From Flow Injection to Bead Injection

BI is a novel approach to assays based on liquid-solid interactions.

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In this Report, we introduce a new methodology applicable to the entire range of traditional reagent and solid-phase assays. Known as bead injection (BI), this technique influences diverse fields of research, including chemical sensors, microfabrication, bioassays, affinity chromatography, spectrophotometry, and electrochemistry. This is because BI combines the advantages of solid-phase chemistry with the novelty of fluidic handling of microcarrier beads, allowing automated surface renewal and postanalysis manipulations. Surface renewal is an especially critical feature because assay surfaces become contaminated or otherwise dysfunctional with repeated use.

All assays based on liquid-solid interactions use fluidic control of the liquid phase (i.e., the sample, reagent[s], and buffer); however, the solid phase is always viewed as a permanent component of the system, to be replaced only after numerous samples have been processed. Indeed, in chromatography, packed enzyme reactors, and biosensor membranes, the stationary phase is continually being developed to withstand numerous assaults during repetitive analyses. But this is a difficult goal to achieve with solid surfaces—no matter how well constructed—because the loss of surface functional groups or surface deactivation by samples will eventually occur. More critically, such research endeavors are narrowly focused on those select chemistries and materials that demonstrate robustness. The consequence of this approach is that
many useful chemistries must be ignored because they are incompatible with the requirements of longevity and repetitive service.

BI does not have these constraints. The technique is based on the microfluidic manipulation of a precise volume of suspended beads that serve as a solid-phase carrier for reagents or reactive groups. The injected bead suspension is trapped at a strategic location in a flow injection (FI) manifold, where it is subsequently perfused by the analyte solution, buffers, or auxiliary reagents. Chemical reactions occur at the bead surfaces and can be analyzed in real time, either directly on the solid phase or within the eluting liquid phase. A multiparameter approach is also possible, by monitoring simultaneously the changes in the solid and liquid phases. At the end of a measurement cycle, the beads can be automatically discarded, collected, or rerouted as desired. Although in its infancy, BI has been applied successfully in many research studies (1-8).

Both FI and chromatography can be carried out in the BI format. In fact, BI can be seen as the third generation of FI microanalytical techniques, following the development of sequential injection (SI). Indeed, SI is the perfect vehicle for BI, which in turn enhances SI by eliminating the problem of mixing reactants during the loading process. Other advantages of BI include improved assay sensitivity, because the use of surface-bounded reagents minimizes sample dilution; lower limits of detection, because the analyte can accumulate on the beads; expanded analysis schemes using parallel and/or orthogonal detection of analyte(s) in the bead and/or solution phases; and compatibility with a wide range of instruments, including spectrophotometric, electrochemical, and radioactivity detectors. In short, BI is a novel approach to microanalytical instrumentation.

**Experimental protocol and instruments**

The BI procedure comprises five steps, as shown in Figure 1a. In step 1, an exact volume of a bead suspension is aspirated and loaded into a flow cell as beads are trapped into a distinct geometry. In step 2, the beads are perfused with a buffered carrier stream, and the baseline for subsequent measurement (spectroscopic or electrochemical) is established. In step 3, a sample is injected, and the analyte is trapped on the bead surfaces. In step 4, this analyte is treated with auxiliary reagents or eluted from the bead column. Finally, in step 5, the spent beads are automatically discarded from the flow cell at the end of the assay cycle.
Spectroscopic measurements are carried out either by direct probing of the bead layer or, in the case of renewable chromatography, by probing the eluted mobile phase. On-bead detection typically requires reestablishing the signal baseline (e.g., re-zeroing the spectrophotometer), rendering the beads "invisible" to the detector. Maintaining a consistent baseline during the measurement cycle is crucial. In general, reproducible BI analysis requires consistency in the bead packing and the mechanical and/or chemical stability of the bed layer. This can be achieved with a well-designed flow cell and prudent selection of fluidic control parameters on an SI system, as shown in Figure 1b.

The flow cell must trap the beads in a geometry that allows uniform perfusion and, preferably, simultaneous monitoring of the entire bead layer. This can be achieved by retaining beads within a specialized flow cell called a jet ring cell (1), which consists of a tube positioned perpendicular to a flat surface, leaving a narrow circular gap. The liquid can escape through the gap in a radial fashion, while the beads are retained and perfused. At the end of the analysis cycle, the jet ring cell is emptied, and the spent beads are removed from the detection zone.

The SI system currently used is equipped with a stepper-motor-driven syringe pump, a holding coil, a multiposition valve, and a short conduit leading to the jet ring cell. Fluidic control and data collection are orchestrated by computer.

