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Immunoassay: The Ideal Trace Organic Analysis Method [and Discussion]

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Immunoassay: the ideal trace organic analysis method

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Techniques using antibodies as reagents provide the sensitivity and selectivity demanded by modern clinical and environmental analyses. The use of non-isotopic labelling methods allows non-hazardous and homogeneous analysis formats, and offers new opportunities for multi-analyte assays and simple sensors. Some recent optical immunoassays illustrate the potential of such methods. Likely future developments in this area are surveyed.

1. Introduction

The problems of the trace analysis of organic compounds in complex matrices (body fluids, tissue samples, foodstuffs, etc.) are among the hardest that analytical scientists encounter. There is a continually growing requirement for better limits of detection, often at pg ml^{-1} or lower levels; the presence of molecules similar to the target determinand demands highly selective analyses; and the labile nature of biological molecules, and the likelihood of molecular interactions such as protein binding of small molecules, present further problems. At the same time, the high level of demand for such analyses, especially in human and veterinary medicine and in environmental and food science, implies that acceptable analytical techniques must be fairly simple and robust, of low cost, and capable of ready automation and/or performance by relatively inexpert personnel (Miller 1979). Immunoassays – analytical methods using antibodies as reagents – now play a major role in addressing these problems. Technical advances are rapid, and the recent application of immunological methods to environmental problems, in addition to their well-established use in biomedicine, is a major development.

Antibodies are among the most remarkable protein products of biological evolution. The principal structural features of the major human antibody, immunoglobulin G (IgG), are shown in figure 1 (Nisonoff 1982). The glycoprotein molecule has a relative molecular mass (RMM) of *ca.* 150000 and a two-fold axis of symmetry, providing it with two binding sites, one formed by the *N*-termini of each pair of heavy (RMM *ca.* 50000) and light (RMM *ca.* 23000) chains. Variable amino acid sequences in these regions allow the generation of numerous (perhaps 100 million) chemically distinct binding specificities while the overall structure of all IgG molecules remains effectively unchanged. Of great importance is the multi-functional nature of the antibody molecule: its 12 intrachain disulphide bonds divide it into a series of functionally distinct domains, the amino acid sequence of all the domains except the *N*-terminal V (variable) ones remaining effectively constant in all human IgG molecules. The ability of antibodies to bind to the bacterial products Protein A

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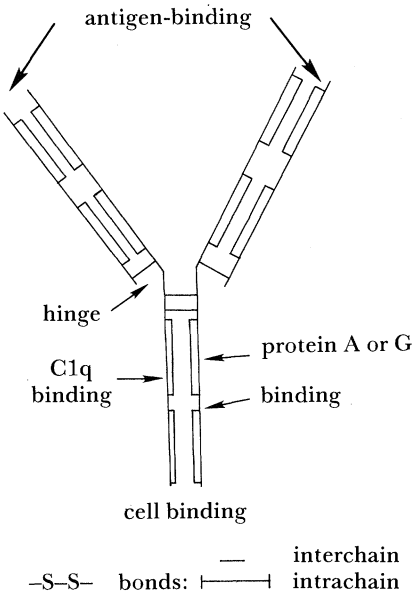
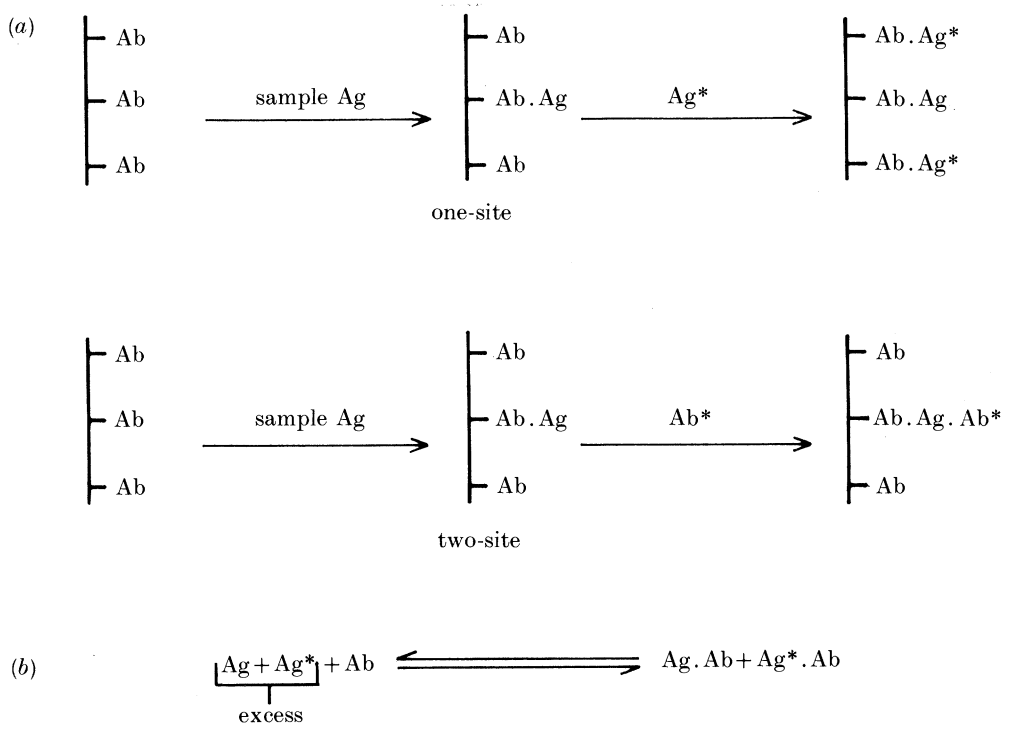


