Sequential affinity chromatography miniaturized within a "lab-on-valve" system

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An automatically renewable microcolumn, subjected to operation by programmable flow, is presented and for the first time used for separation and quantification of biomolecules on Sepharose Protein A beads, with absorbance measurement at 280 nm and a detection limit of 6 ng mouse IgG μL^{-1}.

Affinity chromatography for the assay of biomolecules is traditionally carried out on prepacked columns (volume 1 mL), using a conventional chromatographic setup comprising of a pump, injector, and UV detector.[1] It is based on molecular recognition between a site fixed on a stationary phase and a target analyte that is selectively captured while unwanted species are carried away by the mobile phase. In the second step, a desired biomolecule is released by changing the composition of the mobile phase, eluted into the detector, and quantified.

Since immunoaffinity chromatography is most widely used,[1] it was selected here as a model system to demonstrate advantages of programmable flow for separations of immunoglobulins (IgG) on a microcolumn made of Protein A-coated Sepharose beads.

The use of programmable flow for chromatographic separation was originally proposed by Satinsky *et al.*[2] and termed sequential injection chromatography. It was applied to the assay of methylparaben, propylparaben, and butylparaben in a pharmaceutical preparation. The instrumental setup comprised a sequential injection instrument, where a 5 mL Chromolith column (a reversed-phase), was mounted between the multiposition valve and flow-through detector. Following sample injection and on column adsorption, a nonpolar eluent was aspirated into the holding coil by flow reversal, and then passed, using forward flow, through the reverse-phase column. In this way, nonpolar sample components were gradually released from the hydrophobic stationary phase.

This work presents, for the first time, the use of programmable flow for affinity chromatography in a miniaturized version, whereby the stationary phase can be automatically renewed. Such miniaturization and integration allows substantial improvement of the limit of detection compared to traditional analytical affinity chromatography carried out on 1 mL columns.

Affinity chromatography does not rely on multiple, repetitive processes, which amplify differences in migration velocities, but is based on the release of analyte from the stationary phase caused by a change of the mobile phase composition. Therefore, the size of the column does not have any effect on the degree of resolution and, as a result, the column may be miniaturized, as long as its capacity remains sufficient to retain all of the target biomolecules. This opens door for column downscaling and allows its integration within the lab-on-valve (LOV) module (Fig. 1A). Such a system comprises a 10 μL column, a six-position valve, and a high-precision 1 mL syringe pump, capable of generating high flow rates (up to 200 μL s^{-1}) for column renewal, as well as low flow rates (1 to 5 μL s^{-1}) needed for sample metering, analyte on-column capture, and elution. The instrument relies on software control that allows for versatile and reliable sequencing of events comprising assay protocol and data collection.

The unique feature of the LOV-chromatographic system is the configuration of the column with the flow cell (Fig. 1B). A microcolumn packed with Sepharose beads is held in place by the stainless steel cladding of an optical fiber, which carries light into the flow cell. Flow cell channel dimensions (1.6 mm id) are precisely machined, leaving a gap, 15 μm wide between the channel wall and optical fiber cladding, through which the mobile phase flows but Sepharose beads (size 40–150 μm) cannot pass. Both optical fibers were inserted into the LOV using fittings that closed these channels to flowing liquid, instead forcing it to waste. The length of the optical path (and flow cell volume) is adjusted by moving the fibers into desired positions. For experiments described here, the flow cell volume was 6 μL, optical path was 3 mm, dead volume between column and flow cell was 0.2 μL and column volume was 10 μL. The LOV module was used as purchased, from FIAlab Instruments (www.flowinjection.com). The instrument was

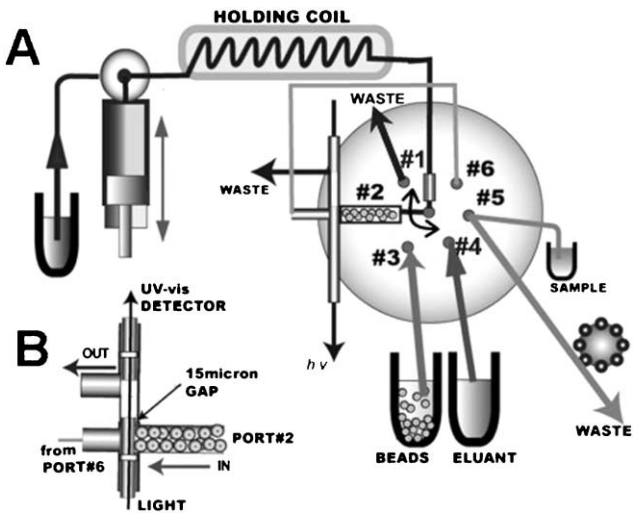

Fig. 1 A: MicroSI lab-on-valve (μSI-LOV) system with integrated renewable microcolumn. B: Detail of fiber optic flow through cell and column packed in conduit leading to port #2. Column is operated *via* port #2, the flow cell is purged between assays *via* port #6.

