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Review

Methods of screening combinatorial libraries using immobilized or restrained receptors

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Abstract

The screening of combinatorial libraries for compounds with high affinity toward drug receptors is currently a major center of attention. We describe methods recently developed for library screening that involve ''constrained'' receptors (either immobilized onto a surface or restrained to a compartment by some physical means). These include affinity selection chromatography, ultrafiltration assays, the scintillation proximity assay, a variety of interfacial optical techniques (surface plasmon resonance and its relatives, among others), the quartz crystal microbalance, the jet ring cell, and new interferometric assays using porous silicon to immobilize the receptor. We note some trends in assay development involving assays of membrane-bound complexes, and the coupling of two analytical methods to expand the assay resolution. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

All biological activity requires binding: enzymes bind substrates, transcription factors bind to DNA, neurotransmitters bind to membrane-embedded receptors, protein subunits associate to form cytoskeletal structures, and so on. The discovery of novel compounds that can interfere with, or augment, these interactions is of vital interest to the pharmaceutical sciences and, ultimately, to the drug discovery industry. Compounds which produce biological activity by binding receptors must have both affinity for the receptor and the ability to produce changes in the receptor to induce the given biological activity (efficacy). In many cases, inhibitors (antagonists) of the process simply bind the target receptor site without efficacy and are sought after as therapeutic modulators of the process.

Given an identified molecular target for drug therapy, a large number of potential drug candidates can be synthesized fairly quickly and easily, thanks to advances in combinatorial chemistry. Screening such libraries for binding to a receptor can provide an important front-line approach to the search for biological activity. However, the increase in the ability to sample larger and more diverse collections of compounds has required a concomitant increase in the ability to screen these (potentially quite large) libraries for active compounds. Manual analysis of single samples is simply too slow and inefficient to cope with the large number of chemical entities that constitute a modern library. Screening for lead compounds now calls for methods that can be automated and have high throughput.

Assuming that one has an adequate supply of receptor, and that library compounds are ready for assaying, some basic requirements for a good high-throughput screening assay are its suitability to automation, its reliability and reproducibility, its rapid turnover of samples, and its high sensitivity. Further desirable qualities include the use of inexpensive reagents and apparatus, a relative insensitivity to solvents and additives used in preparing compounds for screening, the accurate representation of binding behavior in vivo, the minimization of hazardous waste, and the avoidance of techniques that have multiple steps or that are difficult for robots to perform (e.g., centrifugation). The ideal would be an accurate and precise one-step equilibrium assay, done by a robot, with only a short incubation and with minimal use of reagents and of course generating no hazardous waste. The equipment for the assay would be readily interfaced with computers to track samples, to direct robots, to collect results, and to analyze the large volumes of data generated (e.g., to discern structure–activity relations). This ideal assay has so far eluded discovery. Today's assays are a compromise, with one's choice of assay depending on the relative importance of the various factors listed above.

Older, standard methods have been adapted to this effort. For example, the rapid filtration technique,

using glass fiber mats, has been updated for high throughput by the use of robots to handle samples arrayed in the now-familiar 96-well titer plate format. In the last ten years or so, however, a variety of new methods have appeared. Some of these assays, originally developed for basic research quantification of the binding of purified receptors and ligands, are being adopted for large-scale screening programs, and others show promise of being adaptable to such programs. These new assays include affinity-based methods in chromatography and electrophoresis, ultrafiltration methods, application of surface plasmon resonance (SPR) and related optical techniques, and the scintillation proximity assay (SPA). Also, one now sees the coupling of two analytical techniques into a single, more powerful assay; mass spectrometers are now being joined to electrophoretic and SPR assays, for example, which provides an extra analytical dimension for characterization of potential lead compounds.

This is not a comprehensive review of all the methods available for library screening; that is too large a subject to encompass in the space of this review. Our purpose here will be to cover a limited set of methods that are being, or have the potential for being, employed in screening libraries of organic compounds for potential drug candidates. These methods involve the use of ''constrained'' receptors. By ''constrained'' we mean that the receptor species (DNA, RNA, protein, or in some cases, a polysaccharide) either is immobilized by covalent or noncovalent linkage to a solid support of some type, or that the receptor is free in solution but restrained to a compartment by a dialysis or ultrafiltration membrane, a gel layer, etc. No ''biological'' methods are covered (e.g., phage display, cell sorting, immunoassays, etc.); we feel that a discussion of these methods is better left to molecular or cell biologists or immunologists who are expert in those areas. Also, we have elected not to cover such topics as electrophoresis (especially affinity electrophoresis) where the receptor is free in solution and (relatively) unconstrained.

This paper is structured as a series of short reviews on particular screening technologies. We cover new developments in ultrafiltration; optical methods involving evanescent wave physics (e.g., SPR), and some related developments; the scintillation proximity assay; and affinity chromatography applications. After a brief introduction to a particular method (often with references to recent in-depth review articles), we give a short discussion of how that method works. We then turn to recent applications (those within the last five years), where we have deliberately chosen reports of methods that have been, or could readily be, used in high-throughput screening operations. A section of our paper concerns some of the major problems posed by receptor immobilization. We then finish with some predictions of where further developments can be expected.

2. Affinity selection chromatography

A basic screen for a combinatorial library is to pass the library mixture over a surface on which one or another receptor of interest has been immobilized. Viewed simply, the ligands that are retarded in their passage must be engaging in binding interactions with the immobilized receptor molecules, and so these late-eluting compounds are good candidates for development as drugs. The general process may be considered as an extension of affinity chromatography [1], and such applications are underpinned by the vast literature available on affinity chromatography. An excellent review of this general field may be found in the book edited by Ngo entitled ''Molecular Interactions in Bioseparations'' [2] and the article by Jones et al. [3].

The elution profile of ligands from a column of immobilized target molecules may directly provide structure–activity relationships for researchers, particularly in a search for receptor antagonists. That is, the longer the retention time, the higher the affinity for the target molecule, and thus the greater the antagonist potential. This assumes that one is dealing with binding at the receptor site and not nonspecific or other sites on the target molecule under study. The latter can be tested by ligand competition experiments evaluating the elution profile in the presence of ligands known to bind to the desired receptor site on the macromolecule.

A natural evolution of the affinity selection chromatography approach has been to increase the ana-

lytical space by the application of additional chromatography and detection methods. McGuinness et al. [4] have used an automated serial chromatographic technique for screening library compounds for a target molecule. A ''target'' column containing the immobilized target molecule was set in tandem with a reversed-phase column. Peptides from libraries which were retained by the immobilized target column (affinity selection) were transferred automatically to the reversed-phase column for further separation and sequencing. Using a monoclonal antibody against β-endorphin as the target, they were able to show that a single peptide (YGGFL) was selected from approximately 5800 peptides present in the combinatorial library which was being tested.

Another extension of affinity selection is the integration of the affinity selection chromatography with a sensitive detector providing direct structural information on the retained ligands. Kelly et al. have done this with off-line electrospray ionization mass spectrometric detection to screen peptide libraries to the SH2 domain of phosphatidylinositol 3-kinase [5]. Henion et al. described an on-line LC–LC–MS method utilizing immunoaffinity extraction, where a monoclonal antibody to a ligand known to bind to a given receptor (here benzodiazepine to its receptor) is used as the affinity target. The first LC stage separates the antibody/ligand complex from the free ligands, and the second LC stage is a reversed-phase chromatography of the bound ligands with the ligand structure being elucidated by tandem mass spectrometry [6].

In another approach, Evans and Herman used affinity selection chromatography with competitive release by a known ligand to identify library peptides specific for the sugar-binding site of concanavalin A [7]. A column of immobilized concanavalin A was used to capture peptides from a library reported to contain in excess of two million unique peptides. Due to the complexity of the library, it was not possible to select directly for individual peptides. However, identification of peptides which specifically bound to the target was possible using subtractive pool sequencing [8,9]. The latter technique involved comparing elution profiles in the presence and absence of methyl α-D-mannopyranoside, a known ligand for the binding site under consideration, and sequencing the peptides which were retained. This identified a consensus sequence which was used as the basis for a second round of synthesis and screening (ca. 6859 individual peptides). Using this iterative technique, two peptide sequences (HHRSY and HVVSV) were identified which had relatively high affinity for the sugar-binding site on the concanavalin A molecule.

The affinity selection chromatography approach suffers from the same difficulties found with all techniques using immobilized proteins, as summarized in Section 7 below. However, when compared to other trapping methods discussed in this review, the affinity selection chromatography method has the same advantage that chromatography has over batchwise separations, i.e., an increase in the number of theoretical plates. It thus possesses considerable power for resolving hits from a combinatorial library.

3. Filtration methods

3.1. Ultrafiltration

A technique very much like equilibrium dialysis, ultrafiltration uses a semipermeable membrane to separate free ligand from a solution containing receptor, ligand and receptor–ligand complexes. A sample solution that contains both receptor and ligand is equilibrated, then a portion of the solution is driven through the membrane. This filtrate contains only free ligand, the receptor and receptor–ligand complexes having been retained by the filter. One then measures this free ligand concentration and the total ligand concentration (that on the side of the membrane with the receptor); then from knowledge of the total receptor concentration one can calculate the binding density. By repeating the experiment with different total amounts of ligand one can eventually construct a binding isotherm. The technique requires much less time per experiment than the classical equilibrium dialysis method, though one must still be concerned about adsorption of solutes onto the dialysis membrane. Recently the technique has been extended to the study of protein–protein interactions, taking advantage of new membranes with exceptionally high molecular-mass cut-off values (ca. 100 000), to separate low-molecular-mass proteins (with molecular masses below 30 000) from

large proteins (with molecular masses in excess of 100 000) [10,11]. This suggests the possible use of these ultrafiltration membranes in screening for small molecules that may mediate such protein–protein interactions; as yet, however, this possibility remains to be explored.

