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REVIEW

Recent Advances in Homogeneous and Separation-Free Enzyme Immunoassays

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Introduction

The development of the versatile and sensitive analytical technique of quantitative radioimmunoassay (RIA) by Yalow and Berson in 1959 (*1*) has revolutionized diagnostic methodology in clinical medicine (*1–4*) and has provided a powerful analytical tool in biomedical research (*5–8*).

Recently, there has been interest in the use of non-isotopic labels for monitoring the distribution of free or antibody-bound ligands in immunological assays. For example, the following labels have been used in immunoassays: bacteriophages (*9*), free radicals (*10*), fluorescent groups (*11*), chemiluminescent groups (*12*), synthetic particles (*13*), red blood cells (*14*), electron dense materials (*15*), metal (*16*), enzymes (*17–22*), and prosthetic group (*47*).

Major reasons for current interests in replacing the radioisotopic labels with nonradioactive ones are (a) the relatively short half-life of gamma-emitting isotopes (e.g., ^{125}I), which creates logistic problems in the preparation of labeled ligands for immunoassays, (b) the impairment of immunological reactivity and specificity owing to the structural damages caused by the gamma-ray radiation of the isotope, (c) the use of expensive, toxic, flammable liquid scintillants for beta-ray emitting isotopes (effective disposal of scintillants can be

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problematic), and (d) the possible health hazards involved in the use of radioisotopes.

Among the non-isotopic labels mentioned above, enzymes have proven to be the most promising labels in non-isotopic immunoassays (*17–22*). Immunological assays that use enzymes as labels are known as enzyme immunoassays (EIA), enzyme-labeled immunoassays, enzyme-linked immunoassays, enzyme-coupled immunoassays, immunoenzymatic assays, and enzyme-linked immunosorbent assays (ELISA).

There are a number of advantages in using enzymes, prominent among them are: (a) Some enzymes are extremely stable. They retain most of their activity for months or even for years when stored properly; (b) For a number of enzymes, their activity can be monitored easily with relatively cheap and commonly available instruments, i.e., spectrophotometers or colorimeters. Automated hardware for enzyme assay is generally available in most clinical laboratories; (c) Enzymatic reagents can be cheaper than RIA reagents; (d) The catalytic properties of enzymes allow them to act as amplifiers, therefore traces of enzymes with high turnover rate can be assayed with ease even at low concentrations. The sensitivity can be increased further through the use of sensitive substrates, i.e., substrates that give rise to products having high extinction coefficients or substrates that give rise to fluorescent or chemiluminescent products; (e) Assays can be performed in existing automatic devices; (f) No radiation hazards and their related problems are involved; and (g) In some immunological assays using enzymes as labels, the separation of free and antibody-bound labels is not required.

From an operational standpoint, enzyme immunoassays can be divided into two categories: (1) heterogenous and (2) homogeneous or separation-free enzyme immunoassays. In the heterogeneous assays, the quantitation of the proportion of enzyme-ligand conjugates that are unbound or bound by antibodies requires a physical separation of the bound and unbound fractions, whereas the homogenous assays do not require such a physical separation. In homogeneous assays, ligand-labeled conjugates behave differently depending on whether they are free or bound by the antibody. Thus the distinction between free, unbound, and antibody-bound labeled ligands can be made functionally, rather than through physical separation of the bound and unbound fractions.

Heterogeneous enzyme immunoassays have recently been reviewed by Borrebaech and Mattiasson (*23*).

The purpose of this review is to describe various approaches used in the development of homogeneous or separation-free enzyme immunoassays and to discuss their advantages and limitations.

Homogeneous Enzyme Immunoassays (HEIA) Based on Enzyme-Ligand Conjugates

The first HEIA was described in 1972 by Rubenstein et al. (*24*). The principle of the assay is schematically shown in Fig. 1. Here the ligand (a morphine

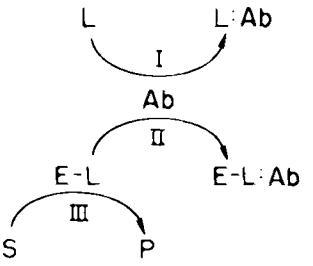


FIG. 1. The principle of homogeneous enzyme immunoassay based on a ligand-labeled enzyme.

derivative) is covalently linked to an enzyme (lysozyme). The enzyme–ligand conjugates (E–L) are enzymatically active and compete effectively with ligands (L) from the sample solution for a limited concentration of antibody (Ab) to the ligand. When antibody binds the enzyme–ligand conjugate (reaction II), a complex, E–L:Ab is formed. The bound antibodies sterically hinder the passage of substrates (bacterial cell wall) to the active site of the enzyme (lysozyme). Hence, the E–L:Ab complex exhibits very little enzyme activity. Figure 1 shows that in the absence of L, reaction I would not take place and E–L and Ab would form E–L:Ab complex (reaction II). The presence of L, however, would compete for the antibody binding sites (Ab), leaving more E–L uncomplexed and free to catalyze the conversion of substrates to products (reaction III). The net result is an increase in enzyme activity with increasing concentration of L.

