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Detection Techniques for Immunoassay and DNA Probing Applications

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A brief review of the major detection systems used in the field of biotechnology is presented. The focus is on immunoassay and DNA probing applications and on systems that show promise of extreme sensitivity. Specific examples are discussed to illustrate approaches which introduce chemical, enzymatic, or exponential amplification. The author's projections for the next 5–10 years in this field are expressed.

KEY WORDS: immunoassay; DNA probes; nonisotopic techniques; enzymes.

Introduction

About 30 years ago, the technique of radioimmunoassay was introduced (1) for the quantitative analysis of molecules of biological interest. The impact of this technique on clinical endocrinology has been enormous: for the first time, it was possible to quantify hormones with specificity and sensitivity. Later, with improvements and newer assay configurations, the technique was used in other medical areas, including: haematology, oncology, microbiology, and pharmacology. The power of this technique arises from: (a) the use of antibodies which confer specificity and sensitivity because of their high binding affinity for the analyte and, (b) the use of a label which can be accurately quantitated at very low concentrations. Currently, the technique is frequently used under the general term 'immunoassay'; it has an annual worldwide market value in excess of $5 billion (2). At least five important developments have been introduced: (a) description of homogeneous immunoassays (3), (b) development of 'two-site' noncompetitive immunoassay formats (4), (c) application of novel solid-phases for easy and rapid separation of bound and free label, (d) introduction of monoclonal antibodies (5), and (e) replacement of the widely used ^{125}I radionuclide with alternative labels (6).

The method has now been successfully automated. Newer systems are expected to have random access capabilities and throughputs similar to the well established clinical chemistry analyzers.

Recombinant DNA techniques have recently emerged and are contributing tremendously to our understanding of the pathogenesis and diagnosis of genetic, neoplastic, and viral diseases (7,8). These new approaches will play an increasing role in the clinical chemistry laboratory, with applications in diagnosis of genetic diseases, in establishing paternity, in forensic medicine, in investigation of neoplasia, and in infectious disease. The principles of these techniques rest upon the selective cleavage of DNA by restriction endonucleases and by the localization of specific sequences of nucleotides after hybridization with known DNA or RNA fragments (probes), labeled with a radionuclide (*e.g.*, ^{32}P) or an alternative label.

Immunoassay and DNA probing techniques have many similarities. In immunoassay, the analyte reacts with a specific antibody; in DNA probing, the analyte (a sequence of nucleic acids) reacts with a specific DNA probe.

Both types of assays need a detection system for quantification. Immunoassay and DNA probing techniques are now in a stage of transition from radionuclide labels: ^{125}I and ^{32}P, respectively. Newer detection methods are being used successfully in both areas (6,9). Many detection systems developed first for immunoassay are now being extended to DNA probing. In some DNA probing experiments, an antibody is also introduced to link the probe with a detection system, thereby, combining the two techniques (10).

This review, describes a number of nonisotopic detection methods, suitable for immunoassay and DNA probing. The sensitivity and detectability of the systems will be emphasized.

High-sensitivity enzyme-linked immunosorbent assay (HS-ELISA)

This technique was introduced in 1980 by Shalev

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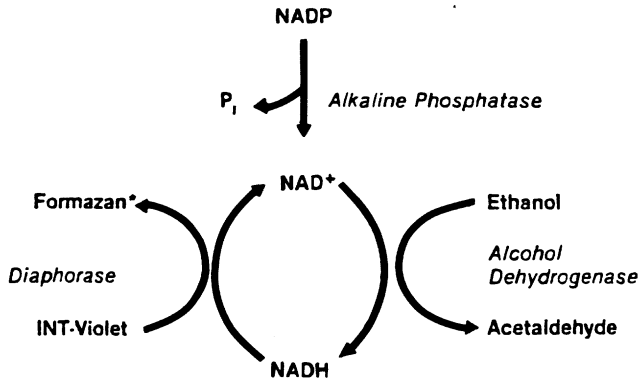

Figure 1—Schematic for the ALP signal amplification. Formazan represents the colored end product. (For more details, see text.)

et al. (11). Their major contribution was the introduction of 4-methylumbelliferyl phosphate as a fluorogenic substrate for alkaline phosphatase (ALP). In comparison to the classical colorimetric substrates for ALP, this new substrate offered about 1000-fold improvement in detection limits. The original authors achieved detection limits of ~ 24,000 molecules of mouse IgG in a model assay. Appropriately modified, this technique is now used in major automated immunochemistry analyzers including the Abbott IMx^R and Baxter's Stratus^R.

