# MOLECULAR DIAGNOSTICS

## Topic: Immunoassays:

BY

DR. ANNIKA SINGH

DEPARTMENT OF LIFE SCIENCES AND BIOTECHNOLOGY

**UNIT I (15 Periods)**

**Enzyme Immunoassays:**

Comparison of enzymes available for enzyme immunoassays, conjugation of enzymes. Solid phases used in enzyme immunoassays.

Homogeneous and heterogeneous enzyme immunoassays. Enzyme immunoassays after immuno blotting. Enzyme immuno histochemical techniques. Use of polyclonal or monoclonal antibodies in enzymes immuno assays.

Applications of enzyme immunoassays in diagnostic microbiology

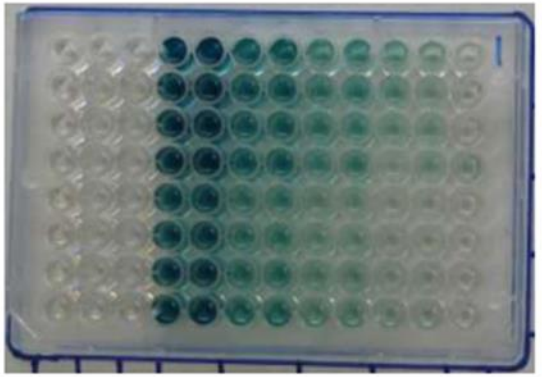
# Enzyme Immunoassays

- **Enzyme immunoassays (EIA)** are used to visualize and quantify antigens. They use an antibody conjugated to an enzyme to bind the antigen and the enzyme converts a substrate into an observable end product.

  **The substrate may be either a chromogen or a fluorogen**

- **Immunostaining**

is an EIA technique for visualizing cells in a tissue (**immunohistochemistry**) or examining intracellular structures (**immunocytochemistry**).





References

- **Enzyme selection** The appropriate enzyme selected to label an immunochemical reagent must have certain qualities as follows:

1) highly purified and high specific activity,
2) inexpensive,
3) stable during conjugation and for a long time in a proper storage condition,
4) easily conjugated, measured, and applied to highly sensitive assay.

- Chemiluminescence and bioluminescence assays generally exhibit more sensitive detection limits than fluorescent or colorimetric assays.



References

- **Enzymes Available For Enzyme Immunoassays** The most commonly used enzyme labels are horseradish peroxidase (HRP) and alkaline phosphatase (AP).
- Other enzymes are β-galactosidase, acetylcholinesterase, Glucse 6 Phosphate dehydrogenase and catalase
- The choice of substrate depends upon the required assay sensitivity and the instrumentation available for signal-detection

**Enzymes used for enzyme immunoassays**

| Enzyme | Source |
|---|---|
| Acetylcholinesterase | *Electrophorous electicus* |
| Alkaline phosphatase | *Escherichia coli* |
| β-Galactosidase | *Escherichia coli* |
| Glucose oxidase | *Aspergillus niger* |
| Glucose-6-phosphate dehydrogenase (G6PD) | *Leuconostoc mesenteroides* |
| Lysozyme | Egg white |
| Malate dehydrogenase | Pig heart |
| Peroxidase | Horseradish |

## Types of Enzyme Immunoassay

Enzyme immunoassays can be of two types depending on separation criteria of immunocomplex

### Homogenous Immunoassay

In these assays, the enzyme coupled to an antigen or antibody retains its activity partially after the reaction. Therefore, separation of the immune complex from the reaction mixture is not required for detection. The change in enzyme activity relates to the concentration of the analyte. Such assays are used mainly in the drug industries and also are known as enzyme multiplied immunoassay technique. The homogenous method commonly is used for the measurement of small analytes like drugs. The absence of a separation step makes it an easier and faster method.



References

## Heterogenous Immunoassay

Here, separation of immune complex from the reactants is a prerequisite for analyte estimation. In such assays, also known as ELISAs, an antibody or antigen is bound either noncovalently or covalently to a solid matrix. The unreacted antigen or antibody is removed, and the bound count is taken.

The solid matrix can be a microtiter plate, nitrocellulose membrane, polystyrene tubes or beads, nylon beads or tubes, or magnetic beads.

It can be of two kinds: competitive and noncompetitive immunoassays.