Figure 1. Antibodies as multifunctional molecules (RMM is 150000).

and Protein G is of value in antibody purification as well as in analytical protocols, and the complement binding and cell-binding capacities of antibodies are also used analytically. IgG molecules, which make up the bulk of the human antibody pool, are chemically very robust, their resistance to denaturation by heating or pH or solvent changes being markedly superior to many other proteins; this attribute is clearly also of analytical value.

Antibodies are classically produced by the repeated injection of an antigen ('foreign' molecule), or a conjugate of a small molecule (hapten, RMM greater than *ca.* 100) with a polymeric carrier, into an experimental animal, with subsequent recovery of the animal's blood serum containing the antibody molecules. Because of the extreme specificity of antigen–antibody interactions, it is often possible to use a simple dilution of this 'antiserum' directly as an analytical reagent, though modern antibody purification methods are simple and effective. Kohler & Milstein's (1975) demonstration of the principles of monoclonal antibody production by fusion of an antibody-producing cell with a myeloma (cancer) cell has led to this alternative approach being used more frequently, with great benefits in quality control. All antibodies from a given monoclonal preparation are regarded as functionally identical, whereas classically generated antibody preparations contain a mixture of antibodies with varying specificities and antigen binding constants.

Immunoassays normally involve the determination of an antigen or hapten, using the corresponding antibodies as reagents (occasionally these roles are reversed), with the detection of the antibody–antigen reaction as the basis of the analytical method. Such techniques were first studied in the 1930s, when the quantitative basis of antigen–antibody reactions was established (Heidelberger *et al.* 1933), allowing macromolecular antigens to be determined by precipitation techniques, and later by nephelometry or turbidimetry. The limit of detection of these methods, which depend on the formation of supramolecular complexes when multivalent antigens and divalent or multivalent antibodies combine, is usually *ca.* 10–100 ng ml^{-1}. The



Scheme 1. Immunoassay methods: (*a*) immunometric, (*b*) competitive binding.

application of labelled antibodies in fluorescence microscopy followed in 1941 (Coons *et al.*). The extension of immunoassays to the determination of small molecules, and of large molecules at concentrations below *ca.* 10 ng ml^{-1}, followed the development of radioimmunoassay by Yalow & Berson (1960). This method, like the many related techniques developed subsequently, uses labelled antigen/hapten analogues or labelled antibodies to overcome the difficulty that precipitation does not occur when small molecules (or very dilute macromolecules) combine with antibodies. Irrespective of the label used, there is a clear distinction between competitive binding methods (scheme 1*b*), in which sample and labelled antigen/hapten molecules compete for a limited number of antibody binding sites, and immunometric methods (scheme 1*a*), in which an antibody excess is used. Immunometric methods are in principle more sensitive than competitive binding techniques (Ekins 1985). They frequently use labelled antibodies rather than labelled antigens or haptens: this has the advantage that, since all antibodies are similar in structure, a very small number of labelling techniques can be applied to numerous assays.

