

Recent developments in automatic solid-phase extraction with renewable surfaces exploiting flow-based approaches

Manuel Miró, Supaporn Kradtap Hartwell, Jaroon Jakmunee, Kate Grudpan, Elo Harald Hansen

Solid-phase extraction (SPE) is the most versatile sample-processing method for removal of interfering species and/or analyte enrichment. Although significant advances have been made over the past two decades in automating the entire analytical protocol involving SPE via flow-injection approaches, on-line SPE assays performed in permanent mode lack sufficient reliability as a consequence of progressively tighter packing of the bead reactor, contamination of the solid surfaces and potential leakage of functional moieties.

This article overviews the current state-of-the-art of an appealing tool for overcoming the above shortcomings, so-called bead-injection (BI) analysis, based on automated renewal of the sorbent material per assay exploiting the various generations of flow-injection analysis. It addresses novel instrumental developments for implementing BI and a number of alternatives for on-line chemical-derivatization reactions, and it pinpoints the most common instrumental detection techniques utilized. We present and discuss in detail relevant environmental and bioanalytical applications reported in the past few years.

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1. Introduction

The complexity of the matrices of samples in environmental, biological, industrial and biotechnological fields along with the low concentration levels of target species that might be encountered pose severe dilemmas for their direct analysis, even when exploiting modern analytical instrumentation. This is a result of the dependence of the analytical read-outs upon concomitant matrix components, and the fact that the concentration of analytes is often below the dynamic linear range of the detection device. Hence, there is a need, prior to the quantification step, for the development of simple, robust and reliable sample-pretreatment procedures, which are aimed at removing interfering matrix constituents and at the same time improving analyte detectability by preconcentration. When performed in a manual fashion, these preliminary operations are labor intensive and time consuming, difficult to control systematically, and a source of major bias and accidental errors (e.g., sample contamination) that might have a decisive impact on the accuracy and the precision of the analytical results.

The advent of the various generations of flow injection (FI) (i.e. FI, sequential injection (SI), lab-on-valve (LOV)) and

hybrid techniques for on-line or in-line sample processing has opened new perspectives as regards automation and miniaturization of sample handling with the extra advantage of saving sample and reagent consumption and reducing waste generation [1,2].

This review illustrates the current state-of-the-art of the so-called bead-injection (BI) technique, which performs the entire solid-phase extraction (SPE) procedure in a renewable fashion in flowing systems. In contrast with earlier reviews dealing with BI [3–6], this article critically discusses the overall platforms described in the literature for BI-based SPE analysis and illustrates alternative approaches for uptake and detection of target species. We also describe in detail pros and cons of BI assays, compared with conventional particle-packed microcolumns, for flow-based SPE, via selection of representative examples in the environmental and bioanalytical fields, as reported within the past decade.

2. On-line solid-phase extraction

Sorptive extraction is the predominant sample-processing method to be automated by resorting to flow systems as a consequence of its straightforward operation, high separation and preconcentration capabilities and minimum consumption of organic solvents. Most often, it is employed by using packed-bed or disk-phase-based microcolumns, which are filled with appropriate sorptive materials and placed within the flow network prior to the detection device.

The ultimate goal is to improve the sensitivity of the analytical procedure and/or overcome the inherently low tolerance of the detection system to sample constituents by preconcentrating the analyte or removing interfering components from harsh milieus (e.g., high-salt or protein-content matrices) with no need for sample dilution. Several solid-phase reactors with different designs, mainly uniformly-bored or conical microcolumns, have been successfully assembled in flow-based set-ups. The temporary retention of low-level concentrations of individual metal ions, nutrients and charged (bio)molecules by electrostatic interactions onto ion-exchange microcolumns or chelating reactors has been a common practice in flow-through SPE methods [7]. Derivatized non-polar metal chelates (e.g., iminodiacetates, dithiocarbamates, dithiophosphates or quinolinates) or hydrophobic species (e.g., organic pollutants, dyes and drugs) have been preconcentrated on reversed-phase materials (e.g., octadecyl-chemically modified silica gel, polytetrafluoroethylene (PTFE) beads or turnings, or multimodal polymeric sorbents) by partitioning, hydrophobic, or π - π interactions [1,7]. The target compounds are either directly retained on the reactive surfaces after appropriate sorbent conditioning or in-line derivatized into a suitable chemical form. This extraction

mode circumvents interfering effects from alkaline and alkaline-earth elements when determining hazardous trace metals via intelligent selection of the chelating reagent [1].

As detailed above, flow-through SPE procedures have been implemented using permanent packed-column reactors. However, these sorptive columns are prone to problems in long-term operation due to the progressively tighter packing or clogging of the column material resulting in increase of back pressure, which might be alleviated to some extent by back-flushing elution protocols. Further drawbacks of on-line SPE with reusable surfaces are analyte carry-over effects, shrinking or swelling of the sorbent bed, malfunctions of the active entities including loss of functional moieties (the last of these being a common problem for reagent-impregnated bead materials) and, finally, surface deactivation due to irreversible sorption of interfering species. All these problems can be eliminated by exploiting the concept of renewable surfaces, or, as it has been termed, BI, i.e. where the solid-phase material, if called for, can be renewed for each analytical cycle.