Henion et al. have recently presented ''immuno-affinity ultrafiltration'', an application of the combined ultrafiltration/centrifugation method to the screening of combinatorial libraries [12]. They used anti-benzodiazepine antibodies to retain high-affinity compounds from a mixture of benzodiazepines, then eluted the tight-binding compounds and separated them by high-performance liquid chromatography (HPLC) for analysis by electrospray ionization mass spectrometry to confirm the identity of the eluted compounds. Competition among the drugs for specific binding to the appropriate antibodies was demonstrated, and the technique could select library components with the greatest affinity for a particular antibody.

A variation on ultrafiltration, dubbed ''pulsed ultrafiltration'' (abbreviated PUF), has been developed by Venton and co-workers for studying ligand–macromolecule binding [13,14]. The PUF apparatus uses standard HPLC components (e.g., pump, connecting tubing, detector), except that a specially-designed ultrafiltration cell replaces the HPLC column, and serves to retain a macromolecular receptor species. A small bolus or ''pulse'' of concentrated ligand solution is passed through the ultrafiltration cell, and the effluent monitored for elution of the ligand by, e.g., UV absorption. If the ligand interacts with the macromolecule, then its elution profile is altered by comparison to the case of ligand injection and elution when no macromolecule is present in the cell. The degree of retardation of the elution can be described mathematically, and it can be related directly to the equilibrium binding constant of the ligand for the macromolecule. The technique is capable of generating nearly an entire binding isotherm with only two experimental runs through the ultrafiltration cell: one without macromolecule, and one with the macromolecule present, of course. The technique has been applied to a variety of different binding systems, including serum albumin with several different ligands, and the enzyme RNase with various mononucleotide inhibitors.

A recent pair of papers from the laboratories of van Breemen, Woodbury, and Venton have described the application of pulsed ultrafiltration to the screening of combinatorial libraries [15,16]. In both studies the screening scheme was the same. A bolus of the library mixture in aqueous buffer was passed through the PUF cell, and the cell thoroughly washed with buffer. Then tight-binding compounds were released by injection of organic solvent (methanol or acetonitrile) in water, and the eluate analyzed by electrospray mass spectrometry (MS).

An affinity gel chromatography technique was reported by Shimura and Kasai in 1985 that has several similarities to the pulsed ultrafiltration technique [17]. These authors used agarose gel beads with covalently-attached trypsin (the receptor here) in a stirred bed, and *p*-aminobenzamidine as the ligand. The ligand was injected as a bolus, and it was eluted with buffer at a constant flow-rate. Two ''runs'' were used, one with active trypsin, and one with trypsin treated with inhibitor, so that it presumably could not bind the *p*-aminobenzamidine. A comparison of areas under the elution curves allowed construction of a binding isotherm, in a process reminiscent of the analysis of pulsed ultrafiltration elution curves.

Of related interest is the report by Kaur et al. on a technique for screening combinatorial libraries through a combination of size-exclusion chromatography, reverse-phase chromatography, and MS [18]. Briefly, receptor is incubated with a solution of the library, and an aliquot passed through a short gel permeation chromatography column; this separates unbound small ligands from the larger receptor species that presumably carries with it any tightly-bound ligands. The ligand–receptor complexes are then dissociated by passage over a reverse-phase column. The eluent from the reverse-phase column is then introduced into an electrospray mass spectrometer, to identify ligands that bound specifically to the receptor.

3.2. General advantages and limitations of the filtration methods

The Henion technique is similar in concept to the PUF screening method in that (1) an ultrafiltration membrane is used to retain a receptor and so to elute

low affinity compounds away from the high affinity compounds held by the receptor, and (2) these high affinity compounds are then eluted with a change in solvent that alters the receptor's conformation. It appears to be generalizable to other protein–ligand systems. It has two drawbacks, compared to the PUF screening method: (1) there is no recovery of the receptor (it is denatured on the membrane surface); and (2) there is an extra step of HPLC to separate the high-affinity compounds before their identification (this latter point, however, may also be regarded as an advantage). The PUF screening method is simpler in that the solvent was chosen so as to permit direct elution to the mass spectrometer, without an intervening HPLC step. (The use of a destabilizing solvent to release ligand after an affinity selection in a screening operation was reported earlier by Zuckermann et al. in 1992 [19]).

The PUF method has the advantages of keeping the receptor in solution, of using readily-automatable technology (e.g., LC and MS), and of using only small amounts of material. Furthermore, the screening technique is relatively rapid; also, the design of the PUF cell offers the possibility of recovering the macromolecular receptor species for use in other experiments. The method of Shimura and Kasai was applied to the binding and retention of a single ligand, but presumably it could be adapted to screening a mixture in much the same way as pulsed ultrafiltration was adapted to the screening of ligand libraries. The main drawbacks, compared to pulsed ultrafiltration, are those associated with the immobilization of the receptor on the gel beads.

4. Scintillation proximity assay (SPA)

SPA arose as an extension of radioimmunoassays, in an effort to develop a homogeneous assay for greater convenience and accuracy [20]. The fundamentals of the technique are described in the articles by Udenfriend et al. [21], by Nelson [22], and in the review by Cook [23]. The technique's diversity is greatly augmented by a number of ingenious sandwich-type assays. The recent review by Cook [23] summarizes an assortment of applications.

4.1. Basics of the technique

The proximity principle of the assay depends on the short penetration range in solution of beta particles or Auger electrons from certain isotopes. If the emitted electrons pass close by a molecule of scintillant, energy can be transferred from the electron to the scintillant, which the scintillant converts to light (photons). The emission of photons can then be quantified by a scintillation counter. If the beta particle is emitted too far from the scintillant then its energy is lost to the surrounding solution and no scintillation occurs. Thus, only labeled particles that are in close proximity to scintillant will be detected.

The assay works well with ^{3}H, ^{125}I, ^{33}P, and ^{35}S; these isotopes emit low-energy beta particles or electrons from internal conversion, and one can label compounds with them to relatively high specific activities. Because the specific activity achievable with ^{14}C is generally too low, this isotope is not commonly used in SPA, even though the emitted beta particle's range is comparable to that for ^{35}S. Also, energetic beta emitters like ^{32}P are not suited for use with SPA, since the pathlength is then too long, which would defeat the principle of close proximity of emitter and scintillant.

In a standard version of the assay the scintillant is embedded inside a microsphere or ''bead''; the hydrophobic polymer polyvinyltoluene is often used here. The bead is coated with a polyhydroxy film, to permit covalent crosslinking of antibodies or other coupling molecules. (Early applications depended on the hydrophobicity of the naked bead surface to bind protein, but the polyhydroxy film coating avoids difficulties with nonspecific binding by hydrophobic ligands, and permits more control over attachment of the coupling species, etc.) Once a suitable receptor is coupled to the beads, radiolabeled ligand may be added and a binding equilibrium established between free and bound ligand. Bound ligand is of course held by the receptor at the surface of the bead, where a substantial fraction of the emitted beta particles or Auger electrons are readily captured by the bead (some are of course directed away from the bead, out into solution, and are thus lost to detection). The free ligand molecules are, on the average, too far from the bead surface for much of their emitted electrons to be captured by the bead, and so essentially the

assay ''sees'' only the bound ligand. The method thus provides a true measure of the binding equilibrium in a homogeneous format. The beads are easily handled as an aqueous dispersion by automated liquid dispensers, and the whole assay can be performed using the common microtiter plate format.

The SPA is readily adapted to enzyme assays. Cook [23] has given a general categorization of enzyme assays by SPA: (1) signal removal; (2) signal addition; and (3) product capture. The first category is suitable for hydrolase activities (e.g., proteases, nucleases, phospholipases, esterases) where the action of the enzyme removes the radiolabel from the substrate; here one follows a loss of signal. The second category is useful for polymerases, kinases, transferases, etc., where the label from a substrate is incorporated into an acceptor that is attached to the bead; here one follows an increase in signal. The third category exploits specificity in binding to capture the reaction product by an antibody, DNA oligonucleotide, etc. that is bound to the bead.

Recently a new format for the assay has been developed that does not use beads. Instead, the coupling molecules are attached directly to the surface of the well of a microtiter plate, whose walls contain embedded scintillant. This simplifies the assay further by eliminating the bead-dispensing step, and the new format has proven to be quite popular.

4.2. Methods of receptor attachment

Given the descent of SPA from radioimmunoassays, it is no surprise that many of the techniques of radioimmunoassays may be carried over to the SPA format. In particular, there is a recurrent theme of sandwich assays, in which a surface is derivatized to bind some intermediary species (e.g., an antibody), to which a receptor is attached, which binds an analyte. In the early development of the assay, antibodies were attached to the surface of the beads by chemical crosslinking [20]. This was quickly followed by studies utilizing direct adsorption of proteins (e.g., antibodies) or membranes with embedded receptors onto the surface of the bead [22]. This method was limited by the poor stability of the bound proteins; there was also a tendency of hydrophobic ligands to bind directly to plastic beads [24].