2. Non-isotopic immunoassays

Although assays involving radio-labels remain in common use, there is a very substantial and still growing trend to replace such labels with non-isotopic markers. Non-isotopic immunoassays are not necessarily more sensitive than radioimmunoassay methods, but they have several other advantages, not least their freedom from the hazards and precautions of preparing, using and disposing of radio-labelled reagents. Further benefits, to be discussed in detail below, include the potential for homogeneous, i.e. separation-free assays; the ability to detect two or more analytes simultaneously; and the possibility of developing simple and robust 'immunosensors' for bedside, field and even *in vivo* use. Studies of alternative labels began soon after radioimmunoassay was introduced (Dandliker & Feigen 1961) but only three such approaches to labelling, using enzymes, fluorescent groups and chemiluminescent groups, have found widespread acceptance.

Enzyme immunoassays have been frequently reviewed (Kricka 1985; Voller & Bidwell 1985), and remain much favoured. This is partly because clinical chemists in particular are routinely familiar with enzyme assays, but also because the amplifying effect of the enzyme label allows the development of many sensitive assays with only simple colour detection (qualitative) or absorbance measurement (quantitative) as the final analysis step. Many practical enzyme immunoassays utilize the (enzyme linked immunosorbent assay (ELISA)) approach (Engvall & Perlmann 1971), in which the antibody (sometimes antigen) molecules are immobilized on a solid surface (most frequently by adsorption on a plastic microtitre plate). Antibody-bound molecules can then be separated from unbound species by simple decantation or suction, thus minimizing the inconvenience of an intrinsically heterogeneous assay method. A closely related approach of growing popularity uses antibody bound to gel particles with magnetic cores. Separation of antibody bound and unbound material is simply achieved by placing a rack of reaction tubes on a permanent magnet.

The second commonly used enzyme immunoassay (the enzyme multiplied immunoassay technique (EMIT)) was the first homogeneous immunoassay technique to become commercially available (Rubenstein *et al.* 1972). The advantages and drawbacks of homogeneous methods are surveyed below, but it is worth noting at once that EMIT methods are still in common use for therapeutic drug monitoring and peptide hormone determination at the nanomolar level.

3. Homogeneous fluorescence immunoassays

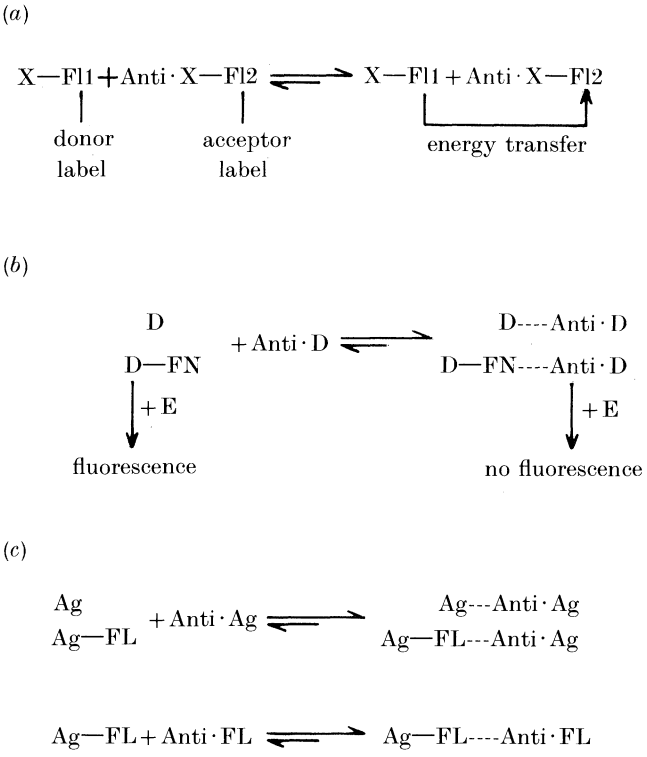
Fluorescence is the emission of a light photon by a singlet molecule previously excited at higher energy (lower wavelength) by absorption of an incident photon. Labelling methods based on this principle were also studied soon after the introduction of radioimmunoassay (Dandliker & Feigen 1961). Their two principal attractions are the very low limits of detection potentially available, and the sensitivity of fluorescence phenomena to the molecular environment. The excellent limits of detection are the result of experimental layouts that permit low-level fluorescence signals to be measured against a more or less 'dark' background, in contrast to absorption spectrometry, in which trace analysis involves measuring a small difference between two large light intensities. Moreover, whereas the use of higher powered light sources does not bring significant benefits to absorption spectrometry, increasing the light source intensity in a fluorescence spectrometer

should lead to a proportional increase in the emitted fluorescence signal (though in practice use of this principle is limited by photodecomposition effects). It follows, however, that if the background signal in a fluorescence assay is not close to zero, much of the method's sensitivity will be lost. As will be seen, this is a crucial consideration in the application of fluorescence immunoassays (especially homogeneous ones) to biological matrices.

