DUAL-ANTIBODY SYSTEMS FOR THE CONSTRUCTION OF BIOSENSORS

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SUMMARY

We are reviewing a dual-antibody analytical system based on competitive immunoassay methodologies. Two antibodies are immobilized at separate locations: one antibody (Ab-1) recognizing the analyte and analyte-enzyme conjugate, and the other antibody (Ab-2) recognizing the enzyme component in the conjugate. This dual-antibody system provides two signals, originating from the analyte-enzyme conjugate bound to Ab-1 and Ab-2, in response to different analyte concentrations in samples. A competitive immunoassay can be performed without separation of bound and unbound analyte-enzyme conjugate by the user. Three different modifications of the dual-antibody system are described: 1) operation in the irreversible mode under equilibrium conditions, 2) measurement in the irreversible mode under non-equilibrium conditions, and 3) continuous monitoring in the reversible mode. The first two modifications can be used for the development of disposable probes. The second system is particularly suitable for immunochromatography with either colorimetric or amperometric signal readout. The third principle makes use of the reversibility of binding for antigen-antibody complex formation and can be adapted for slow-response sensors for the continuous measurement of fluctuating concentrations of small analytes in aqueous media streams.

1. INTRODUCTION

Most of the analytical methods for small analytes using antibodies as specific binding proteins are based on the principle of the competitive immunoassay. A signal is generated as a result of the competition between an analyte and an analyte equivalent (e.g., an analyte-enzyme conjugate) for a limited number of binding sites of an antibody. For this type of immunoassay, a separation step is required: the analyte-enzyme conjugate that is not bound to antibody needs to be removed before the signal representing the amount of analyte in the sample is generated (Figure 1).

The separation of bound from unbound antigen is a shortfall for the development of simple immunoassays of this type that can be performed outside the laboratory. Efforts to circumvent the

separation in the procedure (e.g., in homogeneous immunoassays (1, 2)) have only limited application for the measurement of low analyte concentrations because of an unfavorable signal-to-noise ratio.

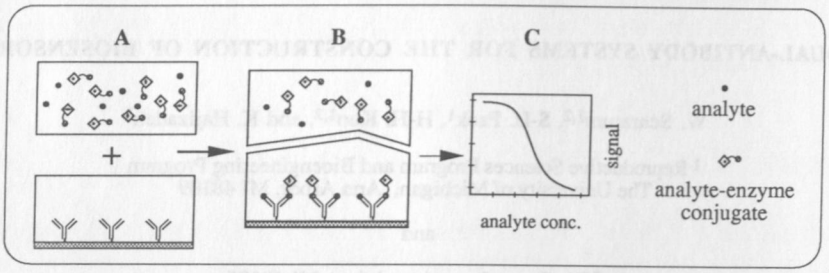


Figure 1. A sample containing the analyte and a constant amount of tracer (e.g., analyte-enzyme conjugate) is added to an immobilized antibody (A). The antigen is in excess to the antibody binding sites. In this type of assay (competitive immunoassays), the amount of free analyte in the incubation medium determines the number of tracer molecules bound to antibody. A separation step, removing the unbound tracer, is required (B) to obtain a signal inversely proportional to the analyte concentration (C).

We have investigated analytical systems based on the competitive immunoassay where two antibodies are involved: an antibody against the analyte (Ab-1), and another antibody (Ab-2) against the enzyme used in the analyte-enzyme conjugate for signal generation (Figure 2).

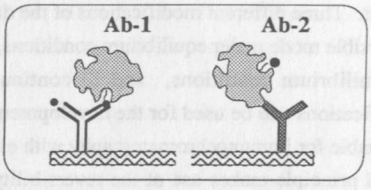


Figure 2. In the dual-antibody assay, two immunoglobulins are used: one that specifically recognizes either the native analyte in the sample or the analyte component in the analyte-enzyme conjugate (Ab-1), and another antibody that binds to the enzyme (Ab-2).

By capturing the bound and the unbound tracer (i.e., displaced from Ab-1), two signals can be obtained for the measurement of analyte concentrations (Figure 3). We have studied three variations of this dual-antibody system: 1) operation under equilibrium conditions in the irreversible mode, e.g., for disposable electrodes reading a differential signal; 2) operation under non-equilibrium conditions in the irreversible mode (combination of the dual-antibody system with immunochromatography); and 3) operation under equilibrium conditions in the reversible mode for continuous monitoring of analytes in a medium stream. In this paper, we will provide an overview of these three modes of operation.