3. The concept of bead injection

The BI approach originally adapted to optical sensing in SI analyzers was introduced as a powerful tool for the automation of immunoassays using diffuse reflectometric detection [4]. The miniaturized BI chemosensors use minute amounts of beads carrying the derivatization reagent or functional moieties, whereby the analyte becomes preconcentrated and can be monitored via optical fibers in custom-built flow-through cells that capture the active sensing surfaces while allowing the perfused liquid to flow freely [4]. After each analytical run, the sorbent particles are disposed by flow reversal and the sensor surface is renewed by injecting a new plug of a fresh bead suspension. This concept of optical sensing at solid surfaces, also termed solid-phase optosensing or BI spectroscopy, constitutes an attractive alternative to on-line SPE procedures with eluate detection for preventing the partial loss of the preconcentration capabilities gained during the sorption step as a result of dilution of the eluate plug when delivered to the detector [8]. Moreover, BI fosters real-time monitoring of the sorption process and thus detection relying on initial rate measurements can be readily accomplished. A further advantage of BI derived from its characteristic renewability is its inherent flexibility in accommodating reagent-based SPE assays with no stringent demands as to the full reversibility of the sorption or elution procedure. This is the case in uptake of Fe(II)-1,10-phenanthroline onto chelating beads because eluents, such as nitric acid or EDTA, are inefficient for quantitatively stripping out the chelate from the beads [9].

Notwithstanding the fact that both optical paths and reaction times in BI-based schemes are shorter, compared with steady-state liquid-phase spectrophotometry, no deterioration in sensitivity is observed as a result of immobilizing the analyte on a small surface area of the sensor wherein the reaction is driven by an excess of solid reagent [4].

In order to handle the bead material reproducibly within the automatic system, it is imperative to ensure bead-size homogeneity and the spherical shape of the reagent-supporting entities so as to prevent compact settlement into the conduits of the flow set-up. Hence, reversed-phase, chemically-modified, silica-gel lumps are not really suited for this purpose as a result of their irregular shape and size distribution [5]. However, bead material with a backbone of poly(styrene-divinylbenzene), poly(vinylpyrrolidone) or agarose (e.g., Sephadex or Sepharose-type beads) fulfils the foregoing demands, inasmuch as they are perfectly spherical and have a uniform size. The use of either micellar media or ancillary continuous recirculation schemes for the bead suspension [10] might be needed for reliable manipulation of hydrophobic sensing entities with higher density than water within the flow manifold.

4. Development of instrumentation

4.1. Jet-ring cell and rotating-rod flow cell

The instrumentation development for BI, initiated by Ruzicka and co-workers [4], is based on an SI network furnished with the so-called jet-ring cell. The name “jet-ring” cell comes from the configuration and operation of the bead-retention cell made as part of the flow conduit by inserting a solid rod to block the beads in the assigned space. As shown in Fig. 1, the channel of the cell has an inner diameter slightly larger than the outer diameter of the blocking rod, forming the O-ring gap between the rod and the wall of the channel all around the rod. Only solutions are able to flow out while beads whose sizes are

larger than the gap are packed due to the continuous incoming flow pushing the beads against the blocking rod. Used beads are discarded by the jet of solution reversing and rapidly flowing upstream.

For capture and release of beads as small as 5–7 μm in SI manifolds, a novel bead-trapping strategy, the so-called rotating-rod flow cell [11], was designed. The renewable sorbent microcolumn is created by first trapping larger beads ($>15\ \mu\text{m}$) in the flow cell, and then smaller beads bearing functional moieties are captured either within or on top of larger beads, thus acting as a filter layer [12].

4.2. Lab-on-valve

The LOV approach hyphenated to SI set-ups should be viewed as a judicious advance towards automation and miniaturization of BI schemes but within integrated microbore units [13]. The microconduit unit, made initially of Perspex, but more recently of hard polyvinylchloride, polyetheretherketone (PEEK) or polyetherimide (ULTEM), is a single monolithic structure mounted atop the multiposition valve of an SI assembly, as illustrated in Fig. 2. Designed to incorporate all necessary laboratory facilities for a variety of analytical chemical assays, including in-valve manipulation of sorbent materials, hence the name *lab-on-valve*, it is made to contain microchannels working as the bead-retention cell along with mixing points for chemical derivatization of the analyte, if needed, and a multipurpose flow-through cell for real-time interrogation of analyte-containing beads [13,14].

In short, packed-column reactors are generated in situ by aspirating beads from a peripheral port of the valve, advantage being taken of the fact that the sorbent in the LOV unit can be manipulated in exactly the same way as when handling liquids. The solid entities can even be automatically transported between different column positions within the micromachined unit, their retention within the columns being facilitated by fitting the column positions with appropriate stoppers (see Fig. 2), namely, up-down movable rods or optical fibers themselves, which will keep hold of the beads, yet allow solutions to flow freely. In-valve detection could be realized at the eluate in the same way as conventional SPE or by solid-phase optosensing [15].

As opposed to lab-on-chip (LOC) microdevices, the open architecture of the SI-LOV approach facilitates implementation of BI protocols at will with no need for redesign of the unit. Readers are referred to a recent comprehensive review by Miró and Hansen [16] for a critical discussion of the pros and cons of LOV compared to LOC microsystems for automated SPE.