In heterogenous competitive immunometric assay, the antibody is immobilized on a solid surface. An analyte consists of a mixture of antigens that compete for common binding site and one of the antigens is labeled for quantification.



References

An **enzyme immunoassay** is any of several immunoassay methods that use an enzyme bound to an antigen or antibody. These may include:

- Enzyme-linked immunosorbent assay (ELISA)
- Enzyme multiplied immunoassay technique (EMIT)
- Fluorescent enzyme immunoassays (FEIAs)
- Chemiluminescent immunoassays (CLIAs)
- Radioimmunoassays (RIAs)



References

# Conjugation of enzymes to antibodies

Conjugation of enzymes to antibodies involves the formation of a stable, covalent linkage between an enzyme [e.g., horseradish peroxidase (HRPO), or alkaline phosphatase] and an antigen-specific monoclonal or polyclonal antibody in which neither the antigen-combining site of the antibody nor the active site of the enzyme is functionally altered.

Antibody conjugation is usually achieved through chemical reaction, although specific enzymatic conjugation, such as sortase-based protein terminal conjugation, or even through protein engineering.

The preferred site for chemical conjugation on the antibody is the $-NH_2$ (amine) group of a lysine or the free –SH (sulfhydryl) group of cysteine

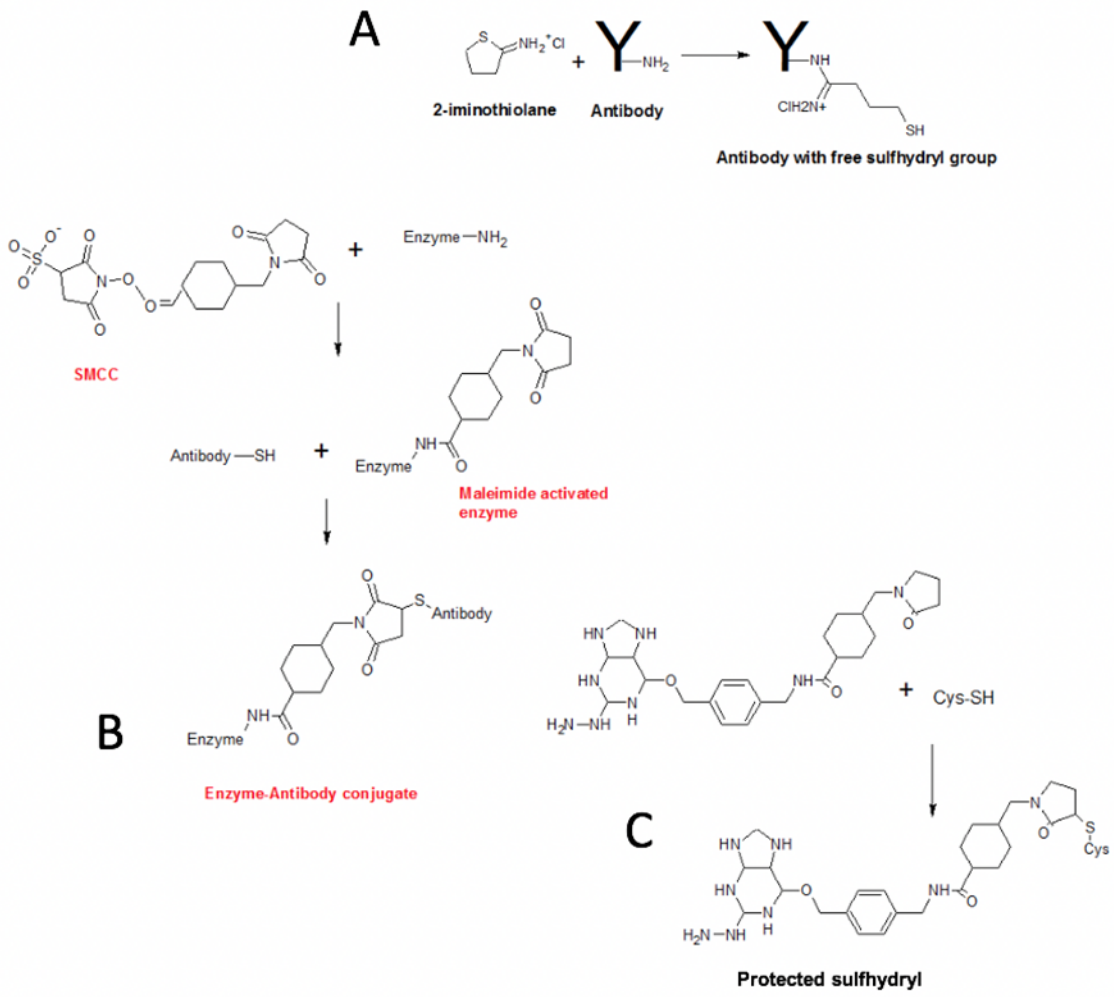
The ratio of Antibody to enzymes may be restricted to just two molecules per antibody.