Methods for attachment of the receptor to the bead have become more robust and sophisticated. Hoffman and Cameron [25] have used a polylysine coating to capture cellular membranes containing the selected receptor; the membranes carry a net negative charge and are attracted to the polycationic bead coating. This coating has the drawback, however, of potentially binding anionic ligands as well, thus limiting its application to neutral or cationic ligands.

Berry et al. [26] introduced the use of beads coated with wheat germ agglutinin (WGA) to bind membrane receptor preparations. The WGA coating will bind to glycoproteins present on the membrane's surface, and so help to hold the receptor in proximity to the bead or plate surface, while maintaining the membrane-embedded receptors in a state close to that found in vivo. The multiple contacts formed between membrane and bead or plate also serve to increase the stability of the attachment.

Commercial vendors have developed sandwich-type attachments for use with receptors in membranes. Suppose that an assay for epidermal growth factor (EGF) is desired. One can obtain commercially SPA beads coated with a polyclonal anti-mouse antibody preparation, which is relatively inexpensive. Then one prepares a monoclonal Ab (MAb) in the mouse that is specific to the (membrane-bound) receptor for the compound of interest, which here are EGF and the EGF receptor. This is expensive, but relatively small amounts of the MAb will be used per assay. To perform the assay, one then mixes the coated beads with the monoclonal antibody, and adds the receptor (membrane) preparation along with labeled ligand (here EGF). This results in a multilayer sandwich of bead/anti-mouse Ab/MAb/EGF receptor (in membrane)/EGF. This approach can also be applied to the binding of one specific receptor present in the membrane, to immobilize the membrane while leaving the other receptor types available for study.

For investigators who prefer to develop their own SPA variants, beads are now available with a polyhydroxy coating that can be used to attach soluble receptors like proteins, nucleic acids and carbohydrates. The linkages are covalent, involving amines, aldehydes, or carboxyl groups, and the chemistry is

well worked out. Additionally, the polyhydroxy coating reduces the hydrophobicity of the polymer surface, allowing a greater range of ligands to be assayed. (Similar means are used to prepare surface-bound receptors to dextran-coated sensors in instruments using optical evanescent wave physics.)

Another common means of immobilizing a soluble receptor is to use a surface coating of streptavidin in conjunction with a biotinylated receptor. Streptavidin-coated beads and 96-well titer plates are commercially available. This assay format requires attachment of biotin to the receptor species, an extra chemical step which may not always be desirable. However, the noncovalent binding of biotin to streptavidin is very tight and stable under the usual assay conditions.

4.3. Applications

An example of careful development of a sandwich-type assay for high-throughput screening is the recent study by Banks et al. [27] of interleukin 5 (IL-5) binding to its receptor. Since the intention was to develop a high-throughput assay for detection of antagonists of IL-5 binding to its receptor, many details of the assay were explored in order to optimize the assay. These included the effects of microsphere concentration, the amount of IgG in solution (needed for the sandwich-type format), and a comparison to a different assay using a biotinylated ligand and immobilized receptor in a 96-well titer plate format with chemiluminescent detection.

As mentioned above, SPA may be combined with enzymatic assays, and can provide a means of screening libraries for inhibitors. Cook has compiled a list of at least 22 different enzyme assays developed with SPA, current as of 1996 [23]. To this we can add recent assays for nitric oxide synthase [28], RNA helicase [29], calcineurin phosphatase [30], p(34cdc2)/cyclin B kinase [31], DNA topoisomerase I [32], and GalNAc-transferase [33].

The SPA is of course well suited for the study of protein–protein interactions and their inhibition. Some ingenious variants on the sandwich-type assay have been developed to follow such interactions [34,35]. Recombinant DNA technology now permits investigators to create new and useful combinations of proteins in the laboratory, by joining together segments of proteins from disparate sources. These fusion proteins can contain domains for enzymatic or binding activity of interest to the investigator, covalently linked to domains useful in creating sandwich-type assays. An example is the report by Jenh et al. on their development of a screen for inhibitors of the CD28 costimulatory receptor, important in the activation of T-cells [36]. In another recent report, Sonatore et al. describe the use of FK506-binding protein fused to SH2 domains, with binding to a biotinylated phosphopeptide ligand [37]. In seeking inhibitors of protein–RNA interactions, Mei et al. adapted SPA to follow the binding of the HIV-1 regulatory protein, Tat, to the TAR RNA of the HIV-1 viral RNA [38]. As for assaying protein–DNA interactions, Carlsson and Häggblad have reported an assay for site-specific DNA binding by the human estrogen receptor [39]. This report suggests that, as with protein–protein interactions, it should be possible to use SPA to screen for small molecule modulators of these macromolecular interactions.

4.4. General advantages and limitations of SPA

The principal advantages of SPA are that it is a homogeneous assay (separation of bound ligand from free is not needed), and that it can be adapted to so many different receptor–ligand systems. The main mechanical operations involve simple pipetting of solutions, and transfer of the sample well plates from one workstation to another. There is no need for liquid scintillant and organic solvents, since the scintillant is embedded either in beads or in the plastic of the well plates. This reduces the number of steps in the assay and improves assay throughput and accuracy, while also reducing waste and the handling of hazardous materials. The assay is easily automated and so lends itself well to the high-throughput screening of libraries.

The derivation of SPA from immunoassays gives it a rich background of sandwich-type assays to draw on, to developing screens for a wide variety of receptor–ligand systems, including soluble proteins, enzymes and membrane-bound receptors. Ingenious methods of attachment keep the membrane-bound receptors in an environment much like that in vivo,

and genetic engineering techniques can now be used to introduce tethering moieties into the receptor.

This last points to the main drawback of SPA, that it requires surface immobilization of one or another of the binding partners. A lesser drawback is that it requires radiolabelling of the ''free'' partner, and the introduction of the label can alter the binding behavior of that species. There is of course also the matter of handling and disposing of radioactive waste.

5. Interfacial optical assays

Optical wave physics at interfaces has recently received intense attention by those interested in characterizing interactions between biomolecules. A multiplicity of optical signal transducers has been devised, variously based on reflectance, ellipsometry, SPR and optical waveguides. Biosensors that exploit one facet or another of interfacial optics have demonstrated a wide range of capabilities, including both equilibrium and kinetic characterization of protein–protein interactions, protein–carbohydrate interactions, DNA–DNA interactions, DNA–protein interactions, receptor–ligand interactions and many others. Recent reviews, pertinent to biomolecular interaction studies, include those by Morgan et al. [40], Schuck [41], and Garland [42]. There is also an older but still relevant review by Axelrod et al. [43] on total internal reflection fluorescence, a related technique using optical waveguides, which has been widely applied to ligand–macromolecule interaction studies.

Instruments for macromolecular binding studies by interfacial optics are available now from commercial vendors. Though the instruments differ in details of design, there is generally the same order of operations for each instrument. To perform a binding assay one first immobilizes one of the binding partners at the sensor's surface. Then one passes analyte solution over the sensor surface, perhaps with stirring, and monitors binding through a change in an optical signal (see below). When finished, one can either replace the sensor surface or regenerate the sensor by means such as those used for affinity chromatography.

5.1. Background

A detailed discussion of the physics of the various devices is beyond the scope of this review, and the interested reader is referred to the above review articles for references on the technical aspects of the physics of the devices. We give here only a brief summary.

5.1.1. Total internal reflectance fluorescence (TIRF)

Consider a beam of light passing through a dielectric medium of high refractive index n_1 (e.g., glass or quartz). When this beam meets a planar interface with a second dielectric medium with a lower refractive index n_2 (e.g., water or aqueous solution), then the beam may be totally internally reflected if its angle of incidence θ is greater than a critical angle θ_c, where $\theta_c = \arcsin(n_2/n_1)$. Although the beam is totally reflected, it establishes an electromagnetic field, or evanescent wave, that extends a short distance (on the order of a wavelength) into the second medium. The intensity of the evanescent electric field depends on both the angle of incidence and the polarization of the incident beam. The evanescent wave can excite fluorescence in molecules in the second medium, provided those molecules are sufficiently close to the interface; molecules that lie more than a wavelength or so from the interface will not be excited. This is the basis for total internal reflectance fluorescence measurements of binding. Suitable receptors are attached to the solution side of the optical interface, and the binding of (fluorescent) molecules from bulk solution is then followed by the evanescent wave-stimulated fluorescence as these molecules enter the thin volume just at the interface. Unbound molecules do not contribute appreciably to the signal.

TIRF has not yet been implemented commercially, but there are a variety of ''laboratory-made'' instruments and devices that exploit this and other aspects of the physics (see Table 1). See the reviews by Morgan et al. [40], by Axelrod et al. [43], and by Garland [42] for further references.

