

Bead injection for biomolecular assays: Affinity chromatography enhanced by bead injection spectroscopy†‡

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Selective capture of target biomolecules by ligands immobilized on a solid support is a cornerstone of two seemingly unrelated techniques: micro-Affinity Chromatography (μ AC) and micro-Bead Injection Spectroscopy (μ BIS). This work shows, for the first time, how these techniques can be carried out using the same instrument and how the data obtained this way complement each other, yielding complete information on retention and elution of target biomolecules. Biomolecular association and dissociation were investigated by μ AC and μ BIS, using computer-controlled programmable flow and the same instrument for automated bead transport, packing of a micro-column, assay of the analyte, and bead disposal. The absorbance of the analyte was monitored within the fiber optic flow cell configured either for monitoring directly on the beads or post-column after elution. The separation, binding, and elution of immunoglobulins (human IgG, rabbit IgG, and horse IgG) on protein G-coated Sepharose beads were studied as model systems. The limit of detection of the μ AC technique was determined to be $5 \text{ ng } \mu\text{L}^{-1}$ IgG, and that of the μ BIS technique was $50 \text{ ng } \mu\text{L}^{-1}$ IgG.

Introduction

Microscale Affinity Chromatography (μ AC) uses the Bead Injection technique for assembly, perfusion, discharge, and renewal of a micro-column that is integrated within a “lab-on-valve” module.¹ Similar to traditional Affinity Chromatography (AC), μ AC uses Sepharose beads furnished with an immobilized bioligand to selectively capture a target biomolecule from the sample and, when the composition of the mobile phase is changed, releases it for quantification by UV-vis spectroscopy. The differences between traditional AC and μ AC are in the scale (millilitres *versus* microlitres), speed of operation, and the mode of operation, as the micro-column is automatically packed, perfused, eluted, and discarded. Automated handling of Sepharose beads and the formation of a renewable micro-column are also used in micro-Bead Injection Spectroscopy (μ BIS),^{2–5} with the difference being that the stationary phase, instead of the eluate, is interrogated by UV-vis spectroscopy. Since both techniques rely on the selective interaction between target biomolecules and ligands immobilized on a solid support, and since the micro-Sequential Injection instrument has been used as a platform for both techniques,^{1–5} it is useful to compare their advantages and limitations for the separation and assay of biomolecules. While the ultimate goal of our research is to develop an improved method for the assay of telomerase,^{6–8} this work represents the first step toward this goal, since using immunoglobulins (IgG) as a model system will allow us to perfect Bead Injection

methodologies and to compare them in a way that has never been done before. Briefly, while chromatography can only assay species that have been *eluted* from a column, μ BIS is best suited for the assay of species that are firmly *bound* on the solid phase. This makes μ AC and μ BIS complementary in a unique way, since the weakness of one technique is the strength of the other.

Experimental

Instrumentation

The Sequential Injection system (FIALab-3000, FIALab Instruments, Inc., <http://www.flowinjection.com>) used for μ AC (Fig. 1a) and μ BIS (Fig. 1c) was controlled by FIALab software, version 5.9.182. The instrument was configured with a 500 μL syringe, holding coil (0.76 mm I.D. tubing, 160 cm long) and “lab-on-valve” (LOV) unit mounted on a six-port multi-position valve. The fiber-optic cables (600 μm diameter from Ocean Optics, Inc., <http://www.oceanoptics.com>) connected the flow cell to the light source (deuterium lamp, Model D 1000 from Analytical Instrument Systems, Inc., <http://www.aishome.com>) and the spectrophotometer (USB2000 from Ocean Optics, Inc.). By adjusting the distance between the tips of the fiber-optic probes, the light path of the flow cell was set to 6.3 mm (volume 13 μL) for μ AC and 1.0 mm (volume 2.0 μL) for μ BIS. The flow cell configurations for μ AC and μ BIS are shown in Fig. 1b and 1d, respectively. The bead column was packed prior to the start of each assay by aspirating bead suspension into the holding coil and, by flow reversal, carrying the beads toward port #2.

For μ AC mode, a micro-column (volume 13 μL) was packed upstream from the flow cell and held in place by the fiber optic probe (Fig. 1b).¹ The flow cell was rinsed through the conduit between port #6 and the outlet of the flow cell. The assembled

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‡ The HTML version of this article has been enhanced with colour images.

micro-column could be perfused at moderate flow rates up to $40 \mu\text{L sec}^{-1}$. At the end of an assay, beads were aspirated back into the holding coil from port #2 and disposed through the

waste port. The loading and removal of beads were part of the software-controlled chromatographic protocol. See Method section for details.