Because of lysozyme's relatively low turnover rate and of its presence in some biological fluids, this enzyme has been replaced by bacterial glucose-6-phosphate dehydrogenase (*25*), malate dehydrogenase (*26–27*), and β-galactosidase (*28*).

Practical, rapid, sensitive, and convenient assays for the determination of therapeutic drugs and hormone levels in serum or plasma have been developed and commercialized (*29*). The precision and accuracy of these HEIAs are comparable with other immunological, e.g., RIA, and non-immunological methods, e.g., GC, HPLC, and TLC (*29*).

An interesting variation of HEIA based on enzyme–ligand conjugation was reported by Ullman et al. (*30*). They observed that covalent attachment of thyroxine (T_4) to malate dehydrogenase (MDH) gave T_4–MDH conjugates having very low enzyme activity, and that the binding of T_4 antibody to the T_4–MDH increased the enzyme activity of the conjugate. Based on these observations and the ability of free thyroxine to nullify the effect of the antibody on T_4–MDH, they developed a practical assay for thyroxine.

Separation-Free EIA Based on Enzyme Channeling

Goldman and Katchalski in 1971 (*31*) showed, theoretically, that when two enzymes catalyzing two consecutive reactions were brought into close proximity by co-immobilization on the same surface, the rate of the overall

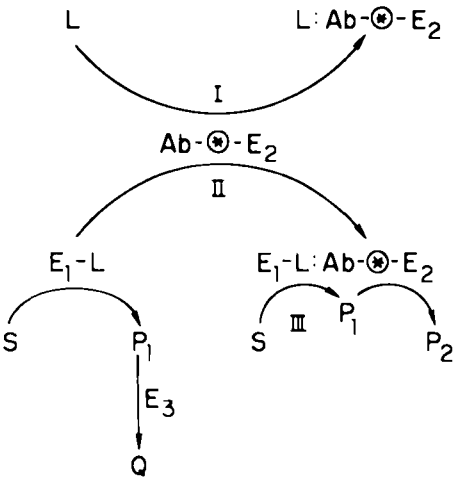


FIG. 2. The principle of separation-free enzyme immunoassay based on enzyme channeling.

reaction was faster than those of the correspnding homogeneous, separate, soluble enzymes of the same activities. They attributed this enhanced reaction rate to the existence of an unstirred layer at the interface between the support and the bulk solution.

Several reaction sequences catalyzed by two or three coimmobilized enzymes have been studied (*32–40*). The results agree with the theoretical formulation of Goldman and Katchalski (*31*).

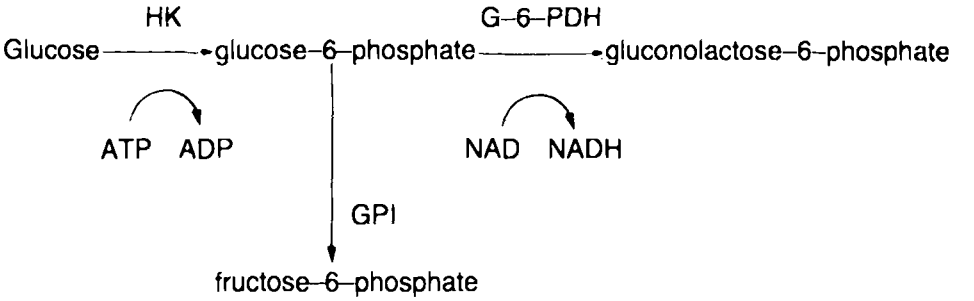
Ullman (*41*) and Litman (*42*) developed a separation-free EIA using the rate enhancement produced by two enzymes immobilized in close proximity to each other. The principle of enzyme channelling immunoassays (ECIA) is schematically shown in Fig. 2. The two enzymes, E_1 and E_2, catalyzed two consecutive reactions that transformed the substrate (S) to P_1 and subsequently P_1 to P_2.