5.1.2. Surface plasmon resonance (SPR)

Again we have an optical beam impinging on an interface between media, but a thin metal layer (e.g.,

Table 1
Interfacial optical assays and related techniques

	Technique or device						
	Jet ring cell	TIRF	SPR	RM	GCS	Porous silicon interferometer	Ref.
Optical effect							
Absorbance	+						[73]
Fluorescence		+					[43]
Interference						+	[65,66]
Evanescent wave		+	+	+	+		[40–43,47]
Resonance			+	+	+		[40–43,47]
Waveguide		+		+	+		[40,42–43]
Prism coupling				+			[42]
Optical grating coupling					+		[40,49]
Other notable points							
Commercial instrument			+	+	+		
Application to soluble receptors	+	+	+	+	+	+	
Application to membrane-bound receptors		+	+	+	+		

gold) is introduced between the two dielectrics. Again the angle of incidence is chosen so as to result in total internal reflectance of the light. This reflection of the light beam, and the creation of the attendant evanescent wave, now excites waves of oscillating surface charge density (propagating electron modes) in the metal, so-called surface plasmons. At a particular angle of incidence a large fraction of the energy of the incident beam is transferred to the surface plasmon, and the intensity of the reflected beam falls; resonance is achieved. The angle at which this resonant condition is obtained is very sensitive to changes in the refractive index at the far side of the thin metal layer, that is, to changes in n_2 just at the metal–dielectric interface. Binding of ligands to receptors at this interface may cause a sufficient change in n_2 so as to permit their detection by the shift in the resonant angle of the optical beam. Since it is the change in n_2 in a thin layer next to the interface that is sensed, the technique essentially registers only bound ligands; those in bulk solution are not detected.

5.1.3. Resonant mirror (RM) and optical grating coupler devices

The resonant mirror technology uses much the same instrumental design as the SPR technology but does not depend on a surface plasmon effect. A beam of light is introduced via a prism to an optical layer with low refractive index that is coated with a thin high-index layer; the high index layer in turn carries a surface for attachment of receptor. At a certain critical angle (the resonant angle), the light passes through the low index layer and is reflected multiple times in the high index layer, and then emerges back through the prism and passes to the detector. The reflected light also undergoes a phase shift. The multiple reflections generate evanescent waves that extend into the solution. The resonant angle is sensitive not only to the refractive indices of the respective optical layers but also to the refractive index experienced by the evanescent wave at the interface with the solution. The polarization of the beam is another variable; the critical angle is appreciably different for the TE vs. TM modes, and in fact one can sensitively detect the resonance angle by using polarized light.

The optical grating coupler sensor (GCS) uses a waveguide, coupled with an optical grating, to cause multiple internal reflections of a light beam. The waveguide has a surface to which receptor is attached and which is exposed to solution. Again, there is a critical angle for the total internal reflection process, and this angle is sensitive to the mass

adsorbed at the solution interface. One can also follow changes in the phase shifts of the TE and TM modes of the beam.

SPR and changes in intensity of the reflected light are exploited directly in the BIAcore instrument from Pharmacia and in the IBIS biosensor from Windsor Scientific. The IAsys instrument from Fisons uses the RM technology and follows changes in the polarization of the reflected beam (actually, differences in the relative phases of the TE and TM modes of the reflected beam). An optical grating coupler system is used by the BIOS-1 instrument from Artificial Sensing Instruments ASI AG to sense phase shift differences.

5.2. Equilibrium and kinetic studies

To perform a binding assay with a suitably-immobilized receptor, one flows a solution of the ligand past the sensor's surface and follows the gain in mass at the sensor's surface by the change in optical signal [44–47]. The flow-injection design of some instruments allows interaction analysis to be done in 5–10 min, so this is a relatively rapid assay compared with other techniques that call for long incubation times. Small volumes are used, in the microliter range for some instrumental designs, with liquid handling by microfluidics. One can amplify the signal by incubating the ligand with an antibody specific to it, then capturing the ligand on the sensor surface through ligand–receptor interactions with portions of the ligand left accessible by the bound antibody. This sandwich-type design is also useful for amplifying signal when the concentration of ligand is very low (e.g., in the n*M* to p*M* range).

Binding assays can also be set up in a competition format, where the surface carries immobilized ligand. The sample to be analyzed is mixed with a suitable antibody and passed over the sensor surface; binding of the antibody to the surface is inhibited by the complexation with the non-immobilized ligand. This can also be amplified by use of a second antibody which recognizes and binds to determinants on the first antibody. This format is useful for detection of small ligands (below a few thousand in molecular mass) which in binding by themselves would not generate a large enough signal.

In addition to equilibrium binding, the rates of ligand–receptor association and dissociation can be followed with these instruments. The time resolution is limited by the mass transport of molecules past the sensor surface. One must also consider diffusion within derivatized surface layers on the sensor surface, such as are commonly used to immobilize various receptors. (Similarly, two-phase diffusion effects should be also considered when using polymer-derivatized SPA beads.) With due consideration for mass transport and diffusion effects, enzyme assays may also be devised and implemented. The extraction of kinetic parameters is dependent on the model chosen, and the influence of mass transport and diffusion must be considered carefully here. The review by Schuck [41] presents a useful summary of these effects, with a comparison of theory and experiment.

5.3. Surface immobilization

A great advantage of the interfacial optical biosensors is that no labeling of either binding partner is required. However, while one of the partners in the binding equilibrium is free in solution, and is introduced by flowing a solution of it past the interface, the other partner must be immobilized at the sensor's surface. As with other binding techniques that depend on surface attachment of one of the components, the immobilization process can yield a nonuniform population of binding partners, with consequences for sensitivity and interpretation of the assay.

Both direct and indirect attachment to the surface of the biosensor are possible. We concentrate here on the commercial instruments; other methods are used with, e.g., optical waveguides with silica surfaces. The metal surface of the SPR biosensor can be coated directly with protein, the protein being held in place by nonspecific adsorption to the hydrophilic surface or through covalent cross-link to the metal atoms. These surfaces, however, tend to be unstable and to lose protein during assays or during regeneration of the surface after the assay. Also, the uncontrolled deposition often results in much inactivation of the deposited protein, reducing the sensitivity of the sensor as well its selectivity. Instead, controlled covalent linkage to a suitably derivatized

surface is to be preferred (see Section 7 for further discussion).

Available with the commercial SPR and RM devices are such derivatized sensor surfaces, with a popular choice being a permeable carboxylmethyl dextran (CMD) layer [48]. This layer is approximately 100 nm thick, the range of the evanescent wave. The layer can be used for adsorption or covalent attachment of the binding partner. The attachment chemistry for dextran is well understood, and one can link the receptor via amino, thiol, or aldehyde groups. Multiple layers of complexes can thus be created within the range of detection at the sensor's surface. This helps avoid crowding (and consequent steric hindrance to binding) among complexes. The surface is also relatively stable so that it can be regenerated by simple acid or alkali washes, for multiple assays. The dextran layer fibers will exclude very large ligands (e.g., very large protein assemblies, lipid bilayer vesicles, membrane fragments, etc.) which may be a consideration when dealing with crude tissue extracts, serum samples, etc. Indirect capture of the receptor can also be effected by first attaching to the dextran a protein that will specifically recognize and tightly hold the receptor of interest. Examples include the use of staphylococcal protein A for capturing immunoglobulins (for subsequent use in a sandwich-type assay with the RM instrument) [49], and streptavidin for capturing biotinylated molecules of various types [50].

The RM, GCS and TIRF devices do not have a metal film but instead have a silica or SiO_2/TiO_2 surface to which the receptor is attached. The chemistry of attachment to silica of proteins and nucleic acids to silica is understood (see Section 7 below on immobilization), and lipid monolayers and bilayers can also be constructed [51,52]. The biosensor surfaces can also be derivatized to hold a carboxymethyl dextran layer, as with the commercial chip offered for the SPR instrument.

5.4. Applications

Most of the literature reports on the use of SPR, RM or GCS instruments deal with the detection of binding of large ligands, since that is what these instruments are best suited for. Only recently have reports of binding of relatively small organic compounds appeared.

An important paper by Nilsson et al. from 1995 described the use of SPR in several different enzymatic assays involving DNA, e.g., capture by ligase action of sticky-ended oligonucleotides, polymerase action by T7 polymerase and by the Klenow fragment of DNA polymerase I, and the cleavage of a double-stranded oligonucleotide with an internal site for the restriction endonuclease *Xho*I [50].

Cheskis and Freedman applied SPR to small molecule modulation of DNA–protein and protein–protein interactions, using the vitamin D_3 receptor and the retinoid X receptor and their cognate ligands [53]. Although not a screening study, this work clearly establishes that SPR may be used for screening for small-molecule modulators of macromolecular associations.

In one of the few literature reports on a screening application of SPR, Wiekowski et al. cloned and purified the a-chain of both the human interleukin 4 (IL-4) and interleukin 5 (IL-5) receptors [54]. They used amine coupling to immobilize the IL-5 receptor chain onto a carboxylmethyl dextran layer on the sensor surface, and then followed the binding of human IL-5. They also employed the reciprocal format, with immobilized IL-5 and soluble receptor. They additionally studied immobilized IL-4 with the soluble a-chain of the IL-4 receptor. Through SPA screening for inhibition of binding of interleukins to receptors, two small molecule inhibitors were found. These were tested in the SPR assay with immobilized receptor. Treatment of the immobilized IL-5 receptor with these compounds blocked the binding of IL-5, apparently through irreversible binding of the inhibitor to the receptor [54].

