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Biotechnological Potential of TRIS (2, 2'-bipyridyl) ruthenium (II) Chemiluminescence Using Immobilized Enzymes with Flow Injection Analysis

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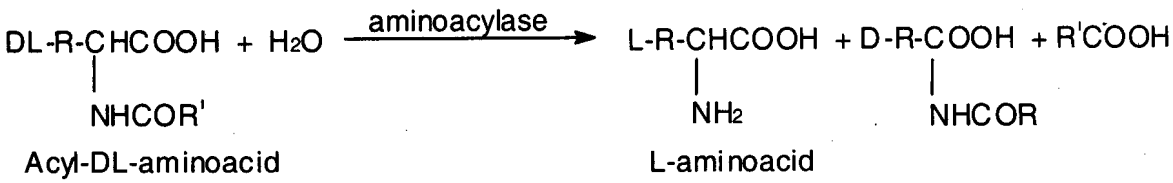
ABSTRACT

Chemiluminescence (CL) in conjunction with flow injection analysis (FIA) shall be used to investigate the biotechnological potential of Tris(2,2'-bipyridyl) ruthenium (II) system. CL using the Tris(2,2'-bipyridyl) ruthenium (II) system allows the development of methodologies for the analysis of nicotine amide adenine dinucleotide (NADH) and NAD-NADH converting biocatalyst (enzymes, dehydrogenases) and their substrates using immobilized biocatalyst. For example the analysis of lactate dehydrogenase and its isoenzymes is important for the diagnosis of myocardial infarction. Certain hormones e.g. thyroxine inhibit glutamate dehydrogenases activity and this effect could be used to develop a highly sensitive method for the determination of Thyroxine and intern provide ways to treat goiter and thyroid cancer which is common in Pakistan. CL-Fl manifold provide the ways to improve the sensitivity, speed and cost effectiveness of clinical assays for hormones/drugs without using radioactivity.

INTRODUCTION

Biotechnology encompasses all of the basic and applied sciences as well as the engineering required to fully exploit living systems and bring their products to the market place [1]. The technology that develops is eventually expressed in various methodologies and types of equipment and instruments built up along a bioprocess stream. For example the commercial product of an enzyme bioreactor "Enzyme Engineering" is a technology based on the applications of immobilized catalyst and is utilized in many different areas. This technology originated in Europe and US has been developed extensively in Japan. The first industrial application of an immobilized enzyme appears in 1969 in Japan e.g., industrial production of L-amino-acids by aminoacylase from acyl-DL-aminoacid [2] according to the following reaction:



Immobilization of enzymes

Immobilization of enzyme is the localization of enzyme molecule to solid support by physical or chemical means [3]. Enzymes are expensive reagents, to use enzyme it has to be immobilized. The advantages of immobilized enzymes are:

1. Economy
2. Convenience
3. Greater stability &
4. Re-usability

Immobilization techniques

During the last thirty years several methods have been reported [4]. The majority of these methods are grouped into the following classes:

1. Immobilization of Enzymes by adsorption
2. Entrapment of Enzymes within polymer matrices
3. Encapsulation of enzymes in semi permeable membrane &
4. Covalent attachment of enzymes with solid supports

Covalent attachment of enzymes with solid support is one of the most important method involving formation of a chemical bond between amino acid residues of the protein and water insoluble solid support. The solid support can be organic and inorganic. Controlled porosity glass (CPG) is one of most important solid support for enzymes [5] which needs a cross linking reagent (Glutaraldehyde) according to the reaction (Fig.1):