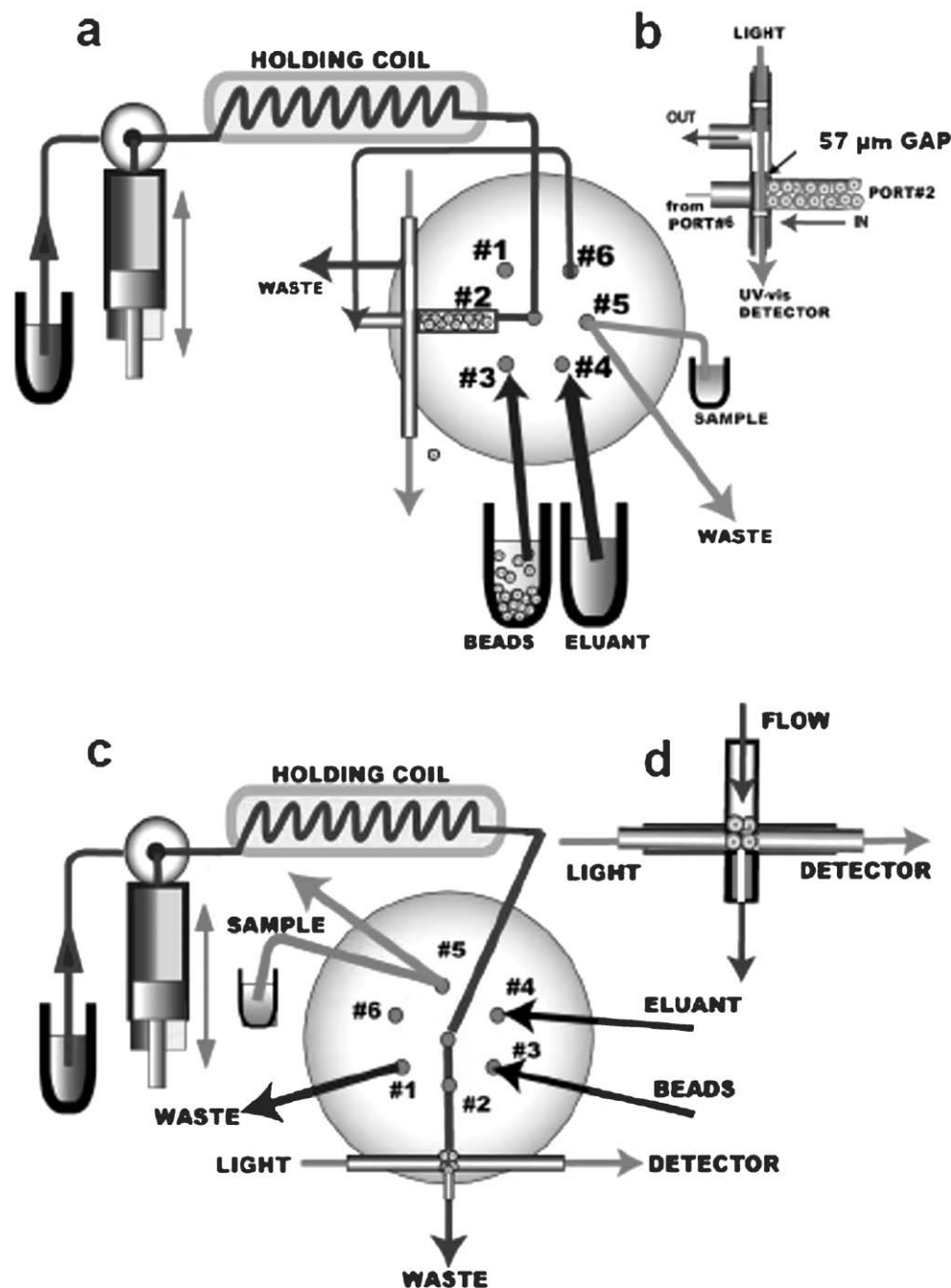


Fig. 1 Schematics of micro-Affinity Chromatography (μAC) and micro-Bead Injection Spectroscopy (μBIS) systems. Both μAC (a and b) and μBIS (c and d) are based on a micro-Sequential Injection (μSI) instrument composed of a $500 \mu\text{L}$ syringe pump, two-way valve, holding coil, and lab-on-valve (LOV) manifold mounted on a six-port multi-position valve. To pack a column, beads are aspirated from port #3 into the holding coil and subsequently delivered to port #2, where they are retained. Sample or eluant solution is aspirated from port #5 or port #4, respectively, into the holding coil and subsequently perfused through the bead column through port #2. (a) The μSI configuration for μAC . The flow cell is rinsed through the conduit between port #6 and the outlet of the flow cell. (b) The flow cell configuration for μAC shown in detail. Beads are packed upstream from the flow cell. The fiber optic probes inserted into the flow cell monitor the absorbance of the eluted analyte at 280 nm post-column. The gap ($57 \mu\text{m}$) between the fiber optic probe and the channel wall allows the beads to be retained but liquid to flow past. The volume of the bead column is $13 \mu\text{L}$ and the length of the optical path is 6.3 mm . (c) The same μSI instrument is reconfigured for μBIS by removing the tubing between port #6 and the flow cell and by rotating the LOV so that the flow cell lies horizontal. (d) The flow cell configuration for μBIS shown in detail. Beads are packed in the flow cell, between the two fiber optic probes that monitor the absorbance on-column at 280 nm . A piece of red PEEK tubing is used to retain the beads. The volume of the column is $2.0 \mu\text{L}$ and the length of the optical path is 1.0 mm .

For μ BIS mode, the manifold shown in Fig. 1a was reconfigured, as shown in Fig. 1c, by removing the tubing between port #6 and the flow cell. In μ BIS, beads were packed into the flow cell (Fig. 1d), where they were retained by a plug fashioned from PEEK tubing (1.59 mm O.D., 0.13 mm I.D., and 2.0 mm long). Similar to the μ AC mode, beads were discarded by aspiration back into the holding coil.