Ultrasensitive enzyme radioimmunoassay (USERIA)

This assay design was introduced by Harris *et al.* in 1979 (12). The basic idea was to use a radioactive enzyme substrate (^{3}H-adenosine). Described originally for quantifying cholera toxin by a 'sandwich' type methodology, this assay detected 600 molecules of cholera toxin per cuvette (close to the theoretically achievable detection limit as predicted by the antibody affinity), if an incubation time of 1000 min for the detection step was used. Under the same conditions, an ELISA methodology was 10,000 times less sensitive. Although extremely sensitive, USERIA has no practical applications, presumably because it relies on radioactivity counting, which is now undesirable, and because a separation step is needed to remove unreacted radioactive substrate before the final measurement to the hydrolyzed product.

Enzymatic cycling

This assay was originally proposed by Johannsson *et al.* (13) (Figure 1). In a typical two-site immunoassay system, ALP is used as a label. The substrate nicotinamide adenine dinucleotide phosphate ($NADP^+$) is dephosphorylated by bound ALP to produce NAD^+. NAD^+ activates a secondary enzyme system which comprises a redox cycle driven by alcohol dehydrogenase and diaphorase. In this cycle, NAD^+ is reduced to form NADH, and ethanol is oxidized to acetaldehyde. In the presence of diaphorase, the NADH then reduces a tetrazolium salt, iodonitrotetrazolium violet, to form an intensely colored soluble formazan dye and NAD^+ is regenerated. The rate of reduction of the tetrazolium salt is directly proportional to the concentration of NAD^+ originally formed by the bound enzyme. This system is very sensitive, even with spectrophotometric detection, because of the very high amplification introduced (more than 10^6 times per min). The practical application of this assay for TSH yielded a detection limit of 0.0013 mU/L which is equivalent to ~ 280,000 molecules per assay cuvette. The principle is applicable to any assay where ALP is the primary label. The detection reagents are now commercially available in a kit.

Time-resolved fluorescence

The technique has been reviewed recently (14,15); it will only be discussed briefly. The label in such systems is either Eu^{3+} or a europium chelator. A fluorescent europium chelate can then be formed by adding either suitable organic chelators (the DELFIA^R system, LKB-Pharmacia), or Eu^{3+} (the FIAgen^R system, CyberFluor Inc., Toronto, Canada), respectively. The fluorescent europium chelates (and some other lanthanide chelates) possess certain advantages, in comparison to conventional fluorescent labels like fluorescein (*i.e.*, large Stokes shifts, narrow emission bands and long fluorescence lifetimes). The fluorescence lifetimes of most conventional fluorophores is approximately 100 ns or less; the lifetime of lanthanide chelates, on the other hand, is 100–1000 μs. Using a pulsed light source and a time-gated fluorometer, the fluorescence of these compounds can be measured in a window of 200–600 μs after each excitation (Figure 2). This method decreases the background interference from the short-lived fluorescence of natural materials in the sample, cuvettes, optics, etc. Commercially available instruments for time-resolved fluorescence immunoassays have been described (14,15). The chelates used by the DELFIA^R system are complexes of the type $Eu(NTA)_3(TOPO)_2$, where NTA is naphthoyltrifluoroacetone and TOPO is trioctylphosphine oxide (14). The immunological label, Eu^{3+}, is introduced into antibodies or streptavidin by using a strong Eu^{3+} chelator of the EDTA type. Similarly, Eu^{3+} can be incorporated into DNA probes. Release of Eu^{3+} and recomplexing with NTA and TOPO can be achieved by lowering the pH to approximately 3.0. DELFIA is well established in the field of nonisotopic immunoassay; it is characterized by high sensitivity and broad dynamic range. It has been criticized because of its vulnerability to Eu^{3+} contamination effects (14). The label, Eu^{3+}, can be measured down

