Instrumentation for Chemiluminescence and Bioluminescence Assays: A Continuous Flow Analyzer (Part-II)

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Summary: A purpose-built continuous flow analyzer for chemiluminescent and Bioluminescent detection is described. The advantages of using continuous flow systems for monitoring these reactions and the detector design are also discussed e.g., good reproducibility due to controlled mixing of sample and reagent, flexible manifold design, high sample throughput, low sample and reagent consumption and simple, low-cost instrumentation.

Introduction

The conventional instrumentation for monitoring CL and BL reactions are based on batch procedures. They are associated with certain disadvantages e.g., low sample throughput, poor reproducibility and high sample and reagent consumption [1-2]. In addition these luminometers are designed to be used only with soluble CL and BL reagents. To overcome the above disadvantages a new CL and BL detector based on flow injection analysis (f.i.a.) that can accommodate mobilized and immobilized CL and BL reagents is designed and constructed. The advantages of flow injection detector as compared with batch procedure is that the reagents can be mixed rapidly and reproducibly in front of the detector [3-4].

The first example of segmented continuous flow manifold incorporating immobilized enzymes used conventional batch luminometers modified to include a flow cell made of either a tygon [5], or glass tube [6]. In one system [5] an assay buffer stream was continuously merged with a second stream containing either sample or wash solution and in the other system [6] samples were injected into a continuously flowing stream of substrate. The species analyzed using continuous flow manifolds include NADH, glucose-6-phosphate and ATP. Attempts have also being made to automate the BL assay by immobilizing bacterial luciferase and oxidoreductase on nylon coils placed in front of the PMT of commercial photometer [7]. This paper

describes in detail the construction of a purpose-built continuous flow analyzer not available commercially for the assays of luminescent compounds. This detector can be designed and constructed locally and can be used in teaching and research.

A flow-through CL and BL detector

In designing a purpose-built flow injection analyzer one has to take into account several points:

1. Photomultiplier tube (PMT) characteristics

The detector characteristics determines the ultimate sensitivity that can be achieved. Due to the variety of photomultiplier tubes available, it is possible to choose a detector to fit the required application. The type of PMT chosen for BL and CL measurement was the Thorn EMT 9789QB (52 mm dia. end window PMT). The characteristics of the PMT are given in Table 1.

Table 1: Chracteristics of the PMT Model Thorn EMI 9789QB.

PMT components	Chracteristics
Tube type	End-window configuration
Photocathode	bialkali (K_2CsSb)
Spectral response	160-600 nm
Dynodes	Venetian blind type with 13-stages
Dark current	0.2nA
Mu-metal shields	Thorn EMI (PS8A)

The photocathode is at the end of the tube with an effective cathode diameter of 10 mm. The end window configuration provides uniform collection of light over the entire front surface of the tube. This type of photocathode was selected on the basis of quantum efficiency at a certain wavelength. The quantum efficiency is defined as the number of photoelectron emitted from the photocathode per incident photon [8]. This ratio is expressed as a percentage, the greater the quantum efficiency, the greater the sensitivity. The Thorn EMI 9789QB tube with cathode type (biaalkali) and cathode composition (K_2 CsSb) has a quantum efficiency of 27% at 390 nm.

The window material of the photocathode influences the spectrum of light reaching the photocathode. The end window of tube type 9789QB is made of quartz, extending the spectral range from 600 nm to 160 nm as shown in Fig. 1

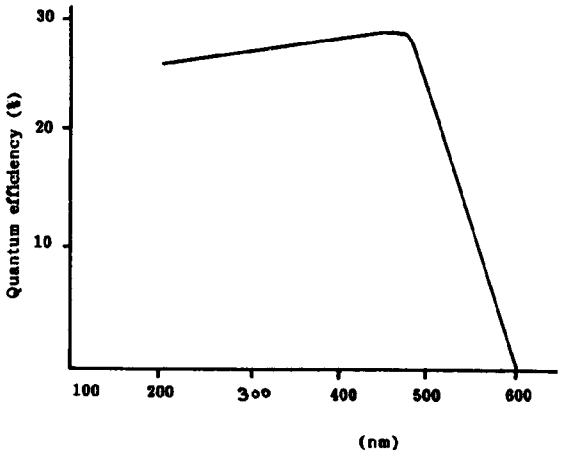

Fig. 1: Spectral response of Bialkali photocathode.