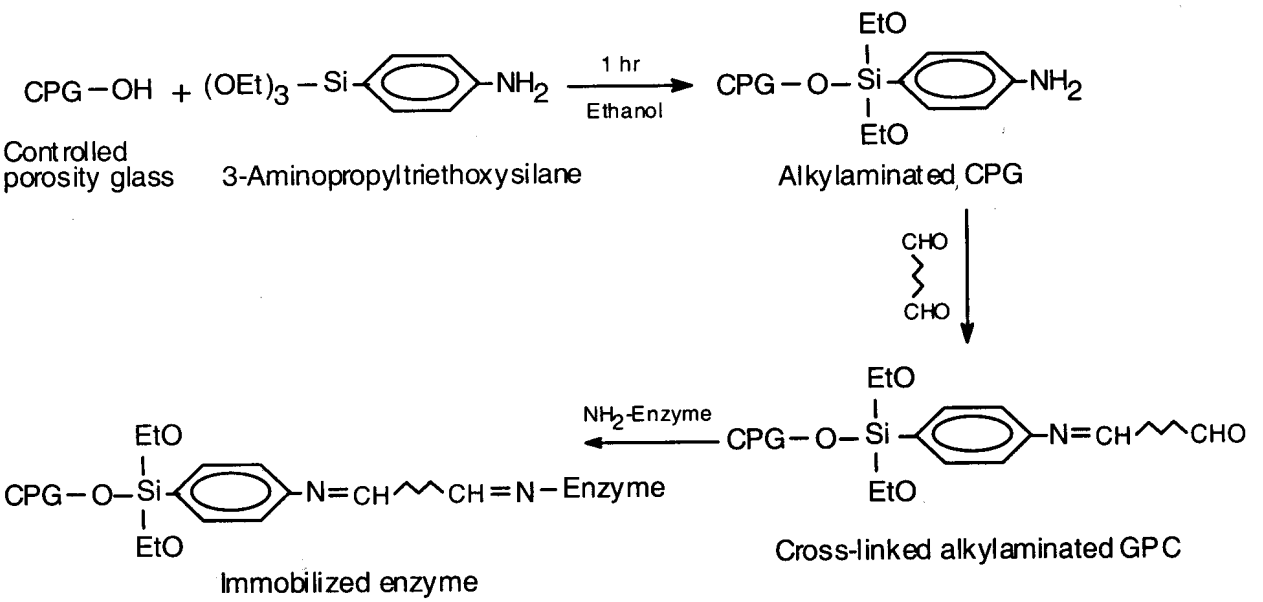


Fig. 1: Cross-linking of an enzyme to controlled porosity glass (CPG).

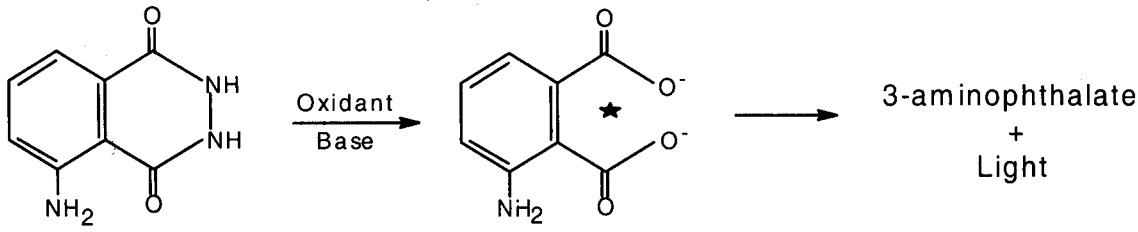
Immobilized enzymes techniques could be used for the analyses of clinically important enzymes/ substrates/ hormones. We would like to present method for the determination of Thyroxine (Hormones) involving the technique of Chemiluminescence.

Chemiluminescence

It is the production of light from chemical reaction without using any source [6], according to the following reaction.

$$A + B \longrightarrow C^* \longrightarrow C + hv$$

The excitation energy comes from the chemical energy of the reactions. All the CL reaction involves the oxidation of molecules followed by de-excitation process with the emission of photons [7,8]. A range of molecules are involved, the best known of these being Luminol (5-amino-2,3-dihydrophthalazine 1,4-dione) is oxidized in aqueous solution to 3-aminophthalate which in tern produce light [9] according to following reaction:



Luminol 3--Aminophthalate

Electrochemiluminescence

Electrochemiluminescence (ECL) is the process where species generated at electrodes undergo electron transfer reaction to form excited state that emits light [10]. Application of voltage to luminophore such Tris(2,2'-bipy) ruthenium (II) results in light emission and allows detection at 10^{-11} M.

$$Ru(bby)_3^{2+} \xrightarrow{\text{electrode oxidation}} Ru(bpy)_3^{3+} + e^-$$

$$Ru(bby)_3^{3+} + \text{reductant} \longrightarrow [Ru(bpy)_3^{2+}]^* + CO_2$$

$$[Ru(bpy)_3^{2+}]^* \longrightarrow Ru(bpy)_3^{2+} + hv$$

ECL has found applications for immunoassays, DNA analysis, hormones, environmental areas [11,12], we would like to concentrate in the determination of Thyroxine.