Membrane-bound proteins present more of a technical challenge for developing a screening assay, since the receptor must be maintained in an environment like that of its native lipid bilayer biomembrane, for it to maintain activity. One cannot simply extract the embedded receptor onto dextran without seriously perturbing or even completely destroying its tertiary folded structure. Better strategies would be either to build bilayers on the sensor's surface and transfer receptors (from, e.g., membrane preparations) into these supported bilayers, or to capture bilayer vesicles (with embedded receptors) onto the

surface of the sensor through tethers of one kind or another.

In a very innovative demonstration from 1993, Schuster et al. showed how to accomplish the latter [55]. They used a ''tethering'' strategy to assemble a signal transduction complex (that for chemotaxis in *E. coli*) onto the surface of an SPR device. They immobilized the CheY protein on the carboxylmethyl dextran layer through a genetically engineered cysteine residue, chosen so as not to interfere with CheY interactions with the CheA protein. They then prepared the histidine kinase CheA protein, and vesicles with membrane-bound Tar protein and the associated CheW protein. They observed specific interactions with the CheA protein but not the vesicle preparation by itself. However, preincubation of the Tar–CheW vesicle preparation with CheA produced a complex that interacted specifically with the bound CheY. Furthermore, the complex dissociated under conditions that would cause phosphorylation of the CheY protein, as expected. This study shows the potential for immobilizing membrane-bound complexes at an optical surface, such that the embedded proteins remain in a lipid bilayer.

In another application of the tether approach, Masson et al. captured vesicles containing a biotinylated phospholipid on a derivatized surface composed of rabbit anti-biotin immunoglobulin, immobilized on CMD [56]. MacKenzie et al. used artificial liposomes that carried a small amount of lipopolysaccharide and one or another of a set of glycolipids that act as receptors for the toxins [57]. They employed an anti-lipopolysaccharide immunoglobulin, immobilized on a CMD layer in an SPR sensor, to capture the liposomes. They then used the captured liposomes, with the various embedded glycolipids, to study the oligosaccharide binding specificity of several bacterial toxins (cholera toxin, *E. coli* heat-labile enterotoxin, or tetanus toxin). Interestingly, this approach demonstrated more restricted binding specificities for the toxins than had been determined previously in another SPR study [58], where the lipid bilayer was formed directly on the sensor's surface.

Since the embedded receptors remain in a lipid bilayer they enjoy conditions approaching those found in vivo, and their binding activity should be correspondingly more natural, as opposed to the unnatural circumstances of direct attachment to a hydrophobic sensor surface. The extension to screening libraries for interactions with these tethered vesicle systems seems obvious. The main limitation here would be in setting up the assay such that a suitably massive complex is assembled (or disassembled) for adequate sensitivity in the detector. Thus far, we have not uncovered any reports from the literature of such applications to library screening.

Karlsson and Ståhlberg report that with a newer model of the BIAcore instrument (the BIAcore 2000), it is possible (in favorable circumstances) to detect analytes as small as M_r 180 by SPR [59]. This new instrument has a much lower noise level than the previous design, and so lower molecular analytes may now be detected. The flow system also now permits four different concentrations of receptor to be immobilized in different spots on the sensor, creating a ''gradient'' binding surface. This allows the instrument to detect low affinity ligands, by using a high concentration of immobilized receptor at one spot, and ''blanking'' it with another spot. Using a modified dextran surface, to which antibody was bound, Karlsson and Ståhlberg were able to detect the binding of theophylline (M_r 180) and aminotheophylline (M_r 308) at concentrations of about 25 n*M* and above. The weak binding of an oligopeptide to a mouse immunoglobulin was also characterized, with an equilibrium binding constant of $2.5 \cdot 10^6$ M^{-1}, indicating the instrument's ability to detect ligands with affinity in the micromolar range.

In addition to DNA–protein binding, drug–nucleic acid binding may be studied by interfacial optical means. There are, however, few literature reports of this type. To sense DNA–drug interactions by SPR, Yang et al. conjugated doxorubicin with polymeric dextrans of various molecular weights, and followed both the equilibrium and kinetics of the conjugate's binding to DNA immobilized on the biosensor's surface [60]. Hendrix et al. used an SPR sensor with immobilized RNA to detect RNA–peptide and RNA–aminoglycoside interactions [61]. This study was done using the newer BIAcore 2000 instrument, which permits detection of binding of low molecular mass analytes. The work in general shows the possibility of screening for low M_r analytes that bind to nucleic acids, using the more sensitive SPR instrument.

Pandey and Weetall have applied TIRF to characterization of the binding of intercalative compounds to immobilized DNA [62]. Using as a reference the highly-fluorescent compound ethidium bromide, they could follow the drop in fluorescence as the bound ethidium was competed off the DNA by a non-fluorescent intercalator. This could lead to standard screening-type assays for DNA-binding compounds. The main drawback is its format in which signal loss, not gain, is followed.

This general area shows much promise for developing assays to screen for inhibitors of enzymes that act on DNA, as well as for use in DNA-based diagnostic assays using hybridization.

5.5. General advantages and limitations of interfacial optical methods

With the SPR, RM and GCS techniques the ligand need not be labeled, which is a significant advantage over other methods that require attachment of either a fluorophore or a radioactive moiety. In the commercial instruments, the change in refractive index is due to a local change in mass density in a surface layer, from protein binding, DNA hybridization, or some other interaction involving macromolecules. In older studies, successful application of SPR required binding of relatively large molecules from solution, in order to create sufficient change in refractive index. A lower limit of M_r 5000 for the ligand was originally suggested by Karlsson et al. [44]. Hence most of the earlier reports deal with protein–protein and DNA–protein interactions or DNA hybridization. Recently the BIAcore 2000 instrument was introduced, with the manufacturer claiming a 10-fold improvement in sensitivity over older designs. Current reports show that in favorable circumstances (using a chip with a high density of receptors) this instrument can be used to detect binding of compounds with molecular masses below 1000 [59,61]. However, for adequate sensitivity it is still necessary to have a high concentration of complexes at the surface, which can lead to other difficulties, particularly in achieving a high enough density of captured receptor molecules that are both active *and* uniform in their activity. Also, temperature control is an important issue, since the refractive index of water is sensitive to temperature, and one can easily get baseline drift or noise due to a change in resonant angle with temperature gradients across the sensor surface.

For membrane-bound receptors and their ligands, the geometry of the bilayer and its means of preparation may affect binding affinity in several different ways. First, apparent affinity may depend on the homogeneity of receptor or ligand preparation; a heterogeneous population of ligands or receptors (as may be found naturally) may have a range of affinities, resulting in a different average affinity than a homogeneous population constructed by chemical synthesis, for example. Second, electrostatic interactions in the binding may depend on the local geometry (planar vs. spherical) and so result in differences in affinity. Third, avidity effects (simultaneous multiple contacts between a single receptor and several membrane-embedded ligands) may result in an apparent increase in binding affinity. Fourth, lipid packing effects in a curved vesicle surface vs. the planar surface of a supported bilayer may alter the conformation or the freedom of motion of either or both the receptor and ligand, with consequences for binding affinity. Fifth, the process of depositing an embedded receptor into a lipid bilayer on the planar surface of a sensor may perturb the receptor's structure and its affinity.

For kinetic studies, it appears that the best results are achieved with a *low* density of receptors at surface, to avoid problems with crowding and steric hindrance. There are also a number of problems in the interpretation of kinetic binding curves, as discussed by Schuck [41] in his review. These involve mass flow past the sensor surface, heterogeneity of binding sites on the surface, diffusion of the analytes through immobilizing polymer layers, possible conformational changes in the receptor upon binding ligand, avidity effects in the binding of multivalent ligands to dense arrays of binding sites, and more. The complexity of the kinetics calls for careful experimental controls in any kinetic study, with careful characterization of the immobilized species, and kinetics monitored at different surface densities.

If the aim is to simply screen samples for the presence of an analyte with high affinity for the immobilized receptor, then the SPR and RM techniques offers a significant advantage over the competing technologies, in that one can employ SPR or

RM with ''dirty'' samples, containing cell debris, viruses, etc. These techniques do not depend on having an optically-clear sample. Thus, fairly ''raw'' samples may be used. Also, despite the problems with interpreting kinetic experiments, there is evidence that relative binding affinities can be determined accurately, when using a competition assay format [63]. Still, one must exercise caution in interpreting results; the report from Ladbury et al. [64] details how ligand dimerization and avidity effects with a high surface density of receptors can lead one astray.

Further considerations are that the commercial instruments are expensive, and involve fine optics, microfluidics and expensive biochemical reagents (monoclonal antibodies especially). There is a general need for cheap, disposable sensor elements with reliable reproducibility.

6. Other surface-based assays

6.1. Porous silicon-based interferometric sensors

Two recent reports on interferometric assays for binding of small ligands show that this area has considerable potential for screening libraries. These assays use a derivatized microporous silicon surface that gives an increase in available surface area, so that one can immobilize more of the receptor species and so capture more ligand with a consequent increase in assay sensitivity. The prepared surface is illuminated from above (not below, as in SPR or RM), and the reflected light is detected using a charge-coupled device camera or diode array. These interferometric assays use light reflected from the surface and from the bottom of the porous layer to generate an interference pattern related to the optical thickness and refractive index of the microporous layer. Binding of a ligand to a surface-immobilized receptor changes the interference pattern; the pattern is quite sensitive to small changes in mass at the surface.