with a maximum of 490 nm. The spectral range covers the wavelengths of interest emitted by CL and BL reactions [9-10]. The dynode system of the PMT provides the gain that makes the PMT a sensitive detector. The present tube has a venetian blind configuration of dynodes. Apart from the configuration, the number of stages (dynodes) and the secondary emission surface must be considered. The more the this stages the greater the gain obtainable for a given voltage. Venetian blind dynodes with 13-stages of type (CsSb) given the highest gain of 10^8 in 10 ns (nano second).

The dark current as measured at the anode, represents the combined contribution of electrons emission from the cathode and the dynodes plus electrical leakage from the tube. Thermally generated electrons usually make the major contribution to dark current, which can be reduced by cooling. The dark current in (bialkali) surface type is 0.2 nA at ambient temperature.

PMT are sensitive to magnetic fields which can cause reduced gain. A metal shield Thorn EMT (PS8A) was used to protect the tube from background magnetic fluxes.

The gain linearity of the tube refers to the functional dependence of the output current on the input light stimulus. Departure from linear amplification occurs through poor dynode chain design. Fig. 2 shows the effect of input voltage on the output current.

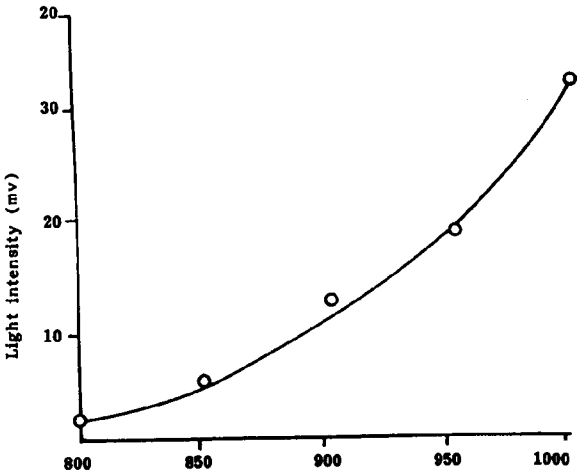

Fig. 2: Effect of varying input voltage on the output current.

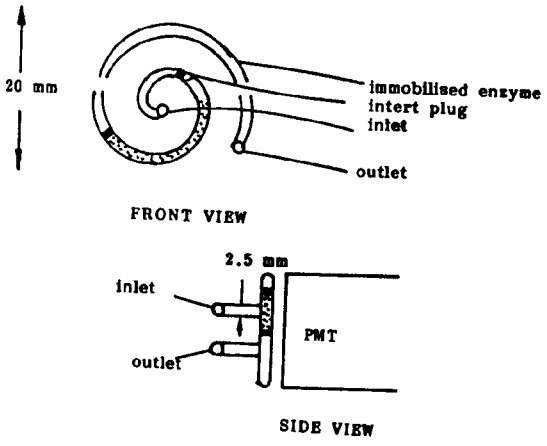

Fig. 3: Immobilized enzyme coil.

The secondary emission coefficient of a dynode is a function of kinetic energy of the primary electrons and therefore, of the accelerating voltage [11]. The PMT power supply used Thorn EMI model (PM 28B) provided a stabilized output voltage over the range 100-2800V at a maximum of 5 mA. The design incorporates high resolution adjustment and excellent accuracy.

2. Flow through reactor

The most common approach to BL and CL analysis in conventional instrumentation is to mix the analyte with an excess of other reactants and measure the light intensity versus time. The disadvantages of such an approach are that it is time consuming, expensive and has poor reproducibility. We have designed a flow through reactor consisting of single glass coil that can incorporate immobilized firefly luciferase or co-immobilized bacterial luciferase oxidoreductase [12-13] (Fig. 3). The glass coil can easily be automated and this is one of the major benefits of this type of reactor. For the chemiluminescent determination the single coil glass flow cell can be replaced by a six coil glass flow cell to avoid sample dispersion [14-15].