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a μSIA-LOV system, controlled by FIAlab software for Windows, Ocean Optics spectrophotometer, deuterium lamp, and 600 μm quartz optical fibers.

Since affinity chromatography is used in over 60% of all purification protocols,[1] we have chosen biomolecular separation that has an immediate practical application, such as monitoring of production of monoclonal antibodies exuded by a cell culture. Protein A, from Staphylococcus Aureus, is a polypeptide in the form of a cylinder that has multiple antibody-binding domains recognized by the Fc portion of G immoglobulins. At pH 6 or higher, histidine residues on the Fc portion of IgG and on Protein A are uncharged and hydrophobic, while at lower pH they become charged and IgG is released. To demonstrate the separation of biomolecules with different affinities for Protein A, mouse IgG with a high affinity, chicken IgG with a low affinity, and bovine serum albumin (BSA) with no affinity were used. The reagents were: phosphate buffer/saline[3] as a mobile phase, 0.5 M HCl as the eluant, and beads were obtained by breaking the prepacked 1 mL HiTrap Protein A HP column.[4]

The assay protocol comprised the following steps: 1. purging of the flow cell by forward flow of 100 μL at 200 μL s^{-1} *via* port #6 to remove possible air bubbles, 2. zeroing the baseline absorbance, starting data collection, and sample aspiration into the holding coil from port #5 by flow reversal, 3. sample on-column transport by forward flow using 200 μL at 5 μL s^{-1} through port #2, followed by syringe refill, 4. aspiration of 5 μL of HCl into the holding coil from port #4 followed by forward flow of 65 μL of mobile phase at flow rate of 2 μL s^{-1} through port #2, while monitoring elution of the target molecules. A series of chromatograms of a mixture of BSA and mouse IgG obtained using this protocol is shown superimposed in Fig. 2. Note how the peaks of nonretained analyte broaden with injected sample volume, while the peaks of adsorbed and eluted IgG increase in height. Using the same column and assay protocol, a mixture of mouse IgG and chicken IgG was analyzed next (Fig. 3) and compared with chromatograms obtained with individual m-IgG and ch-IgG samples. The results demonstrate linearity of responses of individual sample components (including unknown impurity, Im, in m-IgG preparation) and perfect repeatability of chromatographic runs.

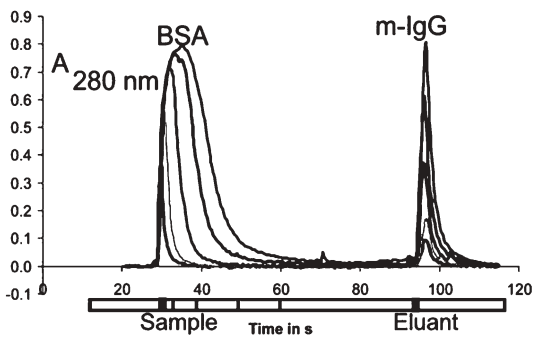

Fig. 2 Five superimposed chromatograms obtained by injecting increasing volumes (5, 10, 25, 50 and 75 μL) of sample comprised of a mixture of 2 mg mL^{-1} mouse IgG (m-IgG) and 4.6 mg mL^{-1} bovine serum albumin (BSA). Column: Protein A, capacity 20 mg mL^{-1} of human IgG, 6% crosslinked Sepharose. Eluant: 5 μL of 0.5 M HCl injected into the mobile phase (PBS). On column flow rate: 5 μL s^{-1}, eluting flow rate: 2 μL s^{-1}.

