ELECTROCHEMILUMINESCENCE – IMPROVING THE PERFORMANCE OF IMMUNOSSAYS

Nicholas Piramal India Ltd., Vinod House, Ground Floor, Kasturchand Mills Estate, 228, Senapati Bapat Road, Dadar (W), Mumbai 400 028*

Introduction

Commercial immunoassay development began with radio isotopically labelled assays in the early 60's. They have proved very attractive since, for the first time, a large variety of small molecular weight proteins and similar compounds could be detected with a high degree of specificity and sensitivity. However, radio isotopic labels show significant and well known shortcomings. In the course of the ensuing immunoassay evolution a variety of other assay techniques were developed, many promising high sensitivity and/or stable reagents, short convenient assay methods and claiming economy and environmental acceptability in use. In the early 80's a new assay category was born-which was based upon the emission of light, produced as a result of a luminescence reaction or activation.

The final breakthrough into a new dimension of immunoassays came at the beginning of the nineties with the development of electrochemiluminescence detection systems. This novel technique is based on the chemiluminescent reaction of species that are generated electrochemically at the surface of an electrode. The superior properties of electrochemiluminescence make it the leading technology for immunoassays.

Strategies for improving Immunoassays

The focus of the new Immunoassay strategies has continued on improved detection limits and the ability to quantify wider analyte ranges. The sensitivity of an immunoassay is usually governed by several factors, among which the detection limit of the label is the most critical factor. Accordingly, considerable effort has been expended in the search for more sensitive labels and the label detection methods.

However, even with a highly sensitive label, other factors may serve to reduce the overall assay sensitivity such as interferences in the sample that contribute non-specifically to the signal, non-specific binding of the labelled reagent or detection noise. Thus the advantages to be gained from the use of more highly sensitive component as immunoassay label do not only derive solely from the detection system. Better assay design, the use of improved binding reagents and the development of well-optimized modern instrumentation contribute significantly to achieve immunoassays with improved performance and greater ruggedness. The main benefits which derive from progress in any one area are generally only realized through synergistic developments in another. Different strategies were employed in the development of electrochemiluminescence-based immunoassays to enhance assay sensitivity and measuring range by using a more sensitive electrochemiluminescence label and label detection method, to eliminate or reduce sources of interference that contribute non-specifically to the signal, to improve the reliability of the immunoassays by devising more sensitive and reproducible key reagents (e.g. monoclonal antibodies, fusion conjugates) and to simplify the assay by eliminating separation of bound and free fraction.

Electrochemiluminescence detection

The new detection method for immunoassays is based on the interaction between a ruthenium complex (ruthenium-trisbipyridyl) and tripropylamine on the surface of a platinum electrode. Ruthenium-Trisbipyridin yields very stable salts that are readily coupled to a variety of biologically interesting species including proteins, haptens, peptides and nucleic acids. The electrochemiluminescence reaction requires a simple electrical "excitation" the magnitude and wave form of which is designed to optimize light yield from the ruthenium/trispropylamine system. The light emission is then measured by a photomultiplier placed above the

*Tel: 4312312, 4328404, 4327510,
Fax: 4328412

excitation cell. The water soluble ruthenium-tribipyridyl salt is a highly stable marker molecule, whereas many chemiluminescent labels, by their very nature, are unstable and those that involve the use of enzymes exacerbate these limitations. Unlike other chemiluminescence techniques, the emission of light does not require the precise dosing and timing of an admixture of co-reactants but merely the application of a small electrical signal to the electrode. One important feature of the electrochemiluminescence process is the potential for a cyclic amplification of the signal. Ruthenium-trisbipyridyl is merely cycled between oxidation states and although tripropylamine is broken down, it is present in a molar excess. Therefore the magnitude of the detected electrochemiluminescence signal does not depend upon a single emission from a single ruthenium molecule. With regard to chemiluminescent systems, most struggle to reach a linear dynamic range across 5 orders of magnitude. The electrochemiluminescence emission/detection method has demonstrated a very linear response across more than 6 orders of magnitude. This, coupled with the low molecular weight of the ruthenium-complex allows generation of multiply-labelled antibodies or other conjugates having very high specific activities.

