectively by derivatizing the molecules with N-(4-aminobutyl)-N/ethylisoluminol, followed by reversed-phase chromatography (C_{18} column) and postcolumn detection with a mixture of ferricyanide - NaOH - peroxide -β - cyclodextrin[57].

Alternatively, dansyl derivatives can be prepared and detected postcolumn by means of the chemiluminescence of the bis (2,4,6 - trichlorophenyl) oxalate - peroxide reaction[58]. If the drug is fluorescent (e.g., the coronary vasodilator, dipyridamole), than it can be detected directly using hydrogen peroxide and a peroxioxalate reagent[59].

Some chemiluminescence assays have combined and immobilised enzyme reactors for various analytes in serum including glucose, urate (10 pmol/20 µl injection) and lactate (10 pmol/20 µl) [60-62].

10. SENSORS AND IMAGING

A. chemiluminescence or bioluminescence sensor can be constructed by immobilising reagents onto a nylon membrane attached at the end of a fibber optic bundle linked to a photomultiplier tube[63-65] e.g. alcohol can be measured using bacterial luciferase, NAD(P)H: FMN oxidoreductase, and alcohol dehydrogenase coimmobilized at the end of the fibber optic probe[66].

Photon counting video-imaging using charged coupled device cameras permitted simultaneous analysis of multiple samples[67,68] and spatial resolutions of chemiluminescence and bioluminescence emission from reactions occurring in tissue samples e.g. imaging of ATP and lactate distribution in human melanoma tissue[69]. Another application was a combination of firefly luciferase reporter gene and firefly luciferin esters that readily enter cells, to study HIV-1 virus that transactivator protein expression in cells[70]. Imaging was also successfully used to determine metabolite distribution in tissue samples[71] using a viscous solution of reagents applied as a frozen layer to the tissue section in order to minimise loss of resolution due to diffusion of products.

11. DETERMINATION OF NITRIC OXIDE

Endothelial cells, smooth muscle cells, macrophages, neutrophils, Kupffer cells and other cell types generate superoxide ($O_2^{\cdot -}$) [72] and nitric oxide (NO) [73]. The simultaneous mediators like interferon γ [74], calcium ionophores [75], lipopolysacharide[76]

and phorbol ester[77]. Thus concomitant NO and $O_2^{\cdot-}$ generation may be enhanced in a variety of pathophisiological situations such as ischemia - reperfusion, acute inflammatory processes, atherosclerosis, bacterial infections and sepsis.

Both NO and $O_2^{\cdot-}$ are free radical species that rapidly react with each other in aqueous solution at pH = 7.4 , yielding peroxynitrite anion ($ONOO^-$) [78]. Peroxynitrite is an unstable species at physiological pH, protonating to peroxynitrous acid (ONOOH) which spontaneously decompose to NO_2 and $^{\cdot}OH$ in 20-30% yield.

$$NO + O_2^{\cdot-} \rightarrow ONOO^- \Leftrightarrow ONOOH \rightarrow {}^{\cdot}NO_2 + {}^{\cdot}OH \qquad (13)$$

The remaining ONOOH will directly isomerize to nitrate (NO_3^-) [79].

Luminol chemiluminescence has been widely used to detect the production of reactive oxygen species ($O_2^{\cdot-}$, H_2O_2, $^{\cdot}OH$) from enzyme, cell and organ systems [80-82] and has been useful for examining the kinetics and reaction mechanisms of the oxygen radical process.

In order to yield light, luminol has to undergo a two-electron oxidation and form an unstable endoperoxide. This luminol endoperoxide decomposes to an excited state, 3 aminophthalic acid, which relaxes to the ground state by emitting photons[81, 83]. In most cases of luminol chemiexcitation in biological systems $O_2^{\cdot-}$ is a key intermediate[84], but alternative pathways of chemiexcitation not requiring $O_2^{\cdot-}$ have been described[85-87].

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