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# Demonstration of a direct capture immunoaffinity separation for C-reactive protein using a capillary-based microfluidic device

Michael C. Peoples [a], Terry M. Phillips [b], H. Thomas Karnes [a,*]

[a] *Department of Pharmaceutics, Virginia Commonwealth University Medical Center, P.O. Box 980533, Richmond, VA 23298-0533, United States*
[b] *Nanoscale Immunodiagnostics, National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892, United States*

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**Abstract**

C-reactive protein (CRP), a biomarker of inflammation and cardiovascular disease (CVD) risk assessment, was selected as a model antigen to demonstrate a direct labeling/direct capture immunoaffinity separation. The miniaturized device for immunoaffinity chromatography was constructed from two syringe pumps, a gradient mixing microchip, micro-injector with 250 nL capillary injection loop, a capillary column, and a diode laser-induced fluorescence detector fitted with a fused-silica capillary flow cell. Monoclonal anti-CRP was biotinylated and attached to 5.0 μm streptavidin-coated silica beads to make the solid support for separation columns. CRP in simulated serum matrix was fluorescently labeled in a one-step reaction and directly injected onto the immunoaffinity capillary. The purified antigen was then eluted in an acid gradient and measured. The antibody binding of CRP was evaluated in two physiological buffers, phosphate buffered saline (PBS) and Dulbecco's PBS (DPBS). A quadratic calibration model produced % relative errors of −15.9 to 12.6 for CRP concentration levels ranging from 0.47 to 95.0 μg/mL. The accuracy (% difference from nominal) and precision (% relative standard deviation) of replicate injections were within 17.0%. The limit of detection was 57.2 ng/mL and chromatographic run times were less than 10 min. The instrument design is simple, and potentially portable, while the assay procedure may be modified for other clinically relevant markers by changing the capture antibody.


*Keywords:* C-reactive protein (CRP); Biomarker; Inflammation; Cardiovascular disease; Immunoaffinity; Microfluidic separation

## 1. Introduction

Immunoaffinity separations rely upon antibody–antigen reactions to selectively remove target analytes from sample matrices. The combination of immunoaffinity techniques with miniaturized analytical instrumentation provides a powerful tool for separating analytes of interest from complex matrices, while consuming minimal quantities of samples and reagents [1]. Microchip and capillary-based systems offer a reduction in diffusion distances that allow for faster antibody–antigen recognition versus traditional microplate immunoassays, especially when packed bead beds are used [2–5]. Two common immunoaffinity techniques include competitive and noncompetitive immunoassays. In a competition assay, sample antigens and labeled antigens compete for a limited amount of antibody sites. Large antigens, capable of possessing two distinct epitopes, may be analyzed by a noncompetitive immunoassay [6]. An immobilized antibody is used to capture the antigen and a detection antibody is introduced to form a sandwich complex. Competitive and sandwich immunoassay techniques require the labeling of secondary compounds, such as analogous antigens or detection antibodies prior to sample analysis. A direct capture immunoassay uses an immobilized capture antibody and directly measures the signal of the antigen either bound to the antibody or after dissociation from the antibody. In many clinical applications the antigen is present in trace amounts or does not readily provide a measurable signal; labeling of the antigen is then performed to enhance the response. In direct capture immunoaffinity chromatography, samples are applied to a separation column and the target antigen binds to a stationary support of immobilized antibodies. Extraneous components in the matrix, including unused labeling reagents, are pumped to waste and the antigen is then eluted in a dissociation buffer for detection [7].

* Corresponding author. Tel.: +1 804 828 3819; fax: +1 804 828 8359.
*E-mail address:* tom.karnes@vcu.edu (H.T. Karnes).

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C-reactive protein (CRP) was selected as a model antigen for proof-of-concept demonstration of direct capture immunoaffinity using a miniaturized chromatography device. CRP is a serum protein and an acute phase reactant whose levels become elevated during injury or infection [8]. The native conformation of CRP is a 115 kDa pentamer made of five noncovalently bonded 23 kDa subunits [9,10]. CRP has been measured as a general biomarker of inflammation and may be used to assess cardiovascular disease (CVD) risk [11]. Low, medium, and high CVD risk categories have been identified corresponding to CRP levels of less than 1.0, 1.0–3.0 μg/mL, and greater than 3.0 μg/mL, respectively. CRP concentrations greater than 10 μg/mL indicate trauma, infection, or other sources of inflammation [11].

