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# Competitive, non-competitive, and mixed format cleavable tag immunoassays

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ABSTRACT

Immunoassays are one of the most useful diagnostic techniques in disease assessment, drug metabolite analysis, and environmental applications due largely in part to the selectivity and sensitivity provided by antibody–antigen interactions. Here, a multiplexed immunoassay termed cleavable tag immunoassay (CTI) was performed in competitive, non-competitive, and mixed formats for the analysis of proteins and small molecule biomarkers of inflammation and tissue damage. Microchip capillary electrophoresis (MCE) with fluorescence detection was employed for the analysis of fluorescently labeled tags corresponding to the analytes of interest cleaved from the detection antibodies. For this work we have selected 3-nitrotyrosine (3-NT) a molecule indicative of reactive nitrogen species (RNS), thyroxine (T4) a molecule used to monitor thyroid gland function, and C-reactive protein (CRP) a marker of chronic inflammation as model analytes to demonstrate the assay principles. The simultaneous detection of 3-nitrotyrosine (3-NT) and thyroxine (T4) was carried out as a proof-of-principle for the competitive CTI while non-competitive CTI performance was demonstrated via the analysis of C-reactive protein (CRP). Limit of detections (LOD) and dynamic ranges were investigated. LOD for 3-NT, T4, and CRP were 0.5 μg/mL, 23 nM, and 5 μg/mL, respectively thus demonstrating the ability of the CTI to detect proteins and small molecules within clinical reference ranges. Moreover, this is the first report of the use of mixed format CTI chemistry for the simultaneous detection of proteins (CRP) and small molecules (3-NT) in a single assay. The success of this work demonstrates the ability of CTI to analyze intact proteins and small molecule biomarkers simultaneously.



## 1. Introduction

Immunoassays are one of the most useful diagnostic techniques in disease assessment, drug and drug metabolite analysis, and environmental monitoring due largely to the selectivity and sensitivity afforded by antibody–antigen interactions [1]. Since their introduction in 1959, multiple immunoassay formats have been developed, including homogeneous (or solution phase) and heterogeneous (solid supported) formats [2]. Immunoassays have now been developed to allow multi-analyte detection in a single run resulting in reduced analysis times and cost as well as providing the potential for multi-analyte point-of-care screening [3,4]. Heterogeneous immunoassay methods such as enzyme linked immunosorbent assays (ELISA), however, are based on detecting the signal on or near the surface where the analyte is captured making the measurement subject to background noise associated with nonspecific interactions at the surface [5].

Recently, our group reported a multi-analyte immunoassay based on immunoaffinity capillary electrophoresis [6] called the cleavable tag immunoassay (CTI) [7,8]. In CTI, the capture chemistry is similar to traditional immunoassays but the detection step is unique. The analyte signal in CTI is not directly detected on a solid support surface; instead, a fluorescent tag is cleaved from the detection antibody and analyzed via microchip capillary electrophoresis (MCE). This approach allows for the decoupling of the detection and capture steps, eliminating the need for complex imaging systems and reducing noise associated with nonspecific adsorption. It also differentiates CTI from traditional CEIA (capillary electrophoresis immunoassays) where the analyte (or antibody–antigen complex) is detected after electrophoresis [9,10]. In CTI, a specific tag with a unique mobility is coded to a specific antibody and therefore each antigen gives a unique peak in the electropherogram where the peak area corresponds to the antigen concentration in the biological sample. Each tag

*Abbreviations:* CTI, cleavable tag immunoassay; MCE, microchip capillary electrophoresis; 3-NT, 3-nitrotyrosine; T4, thyroxine; CRP, C-reactive protein; RNS, reactive nitrogen species; ELISA, enzyme linked immunosorbent assays; CEIA, capillary electrophoresis immunoassays; PDMS, poly(dimethylsiloxane); FTED, fluorescein thiocarbamyl ethylenediamine; FTHD, fluorescein thiocarbamyl hexamethylenediamine; FEB, biotinylated fluorescein thiocarbamyl ethylenediamine; FHB, biotinylated thiocarbamyl hexamethylenediamine; FAMB, biotinylated FITC-cystamine; MEKC, micellar electrokinetic chromatography.

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is generated synthetically and therefore, has a unique and controllable mobility after cleavage and thus a different migration time when analyzed by MCE. We have previously reported the use of CTI for the simultaneous detection of multiple proteins indicative of acute myocardial infarction using a sandwich immunoassay format [7,8]. These results demonstrated the ability to detect cardiac troponin T (cTnT), cardiac troponin I (cTnI), creatine kinase-MB (CK-MB), and myoglobin proteins over a linear range which included the upper reference range for healthy, non-AMI patients.

Many applications such as disease diagnosis [11], drug metabolite analysis [12], and environmental status assessment [13] require detection of small molecules in addition to proteins. Small molecule biomarkers have also been implicated in carcinogenesis, cardiovascular and neurodegenerative disorders [14]. Since these small molecules have a single epitope, the use of competitive immunoassays is required for analysis [15]. Only a few examples of multi-analyte competitive immunoassays have been reported to date [5,16]. The few existing multi-analyte competitive assays allow for fast analysis with low sample consumption, but typically require expensive instrumentation and can be difficult to automate [5]. As an example, a multi-analyte competitive electrophoretic immunoassay with two-color detection was reported for determination of insulin and glucagon in islets of Langerhans [16]. While this assay has a large linear range for multiple analytes, further increases in the number of analytes and/or the ability to perform simultaneous sandwich and competitive immunoassays will be challenging because they will require increases in instrumental complexity.