$$S \xrightarrow{E_1} P_1 \xrightarrow{E_2} P_2$$

In one version of ECIA, the ligand antigen (L) is covalently labeled with E_1. The resultant E_1–L conjugate competes (Reactions I and II) with L for limited and fixed number of antibodies which have been coimmobilized with E_2 on fine beads (Ab–*–E_2). The results of reactions I and II are the formation of L:Ab–*–E_2, and E_1–L:Ab–*–E_2 in addition to free E_1–L and Ab–*–E_2. The proportion of E_1–L:Ab–*–E_2 to uncomplexed enzyme species determines the overall enhancement in the rate of formation of P_2. Thus in the absence of L, reaction I will not occur and most of E_1–L will be complexed via reaction II to form E_1–L:Ab–*–E_2. Therefore, more of E_1–L:Ab–*–E_2 will be formed, which consequently enhances the rate of P_2 formation from S. To minimize background reactions caused by free, uncomplexed E_1–L and either Ab–*–E_2 or L:Ab–*–E_2 (which together can still catalyze the transformation of S to P_1 and to P_2), P_1 must be removed. A third enzyme, E_3, is added to act as a scavenger to remove P_1, because E_3 is sterically excluded from the

E_1–L:Ab–*–E_2 complex, P_i formed in the complex is much less readily converted to Q by E_3—the soluble, scavenger enzyme. The unstirred layer around the bead E_1–L:Ab–*–E_2 further slows down the rate of P_i diffusing into the bulk solution.

Litman (*42, 55*) reported a separation-free enzyme-channelling immunoassay for the human serum immunoglobulins G (IgG). The enzymes used were hexokinase (HK), glucose-6-phosphate dehydrogenase (G-6-PDH), and glucosephosphate isomerase (GPI). The reaction sequence was:



The assay reactants, in addition to enzyme substrates, i.e., glucose, ATP, and NAD, include: (a) anti-human IgG covalently linked to KH; (b) antibodies to human IgG coimmobilized on sephadex beads with G-6-PDH. Thus in the presence of a high concentration of antigen (L) in a test sample, there would be less of the complex E_1–L:Ab–*–E_2 formed and therefore there would be less enhancement of the overall rate of glucose to gluconalactone-6-phosphate conversion. The rate of the overall reaction was followed by the production of NADH spectrophotometrically or fluorometrically.

HEIA Based on Ligand-Labeled Enzyme Modulator

Recently, Ngo and Lenhoff (*43*) developed a homogeneous enzyme immunoassay called Enzyme Modulator Mediated Immunoassay (EMMIA). The assay is based on the ability of a ligand-substituted enzyme modulator (M–L) to modulate the activity of an indicator enzyme (E). The M–L competes with free ligand (L) from the test sample (the analyte) for a limited and fixed amount of antibody to the ligand (Ab). The Ab-bound M–L is unable to modulate the activity of the indicator enzyme. The principle of EMMIA is presented in Fig. 3. It is clear from Fig. 3 that in the absence of analyte (L), reaction I would not occur and that the M-L and Ab to the analyte (L) would combine through reaction II, making M-L unable or unavailable to modulate the enzyme activity. As the concentration of analyte increases, however, it would complete successfully for binding sites on Ab (reaction I) leaving more modulator free to complex with indicator enzyme (reaction IIa) thereby modulating its activity. Depending on the properties of the modulator, the enzyme activity will be increased by a positive modulator or it will be decreased by a negative modulator. Thus for EMMIA with a positive modulator, the enzyme activity will be directly proportional to the concentration of the

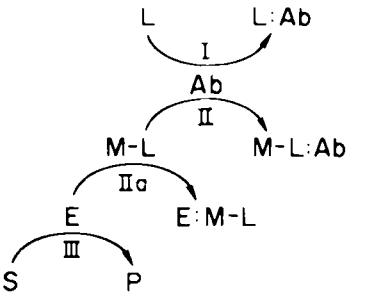


FIG. 3. The principle of homogeneous enzyme immunoassay based on a ligand-labeled enzyme modulator.

analyte. For EMMIA developed with a negative modulator, the activity will be inversely proportional to the concentration of the analyte.

EMMIA for total serum T_4 has been developed by Abbott (Tetrazyme). Acetylcholinesterase was used as the indicator enzyme.