CRP is frequently employed as a model analyte for demonstrating innovative microfluidic and small-scale immunoaffinity separation technologies [12]. A microfluidic sandwich immunoassay for CRP in human plasma was performed using capillary action to generate sample flow [13]. Fluorescently labeled detection antibodies were used to measure CRP from 1 μL sample volumes. Hosokawa et al. [14] developed a microchip immunoassay of CRP in human serum based on dendritic amplification and fluorescence detection. The amplification technique relied upon a sandwich complex that included a biotinylated second antibody. Fluorescein isothiocyanate (FITC)-streptavidin and biotinylated anti-streptavidin were introduced to produce a layered, dendritic structure. A microfluidic competition immunoaffinity separation of CRP was demonstrated with on-chip detection of fluorescein-labeled CRP after an acid elution step [12]. Sandwich immunoassays of CRP have been demonstrated in human serum with on-chip chemiluminescence detection using an imager or photographic film [15]. Kriz et al. [16] applied magnetic permeability detection of anti-CRP nanoparticles for CRP immunoassays in whole blood samples. CRP was sandwiched between antibody-coated silica microparticles and antibody-coated magnetic nanoparticles in a glass vial. The silica particles were allowed to sediment and the magnetic permeability was measured after placing the vial into a magnetic coil. Tsai et al. [17] developed a magnetic-based competitive immunoassay for CRP in serum using a flow-through channel design with a magnetic detection zone. Free CRP and CRP-conjugated silica particles competed for binding of antibody-coated magnetic nanoparticles and the signal was generated by counting the CRP-silica particles under a microscope. Other formats for measuring CRP have included immunochromatographic test strips for whole blood sandwich immunoassays [18] and surface plasmon resonance (SPR) biosensors [9]. The SPR method employed three separate monoclonal antibodies to detect both the native, pentamer conformation and the modified, subunit forms of CRP.

In the current research, a direct labeling/direct capture immunoaffinity separation of CRP is described. The antigen is labeled with a fluorescent dye reagent and separated on a capillary immunoaffinity column for detection with diode laser-induced fluorescence (LIF) detection. The system employs syringe pumps, a microfluidic mixing chip, a capillary injection loop, and a flexible capillary-scale column design to perform immunoaffinity chromatography with low sample and mobile phase requirements.

## 2. Experimental materials and methods

### 2.1. Reagents and materials

Highly pure C-reactive protein and corresponding monoclonal antibody were purchased from Fitzgerald Industries International Inc. (Concord, MA). Streptavidin-coated 5 μm silica beads were obtained from Bangs Laboratories Inc. (Fishers, IN). Sulfosuccinimidyl-6-(biotin-amido) hexanoate (Sulfo-NHS-LC-Biotin), 0.5 mL Zeba Desalt Spin Columns and an EZ Biotin Quantitation Kit were obtained from Pierce Biotechnology (Rockford, IL). Alexa Fluor 647 carboxylic acid, succinimidyl ester ($\lambda_{ex}$ = 650 nm, $\lambda_{em}$ = 668 nm) was purchased from Invitrogen (Carlsbad, CA). SeraSub, a protein-free, buffered polymer solution with the same viscosity, specific gravity, and osmolality as serum was purchased from CST Technologies (Great Neck, NY). Purified water was prepared using a Barnstead Nanopure Diamond system (Dubuque, IW). All other reagents and chemicals were obtained from Sigma (St. Louis, MO). All solutions were filtered through 0.22 μm PVDF Durapore 13 mm syringe filters (Millipore, Billerica, MA) prior to use. Polyetheretherketone (PEEK) capillary tubing of 360 μm outer diameter (o.d.) and PEEK unions were purchased from Upchurch Scientific (Oak Harbor, WA).