Here, we report the development of competitive CTI for the analysis of small molecules, non-competitive CTI for proteins, and a mixed mode competitive/non-competitive CTI for analysis of both small molecules and proteins simultaneously. Very few examples exist of mixed mode immunoassays [17]. For this work we have selected 3-nitrotyrosine (3-NT) a molecule indicative of cellular damage caused by reactive nitrogen species (RNS), thyroxine (T4) a molecule used to monitor thyroid gland function, and CRP a marker of chronic inflammation as model analytes to show the function of CTI for competitive, non-competitive, and mixed format immunoassays.

## 2. Materials and methods

### 2.1. List of materials

#### 2.1.1. Microchip device fabrication and MCE system

- SU-8 2035 photoresist (MicroChem Corp., Newton, MA).
- Propylene glycol methyl ether acetate (Sigma Aldrich, St. Louis, MO).
- Four in. silicon wafers (Silicon Inc., Boise, ID).
- Poly(dimethylsiloxane) (PDMS) (Dow Corning, San Diego, CA).
- Sylgard 184 elastomer curing agent (Dow Corning).
- Power supply for MCE experiment (manufactured in-house) [18].

#### 2.1.2. Tag synthesis

- Fluorescein-5-isothiocyanate (FITC) (Molecular Probes, Inc.).
- Cystamine dihydrochloride (Sigma Aldrich).
- EZ-LinkTM sulfo-NHS-SS-biotin (Thermo Fisher, Rockford, IL).
- EZ-LinkTM sulfo-NHS-biotin (Thermo Fisher).
- EZ-Link maleimide-PEO solid-phase biotinylation kit (Thermo Fisher).
- 1,4-Diaminobutane (Fisher Scientific, Pittsburgh, PA).
- 1,6-Diaminohexane (Fisher Scientific).
- Methanol (Fisher Scientific).
- Triethylamine (TEA) (Fisher Scientific).

#### 2.1.3. Conjugation of BSA and small molecules

- Bovine serum albumin (BSA) (Sigma Aldrich).
- 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) (Thermo Fisher).

#### 2.1.4. Antibody preparation

- EZ-Link maleimide-PEO solid-phase biotinylation kit (Thermo Fisher).
- Biotin Quantitation Kit (HABA assay, Thermo Fisher).
- Avidin (EMD biosciences, San Diego, CA).
- Synthesized tags.
- Toyopearl affinity resin (formyl-650 M, 40–90 μm diameter) (Supelco, Bellefonte, PA).
- Sodium cyanoborohydride (Sigma Aldrich).
- Ethanolamine (Sigma Aldrich).
- $K_2HPO_4$ (Fisher Scientific, 100 mM, pH 7.5).

#### 2.1.5. Competitive CTI

- F96 MaxiSorp Microtiter Plates (Nalge Nunc International, Rochester, NY).
- SuperBlock blocking buffer (Thermo Fisher).
- Phosphate buffered saline (1× PBS).
- BSA-hapten conjugates.
- Detection antibody (Oxford Biomedical Research, Oxford, MI).
- 10 mM TAPS buffer (3-[[tris (hydroxymethyl) methyl] amino] propanesulfonic acid, pH 9).
- TCEP (tris (2-carboxyethyl) phosphine) (Sigma Aldrich).

#### 2.1.6. Non-competitive CTI

- 1.5 mL microcentrifuge tubes (Eppendorf, Hauppauge, NY).
- Capture beads.
- Detection antibody (Fitzgerald Industries International, Acton, MA).
- PBS (1×, pH 7.4).
- NaCl (1 M) containing 0.05% Tween 20.
- SuperBlock blocking buffer (Thermo Fisher).
- TCEP.

#### 2.1.7. Mixed CTI

- Magnabind carboxyl derivatized magnetic beads (Thermo Fisher).
- 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) (Thermo Fisher).
- SuperBlock blocking buffer (Thermo Fisher).
- Phosphate buffered saline (1× PBS).
- Conjugates of BSA and small molecules.
- Detection antibody.
- TCEP.
- 1.5 mL microcentrifuge tubes.
- Magnetic bead separator (Magnabind magnet, Thermo Fisher).
- Capture beads.
- Detection antibody.
- PBS (1×, pH 7.4).
- NaCl (1 M) containing 0.05% Tween 20.

#### 2.1.8. Fluorescence detection

- Photometrics HQ$^2$ CCD (Roper Scientific, Tuscon, AZ).
- TE2000-U epifluorescence microscope assembly (Nikon, Melville, NY).
- Metamorph Imaging System software (Molecular Devices, Sunnyvale, CA).

### 2.2. Microchip device fabrication

A poly(dimethylsiloxane) (PDMS) microfluidic device for MCE was fabricated according previously published methods [19]. The mold master was constructed using SU-8 photoresist and photolithography. A degassed mixture of Sylgard 184 elastomer and curing agent (10:1) was poured on the mold master as well as a bare silica wafer and allowed to cure at 65 °C for at least 2 h. The cured PDMS was removed from the mold and the reservoirs punched using a standard 6 mm hole punch. The surfaces of the two pieces of PDMS were cleaned using methanol and dried in a 65 °C oven for 15 min. The two pieces of PDMS were then placed in an air plasma cleaner and oxidized on high power for 30 s and the two pieces immediately brought together to form an irreversible seal.

### 2.3. Microchip capillary electrophoresis

All MCE separations were conducted using PDMS microchips fabricated as described in Section 2.2. The microfluidic channels were 50 μm high by 50 μm wide with a 5 cm long separation channel. The assembled microchip was pre-treated with separation buffer for 1 h prior MCE experiment. The power supply used for electrophoresis was built in-house [18]. For gated injection, a potential of +600 V was applied at both sample and buffer reservoirs. During injection, the potential at buffer waste reservoir was floated causing a sample plug to be generated in the separation channel. Unless otherwise stated, the separation potential was +75 V/cm and injection time was 1 s. Each well was filled with 50 μL of sample or running buffer. The detection was performed at 4.5 cm from the injection point using an inverted fluorescent microscope.