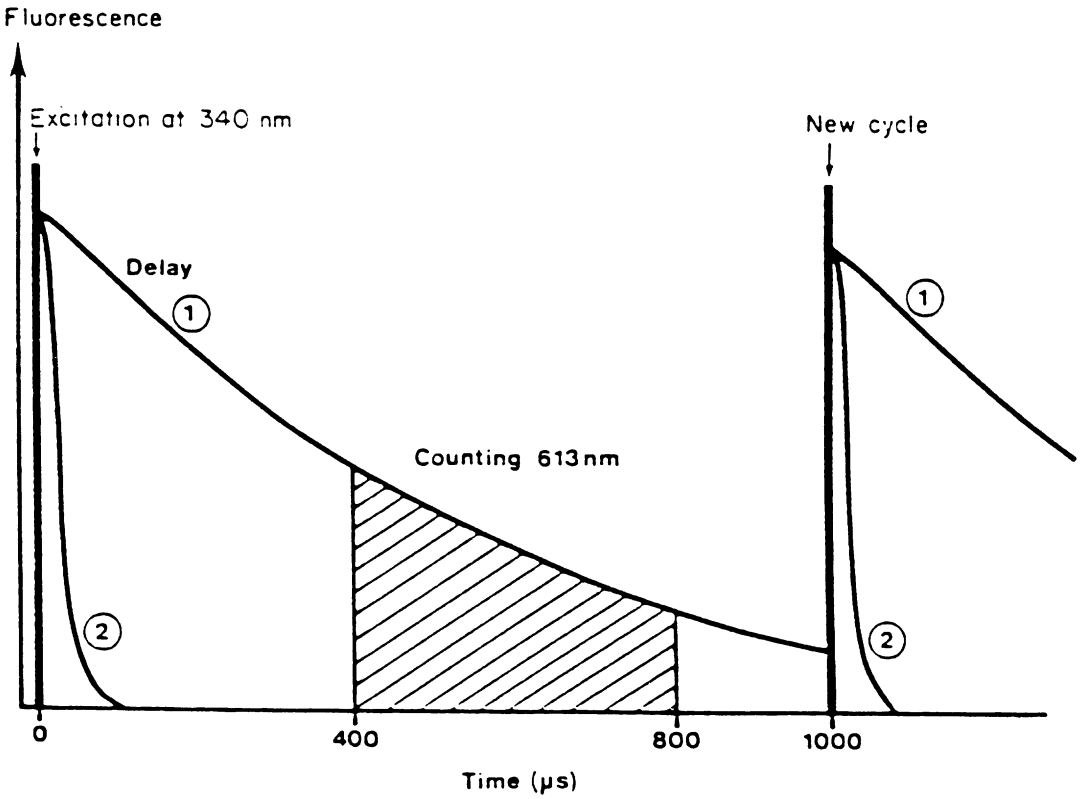

Figure 2—Measurement principle of time resolved fluorescence. The cycle time is 1 ms and pulsed excitation less than 1 μs occurs at the beginning of each cycle. The delay time after the pulsed excitation is 400 μs and the actual counting time within the cycle has the same duration. The total measurement time per cuvette is 1 s. Curve 1 represents the fluorescence of the europium chelate and curve 2 represents the background fluorescence (actual delay time less than 1 μs). Reprinted by permission from Ref. 14.

to 10^{-13} M using time resolved fluorescence and 10^{-17}–10^{-18} moles per cuvette (~ 6×10^6 –6×10^5 molecules) can be detected routinely. Analytes can also be measured down to these levels; in DNA applications, the detection limit is approximately 10^{-18}–10^{-16} mol of probe. This detection limit is at least 20-fold inferior to that achieved by ^{32}P (16).