4.3. FI-BI with home-made and commercial flow cells

BI has also been adapted to FI systems as a cost-effective alternative to more sophisticated SI set-ups. The

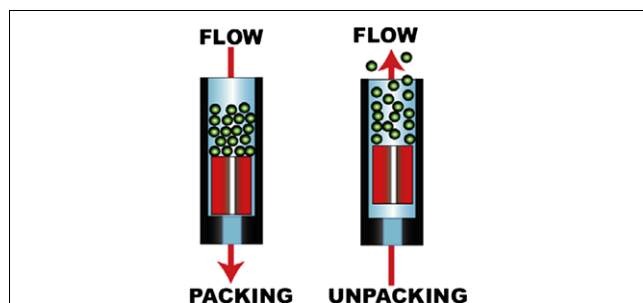


Figure 1. Jet-ring cell and operation of bead injection (packing and renewal steps) (Reproduced with the author's permission from J. Ruzicka, Flow Injection Analysis CD-ROM Tutorial, 3rd Edition, 2004; Published by FIALab Instruments, Inc.).

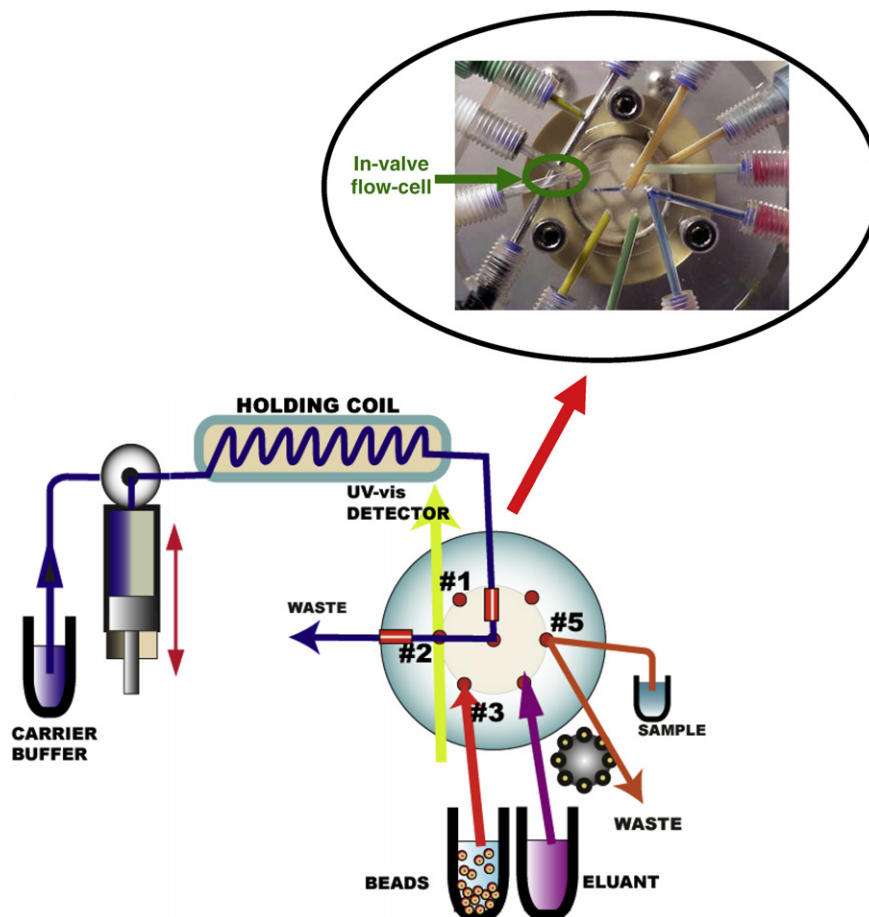


Figure 2. An SI-LOV set-up for BI optosensing. The inset shows a magnified close-up of the micromachined LOV unit (Reproduced with the author's permission from J. Ruzicka, Flow Injection Analysis CD-ROM Tutorial, 3rd Edition, 2004; Published by FIALab Instruments, Inc.).

home-made bead-retention cell introduced by Grudpan's group [9,17] was modified from the jet-ring cell to suit the uni-directional flow normally employed in FI systems. The dedicated cell was furnished with an LED-based light source and a phototransistor detector at the opposite ends of the cell windows. Similarly to the jet-ring cell and LOV unit, the blocking rod leaves a minute space between the rod and the exit channel to let solution flow through while beads are retained. A mechanically movable blocking rod is used to control bead trapping and discarding instead of flow reversal. A solenoid actuator connected to the rod controls its movement. After each analysis, the rod is pulled back far enough to open the outlet and let the used beads flow out.

Commercially-available flow-through cells commonly used in FI liquid-phase spectrophotometric detection have been adapted to BI spectroscopy by Molina-Díaz and co-workers [18–20]. Glass wool is used to plug the outlet of the flow cell to keep beads inside. Bead discarding is done by backward flushing the beads out with a high flow-rate solution using an ancillary uni-directional peristaltic pump. Two Hellma-type flow cells,

namely, 138 OS (1 mm light path, 50 μL inner volume) and 176 QS (1.5 mm light path, 25 μL inner volume), were used for spectrophotometric [18,20] and fluorometric [19] optosensing, respectively. The configurations of both flow cells can be found in Hellma's cell catalog and in works published by Molina Díaz's group [18–20]. However, according to the current Hellma catalog, the flow-cell model 138-OS is no longer available. A drawback of the commercial flow cells is that the detection window is smaller than the bead volume, so not all product sorbed is monitored.