For pinched injection, a 250 μm double-T injector was used for all experiments giving a 625 pL injection volume. During pinched injection a high potential (+221.25 V/cm) is applied to the sample and buffer, and waste reservoirs while a negative bias (−391 V/cm) is applied to the sample waste in order to draw the sample across the separation channel to the sample waste thereby forming the injection plug in the separation channel. During separation the buffer reservoir is switched to +500 V/cm and the buffer waste reservoir set to ground. A pull-back potential of +190 V/cm is applied to both the sample and sample waste reservoirs to prevent leakage into the separation channel. Detection was carried out at 2.5 cm down the channel from the injection using an inverted fluorescent microscope system.

### 2.4. Cleavable tag synthesis

Tags were synthesized according to previously published methods [8]. Cleavable fluorescent tags with an affinity for avidin were selected for the work described here [8]. Shown in Fig. 1, the tag consists of four functional components: (i) detection group: fluorescein (FITC) was used as the detection group for fluorescence detection, (ii) spacer group: necessary for generating tags with unique charge-to-mass ratio to provide resolution during separation, (iii) a chemically cleavable group: in this case, disulfide bonds were incorporated into the tag using NHS-SS-biotin or cystamine, and (iv) conjugation group: avidin–biotin interactions were used to bind the tags to detection antibodies. For the cystamine containing tag sulfo-NHS-biotin was used in place of NHS-SS-biotin. These tags are readily resolved from each other by MCE. In the case of the competitive assays detailed here, tags were conjugated to the 3-NT and T4 antibodies of the target analytes using avidin–biotin chemistry. For all experiments, FAMB (biotinylated FITC-cystamine) was used for 3-NT and FEB (biotinylated fluorescein thiocarbamyl ethylenediamine) was used for T4. For CRP analysis, FHB (biotinylated thiocarbamyl hexamethylenediamine) was used as a tag.

#### 2.4.1. FAMB synthesis

Fluorescein isothiocyanate (FITC) (51 mg) was dissolved in methanol (5 mL) containing 0.1% triethylamine (TEA). The solution was added dropwise over 30 min to the solution of cystamine dihydrochloride (180 mg) dissolved in a mixture of methanol (5 mL), water (2 mL), and TEA (40 μL). The reaction was allowed to proceed overnight at room temperature (22 ± 2 °C) with constant stirring. The solution was evaporated under airflow until the volume was reduced to approximately 5 mL. A 10:1 mixture of acetonitrile/methanol was then added to the solution to precipitate the FITC-cystamine product. The solution was air dried and the product reacted with sulfo-NHS-biotin in a 1:1 molar ratio in phosphate buffer (20 mM, pH 7.4) overnight at 4 °C to create the desired FAMB product. The product was stable for 1 month when stored at 4 °C.

#### 2.4.2. FRB synthesis

The tags synthesized using this process are generically known as FRB where R represents ethylenediamine (C2) or hexamethylenediamine (C6). The synthesis used to produce the intermediates, fluorescein thiocarbamyl ethylenediamine (FTED) and fluorescein thiocarbamyl hexamethylenediamine (FTHD), has been previously published by our group [7]. FITC (100 mg) was dissolved in a mixture of methanol (5 mL) and TEA (50 μL). C2 (200 mg) or C6 (200 mg) was dissolved in methanol (5 mL) containing 0.1% TEA. The FITC solution was added dropwise to the C2 or C6 solution over 30 min and allowed to react overnight at room temperature with constant stirring. The following day, the product was precipitated using a 10:1 mixture of acetonitrile/methanol and the precipitate dried under constant airflow at room temperature. The dried precipitate was then reacted with sulfo-NHS-SS-biotin in a 1:1 molar ratio in phosphate buffer (20 mM, pH 7.4) overnight at 4 °C. Purity of the final products can be confirmed using $^1$H NMR. The final products, FEB and FHB, were stable for 1 month when stored at 4 °C.

### 2.5. Competitive CTI assay

#### 2.5.1. Preparation of BSA-hapten conjugates

Bovine serum albumin (BSA) was used as a protein carrier for small molecules of interest (haptens) to increase their immunogenicity [15]. Both conjugates of BSA-T4 and BSA-3-NT were prepared using carbodiimide chemistry [20]. In brief, a coupling reagent (EDC, 200 μmol) was added to a 2 mg/mL BSA solution (25 mL, pH 5.5). The small molecule solution (5 mL, 5 mM T4 or 3-NT) was added dropwise to the BSA solution and allowed to react for 10 min at room temperature. After 10 min, an additional 50 μmol of EDC was added. This reaction was carried out in the dark at room temperature (22 ± 2 °C) for 24 h. The product was then dialyzed against deionized water (3.5 kD MWCO dialysis membrane, Thermo Fisher) to remove excess free small molecules and excess EDC. The solution was stored up to 1 year at −20 °C.

#### 2.5.2. Detection antibody preparation

The detection antibody was biotinylated using maleimide chemistry in order to better control the number of biotins bound to the