3. Glass coil cover and PMT cover

The output of the PMT is directly dependent on the instantaneous photoflux received at the photosensitive surface [16]. Therefore, important points in the design of a BL and CL detector are,

1. The entire sample should be exposed to the PMT.

2. The source should be as close as possible to the photosensitive surface of the PMT.

3. A mirror should be placed beyond the glass coil in order to reflect the light to the photocathode.

A side view of the PMT housing (type B-2) used is shown in Fig. 4a. Its main features are a quarter turn bayonet lock socket assembly for rapid tube removal, automatic centering of the window and scope for incorporating additional items. Mu-metal shielding and humidity protection of the voltage divider components was provided as standard.

The glass coil cell holder was an aluminium plate with a side view as shown in Fig. 4b. Two holes (3 mm dia.) were drilled in the aluminium plate to hold the glass coil in front of the photocathode. The aluminium plate with glass coil was threaded into the PMT housing, leaving a distance of 2 mm between the photosensitive surface and the glass coil. A reflective mirror (aluminium foil) was placed behind the coil to direct light on to the photocathode. The inlet of the glass coil was passed through a hole at the centre of the aluminium plate and connected to a perspex T-piece (17 x 12 mm) by teflon tubing. The distance between the T-piece and the centre of the coil was

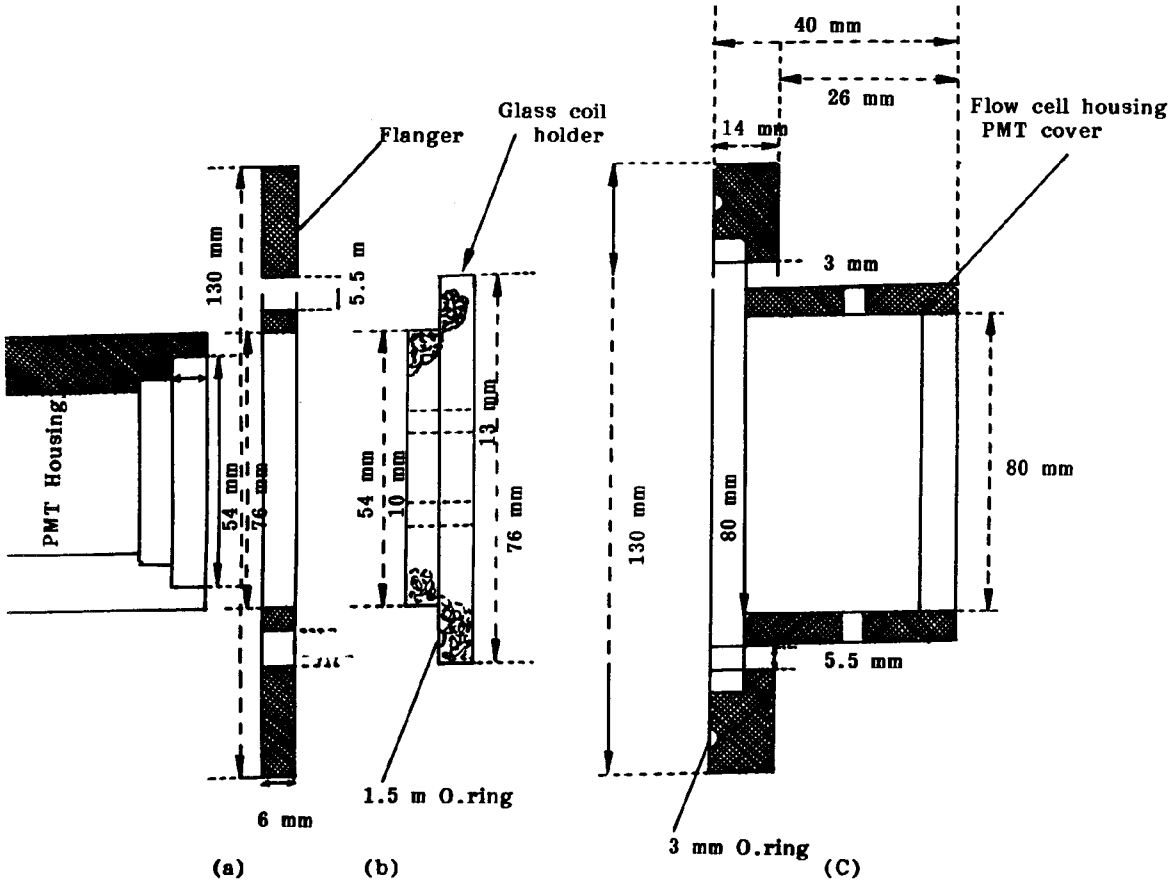

Fig. 4: Photomultiplier tube cover and coil holder.

12.5 cm. The outlet of the coil was connected to polyvinyl chloride tubing to discharge the final solution to waste via another hole in the aluminium plate, which was 10 mm from the central hole. An adhesive (Bostic 1) was used to ensure durable connections.