4.4. Magnetic flow-through cell

Ruzicka and co-workers [21] proposed in the 1990s a tubular BI cell design for magnetic bead-based SI immunoassays with detection of unbound labeled antibodies in the liquid phase, which was later extended to solid-phase optosensing by Imato's group [22,23] using a dedicated configuration for BI with luminescence detection. The magnetic beads involving functionalized agarose-coated iron-oxyhydroxide particles are suitable for assays (e.g., immunoassays) involving several operational steps. A neodymium magnet or magnetic wire

located under the (immuno)reactor is moved up and down to trap the magnetic beads in place or let them flow with carrier solution. Fig. 3 shows the configurations of the magnetic flow cell and SI network for BI-based chemiluminescence bioassays. In this open tubular cell design, the continuously flowing stream of solution is less likely to cause bead packing or clumping during the multi-steps of washing and incubation required in immunoassays. The most important limitation of this flow configuration for BI stems from the metallic nature of the bead core, which impedes monitoring of the development of chemical reactions at low pH values.

5. Analytical procedures for bead injection

5.1. Bead modification

Although commercially-available ion exchangers or (co)polymeric beads can be directly utilized for BI, raw sorbents might be modified in the batch mode to incorporate appropriate moieties by physical or chemical binding with the bead surface or matrix for selective sorption or reaction with the analyte. As a consequence

of the renewable nature of beads, BI is well suited to overcoming the most severe drawback of SPE procedures involving physical immobilization of reactants, i.e. the short lifetime of the loaded sorbent because of the gradual flushing out of the reagent during sample percolation.

For bioassays, antibodies or antigens [15,21–25] have been immobilized on magnetic [21,22] or polysaccharide-based [24,25] beads. The latter have also proved suitable as substrate for cell cultivation [26,27].

For environmental assays of trace metals, polymeric beads have been loaded with ligands (e.g., carbazides or azo compounds) off-line [28–30] because the time frame for quantitative impregnation of the bead surface can then be selected at will regardless of ligand-sorption kinetics. Compared with on-line protocols, higher reagent concentration on the sorbent material is then ensured, which, in turn, should lead to improved enrichment factors and analyte-breakthrough volumes. However, optimization of immobilization protocols (pH and reaction time) by measuring the rate and the yield of reactions could be performed at-line in SI-LOV by attaching a stirred bead microreactor to a peripheral port

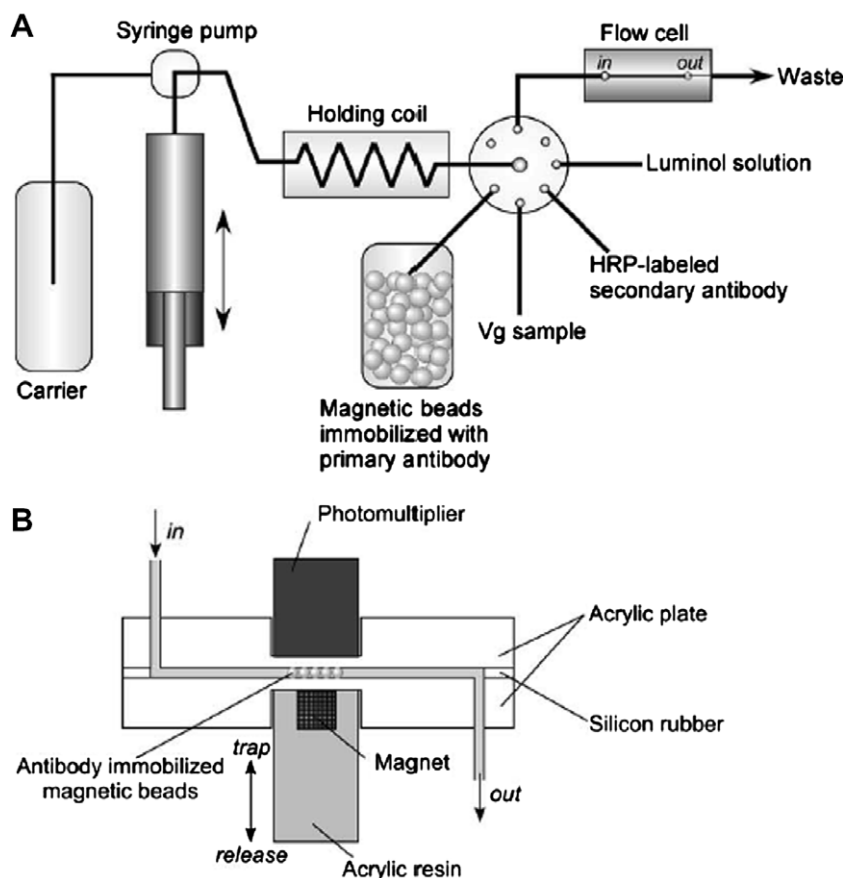


Figure 3. (A) SI manifold for chemiluminescence immunoassay of vitellogenin using magnetic microbeads (B) Dedicated flow-through cell for retention of antibody bearing magnetic beads. Vg, Vitellogenin; HRP, Horseradish peroxidase (Reproduced from [22] by permission of Elsevier Ltd.).

of the selection valve and real-time monitoring of ligand uptake on bead surfaces [31].

5.2. Analyte uptake

Sorption of the analyte itself onto renewable ion-exchange resins has been common practice for SI-LOV-BI determination of trace-metal ions [5], although it could equally be exploited for uptake of charged drugs or biomolecules followed by UV-Vis or fluorometric measurements [32,33].

Analytes could in principle also be retained on the sorbent prior to addition of a chromogenic agent for heterogeneous on-bead derivatization in solid-phase spectrophotometric measurements. For example, copper [34], iron [9] and mercury [35] were separated from sample matrices via sorption onto chelating resins prior to derivatization with ammonium pyrrolidine dithiocarbamate, 1,10-phenanthroline and dithizone, respectively. However, the approach is limited to those applications where the color-development reaction proceeds rapidly with no elution of the chelate formed.