These ruthenium-labelled conjugates are very stable and maintain their usual immunoactivity and affinity. In summary the key advantages of electrochemiluminescence as applied to immunoassays are the amplifying property of the label compound that can be repeatedly excited, thus leading to amplification, and the speed and dynamics of the signal generation and measurement.

The assay design

Using this technique we have designed automated analyzers to perform extremely sensitive, rapid immunoassay measurement of a wide range of analytes of biological interest. The basis of the electrochemiluminescence immunoassays (ECLIA) is the universal streptavidin solid phase which can be coupled to all kinds of biotinylated immunological molecules. The streptavidin-biotin system is very effective in obtaining a high and constant immuno reactivity of the bound antibodies, antigens or haptens without

desorption or steric hindrance problems due to the indirect attachment to the solid phase support. Moreover, it allows the exact placement of immunological reactants and hence the universal solid phase ensures uniform and defined quality for every test parameter. The number of available biotin binding sites on the solid phase is in great excess and guarantees a high binding capacity for biotinylated components.

In recent years, significant improvements in the reaction time needed for immunoassays has been achieved by the use of finely divided particulate suspensions as solid phase supports. Paramagnetic particles are particularly useful in that they can provide a large area for immobilization of antibodies or antigens and hence faster kinetics of the immunological reaction and yet can be removed from suspension in an instant. Prior to the electrochemiluminescence signal generation the streptavidin coated micro particles are "washed" into flow through measurement cell, thus effectively eliminating any effects of the sample matrix.

Elecsys® immunoassays

The favourable characteristics of Electrochemiluminescence detection provides advantages for a broad variety of applications in different indication areas. The signal detection dynamics of the electrochemiluminescence reaction allows a greater analyte range to be measured and the detection limits are significantly superior to those of conventional immunoassays.

Although speed of analysis is not necessarily a major requirement for many immuno assays, there are a number of applications where rapid availability of results is essential for immediate measures such as markers for emergency service.

The range of diagnostic applications under development include thyroid and fertility hormones, infectious diseases as well as various markers of cardiac, malignant and auto immune diseases.

Electrochemiluminescence detection offers the unique advantage for gradation of the assay characteristics as a function of the clinical significance thus increasing the sensitivity or the

working range or reducing the assay time accordingly. Some examples of the immunoassays based on electrochemiluminescence detection in which importance is attached to sensitivity, measuring range or assay time are given below.

Improving sensitivity

Highly sensitive detection methods are required for those analytes in which the recording of the lowest concentrations or concentration changes is of diagnostic significance. Considerable effort has been expended in the development of for example thyroid stimulating hormone (TSH) assays with very low detection limits. TSH has been recommended as the first line of thyroid testing in most clinical situations. In order to be first line for TSH the assay has to be sensitive enough to detect extremely low TSH concentrations in the subnormal range. The continuous improvement in sensitivity of commercial TSH immunoassays in recent years has led to a classification into different test generations, with each new generation being 10 times more sensitive than its predecessor.

The TSH assay based on electrochemiluminescence detection meets the requirements for a sensitive 3rd generation test. The assay can measure down to TSH concentrations as low as 0.005 μ l U/ml. As an additional substantial improvement, the time required for analysis has been reduced to just 18 minutes. Comparable level of performance of conventional TSH assay methods can only be achieved by applying incubation steps of one hour or more.

The determination of the prostate-specific antigen PSA places comparably high demands on the detection limit. PSA was initially only used for monitoring patients in whom prostatic cancer had already been diagnosed. In the last few years, however, it has also been used for diagnosing prostate cancer. The sensitive determination of PSA is helpful for detection of early stages of prostate cancer for follow-up of patients after radical prostatectomy.