The newly developed FIAgen^R system (14) uses

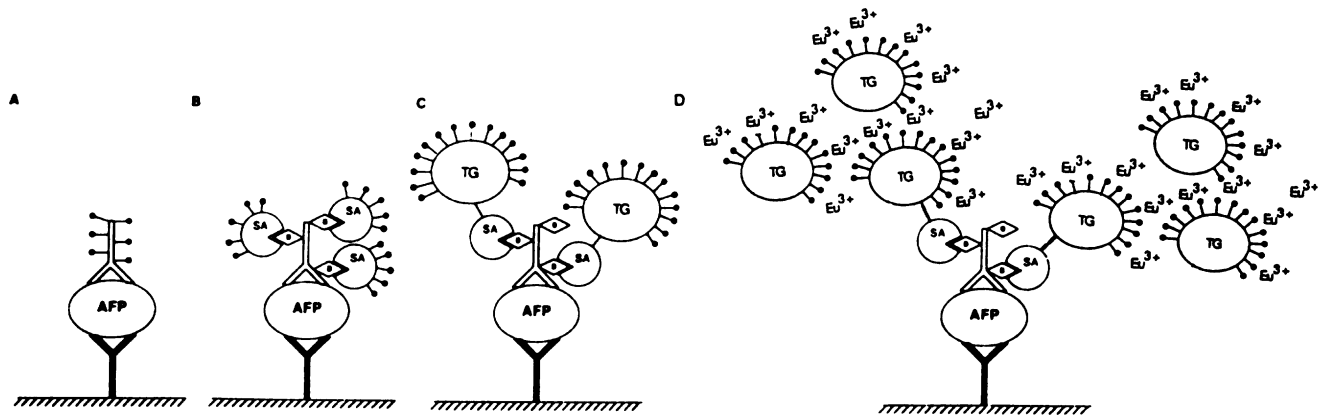

Figure 3—A 'two-site' immunoassay for alpha-fetoprotein (AFP) using biotinylated detection antibodies (panels B, C, D) or BCPDA labeled antibodies (panel A). In panel B, streptavidin (SA) is directly labeled with BCPDA(⊸). In panel C, SA is covalently linked to thryroglobulin (TG) labeled with BCPDA. In panel D, excess BCPDA-labeled TG is complexed to SA-conjugated TG through Eu^{3+} ions. All assays work with excess Eu^{3+} present; this is not shown for panels A, B, and C. (For more details, see text.)

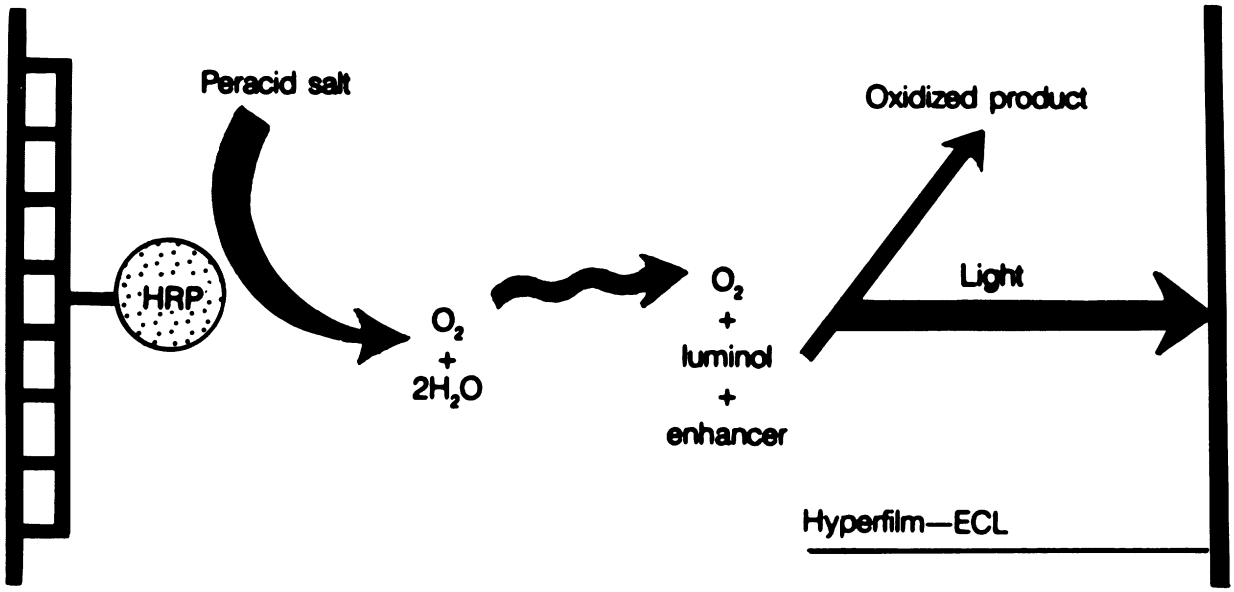

Figure 4—Application of enhanced luminescence for DNA probing. The probe is labeled with horseradish peroxidase and hybridized to a target sequence immobilized on a solid support. After washing out excess labeled probe, the hybrid is soaked with a detection reagent which contains hydrogen peroxide, luminol and an enhancer. (See text.) The light emitted is detected on a special film in less than 1 h.