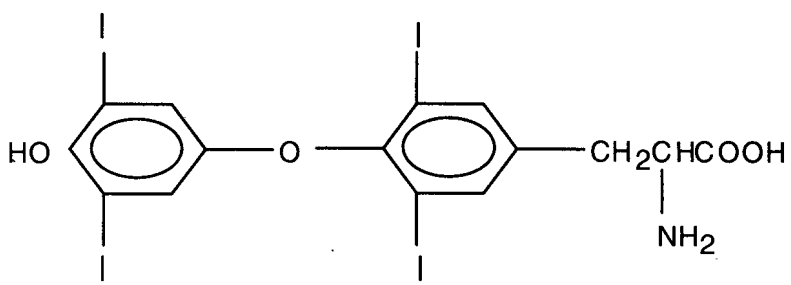
Thyroxine (3,5,3',5'-tetraiodo-L-thyronine, T_4)

Thyroxine is a preparation of natural thyroid hormones [13]. It is formed from protein bound tyrosine and iodine and occurs as protein bound form, thyroglobulin. Thyroxine or tetraiodothyroxine are secreted by the thyroid in to the blood, especially when the gland is stimulated by thyrotropin, and circulate in combination

with a specific protein, thyroxine bound globulin (TBG). The thyroid hormones are essential regulators of the basal metabolic rate. Deficiency of the hormones leads to goiter, myxoedema and critnism, while excessive secretions causes Graves disease (exophthalmic goitre). Thyroxine also acts as a metabolic stimulant on respiring cells in vitro; it uncouples oxidative phosphorylation i.e. Increase oxygen consumption without generation ATP. Thyroid dysfunction is reflected by hypo- and hyper-thyrodism. The important role of thyroid hormone in human physiology, the incidence of thyroid dysfunction and the use of thyroxine to overcome hormonal deficiency have led to increasing the efforts to establish standard analytical methods for the determination of thyroxine. The assay of thyroxine can be accomplished in several ways; competitive protein binding assays, [14] radio-immunoassays (RIAs) [15] and chemical derivatization followed by gas chromatography with electron capture detection, however it is not simple to apply in routine analysis because of the need for derivative preparation.

RIAs offers sufficient sensitivity (down to 10^{-14} M), selectivity and relative insensitivity to variation in the chemical composition of the sample. Owing to its excellent performance RIA became the technique of choice. Yet RIA has some drawbacks, gamma emitting isotopes usually reduce the shelf life of the reagent, health and disposal problems of radioactive compounds are well known [16].

Partly in response to the apparent drawbacks of RIAs, enzyme immunoassays (EIA) have been developed in which CL in conjunction with EIA is a rapid technique. CL immunoassay is thus rapidly growing and has been used for a range of applications such as the thyroid disease, pregnancy and sex functions, tumors anemia, cardiological functions and infectious diseases.



Thyroxine (3,5,3',5'-tetraiodo-L-thyronine, T_4)

Thyroxine determination

Thyroxine can be determined by its inhibition of glutamate dehydrogenase (GLDH) according to following reaction;

$$\text{L-Glutamate} + NAD^+ + H_2O \xrightleftharpoons{\text{GLDH}} \alpha\text{-ketoglutarate} + NADH + NH_4^+$$

NADH produced can be used as a reducing agent and could be coupled with the Tris(2,2'-bipyridyl) ruthenium (II) CL reaction.

Methodology

A variety of different methodologies have been published for CL measurement. These includes commercially available luminometers and a purpose built CL detectors. Most researchers developed their own CL cells [17] to meet their specific requirements. Proposed study also involves a self built CL-FIA detector and its use for the

determination of clinical and biological samples. The methodology involves two approaches, the batch method and flow-through method. The flow-through methodology includes various steps i.e., construction of a cell for the continuous oxidation of $Ru(bipy)_3^{2+}$, the flow-through CL detector, immobilization of enzymes and its incorporation into a manifold for the determination of enzymes and finally to established method for thyroxine determination.

Batch method:

An interesting batch method using a luminometer as shown in Fig. 2 for the determination of common clinical anticancer/immunosuppressive pharmaceutical was reported by Z. He. et al [18]. In batch method either a mixture of analyte/ $Ru(bipy)_3^{2+}$ is taken in the reaction cell and oxidant is added directly in to the mixture, or the oxidant/ $Ru(bipy)_3^{2+}$ is taken in the cell and analyte is injected directly into the cell. The CL is detected by the photon counter in the given time and the result in relative light units (RLU) is displayed on digital meter.