To tackle the above drawback, researchers have exploited homogeneous phase derivatization, i.e. the analyte reacts in-line with the ligand before sorption of the resulted products on beads. This reaction might lead to improved retention selectivity and increased affinity of the analyte to the sorptive material. For example, trace metals after derivatization into a non-charged chelate or (co)precipitate are selectively sorbed on reversed-phase (e.g., PTFE or octadecyl-chemically-modified copolymeric) packing materials [18,36,37]. Indirect determination of drugs (e.g., promethazine and trifluoperazine) could be effected by on-line oxidation with Fe(III) with subsequent complexation of the resulting Fe(II) with ferrozine followed by solid-phase spectrophotometric detection of the chelate onto anion exchangers [18].

5.3. Detection techniques

A notable asset of BI is the inherent capability for hyphenation to many instrumental devices. Though most frequently used in combination with spectrophotometry [9,15,17,20,25,35], BI spectroscopy also has applications involving fluorometric [12,19] and chemiluminescence [22,23] detection. The most important requirement for BI spectrophotometry is the optical transparency of beads to prevent high signal background and light-scattering effects. It should be noted that not merely single-parameter but biparametric BI-based spectrophotometric or spectrofluorometric sensors have been designed for determination of trace elements (e.g., Zn and Cu or Al and Be) using a non-selective chelating reagent and selective elution or sorption protocols [19,20]. Unfortunately, sequential assays were required because of the limited chromatographic resolution of the BI columns.

Coupling BI to electrothermal atomic absorption spectrometry (ET-AAS) with direct introduction of metal-loaded sorbent into the graphite furnace is feasible for polymeric organic beads (e.g., Sephadex or Sepharose) because of pyrolysis of the beads prior to metal atomization [5]. Besides the aforementioned detectors, BI has been coupled to voltammetric [26], inductively coupled plasma-mass spectrometry (ICP-MS) [5], atomic fluorescence spectrometry (AFS) [37,38], MS [39] and radio-metric [40] instruments, although eluate detection was called for in all instances.

6. Applications of flow-through bead injection

In this section, we review relevant applications of BI in both environmental and bioanalytical fields. Table 1 shows the analytical performance of such methods, including the sorbent material, the flow approach, the detection technique, the dynamic linear range, the detection limit, the precision and the sample matrix.

6.1. Environmental assays

Most of the work conducted in this field has focused on determination of trace-level concentrations of inorganic elements in harsh environmental matrices containing high levels of dissolved salts, such as seawater, brines or soils [29,36,41]. To this end, matrix separation with concomitant metal/metalloid enrichment has been effected using SI-LOV-BI preconcentration and eluate detection by ET-AAS or AFS. SI-BI is also a suitable platform for accommodation of trace-metal-speciation assays (e.g., Cr(III) and Cr(VI)) via selective retention of oxidation states in appropriate sorbent materials (e.g., cation and anion exchangers) or via sequential determinations of a single species and total inorganic metal following in-line homogeneous/heterogeneous redox reactions and preconcentration in a given sorptive microcolumn [42]. Readers are referred to a comprehensive review article [43] for further details as to the designs and analytical performance of SI-BI-LOV methods for trace-metal determinations in environmental matrices.

Recent trends are devoted to the exploitation of hybrid flow systems (e.g., multi-syringe flow injection (MSFI) analysis) in combination with BI-LOV to expand the scope of the miniaturized systems [38,44]. It should be borne in mind that SI-BI-LOV manifolds are not well suited to implementation of post-LOV derivatization reactions because of the need in SI-LOV to aspirate the overall solutions in a holding coil. However, interfacing BI-LOV with MSFI enabled the sorptive preconcentration and determination of hydride-generating species by on-line derivatization of eluate with sodium tetrahydroborate prior to AFS detection [38]. Despite the divergent kinetics of analyte sorption and hydride generation,

Table 1. Analytical performance of representative flow-based sorbent extraction methods involving bead injection analysis

Analyte	Flow system	Bead type	Detection technique	Detection limit	Linear range	Precision	Sample/ Application	Ref.
Metal and metalloid species								
Fe	FI	Iminodiacetate chelating resin (Chelex-100)	Spectrophotometry	0.2 µg/L	0.5–6 µg	NR	Beer	[9]
Cd	SI-LOV	C ₁₈ -PS/DVB and PTFE	ET-AAS	5 ng/L (PTFE) and 135 ng/L (C ₁₈)	0.05–1µg/L (PTFE) and 0.2–1.5 µg/L (C ₁₈)	4.3% (PTFE) and 3.4% (C ₁₈)	CRM 279 (Sea Lettuce), CRM 320 (River sediment), NIST 1640 (Natural water) and human urine	[10]
Fe	FI	Chelex-100	Spectrophotometry	25 µg/L	Up to 217µg/L	4%	Spiked tap and drinking water	[17]
V(V)	FI	Sephadex QAEA-25 loaded with ARS	Fluorometry	0.45 µg/L	2–60 µg/L	4.22%	Water, serum, urine and mussel tissues	[19]
Cu and Zn	FI	Sephadex QAEA-25 loaded with Zincon	Spectrophotometry	29 µg/L Cu and 40 µg/L Zn	50–1000 µg/L Cu and 100–1800 µg/L Zn	< 5%	Water, pharmaceutical preparation, soil and hair	[20]
Co	FI	Dowex 50 W loaded with PAN	Spectrophotometry	19 µg/L	50–2000 µg/L	4.16%	Water, pharmaceutical formulation, steel	[28]
Cr(VI)	SI-LOV	C ₁₈ -PS/DVB impregnated with DPC	ET-AAS	0.03 µg/L	0.12–1.5µg/L	3.8%	NIST 1640 (Natural Water), tap water and seawater	[29]
Pb	SI-LOV	Sephadex G-25 impregnated with dithizone	ET-AAS	0.3 ng	1–4 ng	1.9%	Standard solutions	[30]
Cu	FI	Chelex-100	Spectrophotometry	10 µg/L	0.01–1.0 mg/L	NR	Swimming pool water and supplement tablets	[34]
Cu and Hg	SI	Chelex-100	Spectrophotometry	0.63 µg/L Cu and 0.25 µg/L Hg	5–500 µg/L Cu and 2.5–30 µg/L Hg	5.2% Cu and 9.4% Hg	River water	[35]
Ni	SI-LOV	poly-DVB-co-N-vinylpyrrolidone	ET-AAS	0.05 µg/L	0.2–2 µg/L	< 5.6%	Hard tap water, seawater and NIST 1640	[36]
Cd	SI-LOV	C ₁₈ -PS/DVB	Hg-AFS	3.5 ng/L	NR	1.6%	CRM 320 (River sediment), CRM 279 (Sea Lettuce) and GBW 09139 (Cattle blood)	[37]