the europium chelator 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) as label; it does not suffer from any Eu^{3+} contamination effects. This system works best when biotinylated antibodies are used with BCPDA labeled streptavidin. Three streptavidin preparations have been produced (Figure 3), which achieve different detection limits: (a) streptavidin (SA) directly labeled with BCPDA ($SA[BCPDA]_{14}$) for assays with detection limits of 10^{-10}–10^{-11} M, (b) streptavidin covalently linked to thyroglobulin (TG) carrying 160 BCPDA molecules ($SA[TG]\ [BCPDA]_{160}$) for assays with detection limits of 10^{-11}–10^{-12} M, and (c) the preparation under (b) has been activated by an empirical process and is suitable for assays with detection limits of 10^{-12}–10^{-13} M or less. The best detection limit achieved with the latter reagent for a model alpha-fetoprotein assay was ~ 300,000 molecules per cuvette (5 μL sample volume). The system is now being evaluated for DNA probing applications.

Enhanced luminescence

Luminol and isoluminol have been tried as luminescent labels in immunoassays, but not with great success, because the light output is generally poor and its duration is very short. The discovery of a series of compounds which enhance the light output from the oxidation of luminol has optimized the reaction to produce a prolonged (many minutes), high intensity output of light (17,18). In the typical Amerlite^R immunoassay system (Amersham International) the label, conjugated to an antibody is horseradish peroxidase (HRP). HRP catalyzes the oxidation of luminol by H_2O_2 and, the light output is enhanced and prolonged in the presence of an enhancer. Typical enhancers are the firefly luciferin and the benzothiazoles. This system is now well established for immunoassay applications with analyte detectabilities decreased to 10^{-13} M. The same system can be used for DNA probing, as shown in Figure 4. HRP can be easily introduced into a DNA

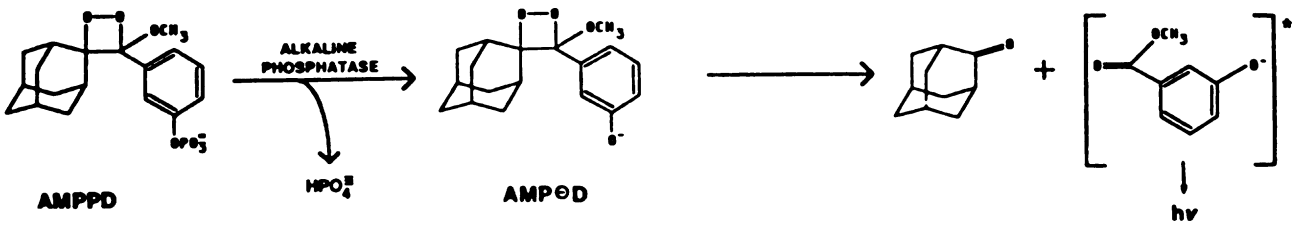

Figure 5—Adamandyl-1,2-dioxetane phosphate (AMPPD) is a substrate for ALP. The intermediate product of hydrolysis fragments into adamantanone and the excited state of methyl meta-oxybenzoate anion which is the light emitter.