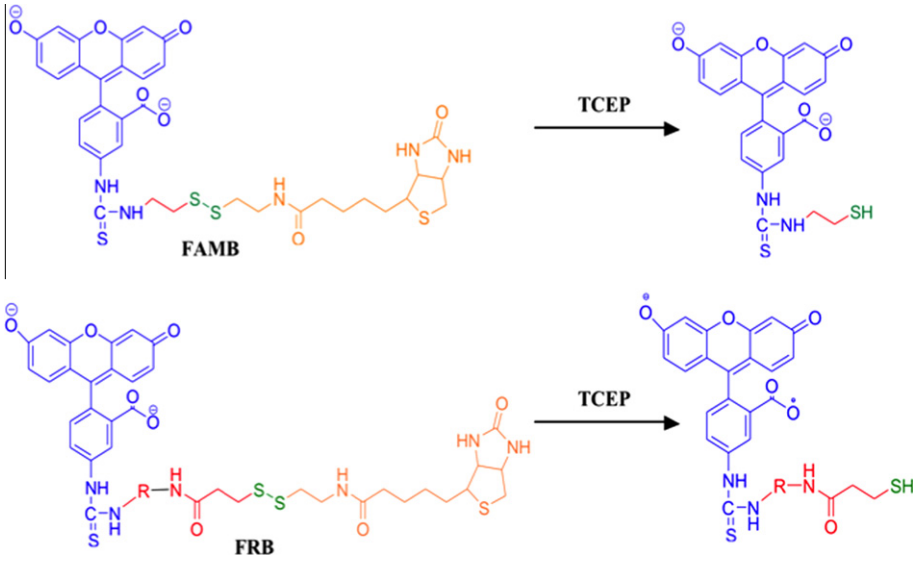


**Fig. 1.** CTI tags and their cleavable products used in this work. Each tag consists of four functional elements (i) detection group (_____), (ii) the spacer group (_____), (iii) a chemically cleavable group (_____), and (iv) the conjugation group (_____). FRB was synthesized based on homo-bifunctional amine spacer. FAMB was produced using cystamine pathway. TCEP cleaves tags at the disulfide bond.

antibody. Typically, 2–4 biotins per antibody can be expected using this chemistry (quantifiable using a HABA biotin quantification kit). This solid phase biotinylation kit also takes advantage of the propensity of antibodies to bind to metal chelate supports. Binding to the nickel chelate support occurs primarily through the histidine rich Fc region of IgG molecules allowing for the reduction of the disulfides within the Fab regions of the antibody, site-directed biotinylation and removal of excess reagents while the antibody is bound to the support. Following antibody biotinylation, avidin was added (1:1 biotin bound/avidin) and allowed to react at room temperature for 2 h. Finally, the biotin-terminated tag was added in three-fold excess (3:1 tag/avidin) to the solution and reacted for 2 h at room temperature. Excess tag was removed using a 3 kD MWCO Nanosep centrifugal concentrator (Pall Corporation). This process results in 2–8 fluorescent tags per antibody.

### 2.5.3. Assay procedure

The steps for competitive CTI are shown in Fig. 2A. As in our previous work, MCE with fluorescence detection was used for analysis of cleaved tag fragments [7,8]. Here, two model analytes, 3-NT and T4 demonstrate proof-of-principle for the multi-analyte competitive CTI chemistry. Conjugates of bovine serum albumin (BSA) and the analyte of interest (BSA-T4, BSA-3-NT) were immobilized onto a microtiter plate surface overnight at room temperature. The solution was removed and the well surface rinsed two times with 1× PBS followed by a single rinse with SuperBlock blocking buffer. Detection antibody (100 μL of 100 μg/mL) and a given amount (10 μL of each concentration/sample) of the corresponding analytes (T4 or 3-NT) were added to the well to run the competitive immunoassay. The reaction was allowed to proceed for 2 h at room temperature. The wells were then rinsed three times with PBS and three times with TAPS buffer (10 mM, pH 9). TCEP (50 μL, 1 mM in 10 mM TAPS, pH 9) was added and allowed to react for 1 h at room temperature. Finally, the solution containing cleavable product was analyzed using MCE with fluorescence detection. The run buffer for MCE was 10 mM TAPS, 5 mM SDS, 0.05% Triton-X 100 pH 9.

## 2.6. Non-competitive CTI

### 2.6.1. Antibody preparation

Capture antibodies for the non-competitive CTI were immobilized on aldehyde functionalized Toyopearl affinity resin

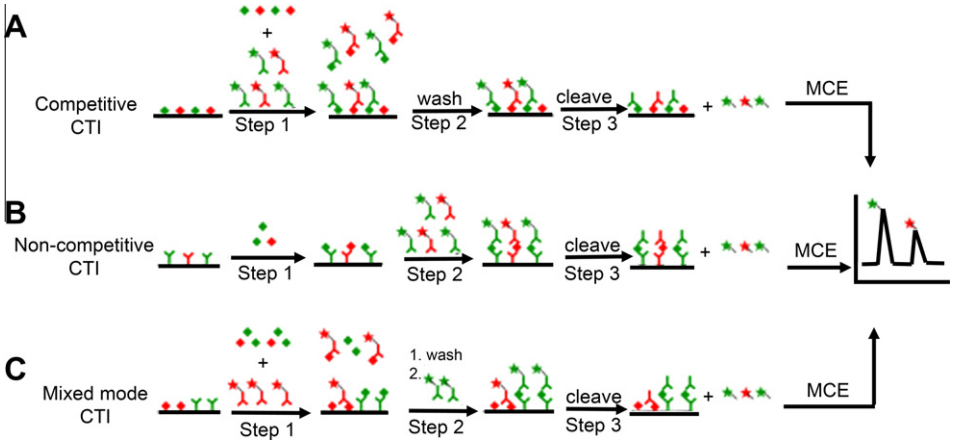


**Fig. 2.** Steps to perform (A) multi-analyte competitive, (B) non-competitive, and (C) mixed competitive/non-competitive mode CTI. Surface to perform both assays can be either bead particles or microtiter plate. The different colors represent different biomarkers. Antibodies and tags have been colored to correspond to the biomarkers.