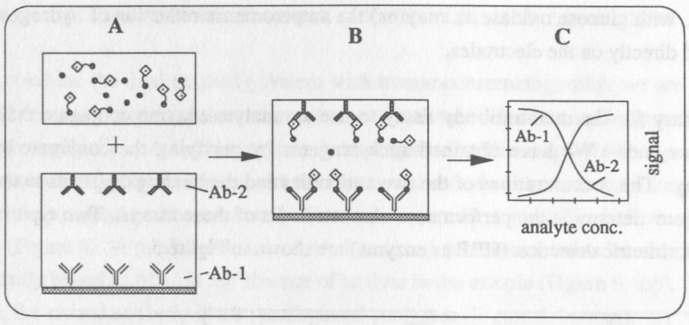


Figure 3. Dual-antibody system using an antibody to the analyte and an antibody to the enzyme. The concentrations and the binding constants of the two antibodies are selected such that the majority of the analyte-enzyme conjugate is bound at Ab-1 in the absence of analyte. In the presence of analyte in the sample (A), the amount of analyte-enzyme conjugate that binds to Ab-1 and Ab-2 (B) is determined by the concentration of analyte. Two dose-response curves are obtained from measuring the signal at the two antibody sites (C).

2. IRREVERSIBLE MODE, EQUILIBRIUM CONDITIONS

For the investigation of the dual-antibody system under equilibrium conditions, we immobilized a monoclonal antibody to the steroid hormone progesterone on polypropylene discs that fit into microwells. An antibody to the enzyme horseradish peroxidase (HRP) was likewise immobilized on separate discs. Discs with different antibodies were incubated in a microwell with varying amounts of progesterone and a constant amount of a progesterone-HRP conjugate. Each disc captures an amount of the analyte-enzyme conjugate that is proportional to the concentration of progesterone in the incubation solution. The enzymatic activity can then be determined, e.g., by a reaction that forms colored products (3).

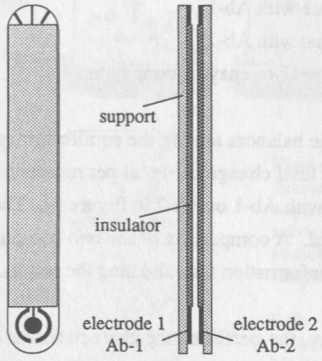


Figure 4. Electrodes for the dual-antibody system for operation under equilibrium conditions (not to scale). *Left:* Front view of an electrode. *Right:* Side view of two juxtaposed electrodes that contain Ab-1 immobilized on electrode 1 and Ab-2 on electrode 2. The electrodes are placed into the sample medium together with the analyte-enzyme conjugate. After complex formation of the conjugate with the antibodies, product generated by the enzyme can be amperometrically measured.

It is required to disassemble the system for the determination of enzymatic activity on each disc by colorimetric measurement. Signal detection can be simplified by electrochemical measurement. To this end, the antibodies are immobilized on two electrodes (Figure 4). With a suitable analyte-enzyme

conjugate (e.g., with glucose oxidase as enzyme) the amperometric reduction of hydrogen peroxide can be recorded directly on the electrodes.

It is necessary for the dual-antibody assay to use an analyte-enzyme conjugate that does not contain free enzyme. We have obtained such reagents by purifying the conjugate by affinity chromatography. The concentrations of the two antibodies and the binding constants to the analyte-enzyme conjugate determine the performance characteristics of these assays. Two typical standard curves with colorimetric detection (HRP as enzyme) are shown in Figure 5.

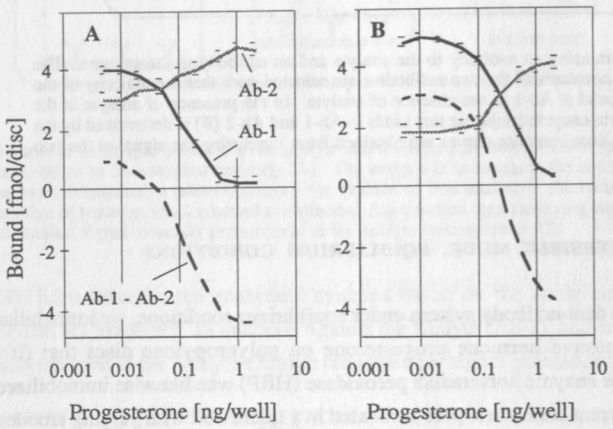


Figure 5. Dose-response curves for two dual-antibody systems under equilibrium conditions. The same antibody (Ab-1) for the analyte (progesterone) was used for both A and B but a different antibody to HRP (Ab-2) was used. The differential signal of the two binding curves is shown (Ab-1 - Ab-2). Reproduced with permission (3).