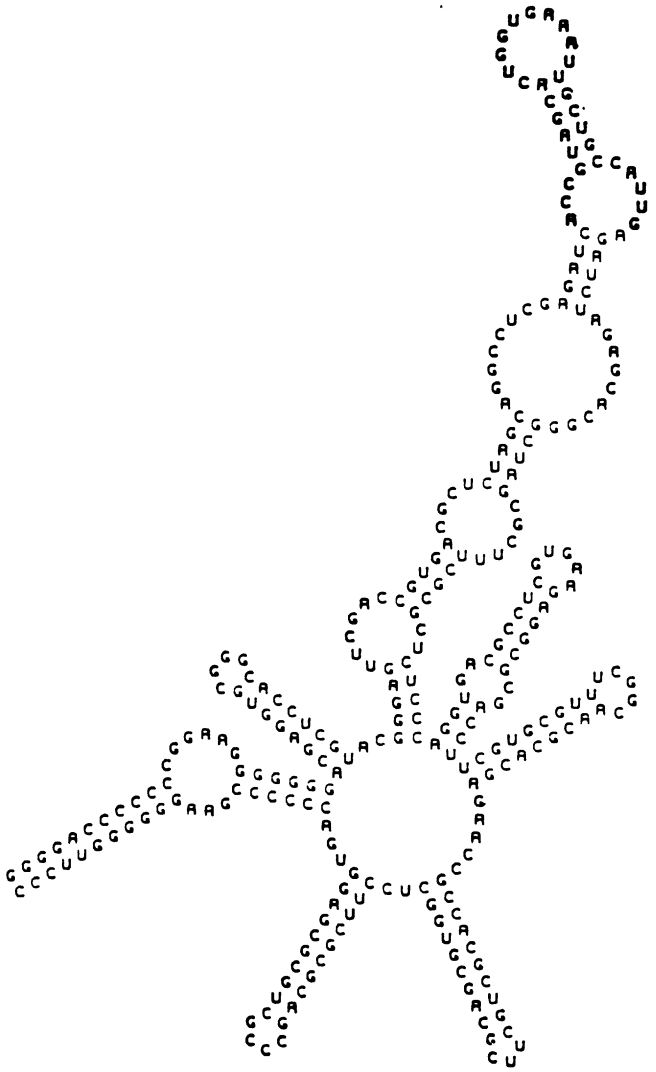

Figure 6—A replicable human immunodeficiency virus (HIV-1) hybridization probe. Bold letters represent the 30-nucleotide long probe sequence that is complementary to nucleotides 4622-4651 in the pol gene of HIV-1 genomic RNA. This sequence was inserted into a replicable RNA piece to obtain a product (shown above) that can serve two functions: to hybridize specifically to complementary target sequences and retain the ability to be exponentially amplified by Qβ replicase. Reprinted by permission from The American Association for Clinical Chemistry (*Clin Chem* 1989; **35**: 1826–31, Ref. 32).

probe using glutaraldehyde. After hybridization, HRP can be detected using the reagents mentioned in the immunoassay application. A specially designed film is applied to obtain a record similar to that of autoradiography, but in much less time (minutes vs many hours). This technique approaches the sensitivity of ^{32}P, with potential detectabilities decreased to as little as 1 pg of DNA.

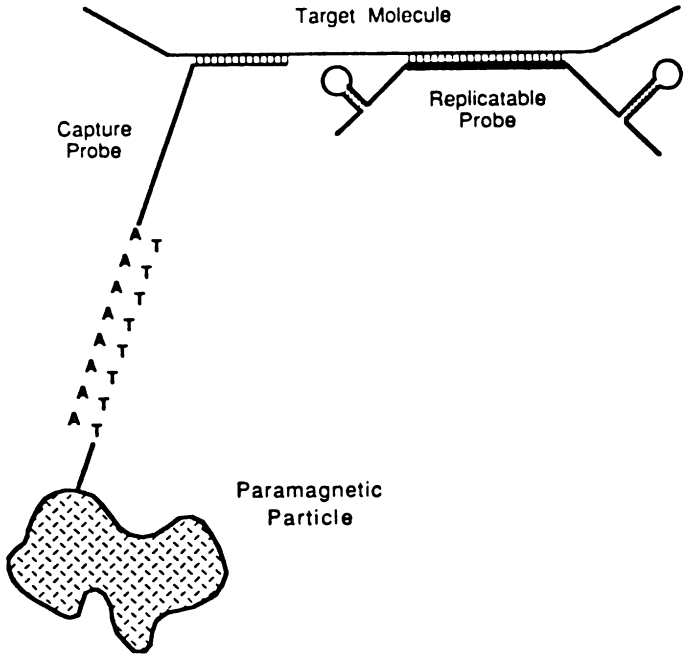

Figure 7—Hybridization assay. The target molecule is shown bound to specific probe carrying a replicable sequence (as shown in Figure 6) and to another probe which is noncovalently linked to a paramagnetic particle (solid-phase). After excess probe is removed by washing, the hybrid is detected as described in the text. The above system can be used with the 'reversible target capture' technique for background signal minimization as described in Refs. 33 and 34. Reprinted by permission from The American Association for Clinical Chemistry (*Clin Chem* 1989; **35**: 1826–31, Ref. 32).