An aluminium cover so designated that easily flanged the PMT housing and screwed into an O-ring placed adjacent to this. The side view of the PMT cover is shown in Fig. 4c. This aluminium housing completely enclosed the T-piece and glass coil and thus prevented any stray light from entering in the housing. The flow entry and exit holes were positioned on the PMT cover so that no stray light could fall directly on the photocathode. Light piping was prevented by enclosing the 0.5 mm i.d. teflon tubing inside black silicone rubber tubing (B.S. components No. 399-423 2 mm bored) and by spraying black paint inside the housing. The distance from the point of injection to the flow through reactor was amll (14.7 cm) to minimize dispersion of the sample in the flow tubes. The whole assembly was easily removable. The detector described above has shown excellent performance for the determination of enzymes and metabolites [17].

Conclusions

The attractive features of a flow injection luminescent detector described above are the simplicity, rapidity, reproducibility and economy. The detector can easily be modified for mobilized

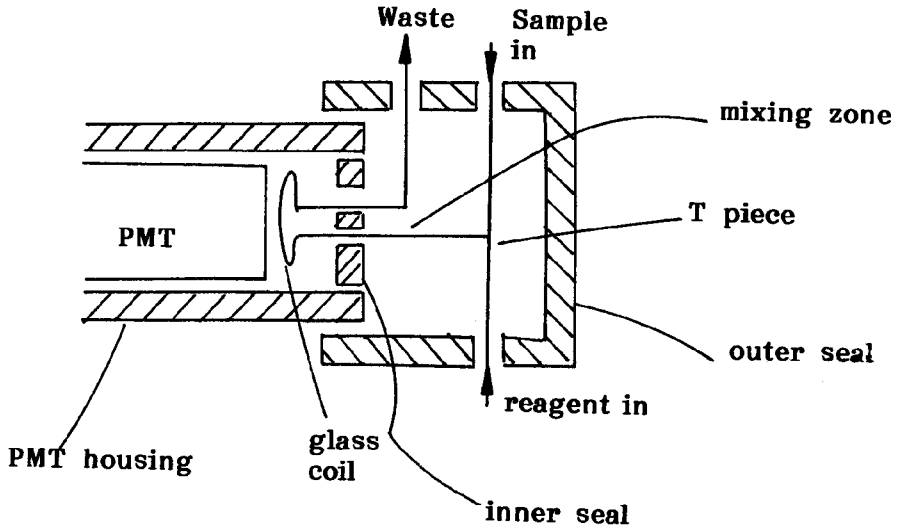

Fig. 5: Flow-through luminescence detector for FIA.

and immobilized bioluminescent and chemiluminescent reagents. The idea can be extended to other BL and CL compounds. The increasing use of CL and BL labels for monitoring immunological interactions could also encourage the developments of homogeneous luminiscence immunoassay using flow-through detector. The introduction of commercial luminometers based on continuous flow techniques rather than batch techniques would undoubtedly stimulate interest in analytical methodologies using chemiluminescent and bioluminescent reactions.

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