This analytical system is characterized by a combination of equilibrium reactions:

- complex formation of analyte with Ab-1,
- complex formation of analyte-enzyme conjugate with Ab-1,
- complex formation of analyte-enzyme conjugate with Ab-2,
- competition of Ab-1 and Ab-2 for binding the analyte-enzyme conjugate.

Different analyte concentrations in a sample affect the balances among the equilibrium reactions, and two signals can be obtained per measurement. The total change of signal per measurement can be increased up to two-fold (e.g., compare Ab-1 - Ab-2 with Ab-1 or Ab-2 in Figure 5). The profile of the two binding curves can be mathematically modeled. A comparison of the two complimentary signals obtained in measurements can provide additional information for validating the results.

In this mode of operation of the dual-antibody assay, the performance characteristics (i.e., the shape and the position of the two binding curves) are determined by the *equilibrium binding constants* for complex formation between antibodies and antigens. This will be different in the system introduced below.

3. IRREVERSIBLE MODE, NON-EQUILIBRIUM CONDITIONS

If we combine the dual antibody system with immunochromatography, we are working under non-equilibrium conditions and the antigens cannot simultaneously form antibody-antigen complexes with the two antibodies. A membrane strip is impregnated with the analyte-enzyme conjugate in a distinct area so that this tracer moves with the aqueous phase when the end of the strip is brought into contact with the sample. The two antibodies are chemically immobilized above the sequestered conjugate (Figure 6). A predefined ratio of Ab-1 and conjugate is maintained so that the conjugate is predominantly bound to Ab-1 in the absence of analyte in the sample (Figure 6, top). In the presence of analyte, the second antibody binds the displaced conjugate (Figure 6, bottom).

The membranes for this system need to be specially prepared to meet certain requirements. Some of these are:

- good capillary action to transport the sample through the solid matrix;
- low non-specific binding of the analyte and the analyte-enzyme conjugate;
- the ability to chemically immobilize the antibodies;
- non-interference with the catalytic activity of the enzyme;
- co-migration of the analyte and the analyte-enzyme conjugate.

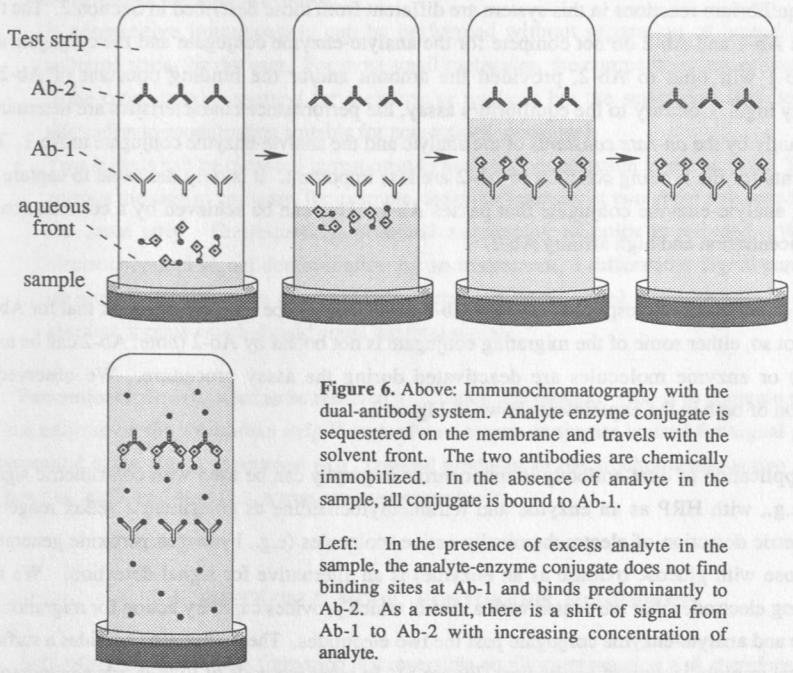
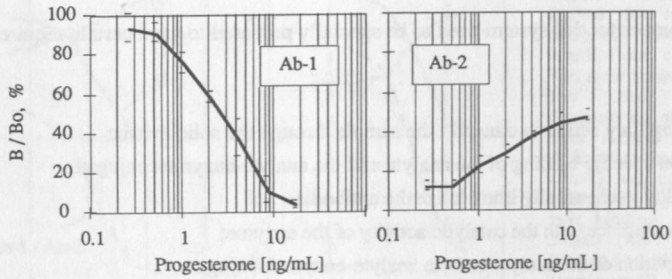


Figure 6. Above: Immunochromatography with the dual-antibody system. Analyte enzyme conjugate is sequestered on the membrane and travels with the solvent front. The two antibodies are chemically immobilized. In the absence of analyte in the sample, all conjugate is bound to Ab-1.