Chemiluminescence using acridinium esters

Acridinium esters produce a flash of light when oxidized in alkaline conditions with hydrogen peroxide. When used as labels, they can be detected at levels of 10^{-18} moles/cuvette or less (19). The advantages of acridinium esters (AE) over luminol and isoluminol derivatives are described in the literature (20). Briefly, AE have a higher quantum efficiency; they do not suffer from serious quenching effects when associated with proteins or haptens; they do not require an enzyme during the oxidation process; and, it is not necessary to release the label before the final measurement is taken (needed if isoluminol is used). Activated acridinium esters, N-hydrosuccinimide derivatives, are commercially available; they are used to produce labeled immunoreactant. Acridinium ester-based luminescent immunoassays are now commercially available from Ciba-Corning (MagicLite™), London Diagnostics, and other companies. Attachment of AE to DNA probes has been achieved using suitable activated AE derivatives and the $-NH_2$ groups of the DNA probe. Model systems seem to work satisfactorily (21). A recent report (22) describes several hybridization

assay formats involving acridinium-ester labeled DNA probes, including homogeneous assays with very good sensitivity.

Chemiluminescent enzyme substrates

Combination of enzymes and chemiluminescent labels can result in a powerful detection system, due to the additional amplification that can be introduced. Recently, a chemiluminescent substrate for ALP, adamantyl 1,2-dioxetane aryl phosphate, has been developed (23,24) (Figure 5). This substrate can be used for the assay of ALP with detection limits as low as 100 molecules/cuvette. Immunoassays and DNA hybridization experiments using this substrate, have been conducted successfully (25–27).

Exponential amplification systems

Biochemical analytical approaches using immunoreactants or DNA probes with exceptional sensitivities will be in demand during the next decade. One of the measuring approaches that should be successful in such problems is that of amplification. Chemical amplifications, similar to the one described by our group (28) (Figure 3), have potential, but amplification usually does not exceed a factor of 10^4. Enzymatic amplification can offer higher factors, but the amplification introduced varies linearly with time; thus, the practicality of an assay is limited if exceptionally long incubation times are used for each measurement. An alternative strategy has been introduced recently, based on exponential rather than linear amplification. This technique can provide amplification factors of 10^6 or more within minutes to hours. In the classical polymerase chain reaction approach (29), a target sequence of nucleic acid is amplified by the enzyme Taq polymerase, using a repeating cycle protocol. At the end of the cycling protocol (usually ~ 30 cycles are used), the target sequence accumulates and can be used for a variety of purposes. In a different, related system, the template is a specific RNA piece (~ 218 nucleotides long), which can be exponentially replicated by an RNA polymerase isolated from Qβ phage infected Esterichia coli (Qβ replicase) (Figure 6). The latter system has been proposed as a potentially powerful detection technique for immunoassay and DNA probing applications (30,31). The system could work as follows (32): the specific RNA piece could be used as a reporter on a specific antibody or DNA probe. After the binding reaction with the ligand or target sequence takes place and excess reagent containing the reporter is washed away, Qβ replicase, nucleotides, appropriate buffers, and salts are added to exponentially amplify the reporter, which can then be conveniently quantified by a classical technique (*e.g.,* absorption or fluorescence spectroscopy) (Figure 7). This approach is very attractive; it has the potential for single molecule detection. The major current limitation (which also compromises the enzyme immunoassay and other detection techniques) is nonspecific binding of the molecule carrying the reporter, because the reporter from this binding is also amplified along with the reporter resulting from the specific binding. Thus, the specific and background signals are similarly amplified without any improvements in the signal to noise ratio. Obviously, novel methods to remove nonspecific binding must be devised if the full benefit from such amplifications is to be realized. One promising method has been reported recently (33,34).

Conclusion

It is now very clear that nonisotopic detection systems are successfully replacing radioisotopes in the field of immunoassay. Many efforts are presently concentrating on solving technical problems in automation and on improving the potential sensitivities to even lower limits. Radioisotopes are still dominant in DNA probing because of better sensitivity. However, it is anticipated that during the next decade, highly sensitive nonisotopic detection systems will replace ^{32}P assays. It is likely that the future detection systems for immunoassay and DNA probing will be the same. These systems will certainly be based on amplifications (chemical, linear, or exponential enzymatic amplification). Exponential amplification of the target molecule, or the signal generated by a reporter, will be applied. Major gains will depend on removing the background signal.

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