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Immunoassay for thyroxine (T4) in serum using capillary electrophoresis and micromachined devices

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Abstract

As part of our ongoing effort to develop electrophoretic assay technology for clinical diagnostics, we describe a competitive immunoassay for the determination of serum thyroxine (T4) based on electrophoresis and laser induced fluorescence (LIF). Measurements of total T4 are useful for the clinical evaluation of thyroid function. A fluorescein thyroxine conjugate was utilized in conjunction with a polyclonal antibody preparation as assay reagents. Capillary electrophoresis (CE) conditions tolerant of the direct injection of serum without extraction or other sample preparation steps were developed and used for quantitation of total T4 in serum. We have been exploring the use of micromachined devices with arrays of channels for high assay throughput. Our assay protocol was carried in a microchip format. The results illustrate that gains in speed can be additionally achieved, with the electrophoretic separation of free from bound labelled T4 being performed in about 15 s for serum samples. © 1997 Elsevier Science B.V.

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1. Introduction

We have been interested in using the high separating power of capillary electrophoresis as a tool to perform immunoassays and other analyses of clinical importance. CE-based immunoassays have been reported by several groups in recent years [1–7]. Our own group has demonstrated a CE-based assay for the determination of cortisol in serum [7]. CE is attractive because of its speed, small scale, high resolution capability and amenability to automation.

To obtain the high sensitivity required for many assays, a detection methodology with low detection limits is required. Fluorescence detection is one option we have explored. This renders the fluorescent labelling of antibody preparations or antigens necessary.

Electrophoresis performed in a single capillary format allows only sequential sample analysis thereby severely limiting the amount of samples being tested. The high throughput required for clinical

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analyzers might be achieved through the use of arrays of capillaries operated in parallel [8,9]. An alternate approach uses micromachining to etch multiple separations channels of identical geometry into a single substrate [10,11]. We have recently described an electrophoretic immunoassay for serum cortisol operated in a single channel microchip [12]. The separation of free and bound labelled cortisol was performed in less than 30 s. We have also performed the assay with multichannel systems operating on multiple samples in channel arrays [13]. Recently an immunoassay for theophylline was presented by Harrison and Cheim [14] on a single-lane microchip. The reported run time was around 40 s and the detection limit for the fluorescein labelled antigen was 5 ng/ml.

In this paper, we describe the development of a CE based competitive immunoassay to determine total T4 (3,5,3′,5′-tetraiodo-L-throxine) in serum. The assay was performed in capillaries and in micromachined electrophoretic analysis devices. The resulting protocol is very similar to that of a cortisol assay recently described by us [7] and to other competitive assays we have studied, indicating that a general competitive electrophoretic immunoassay scheme may be possible.

Thyroid hormones have a major role in the regulation of metabolic processes. Stimulation of the thyroid gland by the pituitary hormone TSH (thyroid stimulating hormone) causes the release of thyroxine (T4) and triiodothyronine (T3). These hormones enter the circulation and exert regulatory effects on target tissues. T4 has direct hormonal activity but can also undergo local conversion to T3 in some tissues. In the bloodstream, T4 (like T3) is predominantly bound to the carrier proteins thyroxine-binding globulin, thyroxine-prealbumin and albumin [15]. Thyroid function is assessed through the measurement of total and free T4 and T3 levels. Measurement of TSH is also useful in diagnosing thyroid disorders. Graves disease is one of several manifestations of hyperthyroidism (elevated thyroid hormones in the circulation) and is typically associated with goiter. Decreased levels (hypothyroidism) can result in anemia, delayed reflexes, liver disease, and reduced metabolism. The normal adult range for the total T4 in serum is 5–12 μg/dl [15].

2. Experimental

2.1. Apparatus

A fused-silica microchip was coupled with a power supply and fluorescence optics for the chip-based assay. High voltage was provided by a Spellman CZE 1000R power supply (Plainview, NY, USA) through a switching circuit and resistor network. Laser induced fluorescence detection used an Omnichrome (Chino, CA, USA) argon ion laser operating with ~3 mW of output at 488 nm, focused into the channel at a 53° angle of incidence with a 10 cm focal length lens. A 20× microscope objective (Edmund Scientific, Barrington, NJ, USA) collected fluorescence emission. The collected light was spatially filtered at a 2 mm I.D. aperture in the image plane and optically filtered by two 520 nm bandpass filters (520DF30 Omega Optical, Brattleboro, VT, USA). A photomultiplier tube (Hamamatsu R928, Bridgewater, NJ, USA) connected to a Keithley 614 electrometer (Cleveland, OH, USA) detected the fluorescence signal. The signal was digitized with a PC-controlled 20 bit data acquisition system (Data Translation 2804, Marlborough, MA, USA) and analyzed using Caesar software (ADI, Alameda, CA, USA).