By using electrochemiluminescence detection and highly specific monoclonal antibodies which enter into equimolar reactions with inactive free

PSA and with active PSA bound mainly to protease inhibitors, it was possible to substantially improve the assay sensitivity to less than 0.005 ng/ml. Sensitivity is also the decisive factor in HbsAg determinations. The surface antigen of the hepatitis B Virus (HBV) was and still is the most important marker of productive HBV infection. Since HBV can be transmitted by blood transfusion or organ transplantation considerable effort has been expended to increase HbsAg assay sensitivity. Low-viraemic HbsAg carriers-usually referred to as healthy carriers-are a major threat since they often have HbsAg concentrations in the subnanogram range and normal liver enzymes. Significant improvements in the sensitive detection of HbsAg were realized by the introduction of electrochemiluminescence labeled antibodies against all HbsAg subtypes. The detection limit (0.005 U/ml, Paul-Ehrlich-Institute units) achieved in a 18 minutes assay time exceeds those of conventional HbsAg assays usually requiring overnight incubation.

Improving working range

Dynamics of the signal response are extremely critical when the assay is applied to the determination of analytes which may exist at very high level.

The rapid rise of human chorionic gonadotropin(hCG) for example in early pregnancy usually requires dilution of the sample in most methods. The method of dilution may cause a systematic error. HCG determination on the basis of electrochemiluminescence detection offers a large signal dynamic range thus allowing the direct measurement of samples within the clinically significant concentration ranges which usually require dilution before measurement.

The same applies also to the determination of antibodies to the hepatitis B surface antigen (anti-HBs) which develop during recovery from hepatitis B and after successful vaccination against HBV. Post-vaccination testing allows assessment of the probable duration of protective level of antibody and minimal dilution effort is an economically attractive goal. The anti-HBs test based on electrochemiluminescence detection yields a broader linear measuring range. Thus anti-Hbs titers occurring in routine practice can be

determined by a maximum of one dilution step.

Characteristics of the signal dynamics also play a role in avoiding or minimizing so-called "High-dose-hook" effect, i.e. a decrease in assay response at high analyte concentration leading to a multi-valued dose response curve. In some pathological situations, for example tumors can secrete much higher levels of peptide markers than those normally found. Such an analytical problem has been reported for the determination of PSA in serum. Another example is high viraemic HB-virus carrier who may develop HbsAg concentrations 10^7 fold above the detection limit of the assays. Accordingly, it is possible to obtain sometimes without warning, measuring curve distortion or even false-negative results. The dynamics of the electrochemiluminescence system in combination with the high-capacity streptavidin solid phase increase the linear response range of the analyte thus preventing the "Hook" at analytically significant concentrations.

Improving assay time

Besides the basic requirements for clinical performance of assays the rapid availability of results and ease of use are critical issues for providing emergency service available on a 24-hour basis. The application of electrochemiluminescence to measure markers of myocardial injury such as CK-MB or Troponin T

significantly reduced the total assay time to 9 minutes. In conjunction with STAT (shortest turn-around-time) utility of automated instruments both assays require minimal sample handling thus providing serum CK-MB and Troponin-T values conveniently at almost any time of the day.

Conclusion

The novel combination of an electrochemiluminescent label used in a magnetic microparticle-based immunoassay format defines a significantly improved level of immunodiagnostic performance. The main advantages of electrochemiluminescent immunoassays are high sensitivity, extended linearity of signal response together with speed of signal generation and measurement. These attributes in conjunction with fully automated random access analyser lead to superior level of user convenience and clinical application. Electrochemiluminescence has been applied today to a wide range of analytes of biological interest including thyroid and fertility hormones, infectious disease, as well as various markers of cardiac, malignant and auto immune disease.

However, the spectrum of applications is far from being exhausted. The capacity of this technology goes beyond current requirements for diagnostic laboratory tests.