### 2.2. Instrumentation

Immunoaffinity chromatography was performed on a laboratory-built system described previously [19]. Two microliter OEM module syringe pumps (Harvard Apparatus Inc., Holliston, MA) fitted with glass gastight 1700 series syringes (Hamilton, Reno, NV) were placed into a 0.22 in. acrylic cabinet enclosure. Mixing of mobile phases delivered by the syringe pumps was performed using a gradient mixing chip (NanoMixer, Upchurch Scientific). Samples were introduced into the system using a micro-injector (Upchurch Scientific) with a 250 nL injection loop made from 50 μm internal diameter (i.d.) PEEK capillary tubing. Fluid transfer lines throughout the system from the pumps to the column inlet were made from PEEK capillary tubing.

A flow cell was made from 360 μm o.d. × 50 μm i.d. square fused-silica capillary tubing (Polymicro Technologies, Phoenix, AZ) and a 2 mm section of the polyimide coating was burned off using a capillary window maker (MicoSolv Technology Corporation, Long Branch, NJ). The flow cell was mounted onto a Zetalif laser-induced fluorescence detector (Picometrics, Ramonville, France) containing a Hamamatsu R928 red-sensitive photomultiplier tube (Bridgeport, NJ). The laser diode system consisted of a 650 nm, 20 mW laser diode (Lasermate Group Inc., Pomona, CA), laser diode mount LDM 4412 with collimating lens (ILX Lightwave, Bozeman, MT), and an ILX Lightwave LDC-3722 laser diode controller. The diode was typically operated at 20 °C and 60 mA and the PMT voltage was maintained at 730 V. A National Instruments (Austin, TX)

USB-9215 data acquisition card with 16-bit resolution was used to interface the detector signal to a PC.

### 2.3. Column design

The column body was prepared from 175 μm i.d. × 1/16 in. o.d. PEEK tubing (Upchurch Scientific) cut to lengths of 19 and 18.5 mm for columns I and II, respectively. Stainless steel external column end fittings (Valco Instrument Co. Inc., Houston, TX) containing 0.5 μm frits (Upchurch Scientific) were tightened onto the columns. The frits were 0.038 in. × 0.030 in. × 0.062 in. made from stainless steel with a polymer ring. Columns were packed with stationary phase under negative pressure using a vacuum pump. Slurries of beads were prepared at 3 mg/mL and added to the top of the column body with the inlet frit removed.

### 2.4. Software

A custom program written in LabVIEW 7.0 (National Instruments) was used to control the syringe pumps to perform step gradients and acquire the detector signal as a chromatogram. SigmaPlot 10.0 (Systat software Inc., San Jose, CA) was used for data analysis and calibration modeling.

### 2.5. Antibody biotinylation

The monoclonal anti-CRP antibody was diluted in sodium phosphate buffer (pH 7.2; 10 mM) and biotinylated using a 30-fold molar excess of Sulfo-NHS-LC-Biotin. The reaction solution was mixed by rotation for 30 min at room temperature, followed by 30 min at 4 °C. Excess biotin was removed with 0.5 mL Zeba Desalt Spin Columns according to the manufacturer's instructions and the antibody concentration was measured at $A_{280\,nm}$ using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The degree of biotinylation was determined with an EZ Biotin Quantitation Kit according to the manufacturer's instructions and a BioTek Synergy 2 microplate reader (BioTek Instruments Inc., Winooski, VT). The kit relies on 4′-hydroxyazobenzene-2-carboxylic acid (HABA)/avidin absorbance at 500 nm. The biotin-labeled antibody displaces the HABA reagent causing a proportional decrease in the signal at 500 nm [20]. A positive control of biotinylated horseradish peroxidase was used to normalize results and the biotin:anti-CRP molar ratio was determined to be 2.25 ± 0.08 ($n = 3$).

### 2.6. Stationary phase preparation

Streptavidin-coated silica beads (5 μm) were washed three times with sodium phosphate buffer (pH 7.2; 10 mM) prior to use. The beads were centrifuged at 1200 × $g$ for 5 min at 7 °C and the supernatant discarded after each wash. Based on the biotin-binding capacity of the beads provided on the certificate of analysis, each mg of beads can bind approximately 2.4 μg of biotinylated antibody. A 7-fold excess of biotin–anti-CRP was added to 1.5 mg of streptavidin-coated silica beads in 0.5 mL and rotated overnight (19 h) at 4 °C. The beads were washed three times as before and reacted with 60 ng/mL biotin for 1.5 h at 4 °C to block any remaining reactive sites. The antibody-coated beads were washed five times and stored in sodium phosphate buffer (pH 7.2; 10 mM) at 4 °C until use.