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Table 1 (continued)

Analyte	Flow system	Bead type	Detection technique	Detection limit	Linear range	Precision	Sample/ Application	Ref.
Metal and metalloid species								
As	SI-LOV- MSFI	Q-sepharose (anion exchange)	HG-AFS	20 ng/L	0.05–2.0 µg/L	5.7%	Tap and underground water and SRMs (TMDA-54.3 Lake water and ERM- CA010-Hard drinking water)	[38]
Cr (VI)	SI-LOV	Q-sepharose (anion exchange)	ET-AAS	NR	0.02–0.6 ng	< 6.8%	SRM 2709 San Joaquin Soil/On-line leaching investigations	[41]
Cr(III) and Cr(VI)	SI-LOV	Chelating Sepharose	ET-AAS	0.010 µg/L Cr(III) and 0.020 µg/L Cr(VI)	0.02–0.28 µg/L Cr(III) and 0.035–0.4 µg/L Cr(VI)	4.7% Cr(III) and 4.5% Cr(VI)	Environmental waters, NIST 1640 and CRM 320	[42]
Radionuclides								
⁹⁰ Sr, ²⁴¹ Am, and ⁹⁹ Tc	SI	Sr-resin, TRU-resin, TEVA resin (Eichrom industries)	Liquid scintillation	NR	NR	3% ⁹⁰ Sr	Nuclear waste	[40]
Bioanalytical applications								
Biotin, DNA, rabbit IgG	SI	Lumavidin-coated beads, peptide nucleic acid-modified beads, Protein A-coated beads	Flow-cytometer fluorometry	NR	1–100 µg/L (biotin) and g/L level for DNA and IgG	< 25%	Binding assays/ sandwich immunoassays/ multiplexed analysis	[12]
IgG	SI-LOV	Sepharose with immobilized protein G	Spectrophotometry	5 mg/L (AC) and 50 mg/L (BIS)	0.100–1.00 g/L (AC) and 0.100–0.400 g/L (BIS)	NR	Human IgG, rabbit IgG, horse IgG	[15]
Mouse IgG	SI	Magnetic beads with immobilized polyclonal sheep anti mouse IgG	Fluorometry	155 µg/L	1–10 mg/L	2.1%	Competitive binding assays of IgG	[21]
Vitellogenin	SI	Magnetic beads with immobilized anti-vitellogenin antibody	CL	2 µg/L	2–100 µg/L	NR	Standard solutions	[22]

Goat, anti-mouse IgG	SI-LOV	Protein G-coated Sepharose 4B and Protein A-coated Sepharose 4B	Spectrophotometry	470 ng	0–0.4 g/L	NR	Interaction of antibodies with protein A and protein G via label dilution	[24]
GAD 65 autoantibody (type I diabetes)	SI-LOV	Streptavidin-coated Sepharose 4B	Spectrophotometry	20 µg/L	100–400 µg/L	< 5%	Human serum/ELISA	[25]
H ₂ O ₂	SI-LOV	Live cells on Cytodex-2 beads	Voltammetry	9 µM	10–1000 µM	NR	Degradation rate of H ₂ O ₂ by wild-type cells or catalase-overexpressing mouse embryonic fibroblasts	[26]
pH	SI-LOV	Sephadex G-50 with impregnated pH indicator/ Cytopore with immobilized live cells	Spectrophotometry	NR	7.2–7.4	< 8.38%	Investigation of extracellular acidification rates of the mouse hepatocyte cell line TABX2S	[27]
Albumin, ovalbumin, lysozyme, human IgG, ribonuclease A and cytochrome C	SI-LOV	Aminolink agarose beads	Spectrophotometry	NR	g/L level	NR	Examination and optimization of protein immobilization protocols	[31]
Biotin-containing conjugates and β-galactosidase	SI-LOV	Streptavidin-coated agarose beads	UV/Vis and ESI-MS	NR	mg/L level	NR	Determination of β- galactosidase activity in human cell homogenates/ Kinetic measurements of dissociation rates of model biotin- containing conjugates	[39]
Lactate and glucose	SI-LOV	Cytopore with immobilized live cells	Spectrophotometry	NR	0.05–1.00 mM (lactate) and 0.1–5.6 mM (glucose)	< 7.3% (lactate) and 7.1% (glucose)	Lactate extrusion and glucose consumption by mouse hepatocyte cell line TABX2S and TABX1A	[47]