The CE–LIF system consisted of a P/ACE instrument Model 5500 (Beckman, Fullerton, CA, USA) fitted with an argon laser source. Excitation was at 488 nm and detection at 520 nm. CE separations were carried out with capillaries having a modified form of the solixanediol/polyacrylamide coating of Schmalzing et al. [16].

2.2. Chemicals and reagents

Polyimide-coated fused-silica capillary columns of 50 μm I.D., 360 μm O.D. were from Polymicro Technologies (Phoenix, AZ, USA). Acrylamide, 2-amino-2-methyl-1,3-propanediol (AMPD), N-tris (hydroxymethyl)-methyl-3-aminopropanesulfonic acid (TAPS), total T4 fluorescence polarization IA reagent set and T4 serum calibrators (optically clear human serum) were all purchased from Sigma. Human serum samples (pooled sera samples stripped of T3 and T4) from Scantibodies Laboratory (Santee,

CA, USA) were spiked at concentrations of 2.0 to 24.0 μg/dl T4 for evaluating the precision and accuracy of the CE assay.

2.3. Micromachining

Microchip separation devices were produced in fused-silica wafers using photolithography and chemical etchants. Enclosed channels were formed by bonding a second fused-silica wafer to the etched wafer. Fluidic and electrical contact to the channels was provided by holes drilled at channel terminals. Each etched wafer carried multiple separation systems which were cut into individual analysis chips after bonding.

Fabrication was carried out as described by Koutny et al. [12]. Briefly, a 400 Å film of chromium was sputtered onto the fused-silica substrate (75 mm dia.×0.4 mm; Hoya, Tokyo, Japan). Photoresist (Shipley 1811, Newton, MA, USA) was spin-coated onto the wafer and baked at 90°C for 25 min. The photoresist was patterned by exposure to UV (365 nm) through a contact aligned photomask (Advanced Reproductions, Wilmington, MA, USA) and development in Microposit developer (Shipley). Chrome was chemically removed in the exposed areas with $K_3Fe(CN)_6$/NaOH (Chrome Etch, Shipley). The resulting resist pattern was etched into the fused silica by immersing the wafer in NH_4F/HF (1:1) etchant at 50°C. The channel depth was 28 μm, yielding a channel width of 66 μm at the top of the channel because of the isotropic etching conditions. Photoresist was removed with acetone and the remaining chrome dissolved using $K_3Fe(CN)_6$/NaOH.

Access holes were laser drilled through the etched wafer at the channel terminals. A second fused-silica wafer was bonded to the etched wafer to enclose the channels. Both wafers were immersed in 50°C NH_4OH/H_2O_2 and rinsed in H_2O. They were then placed in contact and thermally bonded. Bonding was performed at 200°C for 2 h, followed by overnight fusing at 1000°C. We can successfully bond 75 mm diameter wafers free of defects more than 50% of the time using this method. Buffer and sample reservoirs were cut from glass tubing and attached to bonded wafers with silicone adhesive (Dow Corning).

2.4. Assay protocol

In small vials were pipetted 22.5 μl of undiluted serum. To each were added 100 μl of a mixture containing fluorescein labelled T4, T4 releasing agent (each 4.44× dilution of the reagent stock solutions) and 100 μl of polyclonal antibody solution (4.44× dilution). The samples were briefly vortex-mixed and incubated for 1/2 h at 37°C. The incubate was injected directly into the CE system for separation and quantitation.

2.5. Separations

Capillary electrophoresis separations on the Beckman P/ACE instrument were conducted in capillary columns having a total length of 27 cm and an effective length (to detector) of 20 cm. The incubated serum samples were pressure injected for 8 s at the negative electrode. The applied field strength was 30 kV, and 20 m*M* TAPS/AMPD (pH 8.8), was used as the background electrolyte.

The microchip electrophoretic separations were carried out with a field strength of 1200 V/cm in a 20 m*M* TAPS/AMPD (pH 8.8) electrolyte. The offset pinched injection scheme produced an injection volume of ~0.3 nl.

3. Results and discussion

3.1. Standard CE assays

The T4 assay is performed in the competitive format (Fig. 1). Specific amounts of fluorescein labelled T4 and anti-T4 polyclonal antibody are added to the serum sample. The labelled T4 competes with the T4 present in serum for the limited antibody binding sites. Since more than 90% of the T4 present in serum are bound to binding proteins, a T4 displacing reagent is used to allow the determination of total T4. After the equilibrium is established, a small aliquot of the incubation mixture is introduced into the electrophoresis capillary without