Materials

The carrier solution composition was PBS buffer (140 mM NaCl, 16.0 mM Na_2HPO_4 , 2.00 mM KH_2PO_4 , 3.75 mM KCl, 10.0 mM LiCl, 1.00 mM EDTA-2Na, pH 7.4). PBS buffer was also used to prepare the IgG and eluant solutions. Human IgG (catalog #I2511), FITC-labeled human IgG (#F9636), rabbit IgG (#I5006), FITC-labeled rabbit IgG (#F7256), horse IgG (#I4631), Sepharose CL-4B (#CL4B200), and bromothymol blue (BTB, #11,442-1) were purchased from Sigma-Aldrich (www.sigma.com). Sodium borate (#3570-01) was purchased from J. T. Baker (www.jtbaker.com). Blue dextran 2000 (#74415V) was from Amersham Biosciences (www5.amershambiosciences.com). Recombinant protein G-coated Sepharose 4B beads (#10-1241) were purchased from Zymed (www.zymed.com). The beads were washed with PBS buffer several times and suspended (1 : 2) in PBS buffer prior to use. Eluant solution was prepared by diluting 10.0 M concentrated HCl 100 times with PBS buffer. This acidified PBS (pH 1.6) was used as an eluant in order to minimize the difference in refractive index between PBS carrier and the eluant.

Method

The assay protocol for μ AC is comprised of six steps: (1) bead suspension is aspirated into the LOV module and transported towards port #2, where the beads are retained by the fiber-optic probe, forming a micro-column with a volume defined by the geometry of the LOV. Excess beads are sent to waste. (2) The flow cell is purged by carrier *via* port #6 and the micro-column is rinsed with carrier *via* port #2, followed by a reference scan performed by the spectrometer. (3) After absorbance monitoring begins, 20.0 μL of IgG sample are metered into the LOV and applied to the column at $1 \mu\text{L s}^{-1}$, followed by 80.0 μL of PBS buffer. (4) 20.0 μL of eluant are metered and perfused through the column at $1 \mu\text{L s}^{-1}$, followed by 80.0 μL of PBS buffer, to elute the analyte from the micro-column for monitoring downstream in the flow cell. (5) A second elution is performed by repeating step 4. (6) The beads are aspirated back into the holding coil and discarded.