### 2.7. CRP binding

C-reactive protein was labeled with a primary amine-reactive laser dye to assess the activity of columns and develop an immunoaffinity chromatography separation using the microfluidic system. CRP (2.21 mg/mL in a buffer of 100 mM Tris, 2 mM calcium chloride, and 0.1% sodium azide at pH 7.5) was desalted to remove the amine-containing buffer Tris and exchanged into sodium bicarbonate (pH 8.4; 100 mM). A 34-fold molar excess of Alexa Fluor 647 carboxylic acid, succinimidyl ester dye was added to the CRP solution and rotated 1 h at room temperature. After desalting the reaction mixture into phosphate buffered saline (pH 7.4; 10 mM phosphate, 2.7 mM potassium chloride, and 137 mM sodium chloride) (PBS), the absorbance at 280 nm and 650 nm was measured on a NanoDrop ND-1000 spectrophotometer. The degree of labeling was calculated according to the dye manufacturer's instructions and determined to be 5.1 ± 0.7 moles of dye per mole of CRP ($n = 6$ measurements). The extinction coefficient used for CRP was $E$ (mg/mL) = 1.7 (Scripps Laboratories, http://www.scrippslabs.com/datatables/protein_absorbance.html). Labeled CRP at 1 mg/mL in PBS containing 1% bovine serum albumin (BSA) was stored at −20 °C.

Two physiological buffers were evaluated as sample solutions and loading mobile phase buffers, PBS and Dulbecco's PBS (DPBS). Unlike PBS, Dulbecco's PBS is a balanced salt solution containing additional salts of calcium chloride dihydrate (0.9 mM) and magnesium chloride hexahydrate (0.5 mM). The pH of the DPBS buffer was measured at 7.2. CRP was prepared at a concentration of 10 μg/mL in PBS or DPBS containing 0.1% BSA. Each solution was injected (250 nL) into the system using a loading mobile phase (pump A) of PBS or DPBS for the PBS- or DPBS-based sample solutions, respectively. The elution mobile phase (pump B) was glycine–HCl (pH 1.8; 200 mM) for all injections. The flow rate was 2.0 μL/min and the temperature was maintained at 6–8 °C by pumping ice water though a copper coil wrapped around the column. The initial gradient used was 97.5% pump A for 2.5 min followed by a step gradient to 97.5% pump B over 3 s. After 2 min, the mobile phase was returned to pump A for 2 min to equilibrate the column.

C-reactive protein has been shown to undergo conformational changes in the absence of calcium [10] and the PBS and DPBS systems were used to compare common physiological buffers with and without calcium. Fig. 1 shows a typical chromatogram observed for a 250 nL injection of CRP in the DPBS sample solution with DPBS as the running mobile phase. The first peak represents nonreactive material and the second, sharper peak corresponds to immunoaffinity captured CRP. When PBS was used as the running buffer and sample solution, the recovered CRP peak intensity was approximately four times lower than the DPBS system. Therefore, DPBS was selected as the mobile phase used for sample application and also as a sample diluent.

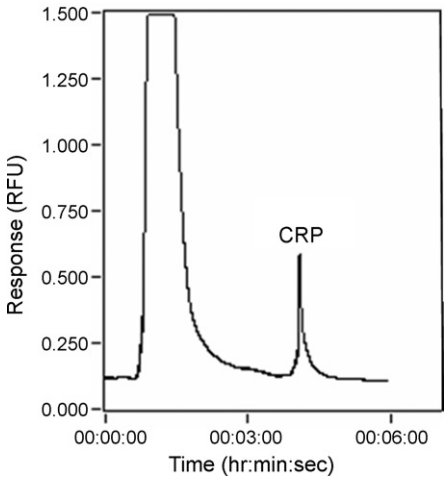


Fig. 1. Typical immunoaffinity chromatogram for a 250 nL injection of C-reactive protein. Conditions are described in the text.