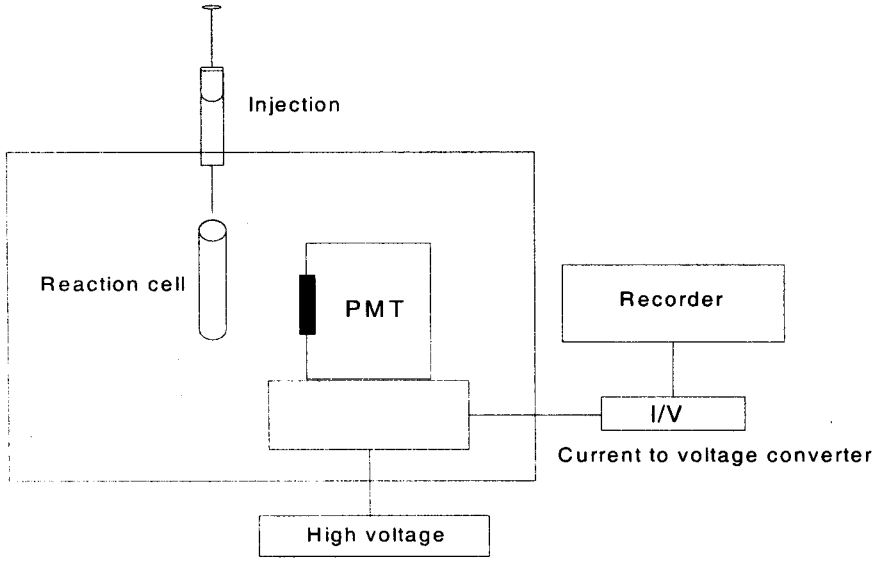


Fig. 2. Batch Luminometer.

Flow-through CL method

FIA is now well established as an excellent technique for rapid, automated, quantitative analysis that combines online chemical and physical samples treatment with a range of flow-through detection system in an enclosed, continuous flow environment. It is particularly well suited to monitoring transient light emission from CL reactions due to rapid and reproducible mixing of sample and reagent in close proximity to the detector [19]. In the proposed study the oxidation of $Ru(bipy)_3^{2+}$ will be done online [20] as shown in Fig. 3 and will be pumped in the system using peristaltic pump. $Ru(bipy)_3^{3+}$ and buffer will be pumped at the same rate, which is then merged down stream with a reducing agent (NADH) at a T piece. The two zones after merging will be allowed to pass through a glass coil positioned in front of PMT, the CL produced as a result of reaction could be fed in to strip chart recorder or computer. For NADH production and for substrate determination, immobilized dehydrogenase mini-columns can easily be incorporated into the FIA manifold between the injection valve and the glass coil positioned in front of PMT.

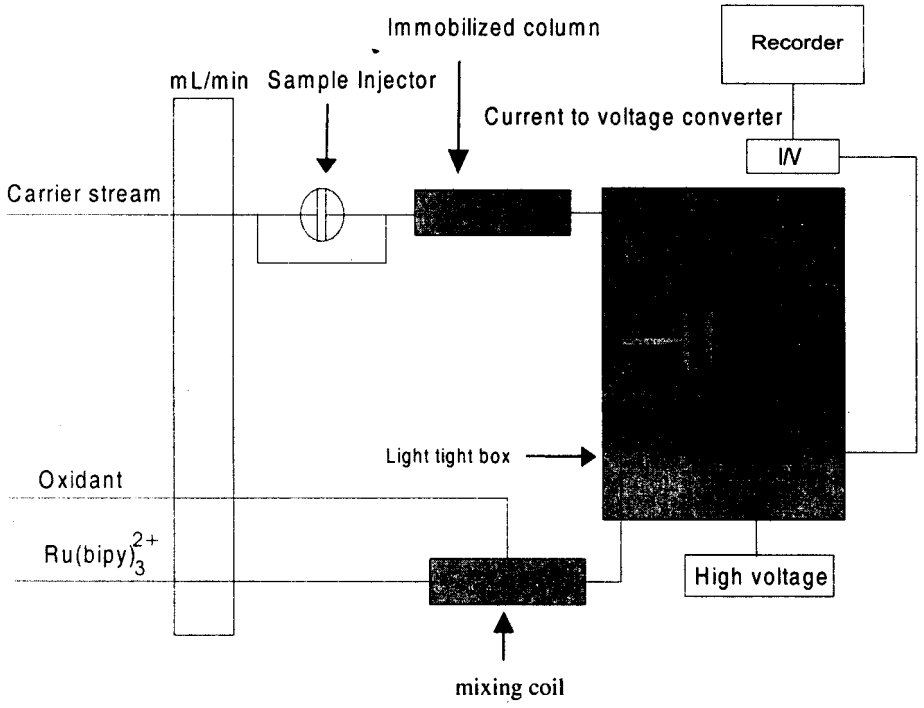


Fig. 3. Flow injection manifold for the Chemiluminescence detection of thyroxine.

CONCLUSIONS

Commercial interest in the areas of clinical diagnostics, process control and environmental monitoring demands for simple, inexpensive and selective sensors to be produced. Tris(2,2-bipyridyl)ruthenium(II) and dehydrogenase enzymes immobilized on CPG can provide ECl sensors. Biosensors described for thyroxine determination is extendible to many other analytes. We are intending to develop a more rugged ECL-based sensor taking advantage of NAD+ recycling.

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