HEIA Based on Ligand-Labeled Enzyme Prosthetic Group

Ngo et al. (*47*) recently developed a homogeneous enzyme immunoassay based on a ligand-labeled enzyme prosthetic group. The principle of the assay is shown schematically in Fig. 4. In this scheme, a ligand (L) is covalently linked to an enzyme prosthetic group, flavin adenine dinucleotide (FAD) forming a stable molecule of FAD–L that serves two functional roles: (a) FAD–L, as a ligand analog, competes with the analyte (L) for a limited binding site of antibodies to L (reaction I); and (b) FAD–L, as a modified prosthetic group, can bind apoglucose oxidase (Apo-G) through a high-affinity binding to form (reaction IIa) an enzymatically active hologlucose oxidase (Holo-G). The binding of FAD-L by an anti-L antibody (Ab) forms FAD–L:Ab, which, owing to the steric hindrance imposed on it by Ab, can no longer combine with apoglucose oxidase. Thus in the absence of L, the analyte, reaction I would not occur and that the antibody to L would combine with FAD–L (reaction II)

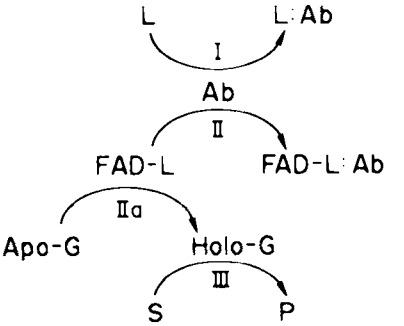


FIG. 4. The principle of homogeneous enzyme immunoassay based on a ligand-labeled enzyme prosthetic group.

making it unable to combine with Apo-G (reaction IIa). Conversely, as the concentration of L increases, it would compete more successfully for Ab (reaction I) leaving more FAD-L free to combine with Apo-G (reaction IIa). Thus, increasing concentrations of L decreases the concentration of FAD-L:Ab and increases the amount of hologlucose oxidase, and therefore increases the observed enzyme activity of reaction III.

HEIA Based on Ligand-Labeled Enzyme Substrate

Homogeneous enzyme immunoassays for therapeutic drug levels in serum using ligand-labeled enzyme substrates were developed by Burd et al. (*49–51*). The principle of the assay is schematically presented in Fig. 5. In this assay, a ligand derivative was covalently linked to an enzyme substrate to form a stable substrate–ligand (S–L) conjugate. The S–L competes with the analyte, L, for a limited concentration of antibody to L. Thus, through reactions I and II, L:Ab and S–L:Ab are formed. The free S–L conjugate is a good substrate for the enzyme, E, whereas S–L bound to the antibody (S–L:Ab) is not. Thus, in the absence of analyte (L), reaction I would not take place, leaving most of S–L free to combine with the antibody to L to form complexes, S–L:Ab (reaction II); neither would reaction III take place. As the concentration of L increases, however, it would compete successfully for Ab (reaction I) leaving more S–L free to act as substrates for E and hence allowing more products to form by reaction III. Thus, the substrate-ligand conjugate (S–L) serves a dual role: (a) as a ligand analog that competes effectively for binding with antibody to the ligand, and (b) as an efficient substrate analog that binds to the enzyme and is transformed into products.

Wong et al. (*50, 51*) and Ngo et al. (*52*) showed that the results obtained with homogeneous enzyme immunoassay based on ligand-labeled enzyme substrate were comparable with other immunological (RIA, EMIT™, RID) and non-immunological methods (GC, HPLC, and microbiological assays), in terms of precision and accuracy.

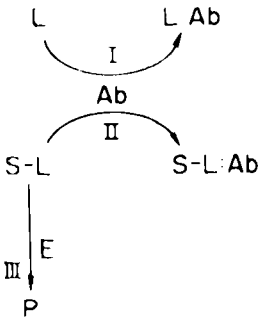


FIG. 5. The principle of homogeneous enzyme immunoassay based on a ligand-labeled enzyme substrate.

HEI for Macromolecules

Homogeneous enzyme or separation-free immunoassays have been developed mostly for quantifying low molecular weight compounds (mw < 2000). The development of HEIA for macromolecules has been slow and skepticism has frequently been expressed on the development of practical HEIA for macromolecules (*18, 53*). Recently, however, a number of different approaches have been used to successfully develop HEIA for macromolecules. For example, Ullman (*41*) and Litman (*42*) used the concept of enzyme channeling in the development of separation-free assay for human IgG.