The assay protocol for μ BIS is comprised of six steps: (1) after beads are aspirated into the holding coil, part of the dispersed zone of the bead suspension is sent to the flow cell and retained by a PEEK tubing plug, with the rest of the zone sent to waste. PBS buffer is subsequently perfused through the bead column to ensure tight packing. (2) The bead column in the flow cell is rinsed with carrier *via* port #2. A reference scan is performed as carrier solution is perfused through the column. (3) 20.0 μL of IgG sample are metered into the LOV and, after absorbance monitoring starts, applied to the column at $1 \mu\text{L s}^{-1}$, followed by 80.0 μL of PBS buffer. (4) 20.0 μL of

eluant are metered and perfused through the column at $1 \mu\text{L s}^{-1}$, followed by 80.0 μL of PBS buffer, to elute the analyte while a decrease in absorbance is monitored on-column. (5) A second elution is performed by repeating step 4. (6) The beads are drawn back into the holding coil and discarded through the waste port.

Full details of protocols can be found online as ESI†.

Results and discussion

Calibration of μ AC and μ BIS systems

The μ AC system was calibrated using human IgG solutions ranging from $0.100 \mu\text{g } \mu\text{L}^{-1}$ to $1.00 \mu\text{g } \mu\text{L}^{-1}$. The results of duplicate runs of each concentration and the PBS blank are shown superimposed in Fig. 2. The average peak height of duplicate runs was used for calibration, yielding a straight line ($y = 1.08x - 0.00638$, $r^2 = 0.999$). The limit of detection (LOD) was calculated according to the equation: $\text{LOD} = 3 \times s_b/m$, where m is the slope of the calibration curve and s_b is the standard deviation of four replicate runs of PBS blank. s_b was found to be 0.002 A and the LOD was determined to be $5 \text{ ng } \mu\text{L}^{-1}$. Note that an initial peak appeared at 50 seconds at concentrations above $0.600 \mu\text{g } \mu\text{L}^{-1}$, indicating the occurrence of non-retained material as the column reaches its capacity.

For the μ BIS system, the calibration was performed using human IgG at concentrations from $0.100 \mu\text{g } \mu\text{L}^{-1}$ to $0.400 \mu\text{g } \mu\text{L}^{-1}$. The results of duplicate runs of each concentration and the PBS blank are shown superimposed in Fig. 3. The average absorbance before elution (point A in Fig. 3) of the duplicate runs was used for calibration. The calibration yielded a straight line, $y = 0.295x - 0.00382$ ($r^2 = 0.999$). s_b was calculated to be 0.004 A and the LOD was determined to be $50 \text{ ng } \mu\text{L}^{-1}$.

The lower sensitivity and higher detection limit of μ BIS compared to μ AC may be due to several factors, such as differences in the optical properties between the mobile and stationary phases, diminished effective path length of the bead-filled flow cell, limited capacity of the beads (the bead column

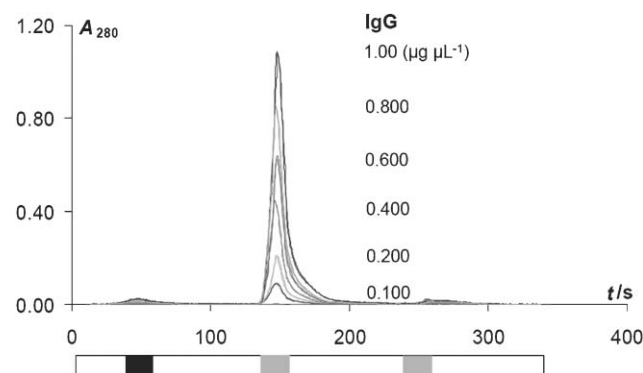


Fig. 2 Calibration of the μ AC system using human IgG at 0.100, 0.200, 0.400, 0.600, 0.800, and $1.00 \mu\text{g } \mu\text{L}^{-1}$. The black bar represents 20.0 μL of IgG sample, tinted bars represent 20.0 μL of eluant, and white bars represent PBS carrier. The results of duplicate runs of each concentration and the PBS blank are superimposed in the figure. The calibration yields a straight line, $y = 1.08x - 0.00638$ ($r^2 = 0.999$).