Because fluorescence phenomena are environment dependent, the binding of a fluorescent labelled molecule to an appropriate antibody often leads to a change in the observed fluorescence signal. Such a change means that a separation step is not necessary to distinguish antibody-bound from free labelled molecules, so a competitive binding assay can be performed with a homogeneous protocol. Such methods are evidently simpler and more easily automated than heterogeneous assays, and several homogeneous methods have been adopted in commercial analysis systems. The approach most widely used at present is the fluorescence polarization assay (Watson *et al.* 1976), in which the fluorescence signal from the labelled species is observed using vertically polarized exciting light, with the emission signal measured alternatively through a vertically and horizontally oriented second polarizer (I_v and I_h respectively). The fluorescence polarization, p, given by $p = (I_v - I_h)/(I_v + I_h)$, is then calculated. It can be shown that p is close to zero for low RMM species, because the oriented molecules selected for excitation by the polarized incident light rotate rapidly and lose their orientation within the lifetime (*ca.* 5 ns) of the fluorescent label. The emitted fluorescence is thus unpolarized, so $I_v = I_h$. When a small molecule becomes bound to an antibody, however, its rotational relaxation time is much increased, so the emitted fluorescence is polarized and $p > 0$ (in theory $p \leqslant 0.5$ in the given conditions). This elegant principle, which requires in practice only minor and simple modifications to a conventional fluorescence spectrometer, has been used to determine many small molecules, but its efficacy disappears once the RMM of the analyte exceeds a few thousand. In my laboratory a fluorescein derivative of lysergic acid diethylamide was synthesized and used in a fluorescence polarization immunoassay for nanogram levels of this drug (Hubbard *et al.* 1983).

A second attractive approach to homogeneous fluorescence immunoassay uses the phenomenon of singlet–singlet energy transfer (Ullman *et al.* 1976). In this assay (scheme 2) an analogue of the analyte is labelled with one fluorophore and the antibodies used are labelled with a different fluorophore. The two fluorescent groups are chosen so that efficient singlet–singlet energy transfer can occur between them when the labelled antigen and antibody molecules are specifically bound. This energy transfer results in the quenching (reduction) of the fluorescence from one fluorophore (the 'donor' group) and possibly the enhancement of the signal from the second 'acceptor' fluorophore. When unlabelled analyte molecules are present, some of the pairs of labelled molecules are dissociated because of the competitive binding reaction, so the observed energy transfer effects are reversed. The crucial feature of this assay is that the efficiency of the energy transfer is proportional to the inverse sixth power of the distance between the label groups, and so is negligible except for specifically bound and labelled antigen–antibody pairs.

This technique requires careful choice of the donor and acceptor fluorophores for its success (Lim *et al.* 1980). The principal photochemical requirement is clearly that the fluorescence emission spectrum of the donor overlaps the absorption (excitation) spectrum of the acceptor. In practice, however, it is also highly desirable that (*a*) the



Scheme 2. Mechanism of fluorescence energy transfer immunoassay. (*a*) Energy transfer enhances Fl2, quenches Fl1. Unlabelled (i.e. sample) X competes with X-Fl1 for Anti·X-Fl2 and reverses the ET effects. (*b*) FN is a fluorigenic substrate for enzyme E; as [D] (drug) increases, I_F increases. (*c*) Binding of Ag-FL to Anti·FL causes quenching. As [Ag] increases, I_F decreases.