Piehler et al. have examined two prototype systems, the high affinity binding of biotin to immobilized streptavidin, and the moderate-to-low affinity binding of intercalators to double-stranded DNA [65]. Lin et al. have demonstrated that their sensor is capable of detecting the binding of biotin (M_r 244) at picomolar concentrations, with a surface modified with streptavidin [66]. Also, they successfully detected the binding of the steroid digoxigenin (M_r 392) with a surface-bound antibody, at micromolar concentrations. Both reports are notable for the direct detection of low M_r analytes from bulk solution at very low concentrations of analyte.

The related technique of ellipsometry was used by van Noort et al. to detect binding of low molecular mass ligands (biotin and an oligopeptide) to receptors bound to porous silicon [67]. However, relatively high concentrations of biotin ligand were needed in this system, on the order of two micromolar; thus the results are not as dramatic as those achieved by Lin et al. [66], with detection of picomolar levels.

6.2. Quartz crystal microbalances (QCMs)

The resonant frequency of a quartz crystal will change with an increase in mass, as for example with the binding of a ligand to a receptor that is immobilized on the crystal's surface. This change in frequency can be measured very accurately, making the QCM device a good candidate as a biosensor. Attachment of biomolecules to the quartz surface follows much the same sort of chemistry as for attachment of these species to other silica sensor surfaces. The quartz surface can be coated with a thin film of gold, thus permitting alternative immobilization chemistries for, e.g., proteins or DNA. As with the other assays covered in this review, the QCM has been applied to protein–peptide interactions, DNA–polypeptide interactions and DNA hybridization [68–71]. These devices, though interesting and promising, need further development before routine application to screening operations will be feasible.

6.3. Jet ring cell with UV–visible absorbance

The jet ring cell [72] is a novel flow cell which retains suspended particles (e.g., micron-sized beads) in an extremely small cell (2.5 μl volume). The cell uses radial flow through a ring-shaped gap of very small dimensions, trapping the particles from a flowing stream. The retained beads can then be

probed optically for surface-bound analyte, and the cell is readily purged for another series of assays. Only small amounts of beads are used per assay.

Ruzicka and Ivaska [73] have reported the application of the jet ring cell to receptor–ligand binding studies, using optical absorbance. In a typical assay, Sepharose beads carrying immobilized receptors are injected into the cell, and the trapped beads are perfused with a buffer stream to establish a baseline absorbance. Then ligand is injected and perfused through the bed of trapped beads and the process observed through the change in optical absorbance. Finally, the beads and free ligand are washed from the cell. In the assay described, Sepharose beads were derivatized with protein G, and their ability to selectively retain immunoglobulins from the carrier stream was observed. Also, the system's ability to capture a first ligand (e.g., an antibody) on protein G–Sepharose beads, and subsequently to trap antigen from solution was demonstrated.

6.4. General advantages and disadvantages

The new interferometric tests, using porous silicon surfaces, constitute a simple detector that may be especially useful for low-molecular-mass analytes. These are label-free techniques, which is greatly to their advantage. However, their sensitivity to variations in sample composition (e.g., solution pH or refractive index) may limit their application. Regeneration of the detector surface is also a concern; for example, a high-salt wash might release substantial amounts of the ionically-retained DNA in the sensor described by Piehler et al. [65] Also, the porous silicon surface may not be suitable for attachment of lipid bilayer vesicles, which would limit the application of this technology in studies of membrane-bound receptor–ligand interactions.

The jet ring cell is a promising technology. The bead format allows construction of many sandwich-type assays, so the range of possible analyses should be substantial. Using absorbance detection, the system can monitor the binding at several different wavelengths, for greater reliability in sensing the binding and for distinguishing one species of ligand from another; this could be important in screening mixtures of ligands from a combinatorial library. In the absorbance–detection format, the assay is of course limited by the requirement for a good chromophore on the analyte. Ruzicka and Ivaska [73] concede that the sensitivity and detection limits of SPR are currently better than the jet ring cell with optical absorbance, but the jet ring cell method has substantial possibilities for future application and improvement.

7. Immobilization of target macromolecules for screening

The purpose of immobilizing a receptor is most frequently to facilitate the separation of bound species (compounds of interest in the library) from free species (those of no interest), or to facilitate coupling of the bound state with a form of detection as in biosensors. The immobilization of target receptors may be accomplished by adsorption, covalent bonding (both to a support and through intermolecular cross-links) or entrapment of the target molecule in and on various polymer matrices. Sections 4, 5 and 6 have dealt in passing with the chemistry of immobilization of receptors (both proteins and nucleic acids) onto surfaces of beads or biosensors. Of course, covalent attachment has been used for years in the field of affinity chromatography; consequently, the literature on this latter subject is far too large to review here. Nevertheless, it is important at least to review the general topic of receptor immobilization and to consider some of the pitfalls. We will examine here selected reports of new methods for receptor immobilization and will provide a brief discussion of some of the problems associated with the functional activity of immobilized proteins that were skirted in earlier sections.

A starting point for the interested reader is the extensive review by Colowick and Kaplan on immobilized enzymes [74]. One of the earliest techniques for protein immobilization involved silane coupling to various inorganic supports. Literally hundreds of laboratories have utilized this methodology for the immobilization of enzymes, antigens, antibodies, receptors, and sundry other compounds. Silane coupling is still one of the most frequently used methods for the preparation of biosensing devices. The reader is directed to the review by Weetall for a discussion of the general preparation

and characterization of silane-coupled proteins and for details of the actual methods for surface silanization and specific chemical coupling of proteins to the silanized carrier [75].

The number of reactive chemical groups for covalent attachment has vastly expanded in recent years, and there are many new methods for immobilization of receptors. Sigma lists no less than six categories of activated matrices for protein immobilization: epoxy-activated matrices; nitrophenyl chloroformate- and *N*-hydroxysuccinimide chloroformate-activated matrices, thio- and thiopropylgels; and polyacrylhydrazido- and oxirane-activated acrylic beads. Taylor provides a general review of commercially available supports for protein immobilization [76].

7.1. Selected new methods for protein immobilization

Several recent reports describing new techniques for immobilizing proteins are worthy of note. Fernandez-Lafuente et al. have described a method for the preparation of aminated agarose gels containing monoaminoethyl-*N*-aminoethyl groups [77]. These gels contain primary amino groups with a low p*K* value (6.8) that are highly activated (e.g., 10% agarose gels containing up to 200 milli-equivalents of primary amines per ml). These two properties make such activated supports suitable for protein immobilizations via carbodiimide activation of carboxy groups. For example, coupling reactions are reported at pH 5.0–6.0 in the presence of relatively low concentrations of activating agent, e.g., 1 m*M*.

Methyl vinyl ether–maleic anhydride copolymer (MMAC) is a water-insoluble polymer with an acid anhydride group that reacts with amino groups of ligands to form stable amide bonds. It has been used to immobilize protein ligands in the wells of plastic microtiter plates for enzyme-linked immunosorbent assays and related methods, and in gels for affinity adsorbents [78]. The immobilization of proteins in wells by this method was efficient and occurred in a dose-dependent manner. In another application of MMAC from the same group, Shodex Et123, a gel having amino groups, was incubated with MMAC, and then the activated Shodex was used to immobilize high concentrations of proteins. Concanavalin A–Shodex thus obtained had high affinities and was successfully used for the high-performance affinity chromatography of sugar derivatives on a short column [78].

New procedures to immobilize high concentrations of protein ligands by reductive amination on two types of formyl-carriers (I & II) having different spacer lengths have been reported by Ito et al. [79]. Formyl-carrier (I) was prepared by reductive amination with glutaraldehyde of the amino-carrier obtained on amination of an epoxy-activated carrier. Formyl-carrier (II) was prepared by sodium metaperiodate ($NaIO_4$) treatment of a glyceryl-carrier obtained on hydrolysis of an epoxy-activated carrier. High concentrations of protein ligands were immobilized on formyl–Sepharose 4B (I) under mild conditions (pH 7.0, 4°C). A series of lectins was successfully immobilized by these procedures; these preparations exhibited an adsorption capacity five times greater than that of commercial preparations, and could be used over twenty times without a significant reduction in their adsorption capacity.

A particularly intriguing reversible method which may hold promise for immobilizing proteins has been reported by Moriya et al. [80]. The technique uses Sepharose derivatized with salicylaldehyde, to form a Schiff base linkage to an α-amino group on the protein; the Schiff base is stabilized by copper (II) chelate formation, and upon removal of the stabilizing metal ion (by, e.g., addition of EDTA) the linkage is broken and the protein released. These workers applied the method to the immobilization first of tryptophan, then of subtilisin BPNN that had been modified with an α-amino acid. Immobilization in either case was reversed by the addition of EDTA.