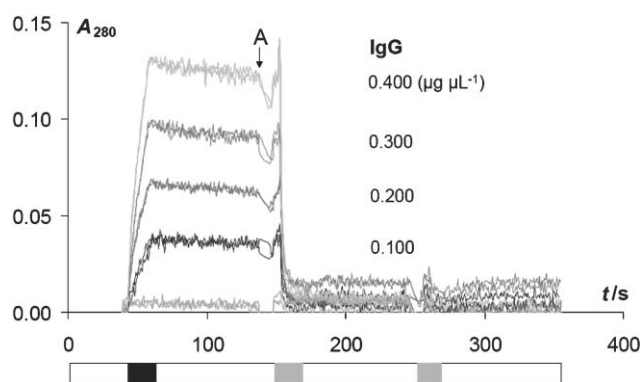


Fig. 3 Calibration of the μ BIS system using human IgG at 0.100, 0.200, 0.300, and 0.400 $\mu\text{g } \mu\text{L}^{-1}$. The black bar represents 20.0 μL of IgG sample, tinted bars represent 20.0 μL of eluant, and white bars represent PBS carrier. The results of duplicate runs of each concentration and the PBS blank are superimposed in the figure. The calibration equation is $y = 0.295x - 0.00382$ ($r^2 = 0.999$).

in μ BIS is a fraction of the size of the column used in μ AC), and the geometry of the μ BIS detection cell, which causes a portion of the target molecules to be captured outside the light path. Since the probing beam is, for practical reasons, aimed through the center of the bead layer, any beads situated above the beam remain undetected, yet these beads must be saturated with target molecules first before any are captured within the sight of the probing beam. In contrast, the geometry of the micro-column in μ AC and the “zero” dead-volume conduit leading to the flow cell, which is large enough to accommodate almost all of the eluted molecules, combine favorably with a low-noise background to give μ AC higher sensitivity and lower LOD.

In regards to the difference between the absorbance of an analyte measured in an empty flow cell *versus* in a flow cell filled with inert beads, the factor that changes is the optical path length ($A = \epsilon bC$, where A is absorbance, ϵ is the absorptivity, b is the path length, and C is the concentration of target molecule). If the flow cell used in this work was filled with solid beads, approximately two thirds of the total volume of the flow cell would be occupied by the beads.^{9–11} However, Sepharose beads contain approximately 80% water, are elastic, and have a fractionation range of 30–5000 kD for dextrans and 60–20 000 kD for globular proteins.¹² Therefore, species with molecular weight less than 30 kD, such as dyes (bromothymol blue, 0.646 kD) will penetrate the beads readily, while globular protein IgG (150 kD) will have more difficulty entering the agarose network, and larger molecules, such as blue dextran 2000 (2000 kD), will have even more difficulty penetrating the beads. The results of monitoring these species in empty and bead-filled flow cells are summarized in Fig. 4. The response for bromothymol blue in a bead-filled cell is 95% (A_1/A_0) of the absorbance value obtained with an empty cell, and the responses for human IgG and blue dextran decrease to 73% and 49% of the empty flow cell values, respectively. The ratio of the absorbance value in the presence of beads to that in the absence of beads (A_1/A_0) is inversely proportional to the molecular weight of the target analyte. This observation also implies the potential application of the Bead Injection

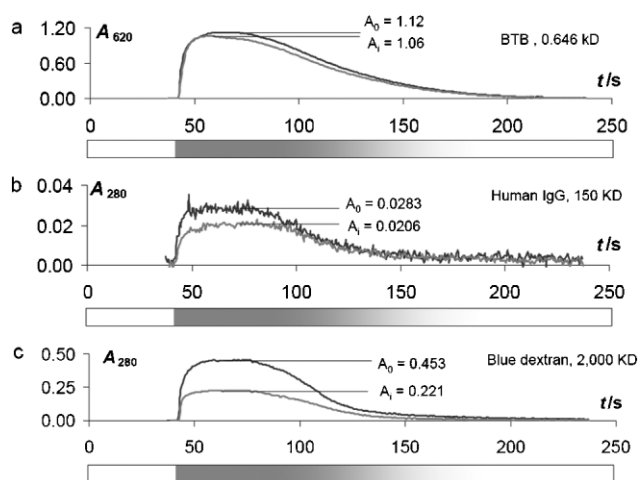


Fig. 4 μ BIS results showing the relationship between the effective path length and the size of the molecule. (a) 0.200 $\mu\text{g } \mu\text{L}^{-1}$ BTB, (b) 0.200 $\mu\text{g } \mu\text{L}^{-1}$ human IgG, and (c) 2.50 $\mu\text{g } \mu\text{L}^{-1}$ blue dextran are analyzed in the absence (A_0 in a, b, and c) and presence (A_1 in a, b, and c) of empty Sepharose beads. 80.0 μL of sample are perfused through the column at 1 $\mu\text{L } \text{s}^{-1}$, followed by carrier buffer. The carrier is PBS buffer for the IgG and blue dextran assays and 5.00 mM borax buffer (pH 9.2) for the BTB assay.

technique for the fast screening of molecular weights of different compounds.

