

# Preparation of antibody conjugates

WOLF D. KUHLMANN

*Division of Radiooncology, Deutsches Krebsforschungszentrum, 69120 Heidelberg, Germany*

*Laboratory Diagnostics & Cell Science, 56112 Lahnstein*

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Enzymes, fluorochromes and other marker molecules are commonly used in a variety of applications to provide specific detection signals. To this aim, they may be conjugated to antibodies, avidin, protein A or G and other proteins in many ways. Fundamental work on chemical modification of proteins and antibody labelings has already been done long before AH COONS and co-workers employed those techniques for the preparation of their first fluorescent antibody conjugates. A popular way to prepare conjugates was to make use of aromatic isocyanates: the phenyl groups become linked through carbamide bonds with the  $\epsilon$ -amino groups of the lysine residues in the protein.

Until the 1990's, the number of fluorochromes for immuno-staining was very restricted. Mainly, derivatives of fluorescein such as fluorescein isocyanate (FIC) and isothiocyanate (FITC), Rhodamine (TRITC) and Lissamine Rhodamine (RB 200) served as fluorophores. Fluorescein isothiocyanate (FITC) is still one of the most employed fluorescent labels. The synthesis of FITC and similar fluorescein derived reagents yields a mixture of isomers. The isomers differ in their physicochemical behaviour and binding properties. For example, the conjugates may elute under different chromatographic conditions, and they migrate differently in an electrophoretic gel. Hence, single-isomer preparations are to be preferred in certain applications. The 5-isomer (Isomer I) of FITC is the most widely used FITC isomer, probably because it is easier to isolate this product in pure form (for more details, see Invitrogen Life Science, <http://www.probes.invitrogen.com/handbook/>).

Conjugation procedures need reagents which form stable covalent linkage. With the continuous development of new marker molecules, a great number of conjugation reagents became introduced. One of the first successfully employed reagents for the preparation of electron dense conjugates (antibody-ferritin) were for example toluene-2,4-diisocyanate and *m*-xylylene diisocyanate. Later on, other bifunctional reagents such as bis-diazotized benzidine, *p,p'*-difluoro-*m,m'*-dinitrophenyl sulfone, water-soluble carbodiimides, cyanurochloride, tetrazotized *o*-dianisidine, glutaraldehyde or *N*-hydroxysuccinimide became popular.

In the mean time, numerous bifunctional reagents were developed. Usually, protein labeling methods involve one of four common strategies,

- Primary amines as target: these are found on lysine residues which are widely distributed and easily modified because of their reactivity and location on the surface of proteins.
- Sulfhydryls as target: they are found in proteins in reducing conditions. Sulfhydryls are less abundant in proteins than primary amines; they may be introduced by reduction of disulfides or by chemical modification of primary amines.
- Carboxyls as target: they are abundant and easily accessible. However, they do not react as readily as primary amines and require cross-linkers.

- Carbohydrates as target: carbohydrate moieties can often be modified without altering protein activity. The labeling procedure needs two steps because carbohydrates are first oxidized to give reactive aldehyde groups.

Conjugation can be carried out in so-called *one-stage*, *two-stage* or *multiple stage* reactions. In the one-stage procedure, bifunctional coupling reagent is added to the mixture of two proteins to be coupled. The disadvantage of this type is that differences in the rate of reaction of the proteins with the coupling reagents can lead to selective polymerization of one of the proteins. Thus, at least two-stage reactions are preferred which will use

- hetero-bifunctional reagents, i.e. reagents containing two different reactive functional groups;
- homo-bifunctional reagents in which the reactivity of one of the functional groups is modified by steric or electronic factors;
- reagents differing in reactivity towards the two proteins to be conjugated (for example by glutaraldehyde in a two-step conjugation procedure; glutaraldehyde is one of the oldest homobifunctional cross-linking reagents);
- specific modifications of proteins to ensure selective reactions during the coupling process (e.g., by chemical blocking of amino groups or protection of antigen binding sites of antibodies with an immunoadsorbent);
- in the case of biotin labeling experiments, biotin derivatives such as biotin-hydrazide and biotin-N-hydroxysuccinimide (with or without a spacer group) are used for conjugation *via* free amino or carboxyl groups, respectively.

For convenience, a number of commercial conjugation kits are now available for easy labeling of antibodies with enzymes or fluorochromes. Some protocols of antibody conjugation and biotin labelings are given in the section “Laboratory techniques”.