Two methods for the immobilization of proteins onto liposomes may be of interest for those involved in screening. The first of these has been described by Goldmacher [81]. The technique consists of covalent coupling of linoleoyl residues to the protein globules and consequent binding of linoleoyl globules to liposomes by a detergent dilution method. Using this approach, the authors immobilized trypsin which in the immobilized form was found to retain specific esterolytic catalytic activity and the ability to bind to a trypsin inhibitor protein. A second method involved covalent immobilization of proteins on the surface of liposomes containing 10 mol% of *N*-

glutarylphosphatidylethanolamine. As described by Bogdanov, Jr. et al. [82], carboxylic acid groups of the liposomal *N*-glutaryl lipid derivative were activated in the presence of water-soluble carbodiimide and *N*-hydroxysulfosuccinimide and reacted subsequently with protein amino groups. The liposome–protein conjugates contained up to $5\cdot10^{-4}$ mol protein/mol lipid. Upon immobilization on liposomes, the lectins RCA1 and WGA retained saccharide specificity and the ability to agglutinate red blood cells. The immobilization of mouse monoclonal IgG in a ratio of $3.5\cdot10^{-4}$ mol IgG/mol lipid was also demonstrated.

Several reviews of potential interest to those in the screening field focus on specific areas of immobilization. Schuck has reviewed issues surrounding receptor immobilization in the use of surface plasmon resonance biosensors [41]. Immobilization techniques, the most commonly employed experimental strategies, and various analytical approaches are summarized. Several sources of potential problems were identified: immobilization of the binding partner, steric hindrance of binding to adjacent binding sites at the sensor surface, and finite rate of mass transport of the mobile reactant to the sensor surface; and the influence of these artifacts on the measured binding kinetics and equilibria, together with suggested control experiments are discussed.

Plant et al. have studied four different nonporous particulate materials (nylon, polystyrene, soda-lime silicate glass, and fused silica glass) for their appropriateness as immobilization supports for immunoglobulins [83]. An interesting older report by Nishio and Hayashi describes the immobilization (by the glutaraldehyde method) of subtilisin BPNN on glass-bead carriers of controlled pore size [84]. These researchers found that the V_{max} and K_m values of the synthetic substrate were similar for immobilized and free enzymes. However, the hydrolytic patterns of immobilized and free enzymes toward casein and carboxymethylated lysozyme were different.

Wu et al. have studied enzymes immobilized on non-porous carriers to control for problems associated with substrate diffusion into the polymer matrix [85]. The use of non-porous beads eliminates the need for a substrate to first penetrate the bead in order to interact with the immobilized protein, providing for much faster response times.

An interesting comparative study of three different methods of protein immobilization has been reported by Butler et al. [86]. Although the work was conducted using antibodies, the results have implications for the general field of protein immobilization. Their data show that less than 3% of the binding sites of monoclonal capture antibodies and approximately 5–10% of those of polyclonals were capable of capturing antigen after passive adsorption. Immobilization of the antibodies via an antiglobulin or a streptavidin bridge resulted in the preservation of antibody binding sites to greater than 70%, with immobilization via the streptavidin bridge resulting in the highest number of functional sites/well. The data presented are consistent with studies on other adsorbed proteins which demonstrate that passive adsorption on polystyrene results in the loss of protein function. In addition, the workers showed that passive adsorption techniques resulted in lower avidity when compared with the non-adsorptive methods. Interestingly, there was some indication that the active antibodies were not evenly distributed on the polymer surface but tended to be active in clusters, implying that the adsorbed and functional antibodies were part of an antibody cluster. Data presented in this report on adsorbed antibodies, and reviewed from the work of others for various adsorbed proteins, indicate that the method of passive adsorption which is widely used in popular microtiter enzyme-linked immunosorbent assays (ELISAs) and which has in many ways revolutionized immunoassays, is really a method of protein denaturation.

Barrett et al. have described a general method for expression, purification, immobilization, detection, and radiolabeling of extracellular domains (ECDs) of type I membrane proteins which should be of interest to those involved in receptor screening [87]. Briefly, the DNA coding for the ECD is fused with a ‘‘tagging’’ sequence coding for a substrate sequence for protein kinase-A, adjacent to the signal sequence from human placental alkaline phosphatase (HPAP), and the recombinant receptor expressed in CHO cells. The HPAP polypeptide tag segment directs the formation of a phosphatidylinositol-glycan anchorage of the protein at the cell surface; this also provides a site for cleavage by phosphatidylinositol-specific phospholipase-C in order to release the recombinant ECD. A 20-amino acid segment of the HPAP tag

remains associated with the ECD, and this segment can be recognized and bound by a specific monoclonal antibody. Thus, one can prepare a bed of immobilized ECD by capturing it via the tag, using an immobilized preparation of the specific monoclonal antibody. In the systems reported, the capture by monoclonal antibody apparently has little or no effect on the ability of the ECD to bind its cognate ligand.

Several researchers have explored ways to orient proteins on surfaces, such that orderly organization, single point attachment, and accessibility of the active site (or binding site) are possible. Rao et al. have reviewed the various approaches available to achieve oriented immobilization of proteins [88]. Ramsden and co-workers [89,90] have explored the immobilization (via specific molecular anchoring moieties of lipid or oligopeptides) of proteins to lipid bilayers on biosensor surfaces.

Tyagi and Gupta have reviewed both monocovalent attachment of target proteins as well as covalent crosslinking as a strategy for protein immobilization [91]. In the case of chemical modification, the authors report that increasing either surface hydrophilicity or surface hydrophobicity can enhance a particular protein's stability. Not surprisingly, the nature, span, and position of the cross-link are important factors in the stabilization achieved. It was also pointed out that in the case of aqueous-organic co-solvent mixtures, protein stability may depend upon the nature of the organic solvents. In the case of polyphenol oxidase and trypsin it was possible to choose optimal solvents on the basis of the polarity index of the solvent [91].

One general approach to the problem of protein orientation on immobilization has been to construct a specific site for immobilization within the target receptor, by chemical means or through genetic engineering. Such a specific site provides a common point of attachment for the receptor and thus presumably produces uniformity in the protein's orientation and hence its biological activity. For example, one could attach a molecular tag to either the C- or N-terminus of a receptor protein, and use a specially-prepared solid support phase to capture the tag and hence the receptor protein. Alternatively, one could alter an internal residue (presumably a unique residue) to a more-reactive form, and then link this residue by chemical reaction onto the support (see, e.g., Moriya et al. [80] for one such method).

A widely-used application of the receptor tag-and-capture approach is to tag the protein with six consecutive histidine residues, a so-called 6His tag. These residues bind with high affinity to metal ions immobilized on chelating resins even in the presence of denaturing agents, but can be released and eluted under mild conditions with imidazole. As an example, Paborsky et al. reported the use of this methodology for the immobilization of six histidine-containing proteins onto microtiter plates [92]. The plate assay format was used to quantitate protein concentrations and determine the affinity of protein–ligand interactions. The technology can also be extended to include high-throughput screening assays for antagonists of protein–protein interactions.

Huang et al. [93] used genetic engineering techniques to immobilize subtilisin BPNN and provide an oriented and active enzyme preparation with several different supports. They introduced a single cysteine residue into the cysteine-free enzyme, by means of site-directed mutagenesis of a selected serine residue. The side-chain sulfhydryl group of the cysteine provided a convenient chemical moiety for coupling to activated thiol Sepharose, thiopropyl Sepharose, and Affi-Gel organomercurial beads, and to silica beads functionalized with amino groups. The chosen site was well separated from the enzyme's active site, and so presumably would not interfere with substrate binding or catalysis. The expectation was that the oriented immobilized subtilisin would show greater enzymatic activity than would subtilisin immobilized in random orientation by a conventional method, and indeed the catalytic efficiency (k_{cat}/K_m) for the randomly immobilized enzyme was significantly lower than that of the oriented enzyme preparation. This particular method of immobilization of course depends on the uniqueness of the introduced sulfhydryl group, and it is not expected to produce uniformly-oriented proteins arrays for proteins containing multiple sulfhydryls. Thus in direct application it is probably limited to a relative handful of proteins. It certainly would not be expected to work with whole antibody preparations, for example, where there are multiple thiol groups present on the polypeptide chains. On the other hand, it may be quite useful in some instances, as with

small polypeptides or proteins (like subtilisin) that naturally lack any cysteine residues.

7.2. Potential problems associated with immobilization

By its very nature, the process of immobilization holds the potential to alter, either dramatically or subtly, the biological activity of the targeted macromolecule. In most cases, immobilization leads to partial or total loss of activity. There are many different possible reasons for this change in activity.

First, simple noncovalent adsorption onto immobilization surfaces can result in the disruption of secondary and tertiary structure. Contacts between domains may be disrupted, with consequences for allosteric properties of the receptor. Immobilization by adsorption of multi-subunit receptors presents even more difficulties in maintaining proper subunit contacts; also, subunits of such receptors may be lost through washing.

Second, adsorption can easily result in randomized orientation of the receptor, with the potential for occlusion of binding sites. In this connection, it is widely believed that much of the loss in protein activity upon covalent immobilization is due to attachment of the proteins to the immobilization support through several different amino acid residues. This results in a random orientation of the immobilized protein and in increased structural deformation due to multi-point attachment. Also, allosteric properties that rely on the mobility of one domain or another may be affected differentially, depending on which domains were immobilized. These effects could easily give rise to a heterogeneous population of receptors, with a distribution of binding affinities and specificities as a probable result.