Fig. 2 shows the effect of mobile phase and sample solution buffer on the immunoaffinity capture of CRP.

### 2.8. Labeling and analysis of samples

Prior to fluorescently labeling CRP in a simulated serum matrix, 10 μg/mL of pre-labeled CRP was spiked into a 1:1 (v/v) DPBS:SeraSub mixture containing 0.1% BSA. The immunoaffinity chromatography conditions were the same as in Section 2.7 with DPBS as the mobile phase in pump A. As shown in Fig. 2, the column was capable of binding CRP in the presence of the sample matrix.

The experimental design for fluorescent dye labeling of the antigen and direct capture immunoaffinity separation is illustrated in Fig. 3. CRP was desalted into DPBS and used to prepare calibration levels of 0.47, 0.95, 3.27, 6.65, 15.0, 23.5, 47.5, and 95.0 μg/mL in the simulated serum sample matrix. Alexa Fluor 647 carboxylic acid, succinimidyl ester dye was prepared at 40 μg/mL in DPBS and mixed 1:1 with each CRP calibrator and a blank. The reaction mixture was incubated for 1 h at room temperature with gentle shaking. Samples were centrifuged at 10,000 × *g* for 1 min prior to injection and stored at 4 °C. The immunoaffinity chromatography conditions for the direct labeling/direct capture assay were adjusted to account for a larger unbound peak response due to excess dye and also a peak due to the matrix. The hold time of 97.5% pump A after sample injection was increased to 5 min, followed by a step gradient to 97.5% pump B over 15 s. The elution buffer of glycine–HCl (pH 1.8; 200 mM) was held for 2 min and the mobile phase was returned to DPBS for 2 min to equilibrate the column.

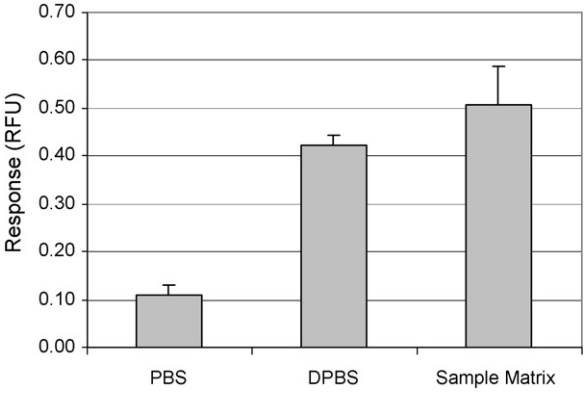


Fig. 2. Effect of mobile phase A and sample buffer solution on CRP-antibody binding. The shaded bars represent the CRP signal ($n = 3$, mean ± standard deviation) in different mobile phases A and sample solutions. The "sample matrix" bar represents a mobile phase A of DPBS and a sample solution of DPBS:serum matrix 1:1 (v/v). Running conditions are described in the text.

Each of the calibrators was injected $n = 3$ times to construct and evaluate calibrations models. Additionally, the 0.95, 6.65, and 47.5 μg/mL levels were injected in duplicate $n = 6$ times and the average of duplicate injections was used to assess accuracy and precision.

## 3. Results and discussion

### 3.1. Immunoaffinity chromatography conditions

Phosphate buffered saline and Dulbecco's phosphate buffered saline were evaluated during method development with respect to CRP binding in the immunoaffinity column. The presence of calcium and magnesium in the sample solutions and mobile phase resulted in a greater degree of antigen recognition by the anti-CRP antibodies in the capillary column. The DPBS buffer may maintain CRP in a favorable conformation for antibody recognition or may provide a more optimal environment for antibody–antigen association. The DPBS solution was used as sample diluent and antigen application mobile phase for all experiments.

### 3.2. Direct capture immunoaffinity demonstration with CRP

Samples were injected onto the immunoaffinity column with a loading/washing step of 5 min in pump A mobile phase. This allowed the large, unbound peak of excess dye to elute from the system prior to the gradient elution step. During the glycine–HCl elution step, the CRP was released from the immobilized antibodies and detected. A comparison of chromatograms for blank matrix and CRP injections is shown in Fig. 4. A small matrix peak is present in the blank just before 6 min and the CRP peak elutes at approximately 7 min.