(formyl-650 M, 40–90 μm diameter) at a concentration 10-fold greater than the upper reference limit for CRP. The particles (800 μL) were washed three times with 1 mL of $K_2HPO_4$ (100 mM, pH 7.5) to remove the azide used to preserve the particles prior to antibody immobilization. The $K_2HPO_4$ was removed and the antibody solution (2 mL) added to the vial. Sodium cyanoborohydride (10 mg) was added to facilitate reductive amination during the antibody attachment and allowed to react with gentle mixing for 2 h at room temperature. After 2 h, the resin was washed three times with $K_2HPO_4$ (100 mM, pH 7.5), and ethanolamine (2 mL, 1 M, pH 8) was added to each vial of particles and allowed to react for 2 h at room temperature to block all remaining aldehyde groups. Sodium cyanoborohydride (10 mg) was again added to facilitate the coupling reaction. The particles were then washed three times with $K_2HPO_4$ (100 mM, pH 7.5). An additional three rinses with SuperBlock blocking buffer were carried out to reduce the amount of nonspecific binding in the remaining steps of the CTI. Particles can be stored in $K_2HPO_4$ (100 mM, pH 7.5) at 4 °C for up to 1 month. Detection antibodies were prepared according to procedures described in Section 2.5.2.

*2.6.2. Assay procedure*

Capture beads (150 μL), prepared as described in Section 2.6.1 were rinsed three times with $KH_2PO_4$ (100 mM, pH 7.5) and the rinse solution discarded. The antigen solution (250 μL) was then added to the beads and allowed to react for 2 h at room temperature. After 2 h, the beads were centrifuged and the liquid discarded. The particles were then rinsed three times with NaCl (1 M) containing 0.05% Tween 20 followed by three rinses with $KH_2PO_4$ (100 mM, pH 7.5). To reduce the amount of nonspecific binding, the beads were rinsed three times with SuperBlock blocking buffer. Finally, the detection antibody (500 μL, 500 μg/mL) was added to the beads and allowed to react for 2 h at room temperature. To remove any nonspecifically bound detection antibodies, the beads were rinsed five times with NaCl (1 M) followed by three times with $KH_2PO_4$ (100 mM, pH 7.5). Cleavage was carried out using TCEP (500 μM, 75 μL) for 30 min at room temperature. Finally, the solution containing the fluorescent fragment was removed from the beads and analyzed using microchip MEKC. The run buffer for MEKC was 10 mM tetraborate, 50 mM SDS, 10 mM DDAPS (N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate). The steps for non-competitive CTI are shown in Fig. 2B.

### 2.7. Mixed mode competitive/non-competitive CTI

*2.7.1. Antibody preparation*

The original non-competitive CTI was carried out on polymer beads. Since the ultimate goal of the CTI is to perform the entire assay, including sample processing, within a microfluidic device, we chose to move to magnetic beads for the mixed non-competitive/competitive CTI. Capture antibodies/BSA-small molecules were immobilized on carboxyl derivatized magnetic beads using carbodiimide coupling chemistry. The functionalized magnetic beads (15 μL for CRP, 30 μL for BSA-3-NT) were first washed three times with 1× PBS. Capture antibody (1 mL, 70 μg/mL) or the BSA-hapten (1 mL, 67 μg/mL) was added along with EDC (100 μL, 10 mg/mL) and allowed to react overnight at room temperature. The EDC was added to facilitate coupling of the protein to the magnetic beads. All dilutions were made in the conjugation buffer (0.1 M MES, 0.9% NaCl, pH 4.7). The solution was then aspirated and the beads washed three times with 1× PBS. The capture beads for either CRP or BSA-3-NT were resuspended in 1 mL 1× PBS and stored at 4 °C for up to 1 month. Coupling efficiency was determined by measuring the absorbance ($\lambda$ = 280 nm) of the protein solution before and after the coupling reaction. The difference in absorbance and the corresponding molar extinction coefficient (IgG = 210,000 $M^{-1}$ $cm^{-1}$, BSA = 43,824 $M^{-1}$ $cm^{-1}$) were used to calculate the amount of protein bound to the particles based on the Beer–Lambert law. Detection antibodies were prepared according to procedures described in Section 2.5.2.

*2.7.2. Assay procedure*

CRP capture beads (300 μL) and BSA-3-NT functionalized beads (200 μL) were combined in a single microcentrifuge tube. The antigen solution (75 μL) containing CRP, 3-NT, and the 3-NT detection antibody (30 μg/mL) was added to the beads and allowed to react for 2 h at room temperature. After 2 h, the beads were separated from the liquid using a stationary magnet and the liquid discarded. The particles were then rinsed three times with NaCl (1 M) containing 0.05% Tween 20 followed by three rinses with 1× PBS. To reduce the amount of nonspecific binding, the beads were rinsed three times with SuperBlock blocking buffer. Finally, the detection antibody for CRP (75 μL, 30 μg/mL) was added to the beads and allowed to react for 30 min at room temperature. To remove any nonspecifically bound detection antibodies, the beads were rinsed three times with NaCl (1 M) followed by three times with 1× PBS. Cleavage was carried out using TCEP (500 μM, 70 μL) for 20 min at room temperature. Finally, the solution containing the fluorescent fragment was removed from the beads and analyzed using microchip micellar electrokinetic chromatography (MEKC). The run buffer for MEKC was 10 mM tetraborate, 50 mM SDS, 10 mM DDAPS (N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate). The steps for mixed competitive/non-competitive CTI are shown in Fig. 2C.

### 2.8. Generation of calibration curves

Calibration curves for all analytes were constructed for concentrations which spanned the reference ranges (Table 1). The curves were fit using a standard 4-parameter logistic model described by the following equation [15]:

$$y = A_2 + \frac{(A_1 - A_2)}{\left(1 + \left(\frac{x}{x_0}\right)^p\right)} \quad (1)$$

where $y$ is the response signal, $A_2$ is the response at infinite analyte concentration (antibody saturation), $A_1$ is the response at zero analyte concentration, $x$ is the analyte concentration, $x_0$ is the inflection point on the curve ($IC_{50}$, center of the curve), and $p$ is the slope factor. The logistic equation fit was also used to determine the limit of detection (LOD), defined as the analyte signal that corresponded to three times the standard deviation of the zero analyte signal, and the limit of quantification (LOQ), defined as the analyte signal that corresponded to ten times the standard deviation of the zero analyte signal [21].