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FI, Flow injection; SI, Sequential injection; LOV, Lab-on-valve; MSFI, Multi-syringe flow injection; ET-AAS, Electrothermal atomic absorption spectrometry; CL, Chemiluminescence; ESI-MS, Electrospray ionization-mass spectrometry; HG-AFS, Hydride generation-atomic fluorescence spectrometry; C₁₈-PS/DVB, Poly(styrene-divinylbenzene) copolymer alkylated with octadecyl groups; PTFE, Polytetrafluoroethylene; ARS, Alzarin Red S; PAN, 1-(2-pyridylazo)-2-naphthol; DPC, 1,5-diphenylcarbazide; AC, Affinity chromatography; BIS, Bead-injection spectroscopy; ELISA, Enzyme-linked immunosorbent assay; NSAID, Non-steroidal anti-inflammatory drug; NR, Not reported.

optimal experimental conditions for both reactions were affixed in a sole flow network due to the discontinuous flow nature of the MSFI technique and the flexibility of the approach for providing variable flow rates at will [38].

BI in an LOV approach has not been merely applied to sorptive preconcentration of metal species but organic pollutants as well prior to reversed-phase liquid chromatographic (LC) separations, as demonstrated by determination of anti-inflammatory drugs in raw wastewaters using hydrophobic/hydrophilic co-polymeric beads with no need for ancillary sample treatment [44]. The marriage of BI-LOV with MSFI proved suitable for quantitative elution of sorbed drugs whilst efficient LC band focusing of eluate was also ensured.

6.2. Cellular assays/activities

Cellular assays are crucial for studies of new pharmaceutical candidates and their reaction protocols. These studies normally involve loading of various doses of a given drug on cells, monitoring the reaction and detecting cell responses in batch mode. These procedures require techniques that can handle living cells reliably by preventing deterioration of cells, while assuring reproducible timing and precise injection of minute volumes of reagents. BI has played an outstanding role in the improvement of cellular assays [45].

In 1999, Hodder and Ruzicka [46] introduced a novel application of SI-BI by using beads as micro-carriers of living cells to measure elevation of intracellular calcium. A high density of cells could be trapped in the small

inner volume of a jet-ring cell, which was placed above a fluorescence-microscope-objective lens. The beauty of this approach is that a fresh portion of cells is exposed to a given concentration of chemical and replaced for each assay. The investigation of cell response to a given drug or chemical is thus done more accurately than classical approaches because of the absence of carry-over effects and loss of cell activity over time. The continuous monitoring of signal changes at the beads provided peak profiles that yielded valuable information on the kinetics of cellular assays and the concentration threshold of reagent, agonist in this case, that causes cell response.

Similar uses of beads as micro-carriers for living cells were extended to LOV [26]. For example, consumption of glucose and extrusion of lactate could be detected sensitively and rapidly due to the high concentration of immobilized cells [47]. The exploration of cellular activities based on the release of protons from cells in LOV was also reported [27]. Though BIs were done manually here, two micro-packed bead columns were uniquely created within one section of the LOV micro-channel, as shown in Fig. 4A. Beads carrying cells were retained upstream to the beads with immobilized pH indicator. Protons extruded from cells were accumulated during the stopped flow and were pushed to react with the immobilized indicator, causing the color change on the indicator beads which could be in-valve monitored by solid-phase spectrophotometry (see Fig. 4D). The flow set-up illustrated in Fig. 4B was proposed as an alternative to more complicated molecular devices and to replace the light-addressable potentiometric sensor for measurement of

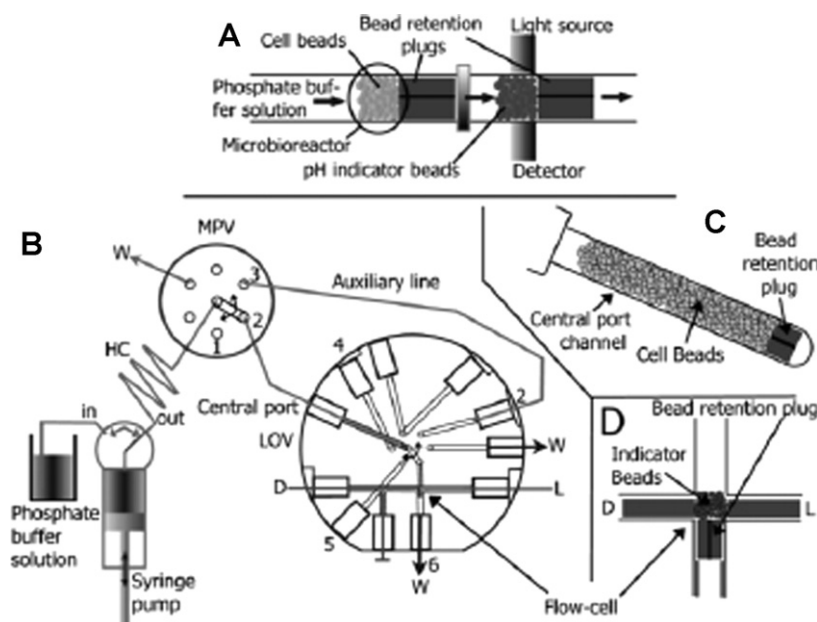


Figure 4. An SI-LOV manifold for measurement of proton extrusion from living cells using two different packed-bed columns. A), C), and D) Magnified views of bead columns (cell beads and pH indicator beads) implemented within LOV microconduits. B) Detailed diagram of SI components for BI spectroscopy of pH indicator beads (Reproduced from [27] by permission of Royal Society of Chemistry).

extracellular acidification rates, which has been utilized frequently for evaluation of metabolic functions, but which is no longer commercially available.