The choice of a coupling reagent is often a matter of personal preference. In certain instances, however, there are important advantages to choosing a particular procedure. The goal of *protein-protein coupling* is limited by the type of functional groups available in the given molecules. The reaction of two different proteins with bifunctional reagents can give rise to a range of products including 1:1 conjugates, polyconjugates and polymers of each of the reactant proteins. Furthermore one must be aware that conjugation products may be generated in which catalytic and immunological activities (enzyme and antibody, respectively) are lost due to steric factors. Labeling reagents which react with  $\epsilon$ -amino groups will be less destructive than reagents (like diazonium compounds) reacting with tyrosine which is within or near the specific combining sites of antibodies. Last but not least, the final conjugate should be small-sized for cell labeling purposes in order to facilitate its penetration into the cells.

Because unconjugated antibodies will interfere with labeled antibodies by competing for antigenic sites, analytical applications of conjugates rely on both (a) the purity of the conjugates and, in the case of enzymes as markers, (b) its enzymatic activity. Thus, purification of conjugates is strongly recommended.

Mixtures of conjugated antibodies, free antibodies and free marker molecules can be separated on the basis of molecular size sieving by passage through a suitable column bed of granulated gels (e.g. Sephadex G 200). Furthermore, purification of peroxidase conjugated antibodies may be obtained by affinity chromatography on the basis of immunological bindings (1<sup>st</sup> step: insolubilized homologous antigen, and 2<sup>nd</sup> step: insolubilized anti-

peroxidase antibodies), or purification of conjugates is performed by use of lectin bindings. In the latter case, one can employ a combination of gel filtration and Concanavalin A.

After purification of conjugates their qualitative and quantitative characterization should be performed, f.e. by immunoelectrophoresis, SDS-polyacrylamide gel electrophoresis, immunohistological testing and quantitative immunoassays including binding studies in affinity chromatography. In the case of enzyme labeling, their catalytic activity should be also measured.

Apart from the use of enzyme labeled antibodies (e.g. covalent linkage between peroxidase and antibody), immunological bridge techniques which do not require covalent conjugation of antibody with marker may be employed. The use of soluble peroxidase-antiperoxidase complexes (PAP, see Immunostaining techniques) is especially popular (STERNBERGER LA, 1979).

Antibodies precipitate with antigens under certain conditions, but when antigens are in excess soluble antigen-antibody complexes will be formed. It was found that immune precipitates of HRP and anti-HRP antibodies do not dissolve readily and that in the antigen-antibody reactions between HRP and its antibody nonionic forces predominate (other forces are generally van der Waals forces and hydrogen bonding). Upon acidification of HRP-anti-HRP complexes ionic interactions become weakened, but separation of molecules will only occur when a small excess of HRP is added at the same time. STERNBERGER's theoretical considerations and experiments have confirmed that under such conditions the added peroxidase molecules possess equal affinity for anti-HRP as HRP in the immune complexes and due to HRP in excess dissociation of the precipitates is achieved; thus, formation of soluble HRP-anti-HRP complexes (PAP) become complete and remain soluble upon neutralisation.

In the original PAP procedure, specific immune precipitates were first obtained by mixing anti-HRP with HRP. Then, precipitates were washed in saline, resuspended by aspiration in saline containing four times the amount of HRP used for precipitation and acidified to pH 2.3. After subsequent neutralisation and removal of undissolved complexes, soluble PAP complexes were finally purified from free HRP by ammonium sulfate precipitation.

Enzyme conjugates can be stored at 4-6°C or frozen at -20°C. In the latter case, avoid repeated freeze-thaw cycles, thus, samples should be aliquoted. Frozen storage may have disadvantages because the conjugates become solid and the buffer salts have nonuniform distribution. Even if the application needs only a small aliquot, the entire sample must be thawed to assure sample uniformity.

Conjugates may be protected and stabilized against a variety of stress factors. Certain additives such as glycerol and ethylene glycol proved useful as cryoprotectants and storage stabilizers by lowering the freezing point of the aqueous solution and, thus, inhibiting the formation of ice crystals. This will insure that the conjugate remains stable and in liquid form. The addition of bioorganic solutes such as *Ectoin* or *Hydroxyectoin* (low molecular, zwitterionic and hygroscopic organic compounds) isolated from the halophilic bacterium *Halomonas elongata* seems to be an attractive alternative to classical chemicals. These tetrahydropyrimidine derivatives proved already useful as protecting and stabilizing agents for proteins against a variety of stress factors (GALINSKI EA et al., 1985; KNAPP S et al., 1999).

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