## 3. Results

### 3.1. Competitive CTI

In competitive immunoassays, as the analyte concentration increases, the concentration of the captured tagged antibody decreases, and hence the detectable signal decreases [15]. Assays for T4 and 3-NT were first carried out individually to demonstrate competitive CTI. Sample electropherograms for the detection of varying concentrations of T4 using competitive CTI are shown in Fig. 3A. Similar results were obtained when 3-NT was used as the model analyte (data not shown). These results show the characteristic competitive behavior. The inconsistent migration times from run-to-run are the result of low electroosmotic flow stability from the polydimethylsiloxane surface used in these experiments [22]. A sample competitive CTI calibration curve for T4 is shown in Fig. 3B.

**Table 1**
Linear range, LOD, and LOQ for CRP, T4, and 3-NT.

| Biomarker | Reference interval | LOD | LOQ | Linear range |
|---|---|---|---|---|
| C-reactive protein (CRP) | <10 μg/mL [24] | 5 μg/mL | 17 μg/mL | 10–25 μg/mL |
| Thyroxine (T4) | Male: 59–135 nM [24] Female: 71–142 nM [24] | 23 nM | 77 nM | 10–100 nM |
| 3-Nitrotyrosine (3-NT) | <1.2 μg/mL [25] | 0.5 μg/mL | 1.7 μg/mL | 0.5–7 μg/mL |

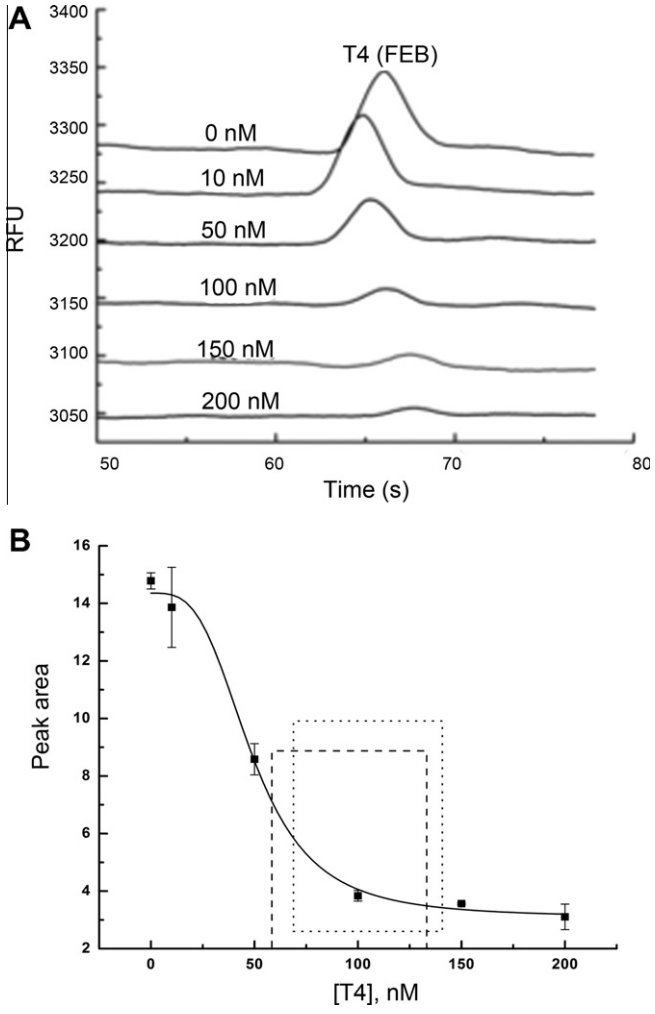


**Fig. 3.** (A) Example electropherograms for competitive CTI of T4. (B) Typical competitive CTI calibration curve for T4. The reference range of T4 is marked on the curve for male (dashed line) and female (dotted line). T4 detection antibody tagged by FEB. Separation conditions are reported in the text. Fluorescence detection was performed using an inverted fluorescent microscope. RFU is relative fluorescence units.

Fitting the T4 standard curve using the 4-parameter logistic model described in Section 2.8 gave a correlation coefficient ($R^2$) of 0.997, a calculated LOD of 23 nM and LOQ of 77 nM while the fit using the 4-parameter logistic model for 3-NT gave an $R^2$ of 0.981, an LOD of 0.5 μg/mL, and an LOQ of 1.7 μg/mL. Differences in units here reflect the differences in reported clinical units. The upper reference level for T4 analytes was marked on curves indicating that our method provided the analysis of the markers in the normal range. This method also gave an acceptable reproducibility with the average relative standard deviation for each analyte concentration of 6.37% and 7.69% for T4 and 3-NT, respectively. All errors were calculated as a standard deviation of the signals from three MCE runs.

After demonstrating the proof-of-concept for competitive CTI for a single analyte, a multi-analyte assay was performed using 3-NT and T4. The BSA-3-NT and BSA-T4 conjugates were co-immobilized onto a well surface. In this case, the 3-NT concentration was varied while the T4 concentration was held constant. The data presented in Fig. 4A demonstrate the detection of these two analytes from a single sample. The cleaved tags are well resolved using the separation conditions described in Section 2.5.3. The dose response curve for 3-NT in the presence of 50 nM T4 is shown in Fig. 4B as a function of 3-NT concentration. A decrease in signal is observed for the 3-NT as the concentration is increased in the sample while no change in the peak area is observed for T4 thus illustrating the selectivity of the system. In addition, these results demonstrate the possibility of multi-analyte biomarker detection in complex biological samples with no need for complicated sample preparation.