Left: In the presence of excess analyte in the sample, the analyte-enzyme conjugate does not find binding sites at Ab-1 and binds predominantly to Ab-2. As a result, there is a shift of signal from Ab-1 to Ab-2 with increasing concentration of analyte.

The last point is particularly important if the hydrophilicity between the analyte and the tracer or the interaction of the two antigens with the membrane differs. While in traditional chromatography a separation of different substance classes is the final goal, this is not so in this application. It is desirable that the analyte and the tracer reach the first antibody together and a separation would be counterproductive. We have, for example, assayed progesterone on membrane strips (Figure 7). Satisfactory results are obtained only in the presence of a carrier substance (e.g., a protein or other complexing molecule). Otherwise, progesterone does not leave the lower part of membrane strips.

Figure 7. Immunochromatography on membrane strips as shown in Figure 6. The tracer was progesterone-horseradish peroxidase. Color development at Ab-1 with tetramethylbenzidine is shown in the left graph, at Ab-2 in the right graph.



The equilibrium reactions in this system are different from those described in Section 2. The two antibodies Ab-1 and Ab-2 do not compete for the analyte-enzyme conjugate and the conjugate that passes Ab-1 will bind to Ab-2, provided the amount and/or the binding constant of Ab-2 is sufficiently high. Contrary to the equilibrium assay, the performance characteristics are determined predominantly by the *on-rate constants* of the analyte and the analyte-enzyme conjugate to Ab-1. The requirements for the binding constant of Ab-2 are less important. It is only desirable to capture all remaining analyte-enzyme conjugate that passes Ab-1. This can be achieved by a combination of excess concentration and high affinity Ab-2.

In this assay, the dose-response curve for Ab-2 ideally should be a mirror image of that for Ab-1. If this is not so, either some of the migrating conjugate is not bound by Ab-2 (note: Ab-2 can be used in excess) or enzyme molecules are deactivated during the assay procedure. We observed a combination of both in the experiment shown in Figure 7.

This application of dual-antibody immunochromatography can be used with colorimetric signal readout (e.g., with HRP as an enzyme and tetramethylbenzidine as colorimetric redox reagent). Amperometric detection of electrochemically active molecules (e.g., hydrogen peroxide generated from glucose with glucose oxidase as an enzyme) is an alternative for signal detection. We are investigating electrodes that are coated with a matrix which provides capillary action for migration of the sample and analyte-enzyme conjugate past the two electrodes. The matrix also provides a surface for chemical immobilization of antibodies (Figure 8). In other methods of immunochromatography (4, 5) or enzyme chromatography (6), a band of varying length is used to determine the concentration of an analyte in a sample. We have two distinct areas for signal generation. Consequently, adaptation to signal quantitation by amperometric methods with an electrode is possible.

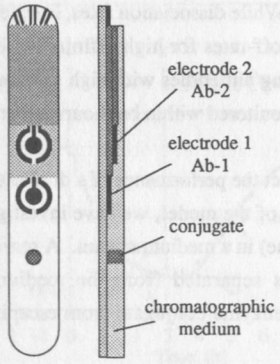


Figure 8. Electrode for the dual-antibody system using immunochromatography (not to scale). The tip of the electrode is placed in the sample. A pre-defined volume of aqueous medium containing the analyte migrates up the chromatographic medium. The conjugate dissolves and is carried with the aqueous phase. Carry-over of hydrogen peroxide from electrode 1 to electrode 2 can be prevented by immobilizing a band of catalase between the electrodes (not shown).

Immunochromatography offers several advantages that are particularly attractive for the design of analytical methods that can be used outside the laboratory.

- Test strips can be manufactured to contain most of the components so that the typical assay steps like adding of reagents, pipetting, and washing by the user are reduced to a minimum.
- The chromatography membrane takes up a defined sample volume so that pipetting or aliquoting of samples is avoided.
- A competitive immunoassay can be performed without separation of bound and unbound tracer by the user. For most small molecules, the competitive immunoassay is the most suitable method for inexpensive analysis but the separation step limits adaptation to modification suitable for non-trained personnel.
- Two signals can be obtained in response to analyte concentration in the sample. This permits the user to compare, for example, the color intensity at two areas developed on the same strip. Consequently, potential misreading of color is reduced. With electrochemical signal determination by an instrument, a differential signal can be measured. This can be electronically compared as a control with the sum of the absolute signals which should equal the total signal.