acceptor is not directly excited at the absorption wavelength of the donor (i.e. it should only be excited by energy transfer), (*b*) the fluorescence of the acceptor is negligible at the fluorescence wavelength of the donor, as the energy transfer may be evaluated by measuring donor quenching, (*c*) the fluorescence of the donor is negligible at the emission wavelength of the acceptor, if the energy transfer is to be assessed by measuring acceptor fluorescence. In my laboratory, several novel donor–acceptor pairs have been studied (Lim 1980; Miller *et al.* 1980; Miller & Thakrar 1982). Among them the use of fluorescamine (Miller *et al.* 1980) derivatives as donors and fluorescein as an acceptor group is of particular interest: in this system donor quenching cannot be used to measure the energy transfer because of a separate enhancement effect (see below), so – uniquely – acceptor enhancement is used, and despite a high background signal from the donor fluorophore, provides assays at the nanomolar level (figure 2). This assay can be used for both small and large molecules though in the latter instance, when several label groups are incorporated into each molecule (analyte or antibody), there may be complications from intramolecular energy transfer between neighbouring fluorophore groups. The most obvious disadvantage of the energy transfer assay is that it requires the preparation of two labelled reagents.

The practical limit of detection in blood plasma samples for most polarization and energy transfer assays, as well as other homogeneous fluorescence immunoassays

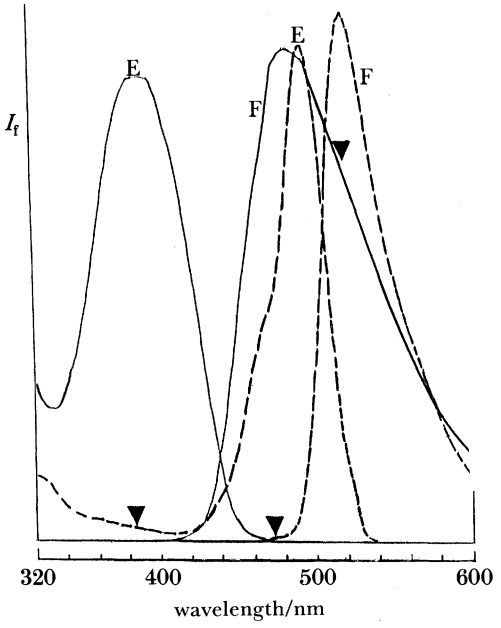


Figure 2. Excitation (E) and fluorescence (F) spectra of fluorescamine (Fluram TM, Roche – continuous lines) and fluorescein (broken lines) conjugates as used in energy transfer immunoassays. The arrows at *ca.* 380, 470 and 520 nm show respectively that there is negligible direct excitation of the acceptor fluorescein groups at the excitation wavelength of the donor fluorescamine groups; that there is negligible fluorescein emission at the fluorescamine emission wavelength; but that there is considerable fluorescamine emission at the fluorescein emission wavelength.

such as the fluorigenic enzyme substrate assay (Wong *et al.* 1979) and the fluorescence protection assay (Nargessi *et al.* 1978), seems to be in the nanomolar range. This is much poorer than would be expected from studies of fluorophores in pure solution, where background signals are very low. Plasma and other biological samples, by contrast, exhibit considerable fluorescence and light scattering signals, and the absence of a separation step in the assay means that these background signals remain in the sample at the time of measurement. The commonest of fluorescent labels, fluorescein, is particularly disadvantaged both by its low Stokes shift (difference between excitation and emission wavelengths), which makes it vulnerable to Rayleigh scattered light from protein containing samples; and by the fluorescence of albumin bound bilirubin, which occurs at almost exactly the same wavelength as the fluorescein emission.

The intrinsic fluorescence of blood serum/plasma has been extensively studied in our laboratory. Table 1 summarizes the main emission bands, the extent to which the signals originate in protein bound material, and the degree to which they can be reduced by simple pretreatment of the sample with a small affinity column. The emission at 360 nm (excited at *ca.* 280 nm), due largely to tryptophan groups, is by far the most intense but hardly interferes with fluorescence immunoassays using visible region labels. The longer-wavelength bands are much weaker but do provide substantial background signals which, along with the scattered light background described above, drastically worsen the accessible limits of detection.

Passing serum samples through small precolumns containing one or more affinity

Table 1. *Principle fluorescence bands of normal human blood serum*

(EX and EM represent excitation and emission wavelengths respectively; AC represents an affinity chromatography column containing a mixed Cibacron Blue, concanavalin A and protein A stationary phase; ND, not determined.)