Maintaining the beads in a well-defined suspension is not a trivial task. Different bead materials require different methods of handling. For example, suspensions of dense beads, such as those made of vitreous carbon (5), are sturdy and can be directly agitated by magnetic stirring. Agarose and Dextran beads have similar densities to buffer solutions, but they are fragile and should be stirred by gently rotating the suspension container (8, 9). Equally challenging is the need to compensate for the optical properties of the beads. Some beads (e.g., Sepharose, Sephadex, and Cytodex) are fully transparent in the visible region, with increased absorbance toward the UV region. Porous glass beads are suitable only in the visible
absorption range, whereas many polymeric beads, such as Polysorb (5), are white and, therefore, suitable only for reflectance-mode monitoring. Amazingly, these beads will not damage or block the channels of a multiposition valve, provided that the original slurries are diluted (at least 1:10) and the quantities used are within the microliter range. It is fortunate that chromatographic technology can provide a wide assortment of solid-phase beads that are suitable for BI experimentation. Bead sizes in the 10- to 150-μm range are suitable for BI as currently practiced.

**Renewable chemical sensor system**

The main reason for the limited success of biosensor technology is that the chemical or biological transduction mechanism must be reversible and indefinitely stable. In other words, the chemical, biochemical, or biological species monitored is expected to be repetitively used with high fidelity for analyte detection and sensitivity of response. Unfortunately, all sensor systems are prone to fouling or aging at the sensing surface--inevitable processes that are the Achilles' heel of many novel sensor devices. In contrast, BI uses microbeads as a carrier of the chemical interaction, with the aim of making this reactive surface replaceable as needed.

In addition to providing a renewable surface, this approach has two significant advantages. First, the BI system uses an open architecture in which the hardware (computer, detector[s], optics, and SI manifold) remains the same while the chemistries (microbeads, analytes, and reagents) and computer control (software) are changeable; the same basic instrument can be easily configured to perform a variety of assays in a dedicated or a sequenced fashion. This open-architecture approach was demonstrated with a voltammetric biosensor detecting hydrogen peroxide, which is generated during enzymatic oxidation of analytes in the presence of dissolved oxygen. The hydrogen peroxide response is then related to the analyte concentration. Electrically conducting glassy carbon beads were used as enzyme carriers, allowing both the electrode surface and the enzyme to be renewed or exchanged. Thus, by using different oxidases, selective assays for ethanol, galactose, lactose, and glucose were performed in samples of beer. Serial response curves for various concentrations of glucose can be seen in Figure 2.
Perhaps the most important advantage of a renewable chemical sensor system is that it opens the door to a wide range of well-established chemistries that are, for the most part, thought to be unsuitable for repetitive assays. For example, an irreversible reagent-based assay was demonstrated (4) using a fiber-optic-based renewable sensor to perform trace Cr(VI) assays with 1,5-diphenylcarbohydrazide (DCP). In an acid medium, DCP forms a strongly colored red-violet product believed to be the chelated Cr(III). In this experiment, DCP was initially adsorbed onto MP-1 Polysorb beads that were kept in suspension by a magnetic stirrer. Approximately 30,000 beads (equivalent to a column of ~70 bead layers) were trapped in the jet ring cell and subsequently perfused by a 500-μL aliquot of analyte solution. Figure 3 shows the reflectance spectroscopy measurements used to monitor the DCP-Cr complex as it was captured on the surfaces of the beads.

Biologand interaction assays

Biologand interaction real-time measurements are an important pursuit in several fields, including pharmacology, immunology, and molecular biology. In biologand interaction assays (BIAs), the first component is immobilized on a solid substrate while the second component is brought in transient contact with the substrate by continuous perfusion. The rates of formation and dissociation of the ligand complex are monitored. A fluorometer can be used to monitor these ligand interactions because fluorescent labels are often used to tag one of the interacting biomolecules. The detection of unlabeled molecules, however, requires surface plasmon resonance (SPR) (10). Fluorescence measurements allow for higher sensitivity and lower detection limits, but the chemistry of labeling a biomolecule may corrupt the molecule or obstruct the reactive site, distorting the kinetic results. SPR, on the other hand, allows the target biomolecules to be used in their native forms.

BI can also be used for biologand interaction assays and was initially used with fluorescent labels in several studies (2, 3, 9, 11). However, the use of UV-vis
absorptiometry with a fiber-optic-coupled flow cell has allowed, for the first time, simultaneous monitoring of both labeled and unlabeled biomolecules (7, 8). This technique—BIA by flow injection absorptiometry (BIA-FIA)—uses Sepharose or Sephadex beads as the carrier of the immobilized biomolecule, which binds with a second ligand (and possibly more). Absorbance measurements in the 260- to 300-nm range allow for the universal detection of amino acids found in unlabeled proteins, and measurements in the visible range allow for the detection of fluorophores found in labeled proteins. The affinity kinetics and the quantities of the biomolecules can be determined by monitoring the UV-vis spectrum of the bead layer during the association and/or dissociation steps.