Two major challenges need to be resolved to advance this method. One is to maintain the stability of the enzyme on the membrane strip if an analyte-enzyme conjugate is used for signal generation. The second is the signal generation (e.g., colored products, or electrochemically active molecules) without an additional step (i.e., a true one-step procedure).

4. REVERSIBLE MODE, CONTINUOUS MONITORING

Antibody-antigen complex formation is a reversible equilibrium reaction and, therefore, inherently suitable for continuous monitoring of changing analyte concentrations (reversible mode). The response time of sensors using binding proteins for continuous monitoring depends on the kinetic

reaction of the antigen-antibody complex formation. While dissociation rates, k_{off}, for antibodies can vary over a wide range from 10^{3} to 10^{-4} sec^{-1} (7, 8), off-rates for high affinity antibodies are often very slow. For reasonably sensitive systems (i.e., using antibodies with high association constants), changes in analyte concentrations, therefore, will be monitored within but hours rather than minutes.

We have established a mathematical model to predict the performance of a dual-antibody system in the reversible mode. For the experimental validation of the model, we have investigated the sensor response to fluctuations of a small analyte (progesterone) in a medium stream. A sensor compartment containing the two antibodies, Ab-1 and Ab-2, is separated from the medium stream by a semipermeable membrane that prevents the analyte-enzyme conjugate from escaping, but permits diffusion of the analyte from the medium (Figure 9).

For our current experimental design, we use progesterone-HRP as an analyte-enzyme conjugate and we measure the enzymatic activity by a colorimetric reaction. This still requires the destruction of the analytical cell for measurement and does not yet permit us to continuously monitor the signals at the two antibody sites. We will achieve this with electrochemical sensors that are under investigation. With these sensors, the formation of hydrogen peroxide produced by the glucose oxidase in the analyte-enzyme conjugate will be measure amperometrically. Hydrogen peroxide released into the bulk solution from one electrode can be prevented from reaching the other electrode by a mesh, containing immobilized catalase, that separates the electrodes.

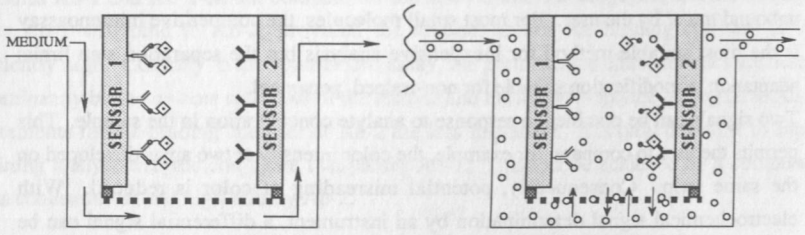


Figure 9. Continuous monitoring of analytes in a medium stream. In the absence of analyte, the analyte-enzyme conjugate binds predominantly to sensor 1 (left). At high concentrations of analyte in the medium, the signal shifts to sensor 2 (right). Reproduced with permission (3).

Potential rate-limiting factors for the time-response of the sensor can be 1) the permeation rate of the antigen through the semipermeable membrane, and 2) the binding constants of the two antibodies to their respective antigens. Another limiting factor is the half-life of the enzyme in the sensor compartment, if enzymes are used as signal generators. This accounts for the deviation of the experimental results in Phase D (Figure 10) from the calculated response (3, 9, 10).

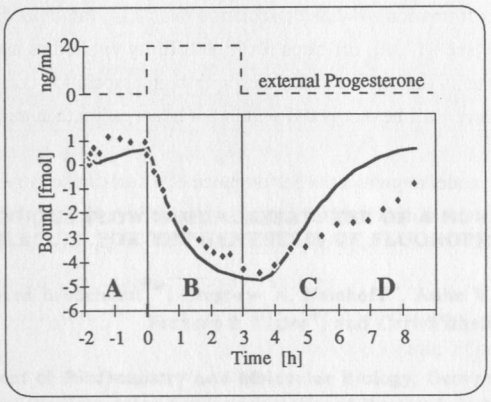


Figure 10. Measurement of progesterone in a medium stream with an equilibrium, reversible dual-antibody system. A differential signal was obtained from both sensors in response to changes in the progesterone concentration (top, dashed line) in the medium. The experimentally determined response (diamonds) is compared with the calculated response (solid line) predicted by our mathematical model. The four phases are: **A** - no progesterone in the medium; **B** - high progesterone; **C** and **D** - no progesterone, recovery of the sensor. Reproduced with permission (3).

5. ACKNOWLEDGEMENTS

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