EX	EM	relative intensity	protein bound?	% reduction by AC
285	340	100	yes	90
350	450	0.7	yes	80
400	470	0.5	yes	81
425	520	0.3	yes	75
530	650	0.1	?no	ND

matrices provides only a partly satisfactory approach to the reduction of background effects. Although the method is rapid, it only involves a slight dilution of each sample, and reduces background light scattering as well as fluorescence (because strongly scattering proteins are removed from the sample), it cannot be used in many cases where the analyte is itself a protein, nor when a small analyte might be protein bound. Several other pretreatment methods have been suggested, but the currently favoured method is entirely spectroscopic and relies on the fact (first pointed out by Wieder 1978) that most naturally occurring fluorophores have lifetimes less than *ca.* 30 ns. Use of a fluorescent label with a lifetime substantially greater than this, along with a time-resolved or phase-resolved method of distinguishing between short- and long-lived emissions, would discriminate against the background signal and in favour of the label fluorophore. A number of commercially available immunoassay systems exploit this approach by using lanthanide labels whose intensities are enhanced by energy transfer from organic chelating groups and whose lifetimes are *ca.* 1 ms, and pulsed source fluorimeters with pulse widths of *ca.* 10 μs. In my laboratory terbium-labelled transferrin has been used as a label: it is *ca.* 50000 times more fluorescent than unchelated terbium chloride (Wilmott *et al.* 1984). Unfortunately none of the lanthanide based assays so far developed is genuinely homogeneous, and in any heterogeneous assay many of the species causing background interference are in any case removed at the separation stage, so the use of lanthanide labels is still not fully developed.

Most of the homogeneous fluorescence immunoassays described above lack complete generality. Thus polarization methods do not work satisfactorily with macromolecular analytes, and energy transfer assays may also suffer problems when large molecules are under study. Some other fluorescence enhancement and quenching effects have been discovered which are apparently unique to individual analytes. A simple approach to homogeneous assays that is universally applicable would be very desirable. Some years ago, it was discovered in my laboratory (Handley *et al.* 1979) that when analytes are labelled with fluorescamine the fluorescent products invariably show enhanced fluorescence (usually about five-fold) when bound to specific antibodies. (This is why fluorescamine–fluorescein energy transfer cannot be monitored by donor quenching measurements, see above.) The effect is exemplified in figure 3. Unfortunately it seems that this approach also is unacceptable in practice, as further study shows that fluorescence enhancements of a similar order occur when the labelled molecules bind non-specifically to other molecules (Mahant 1983). This is an excellent example of the sensitivity of

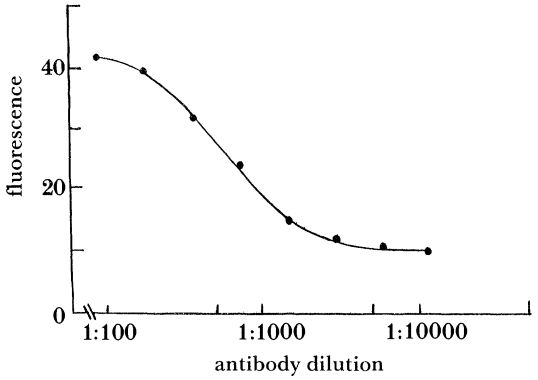


Figure 3. Titration curve showing the enhanced fluorescence of fluorescamine-thyroxine (FL-T4) conjugates on binding to anti-T4 antibodies.

fluorescence phenomena to environmental effects proving disadvantageous. In addition fluorescamine labelled molecules have fairly low molar absorptivities and quantum yields, and are not very stable in solution. None the less the search for a simple but general homogeneous fluorescence immunoassay is well worth pursuing.

4. Chemiluminescence energy-transfer immunoassays

The development of chemiluminescence (CL) immunoassays has been a notable feature in recent years (Kohen *et al.* 1985). The excitation of a molecule to an energetic electronic level via a chemical reaction rather than by external illumination has the obvious advantages of simplicity – a luminometer is fundamentally only a reaction vessel close to a photon detector – and the removal of scattered light interference. There is considerable controversy over which approach has the greater sensitivity. In CL each emitting molecule can clearly only be excited once, but in real samples this excitation often happens very efficiently: in fluorescence spectrometry each labelled molecule can in principle be excited many times, but in practice excitation efficiencies in dilute solution are low, and the use of exceptionally intense light sources often results in photodecomposition. In practice it seems that the best CL systems have thus far proved considerably more sensitive than the best fluorescence ones, though extreme precautions (e.g. rigorous removal of stray light) may be necessary to achieve optimum results.