Third, covalent linkage to the surface may either occlude or chemically alter functional groups that may be important for ligand recognition and binding. This can result in either partial or complete inactivation of the receptor, or a change in specificity. Also, we should note that the altered or blocked residues need not be those directly involved in contacts with the ligand species; they may instead be involved in transmitting allosteric effects, for example.

Fourth, crowding on the support surface between immobilized receptors may hinder the approach of ligands to the binding site, or may hinder motions of regions necessary for ligand binding. A high density of bound receptors may also promote avidity effects in the binding of large ligands, or lead to the association or aggregation of smaller ones. Again, these effects have obvious implications for binding affinity and specificity. Beyond this, there are ligand diffusion and trapping problems associated with porous microspheres or porous media such as the CMD layer used with SPR sensors. One should always consider as well the possible nonspecific binding of the analyte to the support surface.

All of these can cause problems with interpretation of the screen, and one should take due care in drawing conclusions about binding specificity or affinity from results obtained using immobilized species. It appears that site-specific attachment of the receptor to the surface with oriented affixture is much more desirable, though more difficult to achieve, than random adsorption of the receptor. This gives a more uniform receptor population with native or native-like conformation of the receptor, and increases the chances of observing binding behavior nearer that of the in vivo situation. On the other hand, it should be noted that no in vitro assay, or animal model for that matter, is truly representative of the activity of a compound in a clinical trial. The key to success in all these approaches is to recognize this fact and to qualify the results accordingly.

8. Summary

The preparation of soluble receptors in adequate quantities is now not a real roadblock to screening programs. Thanks to recombinant DNA technology, cloning and expression of receptor genes in a suitable host organism is usually a straightforward matter. One can reasonably expect to prepare multimilligram quantities of purified (soluble) receptor protein in a matter of a few months. Cloning of a membrane-bound receptor follows much the same route as that for a soluble one, but handling and purification of the receptor will differ. For full activity, the protein must very often remain surrounded by a hydrophobic environment as in, e.g, a lipid bilayer in a microsomal preparation, or it must

be solubilized by a (mild) detergent. Cloning and expression of a soluble portion of the receptor may also be possible, or one may try a cloning system that ''displays'' a part or all of the receptor on the host cell's surface. If the target protein is of eukaryotic origin and normally undergoes post-translational modification one must naturally be concerned in the choice of host cell. Recent reviews [94,95] provide guidance through these and other difficulties in achieving an adequate supply of authentic receptor for a screening operation.

8.1. Future trends

Three distinct trends in assay development can be seen today: devising smaller assays, capturing intact signal transduction complexes and coupling of different assay technologies to broaden the analytical range.

8.1.1. Further miniaturization of assays

The 96-well titer plate format is a recognized standard for automated assays. To increase throughput and to reduce consumption of reagents, higher sample-capacity plates are being developed, notably a plate with 384 wells [96], having the same footprint as the 96-well plate (a plate with 1536 wells is being developed also). While the miniaturized format reduces consumption of valuable reagents (assay volumes may be reduced to 50 μl in the 384 well plate), it also calls for more sensitive assays capable of reliably detecting hits in the smaller volumes used. There are also more demands on the liquid-handling systems for pipetting and mixing these small volumes.

8.1.2. Capture of signal transduction complexes

Receptors embedded in lipid bilayers are notoriously more difficult to work with than soluble receptors. A main problem in studying these systems with sensors that require binding to a surface has always been that of maintaining the receptor in a state like that in vivo, while holding it on the surface of the sensor. One answer is to use a tether of one type or another to immobilize whole vesicles together with the embedded receptor species, as described in the reports by Schuster et al. [55], MacKenzie et al. [57], and Masson et al. [56]. These reports were concerned with immobilization at the surface of an SPR device, but the general strategy of vesicle immobilization should be applicable more widely, e.g., to RM, TIRF, or SPA, perhaps also to QCM and jet ring devices. Another strategy is to create lipid bilayers directly on the surface of the sensor and to immobilize the receptor in that bilayer, as exemplified in the work by Heyse et al. [97], Plant et al. [98], Ramsden and co-workers [89,90], and Salamon and co-workers [99–103]. We can expect to see many changes rung on these basic approaches, as work proceeds on the complicated protein assemblies involved in signal transduction.

8.1.3. Coupling of techniques

As remarked in the Section 1, when a simple screening assay registers a ''hit'', the assay typically does not give much further information as to the chemical identity or biological properties of the ''hit''; much further analysis is needed to characterize the sample. For example, a drawback to SPR and the other interfacial optical methods is that these techniques do not by themselves identify the ligand species. Coupling the simple initial screening assay with a second analytical technique that is in some sense ''orthogonal'' to the original technique should allow one to spread out a library of compounds over a larger volume of analytical space, with consequent advantage in more rapid and thorough characterization of the sample. This second technique might provide some chemical structural information, for example, or other chemical or biological details that the primary assay does not sense. Ideally, the second method should also be compatible with the primary assay in terms of sample mass and volume, composition (e.g., no interfering substances introduced; solvents and buffer constituents are compatible), and rate of sample turnover. One such analytical method is mass spectrometry, whose coupling to HPLC is well established.

MS offers the special advantages of high sensitivity, parallel detection of different species, speedy processing of spectra to identify compounds covering a molecular mass range from the low hundreds to several tens of thousands, and amenability to automation. We are now beginning to see MS paired with other assays involving flow systems, e.g., with SPR and QCM detectors as well.

Krone et al. [104] and Nelson et al. [105,106] have applied matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS in concert with SPR. Species retained on the SPR sensor surface were identified by direct MALDI-TOF analysis of the sensor surface. The systems so analyzed were a sandwich or composite of antibody/antigen/antibody. Sönksen et al. [107] have also demonstrated this instrumental coupling with antibody–antigen and DNA–protein systems. In their procedure, the SPR instrument senses the binding of the analyte; then the captured analyte is eluted (fmol quantities, in μl volumes) from the sensor surface. The eluate is subsequently analyzed by MALDI-MS. The combination of the two methods allows calculation of the concentration of the surface-bound analyte (by SPR) and the identification of the analyte (by MS). It would be no great leap to combine MS with the optical absorbance system in the jet ring cell.

Garland [42] has pointed out that coupling of TIRF with SPR or RM assays would be possible with only slight modification of present cell designs. Such a combination could provide another channel by which to observe binding interactions, and to identify one or another binding partner in the presence of a mixture. To date, there has been no commercial implementation of this suggestion. A drawback to TIRF is of course its requirement for a fluorophore, which may call for a separate labeling step if the analyte is not intrinsically fluorescent.

Nuclear magnetic resonance (NMR) has not been featured in this review, primarily because of the paucity of the literature on its application to library screening. Only recently have reports begun to appear concerning potential applications of NMR in screening libraries. In an approach they call ''SAR by NMR'', Shuker et al. have described the use of ^{15}N-labeled proteins to identify active compounds from a library of low-molecular-mass ligands [108]. Lin et al. have described ''affinity NMR'', in which they demonstrate a detectable difference in diffusion coefficient for a small compound free in solution and in complex with a model receptor [109].

The main advantage of NMR is the wealth of structural information it potentially offers. Its main drawback is its insensitivity, particularly when compared to mass spectrometry or radiometric detection. However, recent advances in magnet technology and other NMR hardware are steadily reducing the sample size needed while improving the sensitivity. For example, Olson et al. have obtained proton NMR spectra from 5 nl samples containing pmol quantities of simple organic compounds or oligopeptides [110]. Martin et al. have carried out full two-dimensional NMR structure elucidation with samples of less than 5 μmol [111]. These sample volumes and mole quantities are in the range where NMR structure determination could be coupled to flow injection assays like SPR or affinity chromatography, with the potential for a very large increase in information gained during a single screening pass through a library.

8.2. Final remarks

This review has deliberately been limited in scope to those binding assay methods that (1) involve a mechanical or chemical restraint on the receptor–ligand complex, and (2) are applicable (or may shortly be applicable) to screening combinatorial libraries. Restraining the ligand–receptor complex is most commonly done by immobilizing the receptor in one fashion or another. While such immobilization can greatly increase the general utility of the assay, in terms of time, cost, and ease of implementation (especially in assay automation), it also presents some problems, primarily in distorting the conformation of the immobilized species and reducing its capacity to participate in the binding equilibrium.

One might state as an axiom for screening operations that the more complex and difficult the screening assay (e.g., human clinical trials) the greater the information content of the assay (bioavailability, metabolism, side-effects, etc.). Conversely, simple assays convey only limited information (e.g., yes/no answers on receptor binding). By their very nature, the high-throughput assays tend not to yield much information on structure–activity relations in a screened library. One route to greater information content from a primary screening assay is to couple the assay with a secondary technique. This secondary technique, by itself, might not be suitable for analyzing a complex mixture, but when joined with the primary binding assay, it could supplement that assay in giving much more detailed information

about the "hits" made in that primary screen. One such technique already in use is mass spectrometry; another that shows promise (but is not yet quite ready for routine application) is NMR.

Finally, three areas that should receive much more attention in the future are (1) the use of membrane-bound receptors in in vitro screening assays; (2) screening for small molecules that block (or possibly augment) protein–protein interactions; and (3) the development of more sophisticated biological assays, involving whole cells and defining biological responses more closely. We look forward to seeing many new studies in these areas, along with the development of innovative (and perhaps coupled assay) screening methods.

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