Performance of μ AC and μ BIS

The complementary features of μ AC and μ BIS are emphasized when comparing the results obtained using both systems for the interaction of human IgG with protein G immobilized on Sepharose beads (Fig. 5a and 5b). The results of duplicate runs and the PBS blank are shown superimposed in each figure. The experiments in Fig. 5a and 5b were performed at identical flow rates and injected volumes, the only difference being the concentration of IgG in the injected sample (0.100 $\mu\text{g } \mu\text{L}^{-1}$ for μ AC and 0.200 $\mu\text{g } \mu\text{L}^{-1}$ for μ BIS).

Since IgG is firmly retained on protein G, the initial baseline of the chromatogram remains flat during injection of the sample on the column and a peak is recorded only after the eluant is injected on the column. In contrast, the μ BIS output shows a steep rise as soon as the injected sample reaches the bead layer and the absorbance remains nearly constant until the eluant is injected. The subsequent dip observed in the data is the result of the flow stopping as the pump prepares to deliver the eluant. When compared, the leading edge of the μ AC peak (Fig. 5a) coincides with the falling edge of the μ BIS response (Fig. 5b). As a second injection of eluant is perfused through the column, only a minor disturbance of the baseline on the chromatogram is observed, which corresponds to a slight decrease in the μ BIS response.

Since immunoglobulins from various sources interact with protein G with different affinities, the comparison of μ AC and μ BIS responses of rabbit, horse, FITC-labeled human, and FITC-labeled rabbit IgG offers insight into information provided by these complementary techniques (Fig. 6). The μ AC responses for human IgG-FITC (Fig. 6a) and rabbit IgG (Fig. 6c) each contain an initial peak (~ 50 seconds) that

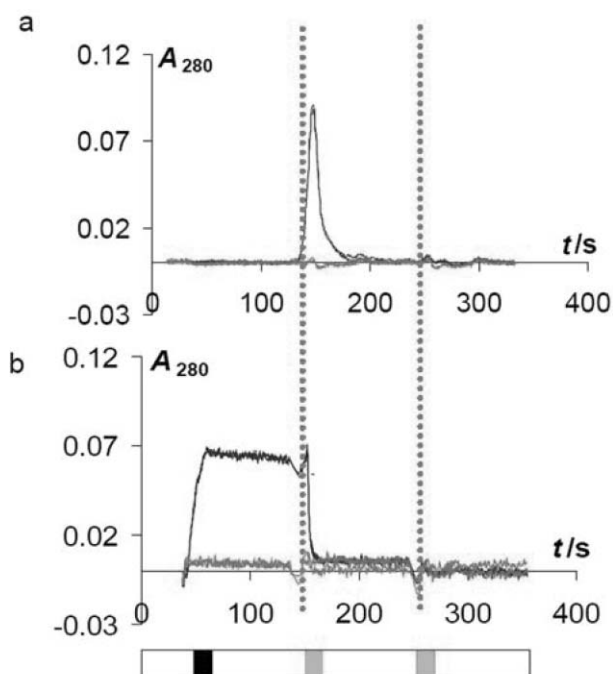


Fig. 5 Results of human IgG assays using μ AC and μ BIS. The black bar represents 20.0 μ L of IgG sample, tinted bars represent 20.0 μ L of eluant, and white bars represent PBS carrier. The results of duplicate runs of each concentration and the PBS blank are superimposed in the figure. (a) Chromatograms of human IgG assays at 0.100 μ g μ L⁻¹. (b) Results of μ BIS assays of human IgG at 0.200 μ g μ L⁻¹. The dotted lines identify where the leading edge of the μ AC peak corresponds to the falling edge of the μ BIS response in the first elution and, in the second elution, where the small disturbance of the baseline on the chromatogram corresponds to a slight decrease in the μ BIS result.

indicates the presence of non-retained material passing through the flow cell, while the same material cannot be observed using μ BIS (Fig. 6b and Fig. 6d). On the other hand, the μ AC response offers no indication as to whether the column has been partially or completely regenerated after two elution cycles. In the chromatograms of rabbit IgG (Fig. 6c), rabbit IgG-FITC (Fig. 6e), and horse IgG (Fig. 6g), a peak near 260 seconds is observed during the second elution, indicating that the first elution is incomplete. However, the chromatograms give no information on whether the second elution is complete. On the other hand, the μ BIS responses show, by the level of the baselines after injections of eluant, that the second elution does not release all horse IgG molecules from the protein G column (Fig. 3h) but is more complete in the case of rabbit IgG (Fig. 3d) and FITC-labeled rabbit IgG (Fig. 3f).