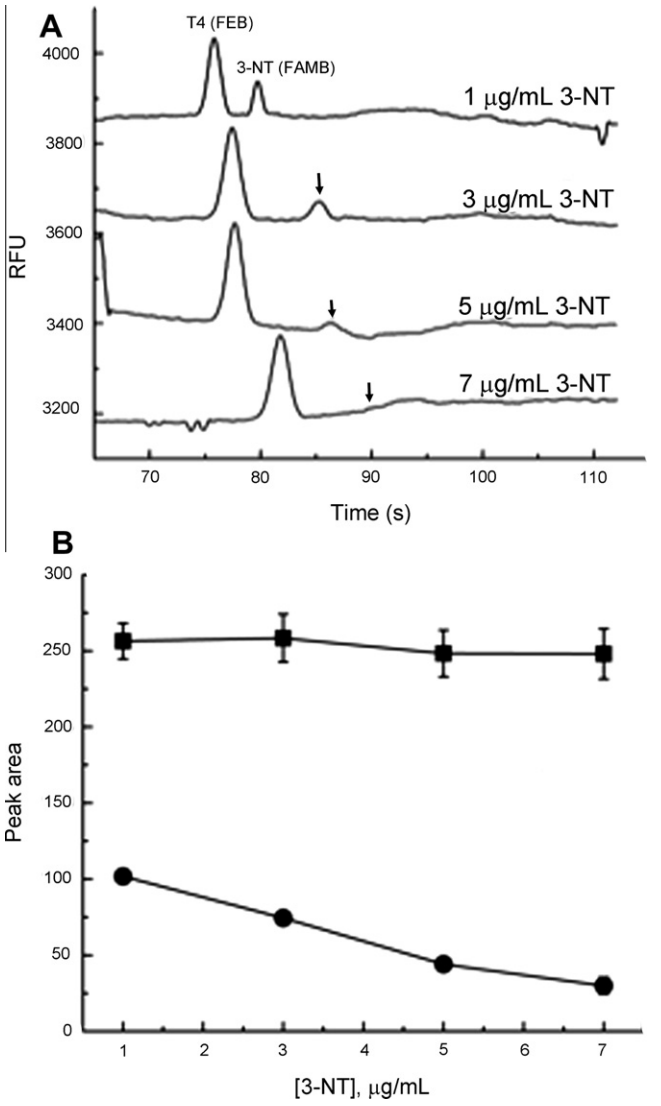


**Fig. 4.** Electropherograms of multi-analyte competitive CTI. (A) Electropherograms showing the multi-analyte competitive CTI of 3-NT and T4, variable concentration of 3-NT (1–7 μg/mL), and constant concentration of T4 (50 nM) were added to run competitive CTI. (B) Resulting dose–response curve for 3-NT (●) and T4 (■). Error bars for 3-NT are contained within the points. Separation conditions are reported in the text.

### 3.2. Non-competitive CTI

Bead-based immunoassays are dependent on both bead volume (solid-phase) and amount of cleavage agent added. By adjusting these parameters, the sensitivity can be shifted to the desired concentration range for the analyte of interest. In this case, assay parameters were adjusted to ensure that the sensitive portion of the curve for CRP bracketed the reference interval. The reference interval is generally defined as the level below which 95% of the normal population falls. As is typical for immunoassays, the data is shown in semi-log format (Fig. 5). The reference interval is marked on the calibration curve in order to illustrate the ability of non-competitive CTI to distinguish between normal and elevated CRP levels. The linear range, LOD, and LOQ for CRP are also given in Table 1 along with the reference interval. The limit of detection is defined here as a signal greater than three times the baseline noise. Furthermore, when a healthy control serum sample was run, no signal was seen for CRP demonstrating the selectivity of this assay in the presence of serum proteins.

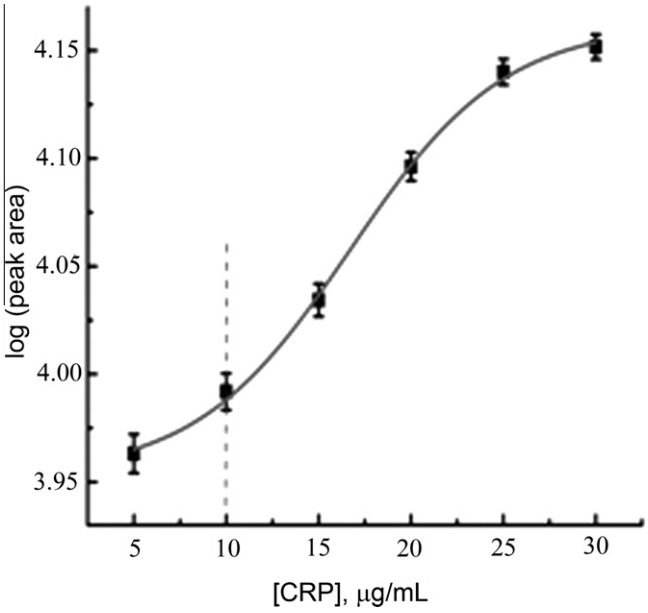


**Fig. 5.** Calibration curve for CRP detected via non-competitive CTI. Concentrations span both normal and elevated levels. The upper reference limit is marked on the curve. CRP was diluted in 1× PBS. Detection antibody for CRP tagged with FHB. Separation conditions are reported in the text. Fluorescence detection was performed using an inverted fluorescent microscope.

### 3.3. Mixed format CTI

After showing viability of non-competitive and competitive CTI individually, a mixed format CTI was carried out with 3-NT (competitive) and CRP (non-competitive) as the molecules of interest. In this two component assay, FAMB was used for 3-NT and FHB was used for CRP. The data presented in Fig. 6 demonstrates the detection of these two entities from a single sample. Two separate samples with differing known amounts of 3-NT and CRP were used for the mixed format CTI. In Fig. 6A the concentrations were 5 and 10 μg/mL for 3-NT and CRP, respectively and in Fig. 6B the concentrations were 3 and 30 μg/mL for 3-NT and CRP. In the resulting electropherograms, the peak height for CRP increased by a factor of 2.8 while the 3-NT peak height decreased by a factor of 0.17. These changes are in line with the stated concentration changes from the samples. Furthermore these changes bracket the clinical reference values for both 3-NT and CRP. This represents the first example of a mixed format immunoassay done using CTI. It should be noted that these calculations were based on the peak height and not peak area as a result of the shoulder on the 3-NT peak. The identity of the shoulder is not known at this time but is currently under investigation.