### 3.3. Calibration models and metrics

Quadratic and four-parameter logistic (4PL) curves were evaluated using the data from the response of the eight calibrators. To calculate figures of merit, including accuracy and precision, the best model was selected by plotting % relative error (%RE) against the concentration levels. The %RE represents the difference between back-calculated concentrations and nominal concentrations divided the nominal concentration and multiplied by 100% [21]. Fig. 5 shows the two models and the plotted %RE versus standard concentration. The quadratic model had an $r^2$ of 0.9875 with %RE ranging from −15.9 to 12.6 for the eight levels. The 4PL model had an $r^2$ of 0.9862 with the

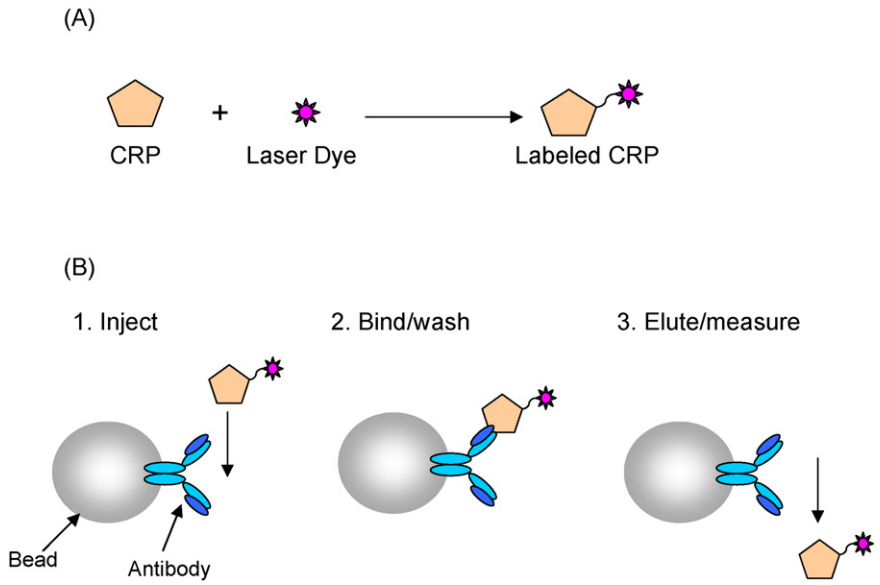


Fig. 3. Procedure for direct fluorescent labeling and direct capture immunoaffinity separation of CRP. Experimental details are described in the text. (A) Fluorescent labeling of antigen and (B) direct capture assay.

%RE between −30.8 and 31.6. The current guidance for model selection of ligand-binding assays, including immunoassays, recommends %RE be within 20% for back-calculated standards and 25% at the lower and upper limit of quantification (LLOQ and ULOQ, respectively) [22]. Based on these guidelines, the quadratic model was chosen to describe the data and calculate accuracy.

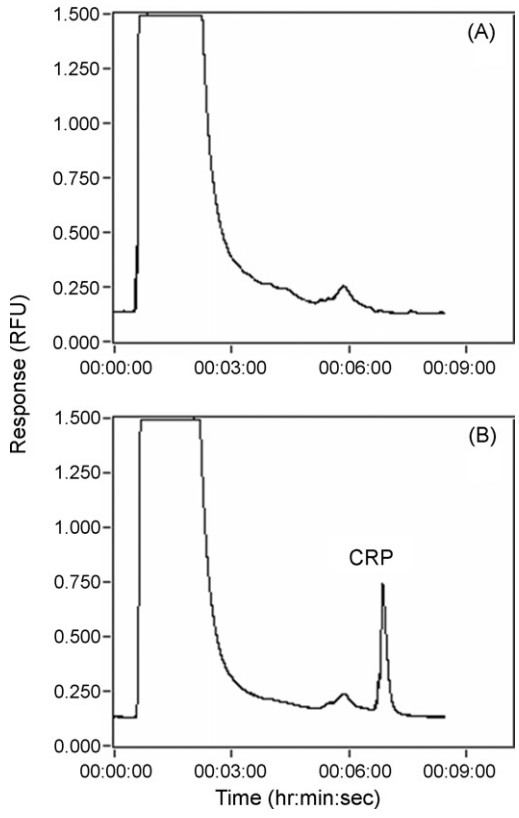


Fig. 4. Typical chromatograms of a blank (A) and 6.65 μg/mL CRP standard (B) in the sample matrix. Running conditions are described in the text.