An approach that has attracted particular interest in the author's laboratory is the use of the peroxyoxalate CL mechanism in conjunction with conventional fluorescent labels. It has been known for many years that oxalate esters with good leaving groups, such as bis(2,4,6-trichlorophenyl)-oxalate (TCPO), give only a very weak luminescence when oxidized with hydrogen peroxide. If, however, this oxidation is carried out in the presence of a fluorophore, the latter emits strongly (quantum yields can reach 30%) at its characteristic wavelengths, the excitation mechanism being energy transfer via the reactive intermediate dioxetanedione. As applied to immunoassays, this method would appear to combine the simplicity and sensitivity of CL methods with the convenience of using routinely available fluorophores. Experience shows that, using only the simplest instrumentation (e.g. a filter fluorimeter with the light source obscured) limits of detection for such fluorophores are often several orders of magnitude better (lower) than those obtained by

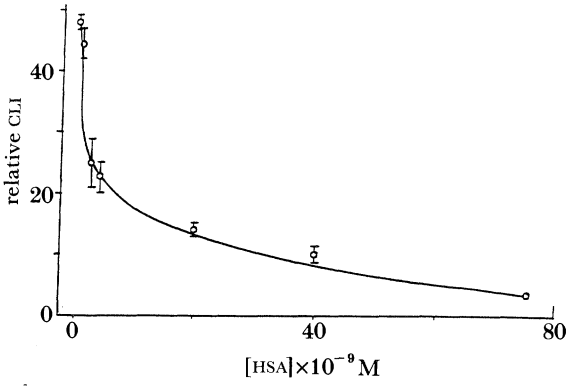


Figure 4. Enhanced chemiluminescence energy transfer immunoassay for human serum albumin (HSA) using TCPO and dansyl labelled albumin (for details see text).

conventional fluorimetry. When dimethylaminonaphthalene sulphonyl chloride ('dansyl' chloride) is used as the fluorescent label, there is an additional possibility of developing homogeneous immunoassays, as the dansyl labelled analytes show enhanced emission intensities after binding to antibody. This principle has been used in the homogeneous analysis of both small and large molecules (Miller *et al.* 1986) at the subnanomolar level (figure 4). The principal problems are the insolubility of some oxalate esters in aqueous media, and the tendency for the CL emission to be quenched in protein containing samples, but both problems can be minimized by judicious choice of buffer solution. This overall approach, already used in other analytical areas such as chromatographic detection, thus seems to hold considerable promise in immunoassays.

5. Future developments in optical immunoassays

Although the future of a range of fluorescence and chemiluminescence immunoassays seems assured, a number of the potential advantages of these methods remain to be fully exploited. Foremost, perhaps, among these is their capacity for multi-analyte assays. The extreme specificity of antibodies has made such assays commonplace in the realm of immunoprecipitation methods, but they have only rarely been attempted in the immunoassay of small molecules or trace amounts of macromolecules. Typical applications would include screens for drugs of abuse, the detection of cancer markers, simultaneous thyroid or steroid hormone assays, etc. Two distinct approaches are available, namely the use of two or more fluorescent labels, one per analyte, with the fluorophore emissions resolved during the analysis, and the alternative use of a single fluorophore but with antibodies of the necessary specificities separately dispensed in (for example) different wells of a microtitre plate. The first approach requires the availability of suitable fluorophores, along with optical and/or software methods of resolving their spectra: the second would require highly sophisticated reagent dispensing instruments (Ekins 1989).

A second important area awaiting exploitation is the use of the very near infrared (IR) region of the spectrum (*ca.* 650–900 nm) for fluorescence measurements. Relatively few compounds show intense electronic absorption transitions and bright fluorescence in this region, but this is a major advantage in the field of immunoassay. If one of these rare compounds could be used as a fluorescence label its determination

could take place against a very low background; the combined fluorescence and scattered light background signal from a diluted blood serum sample excited at 750 nm is only about $\frac{1}{100}$ of that obtained on excitation at 350 nm. Other benefits of working in this spectral region include the reduced risk of photodecomposition of the fluorophore; the availability of very small, robust, low cost, and efficient light sources (e.g. diode lasers) and detectors (e.g. diode arrays, charge coupled devices); and the suitability of low-cost fibre optics. Preliminary studies show that the peroxyoxalate energy transfer system provides exceptional sensitivity in this region, and very recent work indicates that fluorescence enhancement, quenching and wavelength shift effects can be expected with near-IR fluorophores just as with conventional UV–visible fluorophores. There is thus every expectation that this region will provide a range of simple, sensitive and possibly homogeneous fluorescence immunoassays.