To enhance the efficacy of conjugation the antibody may be altered by the introduction of unusual amino acids or chemical modification.

Sulfhydryl groups is that they can be used to form a stable, cleavable disulfide (-S-S-) linkage.

Most of the cysteines in antibodies are already present in the form of disulfides, and the available ones may not be at convenient positions.

Hence sulfhydryl groups can be introduced at the site of primary amines – especially those of lysine residues- by modification with reagents such as Traut's reagent (2-iminothiolane)




Modification of amines to sulfhydryls. A) Reaction with Traut's reagent to generate a free sulfhydryl. B) Generation of a non-cleavable sulfhydryl using SMCC (succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate). C) Generation of a protected sulfhydryl using maleimide crosslinking.

# Enzyme-antibody conjugates and their uses

| Enzyme | Reactive moiety | Conjugation method | Advantages | Disadvantages |
|---|---|---|---|---|
| Horseradish peroxidase | Sugar | Periodate method | Quantifiable results | Cannot be used for whole cells |
| Urease | Amine groups | Glutaraldehyde | Can be used for whole cells since there is no endogenous urease activity | Specificity of crosslink may be uncertain |
| Alkaline phosphatase | Amine group | NHS, maleimide-thiol coupling | Quantifiable results | Cannot be used for whole cells |
| β-galactosidase | Amine group | Sulfosuccinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (sulfo-SMCC) | Can be used for Cell-ELISA Results reflect the true Ab-Ag reaction for higher systems since there is no indigenous β-gal activity | |

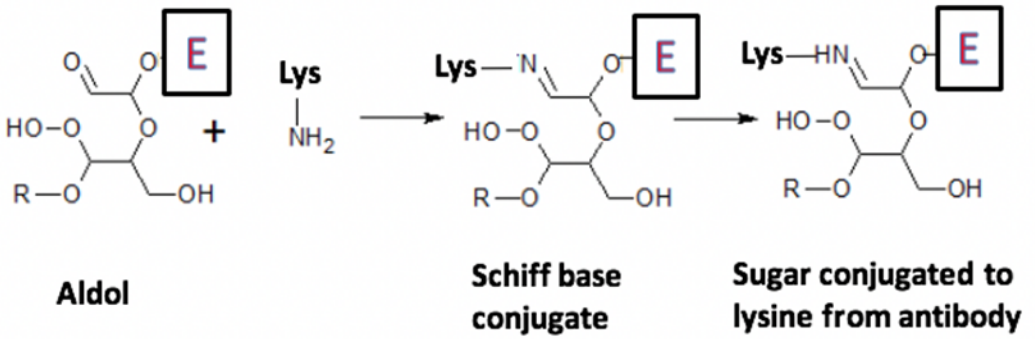
# Methods of conjugation and uses of enzyme-conjugated antibodies

## Sodium periodate oxidation

Conjugation of enzymes to antibodies can be based on the linkage of the sugar or lysine groups in the enzyme.

Sodium periodate oxidation of the sugars present on glycoproteins renders them susceptible to reaction with the terminal amine ($-NH_2$) of lysine residues in the antibody.

This forms a stable covalent link between the glycoprotein and the antibody. This is the method of choice to conjugate enzymes like horseradish peroxidase (HRP) to antibodies




The sodium periodate method results in a Schiff's base between the oxidised sugar of the enzyme and the $-NH_2$ from a lysine residue of the antibody.