The equation for the quadratic model was $y = y_0 + ax + bx^2$ and the parameter estimates were $y = 0.0491\ (0.0347) + 0.0699\ (0.0027)x - 0.0005(2.8360 \times 10^{-5})x^2$, where standard error is shown in parenthesis. The limit of detection (LOD) was estimated to be 57.2 ng/mL (497 pM) at two times the standard deviation of the blank response, $n = 3$. The linear slope term, a, was used to convert the response into a concentration in ng/mL. Based on the 250 nL injection loop size, the on-column LOD was 14.3 pg (124 attomoles). Table 1 summarizes the accuracy and precision data represented as % difference from nominal value (%DFN) and % relative standard deviation (%R.S.D.), respectively.

The equation for the 4PL model was $y = \min + (\max - \min)/[1 + (x/\mathrm{EC50})^{\mathrm{Hillslope}}]$, plotted with a logarithmic $x$-axis scale. The EC50 term represents the inflection point, the Hillslope is a slope factor, and the min and max terms represent the lower and upper asymptotes of the curve [21]. Parameter estimates with standard error in parenthesis for the 4PL model shown in Fig. 6 are as follows: min = 0.094 (0.0497), max = 2.8329 (0.1563), EC50 = 22.2126 (2.1604), and Hillslope = −1.5617 (0.2015). The 4PL model was chosen to estimate the maximum antigen-binding capacity of the columns. The useful range of this model can be extended on the upper end by

Table 1
Accuracy and precision for three levels of CRP ($n = 6$ replicates, duplicate injections)

| CRP concentration (μg/mL) | Precision (%R.S.D.) | Accuracy (%DFN) |
|---|---|---|
| 0.95 | 2.0 | −8.8 |
| 6.65 | 12.3 | −11.1 |
| 47.5 | 17.0 | 4.8 |