Perhaps the most remarkable developments can be anticipated in the area of the analytical reagents themselves: antibodies and related molecules. The advances made possible by the advent of monoclonal antibodies may yet be overshadowed by the production, using the techniques of genetic manipulation, of antibodies with two different specificities in their two binding sites; of antibodies with intrinsic enzyme activities ('abzymes'); and new proteins of the protein A/G type, with better or more refined antibody binding properties, and combined enzyme activities (Atkinson & Sherwood, this symposium).

Some or all of these projected advances may be combined in the development of new immunosensors, designed as simple and robust measurement devices for field, clinic or bedside use. The combination of fibre optic systems with fluorescence based assays seems particularly attractive, as the measurement principles and the optical components are already available. In practice, difficulties arise from the high binding constants of antigen/hapten–antibody interactions, which makes the development of a reusable sensor unexpectedly difficult. It can hardly be doubted, however, that these difficulties will be overcome, and that numerous existing and novel immunoassay methods will further increase their dominance in the field of trace organic analysis.

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Discussion

W. J. Albery (*Molecular Sensors Unit, University of Oxford, U.K.*). I noticed in Professor Miller's experimental plots against concentration that a variety of different shapes were obtained, It also appeared that the lines on his graphs simply joined up the points. It is possible to develop a theoretical model for these systems? If not, why not?

J. N. Miller. Theoretical models have indeed been developed, but they frequently assume simplified experimental conditions which may not be realized in practice.

Thus it is generally assumed that all the antibodies used have uniform binding constants, which is certainly not valid with polyclonal antibodies. In these circumstances (as in many other areas of analytical science) pragmatic curve-fitting methods are frequently used.

D. T. Burns (*Queen's University of Belfast, U.K.*). In general, curved calibration curves tend to be less reproducible than rectilinear ones and suffer from problems of adverse propagation of errors. Can these problems be addressed in immunoassay calibration graphs?

J. N. Miller. To some extent such problems are addressed by transforming the data (e.g. by log or logit functions) to generate rectilinear graphs. Such transformations are common, but carry dangers of their own; for example, they alter the weights given to different points on the graph. It should be noted that many immunoassays are used in circumstances in which extreme precision is not needed.

R. W. Abbott (*Lilly Research Centre Ltd, Surrey, U.K.*). One of the difficulties associated with immunoassay can be the cross-reactivity of antibodies with analytes similar in structure to the species being determined, e.g. the opiates. Can Professor Miller comment on this, and, looking into the future, does he see an increase in interest in the linking of chromatographic techniques, e.g. HPLC, with immunoassay?

J. N. Miller. Antibody cross-reactivities can be both a problem (e.g. when only a single metabolite is under study, but often metabolites and/or the parent compound cross-react), and an advantage (e.g. when screening for a class of related compounds, rather than for a single compound).

Hybrid HPLC immunoassay methods (and perhaps, more favourably, CZE immunoassay methods) may help to overcome cross-reactivity problems, and they have been applied in (e.g.) forensic analysis. On the whole, however, I believe that the trend will be towards the reduced use of separation methods (in this and other contexts), and the increased use of exquisitely selective reagents, as described elsewhere in this symposium.

G. S. Wilson (*University of Kansas, U.S.A.*). The combination of immunoaffinity chromatography and HPLC is useful especially for analysis of families of compounds. An example would be preclinical studies of a drug and its metabolites. One could at great time and effort possibly make monoclonal antibodies that could not cross-react. On the other hand for most applications the HPLC separation following an immunoaffinity class separation would provide a broader and more reassuring overview of the sample. The immunoaffinity separation has the added advantage of considerable sample concentration and clean-up.

J. N. Miller. I agree entirely. The analytical use of immunoaffinity columns is an idea we looked at many years ago in connection with the determination of trace protein levels in bloodstains. It did not come to much at the time, but its applications are now rapidly expanding. Combinations of this approach and conventional HPLC offer a number of interesting possibilities, although to some extent the capabilities of the methods overlap.