6.3. Kinetic affinity studies and affinity chromatography

In 2002, Ogata et al. [39] demonstrated that BI-LOV could be used effectively as an automatic system for investigation of kinetics of affinity capture and release of biomolecules. Model studies were conducted with a well-known non-covalent binding between streptavidin-coated beads and biotinylated conjugates. This group devoted further work to develop the beta-galactosidase assay in human-skin fibroblasts using on-line ion-exchange and electrospray ionization-mass spectrometry (ESI-MS) [39]. This work revealed the flexibility of LOV as a front end to many analytical instruments besides optical-fiber spectrophotometers.

A similar miniaturized set-up was exploited by Ruzicka and co-workers for critical comparison of BI spectroscopy with affinity chromatography (or more precisely "affinity extraction"), which involved eluate detection, in the course of bioassays [15]. Preliminary studies showed that results obtained from the two different modes of detection complemented each other and gave a complete picture of biomolecular association and dissociation between target biomolecules and bioligands immobilized onto bead surfaces. However, while affinity chromatography offers improved sensitivity, compared to BI spectroscopy (detection limits are >10-fold better), BI spectroscopy offers unique possibilities for monitoring biomolecules irreversibly bound to the chromatographic column.

6.4. Immunoassays

The precise micro-fluidic manipulation and timing inherent in SI-LOV, combined with the concept of renewable solid surfaces, make it possible to perform multi-step protocols (e.g., immunoassays) automatically [24,25]. In-valve bead retention was accomplished using jet/nozzle plug and fiber optics, as previously designed for other LOV applications. Both label dilution immunoassay for determination of goat, anti-mouse IgG [24] and ELISA for detection of GAD65 antibodies [25] were demonstrated. Although low sample throughput is a limitation, compared to standard 96-well plate immunoassay, the much shorter time per assay cycle (less than 30 min vs. 5–8 h) is suitable for immediate clinical feedback and low-to-medium numbers of samples. In addition, the micro-fluidic, computer-controlled analyzer requires much lower sample volume (50 μ L or less vs. 200–400 μ L) and precludes the need for experienced medical technicians.

6.5. Purification and quantitation of DNA and specific biomarkers

BI-LOV has recently been proposed as a platform for determination of nucleic acids. The use of silica gel as a

renewable SPE material for purification of DNA from whole human blood was demonstrated [48]. Beads were packed within an LOV cavity to retain DNA, which was later eluted for PCR amplification and electrophoretic separation. DNA purification was monitored on-line by post-column fluorimetry using ethidium bromide as a derivatization reagent. Other researchers used specially functionalized beads for elucidation and quantitative determination of specific DNA sequences [49,50]. The improved sensitivity gained by BI-spectroscopic detection allowed determination of DNA at the pmol level [49]. This system might ultimately be applied to any assay involving identification of life-threatening micro-organisms.

Hartwell et al. [51] incorporated wheat-germ-bearing beads in an FI-BI assembly for determination of bone-specific alkaline phosphatase. Detection can be done easier than with the conventional wheat-germ-agglutination technique. In addition, the increase in surface area of micro-beads for immobilization of reagent with respect to the inner surface of the micro-well used in ELISA permits better differentiation between the osteoporosis-patient group and the normal group.

Readers are encouraged to peruse critical reviews [32,33] for a comprehensive overview of LOV applications within the bioanalytical field.

7. Future trends

It is to be expected that BI in the different formats will be further developed in the years to come for high-throughput analysis or screening in various analytical fields. Coupling of LOV with affinity chromatography with various detection systems, including BI-spectroscopy, MS, amperometry or fluorometry, is expected to be well suited to current demands in bioanalytical sciences, including natural-product research, drug discovery, and protein and DNA analyses, as well as in clinical and medical analyses for rapid screening and diagnostics.

SI-LOV-BI offers significant potential in the environmental field for removal of matrix ingredients in leachates and concomitant analyte enrichment following on-line leaching tests of environmental solids (e.g., soils or sediments) packed in microcolumns attached to the peripheral ports of a multi-position valve [41]. It should be noted that there has not yet been any report on any attempt to investigate the sorptive capabilities of materials (e.g., soil layers for trace elements) by utilizing flow systems furnished with on-line solid-column reactors. Leaching or sorption schemes might be extended to radioisotopes as tracers or essential ingredients of isotope dilution in geochemical studies or as analytes in nuclear technological studies involving nuclear waste storage and management. Applications of BI systems with radiochemical detection for automation in handling radioactive materials in a closed system would also be

very useful practice for nuclear medicine and nuclear reactor management.

It would be also interesting to explore the combination of BI with field-flow fractionation as a tool for gaining knowledge on size and distribution of low-level concentrations of macromolecules. This should be useful for environmental and clinical investigations.

As regards to new sorptive materials, we expect molecularly-imprinted polymeric spheres, prepared by suspension polymerization, precipitation polymerization or multi-step swelling polymerization [52], which feature improved selectivity for a given target analyte or group analogous, to be exploited for BI spectroscopy or BI sorbents prior to LC separations.

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