## 4. Discussion

Chronic diseases typically cause changes in both circulating proteins and metabolites; however, currently available assays are generally capable of screening for one or the other but not both of these classes of biomarkers. CTI chemistry is able, with small

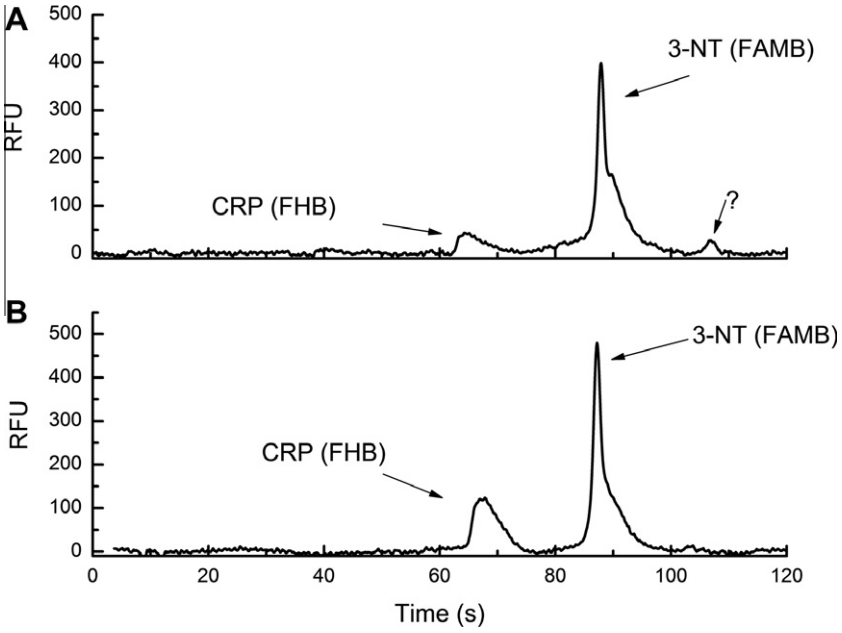


**Fig. 6.** Electropherograms of mixed non-competitive/competitive CTI. (A) 5 μg/mL 3-NT and 10 μg/mL CRP in PBS and (B) 3 μg/mL 3-NT and 23 μg/mL CRP in PBS. Separation conditions are reported in the text. Fluorescence detection was performed using an inverted fluorescent microscope. RFU is relative fluorescence units.

modifications, to measure both small molecule metabolites and proteins in a single run using a mixed format approach to immunoassays. We have successfully shown that the CTI can be used to separately quantify small molecules (metabolites) in competitive format and proteins in the non-competitive format. Furthermore, we successfully demonstrate the ability to use non-competitive and competitive CTI simultaneously in a single assay for the detection of markers related to inflammation, namely 3-NT (competitive CTI) and CRP (non-competitive CTI). To our knowledge this is the first report of a mixed competitive/non-competitive CTI. The concentrations studied fall at or above the reference intervals for both 3-NT and CRP indicating the ability to discern between normal and elevated levels of small molecules and proteins. The results shown here were collected from 3-NT and CRP standards diluted in PBS; however, given that we have not seen any interference from complex samples such as serum when performing non-competitive or competitive CTI separately, we do not anticipate any issues when transitioning to real samples.

We were able to detect 3-NT, T4, and CRP at both normal and elevated levels with LODs in the low μg/mL range. One reason for this is that the final step in the CTI, the cleavage step, not only serves to cleave the tags from the detection antibodies but also as a pre-concentration step as the volume of solution in which the detectable product is present has been reduced by 5–50% compared to the original sample volume. It is important to note that the incubation times currently employed in the off-chip CTI are quite long (30 min–2 h). In our future work, the incubation time will be significantly shortened due to reduced diffusion distances when the entire assay is performed within a microfluidic device and magnetic beads will be used as a solid support in on-chip immunoassay as they can be held within the chip by the external magnetic field [23].

While the use of a microtiter plate instead of polymer beads for the competitive CTI simplifies the rinse steps compared to the non-competitive CTI, the use of well surfaces as a solid support causes lower sensitivity than bead-base materials due to lower surface area [15].

This is the first report of the use of magnetic particles in the CTI. Magnetic particles are known to provide better volume control, ease of sample manipulation and an increase in the solid support surface area compared to polymer microparticles or microtiter plates. For these reasons we chose to use carboxyl derivatized magnetic particles for the mixed mode competitive/non-competitive CTI. Furthermore, the use of magnetic particles will facilitate the transition to performing the entire CTI assay, including sample processing, within a microfluidic device.

## 5. Conclusions

Here we successfully demonstrated the ability to perform CTI in competitive, non-competitive, and mixed competitive/non-competitive formats for the detection of inflammation biomarkers. As model analytes, T4 and 3-NT were selected to show the proof-of-principle for multi-analyte competitive CTI. CRP was successfully analyzed by non-competitive CTI. LOD, LOQ and working range were determined and encompass the upper reference levels for the three molecules studied. Finally, CRP and 3-NT were selected to demonstrate that the mixed competitive/non-competitive CTI can be conducted with successful results indicating that using CTI proteins and small molecules can be determined simultaneously. This assay gives a promising method for screening small molecule and protein biomarkers, and offers an alternative for to clinical applications requiring a multi-analyte point-of-care screening system. This novel assay provides much lower analysis time, sample consumption as well as cost of analysis compared to the traditional analytical methods.

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