The chromatogram of horse IgG (Fig. 6g) shows that the peak near 150 seconds in the first elution is significantly smaller than the corresponding peak for the other IgG samples, even though the amounts of the various IgG samples applied to the columns were held constant. Possible explanations for this observation include incomplete elution of IgG from the column or an IgG content in the purchased preparation less than what was certified by the manufacturer. In the μ BIS response (Fig. 6h), the plateau level is lower than that of the

other IgG samples, indicating a lower IgG content in the horse IgG sample than expected, and the elevated baseline after both elution cycles indicates that the column cannot be easily regenerated.

The μ BIS results for these different IgG samples indicate, by the relative ease of elution, that, among the molecules studied, horse IgG has the highest affinity for protein G, followed by rabbit IgG and human IgG. This observation is consistent with the literature, which ranks the affinity between protein G and IgG molecules raised in these animals in the following order: horse > rabbit > human.¹³

Accumulation of IgG on protein G beads

Results obtained with rabbit IgG, FITC-labeled rabbit IgG, and horse IgG, (Fig. 6d, 6f, and 6h), indicate that a fraction of IgG molecules remain bound to protein G-coated beads after a single elution. In order to confirm this finding, the μ BIS protocol was repeated three times, but with only one eluant injection in each cycle. By injecting a sample of 0.200 μ g μ L⁻¹ FITC-labeled rabbit IgG, and by programming only one reference scan at the onset of the monitoring series (Fig. 7), the results show that the amount of IgG remaining on the column after a single elution increases with the number of assay cycles. With incomplete elution, the amount of IgG retained on the column accumulates over several cycles, resulting in diminished column capacity and deterioration of the column.

Conclusion

Two complementary techniques, μ AC and μ BIS, have been applied to the assay of biomolecules using various immunoglobulins and protein G-coated beads as model systems. Both methods use the same micro-Sequential Injection instrumentation and Bead Injection methodology to form renewable micro-columns. μ AC monitors the eluted analyte post-column, while μ BIS monitors the capture and elution of analyte on-column.

The μ AC method offers higher sensitivity, with a limit of detection ten times lower than the μ BIS method. A longer light path, better geometry, and the absence of light scattering in μ AC contribute to this advantage. The limitation of the μ BIS technique stems from configuration of the flow cell, where, inevitably, a significant portion of target molecules remain outside the optical path, as well as from the light path geometry that must be designed to allow sufficient light to penetrate the bead layer.

While more sensitive, the limitation of chromatography is that it can only monitor species *eluted* from the column, while any analyte remaining on column is not observed. This creates serious problems whenever target or matrix species bind irreversibly to the column, since the column capacity will gradually diminish (see Fig. 7). Even worse, if the composition of the eluant is changed in such a way that species irreversibly captured in previous runs are unexpectedly eluted, “ghost” or carryover peaks will be recorded. With Affinity Chromatography being widely used in the purification of antibody production in pharmaceutical and biotech companies,^{14,15} it is important that the injected antibody is completely eluted and the column is completely regenerated.