Figure 4 shows the simultaneous monitoring of labeled and unlabeled immunoglobulin (IgG) antibodies. The binding and dissociation of dye-labeled goat anti-mouse IgG on protein G Sepharose 4B beads demonstrates the capture and elution process—achieved, in this case, by simply changing the pH of the carrier stream (8). Note that this biosensor system provides not only binding kinetics but also spectral and separation information, because the IgG molecule is released from the bead in a chromatographic fashion.

Several features make BIA-FIA especially attractive for such experiments. First, Sepharose beads are commercially available with a variety of functional groups because they are widely used as a stationary phase in affinity chromatography. Second, literature on affinity chromatography provides a wealth of information on optimum conditions (e.g., pH, ionic strength, and functional group selection) for the separation of biomolecules and serves as a guideline for protocol development. Third, detection of small, unlabeled molecules is particularly difficult for SPR-based systems. However, as shown in Figure 5, BIA-FIA of biotin (MW 244), using avidin-coated Sepharose 4B beads, proved quite sensitive, with a detection limit approaching 12 ng of biotin.
Functional assays

Although BIA provides information on the binding of a molecule to a receptor site, it does not indicate whether such an event provokes a physiological response. Nonetheless, identification and characterization of a potential drug candidate require the determination of its efficacy--information obtainable through a functional assay. Functional assays classify a drug as an agonist or antagonist, depending on whether it invokes or inhibits a biological response. Many different functional assays have been carried out using a variety of eukaryotic cell lines--notably, the Chinese hamster ovary (CHO) cells containing the gene for the rat muscarinic type-1 acetylcholine receptor. In these assays, fluorescent probes (e.g., fura-2 or INDO-1) can be used to monitor the concentration of a transient release of cytosolic Ca\textsuperscript{2+}, which results from the stimulation of a receptor. Ideally, the initial response of fresh cells is measured because the repeated or prolonged cell exposure to drugs will often result in a diminished response.

BI has proved to be a good tool for functional assays because it provides automated and reproducible stimulation of cells cultured on microcarrier beads (11). A significant advantage of using beads as the cell carrier is that a representative sampling can be obtained from the bead slurry, eliminating the biological variability that occurs when cells for each measurement are cultured in separate wells of a Nunc plate. Also, because each injection of stimulant reacts with a fresh portion of cells, the responses are very reproducible. This eliminates the biological variability that occurs when cells become acclimated to agonists within a series of measurements, and it allows for accurate comparisons of efficacy among various stimulants. Figure 6 shows a functional assay on live CHO cells supported on Cytodex beads. Cells are exposed to a stimulant, and the intracellular Ca\textsuperscript{2+} rises, as represented by an increasing fura-2 fluorescence signal ratio. The curves shown demonstrate the strong cellular response to stimulation by carbachol; in contrast, stimulation by atropine does not provoke a significant response, thus establishing a baseline.
In addition, BI provides kinetic information that cannot be gleaned when stimulants are manually added (i.e., via pipette) to a cell culture. BI allows different agonists to be distinguished on the basis of their initial kinetic behavior during the onset of receptor-agonist interaction. The efficacy of acetylcholine, carbachol, and pilocarpine differs with respect to the initial onset, the initial rate, the duration, and the maximum response (11). Furthermore, as the dose levels are increased, these differences are obliterated. None of these features is obvious or easily observed using manual techniques.

Chromatography

Renewable microcolumn chromatography is a logical extension of the BI concept. Being presently restricted (for practical reasons) to relatively short columns, it is focused on separations based on mobile-phase changes, rather than relying on the separatory power provided by a large number of theoretical plates. Thus, recently published applications in renewable chromatography demonstrate BI as a simple means for automated solid-phase extraction (12) or as a truly sophisticated method for nuclear waste separation and analysis of radionuclides (13).

The separation of $^{90}$Sr from other radionuclides using a TEVA resin shows a typical recovery of 95%. This high affinity can leave residual $^{90}$Sr attached to the stationary phase and causes carryover in the runs that follow. By renewing the column material after each analysis, carryover is eliminated. Therefore, accurate measurements can be made from low-activity samples that follow high-activity materials. A similar carryover problem occurs in the separation of americium and technetium. So far, the eluent, rather then the stationary phase, has been monitored in these renewable column experiments. For this reason, BI detection is performed downstream from the trapped beads, and the beads are retained by a fixed frit. Reverse flow is required for bead removal and is achieved by a two-position valve (13, 14).