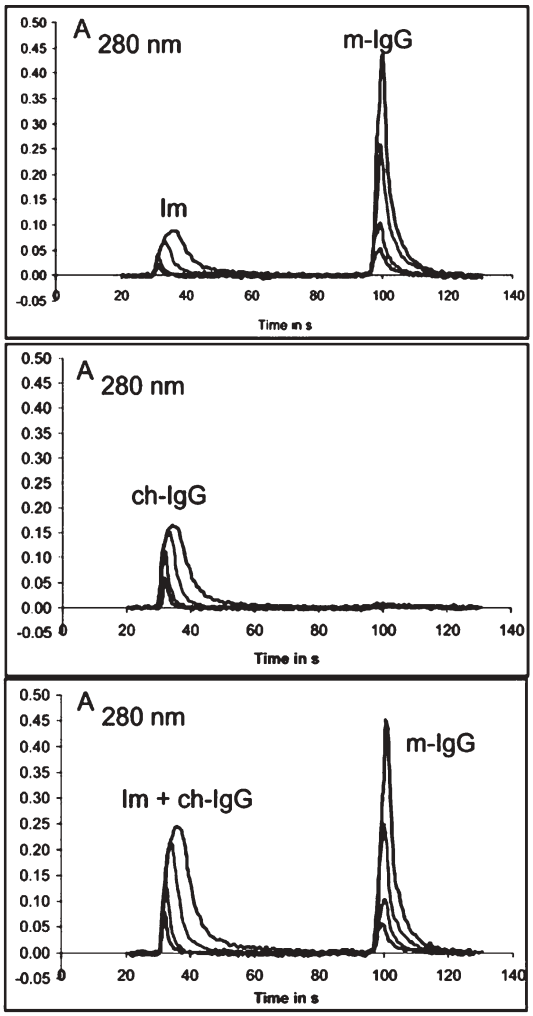

Fig. 3 Four superimposed chromatograms obtained by injecting increasing volume of sample solutions (5, 10, 25 and 50 μL). Protocol identical to that in Fig. 2. Sample composition: Top: mouse IgG (m-IgG) 0.5 mg mL^{-1}. Middle: chicken IgG (ch-IgG) 0.5 mg mL^{-1}. Bottom: mixture of m-IgG and ch-IgG containing 0.5 mg mL^{-1} of each. Note that m-IgG preparation contains an unknown impurity (Im).

A detection limit of 6 ng IgG μL^{-1} was estimated from data in Fig. 4 by assuming that the minimum detectable level of 625 ng m-IgG would produce the same response if contained in 100 μL injected volume rather than in 25 μL, since peak heights are shown to increase linearly with injected sample volumes. Comparison with a conventional 1 mL column (Hi-Trap Protein A) was performed using the same reagents and m-IgG, albeit at higher analyte concentrations, eluant volume (25 μL), and flow rates (elution at 50 mL s^{-1}). The column was mounted on the external fitting of the LOV flow cell and supplied by the tubing leading to port #6. The flow cell volume and light path were the same as in the experiments with the microcolumn. The 60-fold increase in detection limit is due to more extensive dilution of analyte in the volume eluted from 1 mL column (270 μL).

While the microcolumn was used repeatedly for several hundred assays, it is advantageous to recharge it automatically whenever needed. This is done by aspirating bead suspension *via* port #3 (at flow rate of 200 μL s^{-1}, 100 μL volume), switching the valve to

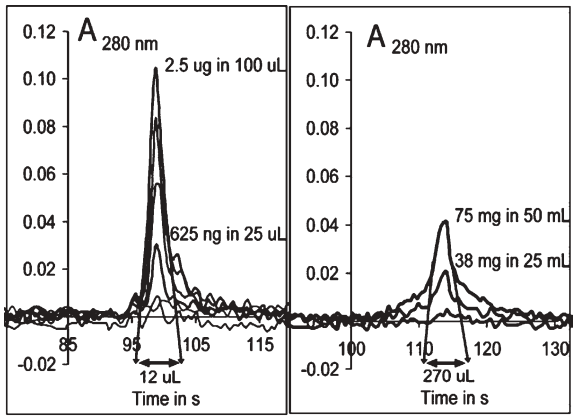

Fig. 4 Limit of detection as obtained with 10 μL microcolumn (left) and 1 mL prepacked externally mounted column (right). Column material Protein A, capacity 20 mg mL^{-1} of human IgG, 6% crosslinked Sepharose. Eluent: 0.5 M HCl. Mobile phase: PBS. Sample: m-IgG in concentrations indicated in the figure. For details see text.

port #2, and packing the column at 100 μL s^{-1}. The column is automatically discarded by reversing these steps with the valve switched to waste (port #1).

In summary, use of programmable flow in the LOV format provides an unprecedented versatility in choice of injected sample volumes, flow rates, and flow directions, tailored to carry out steps of an assay protocol. While this approach was previously used for enzymatic assays and bioligand interaction assays,[5] this work adds immunoaffinity chromatography to the array of assays automated and miniaturized by the μSI technique. Downscaling and integrating a column within the LOV module yields lower limits of detection, shortens assay cycles to less than 2 min, and reduces the volume of waste to 2 mL per assay. In contrast, traditional affinity chromatography[3] on 1 mL HiTrap Protein A column uses 30 mL of mobile phase, takes 30 min to complete, and uses nearly 100 times more column material.

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