## Conjugation by simple glutaraldehyde crosslinking

Glutaraldehyde, like dimethyl adipimate and the homologous dimethyl suberimidate, is homo-bifunctional crosslinking agents.

These molecules have the same active groups on both ends and a sufficiently long 'linker'.

These are generally used in protein-protein crosslinking, and therefore, antibody-enzyme conjugation.

The reactive end groups target terminal amines ($-NH_2$) of lysine and hydroxylysine on both proteins.

It reacts with amino groups of proteins, and when two proteins are mixed in its presence, stable conjugates are obtained without the formation of Schiff bases.

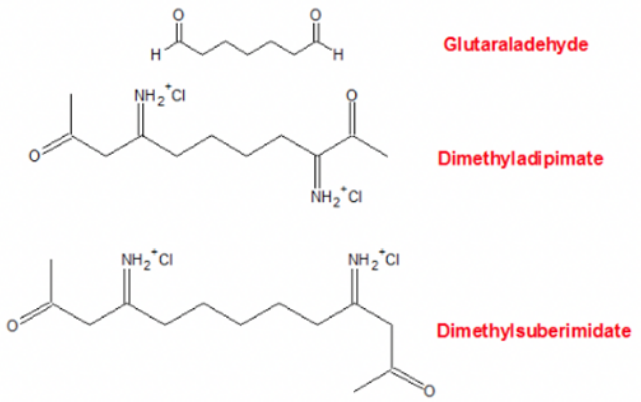
The advantage of using these crosslinking agents is the ease of carrying out the reaction.

The proteins to be cross-linked are incubated with the reagent in a suitable amine-free buffer, and the high molecular weight proteins are separated by column chromatography.

The obvious disadvantage is the non-specific crosslinking that leads to the formation of large aggregates of proteins that does not serve the purpose.

Self-coupling is a problem unless the proteins are at appropriate concentrations.

For example Urease and Alkaline Phosphatase Labeling



- β-Galactosidase Labeling Using m-Maleimidobenzoyl-n hydroxysuccinimide Ester (IWS)
- MBS is a heterobifunctional reagent, and it is of particular value when one of the proteins involved has no free thiol groups, e. g., IgG.
- *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) is a heterobifunctional agent that links a thiol group to an amino group at neutral pH
- In this method , the IgG is first modified by allowing the N-hydroxysuccinimide ester of MBS to react with amino groups in the protein.
- After the removal of unused reagent, the enzyme is added and the modified IgG reacts with thiol groups in the β -galactosidase via its maleimide groups to form thioether links.

# Solid Phase of ELISA Kits

A variety of materials can be the solid phase of ELISA kits such as polyethylene, polyacrylamide, agarose, glass and silicone rubber.

The ideal Elisa plates ought to have good adsorption performance, low blank value, high transparency. In addition, same size of very hole and identical performance are also the quality standard.

The most commonly used substance is polystyrene for it is good at protein adsorption and does not damage the immune activity of a protein. It also have no effect on the immune response and the color reaction in ELISA. What's more, it is inexpensive and is easy to get.

There are mainly three kinds of carriers in ELISA kits: microtiter plates, beads and small tubes.

Microtiter plates are used most widely and the plates used for ELISA are called ELISA plates. The international standard wells of microtiter plates are 8×12.

Elisa plates are characterized for that a large number of samples can be detected at same time and can read out the results in a special colorimeter quickly.

Now there are a variety of automated instruments for microtiter plate detection of elisa, including loading, washing, insulation, colorimetric and other steps, which is beneficial to standardizing the operation.

# Solid Phase of ELISA Kits

Due to different raw materials and production process, there are some gaps between ELISA kits. It is suggested that check the quality before using. The common checking methods: utilize human IgG (10ng/mL) to coat every hole of the ELISA plate, adds anti-human IgG enzyme-labeled antibodies with appropriate dilution into every hole after washing, then washes after incubation, adds substrate.

After the termination of the enzyme reaction, measures absorbance of dilution at every well.

The mean difference between individual readings and all readings should be less than 10%.

For the other solid phase carriers, the beads are typically the balls of 0.6cm diameter and the surface increases adsorption area significantly after grinding treatment.

The other feature of the beads is easy to wash, which is significant in the experimental procedures.

Small tubes also have a larger surface adsorption as the solid phase carrier, thus reaction amount of sample