Assay for Human Serum IgG Based on the Substrate-Labeled Method

Ngo et al. (*54*) developed a homogeneous enzyme immunoassay for human serum IgG. The reagents involved in the assay were: β-galactosidase, μ-galactosylumbelliferone-labeled human IgG, and specific antibody to human IgG. The human IgG labeled with β-galactosylumbelliferone residues served as a macromolecular substrate for the enzyme and as a labeled antigen which competed effectively with IgG from the serum. When the IgG level in the test serum was low, antibodies combined with the labeled IgG forming three-dimensional lattice structures that made the β-galactosylumbelliferone residues less accessible to the enzyme and, therefore, less product formed. As the concentration of IgG in the serum increased, however, the large, three-dimensional lattice structures broke down into smaller units. Consequently more β-galactosylumbelliferone residues were exposed, they became more accessible to the active site of the enzyme, and therefore more product formed. In this assay, the enzyme is used merely to distinguish and to quantify the proportion of free, unbound substrate-labeled IgG. The enzyme does not provide any amplification effect. The maximum signal generated is, of course, dictated by the concentration of free, unbound substrate-labeled ligand. The concentration of the latter is in turn determined by, and should be comparable to, the concentration of the ligand in the test sample. It is therefore necessary to use a fluorogenic substrate such as β-galactosylumbelliferone in the labeling of the antigen. It should be noted that highly purified antigens are the *sine qua* non for the successful development of this assay. In the system developed by Ngo et al. (*54*) the limit of detection for human serum IgG was 500 ng/mL.

Assay for Proteins Employing β-Galactosidase-Labeled Antigen

Gibbons et al. (*28*) reported a homogeneous enzyme immunoassay for proteins employing β-galactosidase. The β-galactosidase was covalently labeled with protein antigens. When the antibody to the antigen bound to the antigen–enzyme conjugate, it did not alter the catalytic action of the enzyme toward its normal, small molecular weight substrate, such as O-nitrophenylgalactoside. The activity of the antigen–enzyme conjugate toward a specially designed macromolecular substrate, however, was inhibited up to 95% because of the steric exclusion of the substrate from the active site of the

enzyme. In assay solutions, the antigen competed with antigen–enzyme conjugates for a limited number of antibody-binding sites. As the concentration of antigen increased, more antibodies are tied up, leaving more antigen–enzyme conjugates free to hydrolyze the macromolecular substrate. The method appears to be very sensitive with a limit of detection of about 25 ng/mL for human immunoglobulin G.

Assay for Macromolecules Based on Prosthetic Group Label

A homogeneous enzyme immunoassay for macromolecules based on enzyme prosthetic group-labeled macromolecules has been developed by Ngo et al. (*47*). In this assay, the macromolecule, human IgG (mw 160,000) was surface labeled with an enzyme prosthetic group, flavin adenine dinucleotide (FAD) forming a stable FAD–IgG conjugate. FAD–IgG conjugate is a double epitopic molecule because it can combine with the antibody to IgG or with apoglucose oxidase. The combination of FAD–IgG with the antibody or with the apoenzyme is mutually exclusive. In the absence of IgG, all of the FAD–IgG combined with the antibody rendering the FAD–IgG incapable of serving as a prosthetic group; hence, no holoenzyme formed and no enzyme activity was observed. As the concentration of IgG increased, however, it competed successfully for the antibody binding and left more FAD–IgG uncomplexed and free to be the prosthetic group of apoglucose oxidase. Consequently the enzyme activity formed is directly proportional to the concentration of Ig G present. The levels of human IgG determined with this method correlated well with those obtained by the accepted radial immunodiffusion method. Furthermore, the within-assay and between-assay precision, analytical recovery, and the accuracy of this method were comparable to those of other immunological methods.

Conclusion

We have described various approaches used in the development of homogeneous, nonseparation enzyme immunoassays. HEIAs for both small molecules (haptens, mw $<$ 1000) and large molecules (antigens, mw $>$ 100,000) have now been developed.

HEIA is gaining increased importance in immunodiagnosis and in therapeutic drug monitoring. The techniques are certain to obtain greater utility as they mature rapidly. They provide a more convenient, rapid, and safer alternative to radioimmunoassay, because the reagents involved in HEIA are more stable, nonradioactive, and their measurements require relatively simple, inexpensive and commonly available instruments. The elimination of the separation step in HEIA improves the precision and accuracy of the assay.

With the exception of HEIA based on ligant-labeled enzyme substrate (*49–51*), enzymes in HEIAs serve as signal generators and as signal amplifiers. Because of the amplifying power, HEIAs are potentially very sensitive immunochemical techniques.

It can be anticipated that future developments will revolve around the following areas: (a) improving the chemical methods used for coupling ligands to enzymes; (b) the selection and use of more stable and efficient enzymes, such as those from thermophilic bacteria; (c) improving the sensitivity of detecting system, such as using more sensitive chromogens and superior optical devices; (d) the use of non-enzymic catalysts in place of enzymes, because non-enzymic catalysts are generally more stable than the enzymes are; and (e) the adaptation of wet, solution-phase HEIA to dry, solid-phase reagent systems such as the multilayer film elements developed by Kodak (*56–59*).

The automation of HEIA through mechanization will also receive much attention. However, progress in this area will largely depend on the test format and protocols of the assays, i.e. would dry chemistry systems be developed rapidly and supersede the wet solution ones?

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