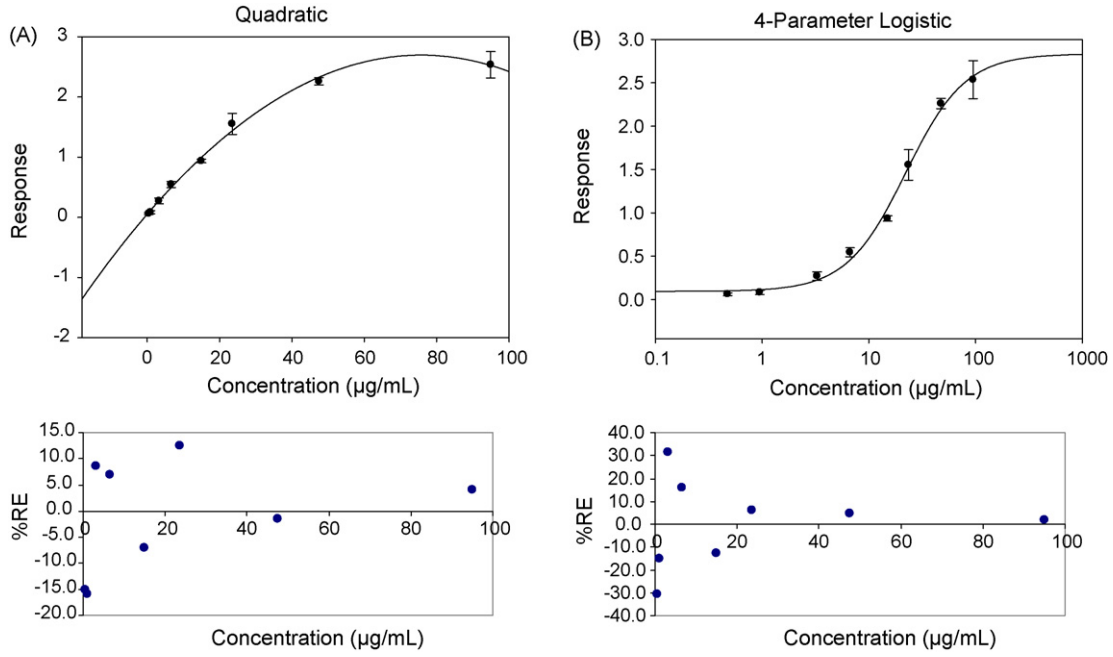


Fig. 5. Comparison of quadratic (A) and 4-parameter logistic (B) models. Each data point represents the mean ± standard deviation of $n = 3$ injections and the response is in relative fluorescence units (RFU). The % relative error vs. concentration (duplicate injections) is shown below each model.

achieving a higher degree of active immobilized antibody on the solid support (i.e., proper antibody orientation) and on the lower end by reducing background and non-specific binding. Blocking agents or surfactants can be used in wash steps or added to the mobile phase to lower non-specific binding of labeled matrix components.

### 3.4. Column characteristics

Using the 4PL model parameter estimates a maximum binding concentration of CRP was calculated. The concentration injected on column was adjusted for the dilution factor of the dye solution and the 250 nL injection volume. Column I (0.175 mm × 19 mm) and column II (0.175 mm × 18.5 mm) could bind approximately 188 and 125 ng of C-reactive protein, respectively.

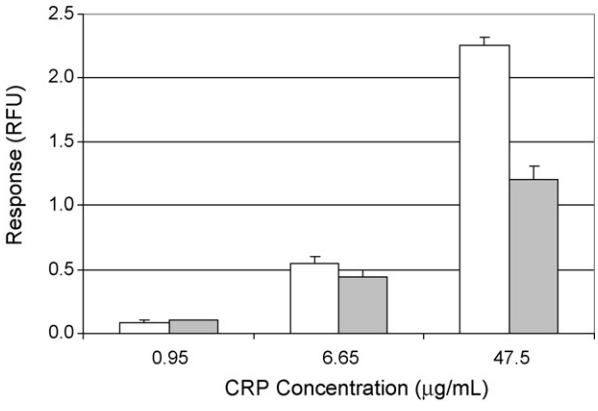


Fig. 6. Effect of injection number on CRP response (at least $n = 3$, mean ± standard deviation). The white bars represent CRP response after 35 elution cycles and the shaded bars represent CRP response after more than 100 injections.

To preserve the working lifetime of the column, a column chiller consisting of a copper coil was pumped with ice water during operation and columns were stored at 4 °C when not in use. At greater than 100 injections, the response of the 47.5 µg/mL standard was approximately 50% of prior injections, while the 0.95 and 6.65 µg/mL standard responses remained constant (see Fig. 6). The useful life of the column is reduced for the higher standard concentrations as the amount of active antibody is compromised. Inactivity of binding sites may be caused by incomplete antigen elution or repeated acid elution cycles that damage antibodies and decrease the overall antigen-binding capacity [23]. On the 4PL model, this would result in a lowering of the upper asymptote and a reduction in range of measurable concentrations. Therefore, the lifetime of anti-CRP columns that could measure the entire calibration range was approximately 100 acid elution cycles stored and operated at refrigerated conditions.

## 4. Conclusions

A direct capture immunoaffinity separation of C-reactive protein was demonstrated using a miniaturized, capillary-based device. This simple, pump-driven microfluidic system was assembled from many commercially available materials. The basic components of the system include two syringe pumps for fluid delivery, a gradient chip for mobile phase mixing, a micro-injector with capillary injection loop, capillary columns packed with immunoaffinity beads, and a diode LIF detector containing a laboratory-constructed, fused-silica capillary flow cell. CRP was directly labeled with a fluorescent dye, bound

to antibody-coated beads in a capillary column, and eluted for measurement by diode LIF. This procedure is straightforward and requires a one-step reaction prior to sample injection. The small injection volume (250 nL) and low mobile phase flow rate (2.0 μL/min) minimize reagent and sample consumption and offer the potential to analyze sub-microliter samples. The small-scale instrumentation and low sample volume requirements are advantageous in clinical applications that require portable systems such as patient point-of-care settings.

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