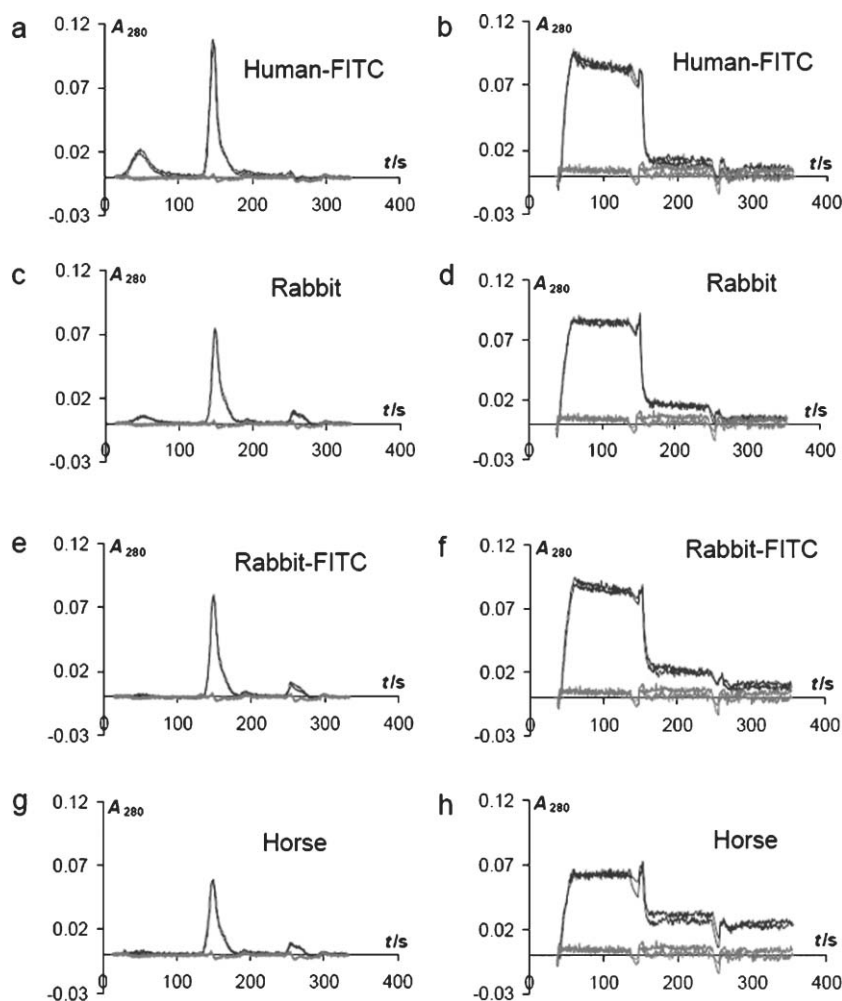


Fig. 6 Results of μ AC and μ BIS assays of different IgG samples. The results of duplicate runs of each concentration and the PBS blank are superimposed in the figure. (a), (c), (e), and (g) are the chromatograms of FITC-labeled human IgG, rabbit IgG, FITC-labeled rabbit IgG, and horse IgG, respectively, at $0.100 \mu\text{g } \mu\text{L}^{-1}$. (b), (d), (f), and (h) are the corresponding μ BIS results for different IgG samples in the same order at $0.200 \mu\text{g } \mu\text{L}^{-1}$.

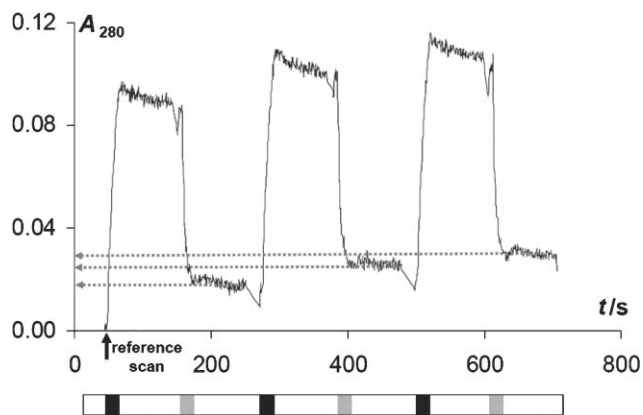


Fig. 7 Accumulation test for carryover on the column after a single elution. The protocol is similar to that in the Method section, except that three assays are performed in a row with only one elution in each run and only one reference scan is performed at the beginning of the first assay. The black bars represent $20.0 \mu\text{L}$ of IgG sample, tinted bars represent $20.0 \mu\text{L}$ of eluant, and white bars represent PBS carrier. The dotted lines show that the amount of IgG remaining on the column after one elution increases with the number of assay cycles.

μ BIS may be used to monitor carryover on the column and optimize elution condition, in order to ensure complete recovery of the column.

In contrast, μ BIS monitors the analyte *retained* on the stationary phase and the quantification of the analyte is done by means of absorbance measured at the plateau. Since beads can be automatically discarded, the target analyte does not need to be eluted. An example of such a situation involves the strong interaction of biotin with avidin or streptavidin routinely employed for the separation of target analytes from complex mixtures. The use of streptavidin-coated beads for the capture of biotinylated DNA is a critical step of a telomerase^{6–8} assay protocol that we plan to develop. The findings presented here indicate that μ BIS offers a feasible approach to the development of this assay.

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