Titrations

Solid-phase titration is a novel technique that uses beads as individual microvessels containing a titrant and an indicator (6). Sepharose beads are composed of 80% solution and 20% matrix and are highly transparent to visible light. The beads can be filled with a titrant, such as a NaOH solution, and a pH indicator can be attached to the surface by ion exchange (e.g., Sepharose Q is an anion exchanger).
A well-defined quantity of beads is trapped in a flow cell, forming a microcolumn. The bottom layer is probed by using a pair of optical fibers in transmittance mode. At a constant flow rate, an injected zone of acid is titrated as it gradually advances through the bead layers to finally neutralize the bottom layer. When this bottom layer of beads is titrated, their absorbance will abruptly change, indicating the end point. The volume of sample needed to reach the endpoint is directly proportional to the analyte concentration. Although originally designed for the titration of sulfuric acid in nonaqueous samples (e.g., 1-butanol and oils), this somewhat peculiar technique works for aqueous samples as well. It has the advantage of allowing for the titration of highly colored samples because the colored sample solution flows through a narrow channel between the beads, forming only a thin layer within the light path (6).

Conclusions and perspectives

BI is a logical extension of the FI concept, and, therefore, it is natural that several features of BI can be traced back to early literature on FI. Suspended matter was first pumped into a flow cell for sulfate determination by turbidimetry (15), and packed reactors were used for the conversion of nitrate into nitrite (16), as well as for the enzymatic conversion of urea and glucose (17). Real-time monitoring of solid-liquid interactions by spectrophotometry in a flow cell was suggested in 1985 (18, 19), and optosensing by fluorescence was introduced five years later (20). However, it was the SI technique, developed in our laboratory, that made it possible to handle suspended beads with precision and reliability. This is why BI originated in our laboratory and thus far has been confined to our group (1-9, 11, 12) and to our close collaborators (13, 14). This course is not without precedent: During the formative years (1975-1997) of FI, only 3 out of 20 publications were from other independent researchers, in contrast to the nearly 10,000 refereed FI papers published to date (see http://www.flowinjection.com/search.html). Although such growth for BI remains to be seen, our aim is to communicate the potential of BI and to inspire further research and development in this area.

The use of beads as a reagent carrier invites modification of nearly all chemical assays into a BI-based format. Presently, our focus is restricted to its use in biology, biotechnology, and drug discovery, in which there is an immediate need for kinetic interaction assays. For example, an important question in the study of bioligand interactions--and indeed all immunoassays--is whether labeled biomolecules behave in the same fashion as unlabeled ones. The answer is not unequivocal, because
the molecular size of the label, its location on the biomolecule, and the chemistry of attaching fluorescent tags may corrupt the bioactivity or the structure of the host. Kinetic comparisons of binding and dissociation between labeled and unlabeled species can answer each individual situation and thus suggest protocols for correct labeling of biomolecules.

In addition, studying the responses of living cells to various chemical stimuli is a central topic of drug discovery and cell biology. By monitoring the initial phases of stimulant-receptor interactions, the functional tests on live cells reveal the true potency of agonists and antagonists in physiologically relevant experiments. Manual techniques can overwhelm the biochemical response through excessive concentration gradients and long durations of dose exposure. BI not only provides uniform dosing and controlled exposure periods but also yields the kinetics of the response in a highly reproducible fashion. The power of such a functional assay makes BI data amenable to mathematical analysis, which can resolve the dynamic interactions of complex physiological processes.

When considering the present trend toward making microscale analytical instruments, it is intriguing to speculate how much BI could be scaled down. At this nascent stage of development, microliter amounts of beads are typically trapped in a jet ring cell, whereas flow channels have 0.5-mm diameters and the flow rate is in the microliter-per-second range. The downscaling of a BI system can be conceptualized in two ways. Conventionally, one could reduce the entire system proportionately—all dimensions, including the beads, would be decreased by a couple orders of magnitude. There is, however, an intriguing alternative: A typical bead of ~125 µm is quite large by microfabrication standards, but its internal volume is <1 nL (a bead of ~25 µm = 10 pL), making it an ideal vehicle for microchemistries. More significantly, a serious caveat to practical microfabricated devices is surface contamination that causes carryover and loss of analyte. This problem can be easily avoided with the use of carrier beads, because they contain both the analyte and the reagent and are replaced for each measurement.

In addition, current microfabricated devices based on semiconductor technology are limited to a few select materials and require a highly specialized facility for their fabrication. Moreover, microfabricated devices are typically interfaced with lasers, detectors, and vulnerable fluidic systems that are anything but miniature or practical. In contrast, BI technology is based on proven technologies and can use a variety of commercial
components already available. In fact, a large variety of beads are currently available and ready to use. Fluidic components for injection, transport, and capture of bead suspensions are easily fabricated in standard machine and electronic shops because they are at a mesofabrication scale instead of a microfabrication scale. Consequently, BI is capable of producing microanalytical results at a mesofabricated level.

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