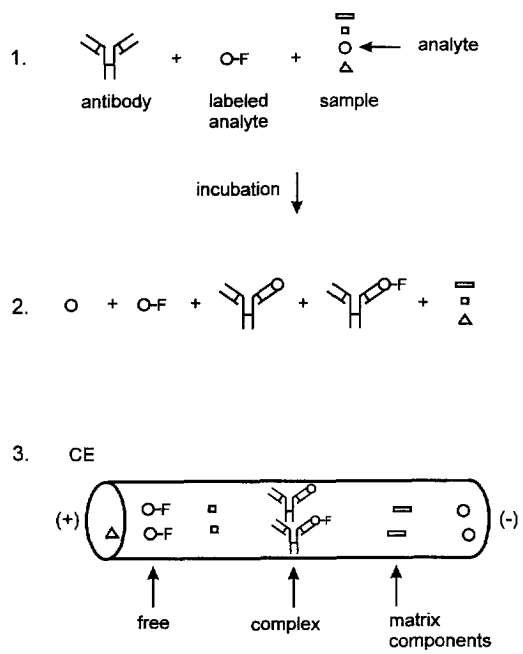

Fig. 1. Competitive electrophoretic immunoassay scheme.

clean up or extraction. Electrophoretic separation resolves the free labelled antigen from the antigen–antibody complex and from other serum components. Typical CE profiles for incubated serum solutions in the relevant clinical range from 3–24 μg/dl are shown in Fig. 2. The fluorescent signal of the free labelled antigen is used for quantitation. It is completely separated from all the other components in less than 1 min. The signal shows the expected behavior: it is high when the concentration of antigen present is high (most of the antibody is complexed to antigen) and it is low when the antigen concentration is low (the tracer takes up most of the antibody binding sites). Fluorescein (the first detected peak) can be used as an internal standard to correct for injection imprecision. The broad, well separated peak of low intensity at around 1.2 min is due to serum protein with intrinsic fluorescence at the excitation wavelength used (e.g., bilirubin). The fluorescent complex peak is not shown. The complex migrates slowly, appearing after 5 min as a very broad peak of low intensity that is unsuitable for quantitation. Due to the use of stable coated capillaries with zero electroosmotic flow, the migration time reproducibilities between both individual runs and different capillaries were around 0.5%. A log-linear calibration curve ($\log [T4] = 0.0397(\text{peak area}) - 7.718$; $r^2 = 0.998$) with a good signal response was obtained when using the free labelled antigen signal for the antigen determination over the range of clinical interest, 3–24 μg/dl. Over a wider concentration

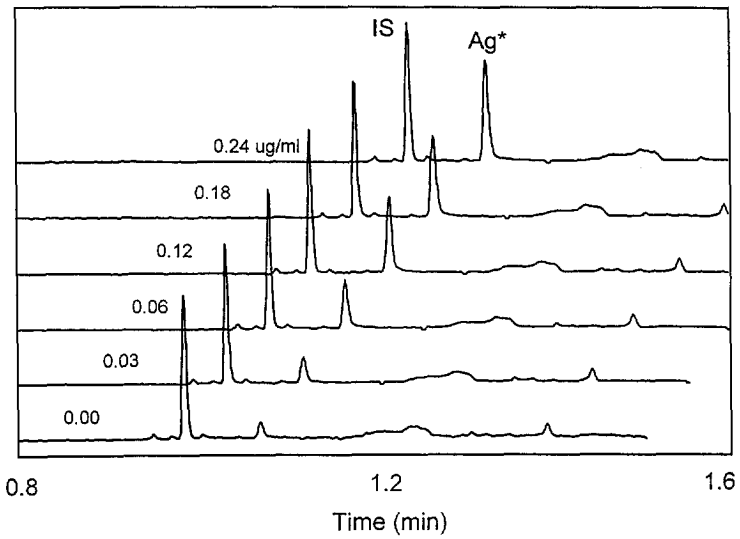

Fig. 2. Electrophoretic immunoassay profiles for serum T4.

Table 1
Accuracy data for T4 assay

T4 present (μg/dl)	T4 found (μg/dl)	Recovery (%)
18.00	17.87	99.3
14.00	14.76	105.4
10.00	10.10	101.0
6.00	6.84	114.0
2.00	2.02	101.0
13.97	13.21	94.6
10.47	10.87	103.8
8.73	8.15	93.4
6.99	7.30	104.4
5.24	5.28	100.8
24.00	21.76	90.7
18.00	17.87	99.3
12.00	14.00	116.7
6.00	6.66	111.0
3.00	2.72	90.7

range the calibration curve has the sigmoidal shape typical for competitive assays.

The accuracy of the method was evaluated with three sets of T4 samples prepared from three different serum sources in spike and recovery experiments. The CE measured recoveries ranged from 90–110% but were close to 100% in most cases (Table 1).

The incubation reproducibility was 5.2% evaluated with 2 different serum samples, each incubated 3 times. This number is satisfactory but might easily be improved once time, temperature and pipetting are better controlled by an instrumental set up specially designed for CE immunoassays. The injection reproducibility was 2.4% (29 samples each injected 5 times), in the range typical for CE.

3.2. Microchip assays

As already discussed in Section 1 we are currently exploring the use of micromachined devices to increase the throughput of CE based immunoassays. The experimental set up for such a device operated in the single channel mode is depicted in Fig. 3. Representative data obtained with the microchip is

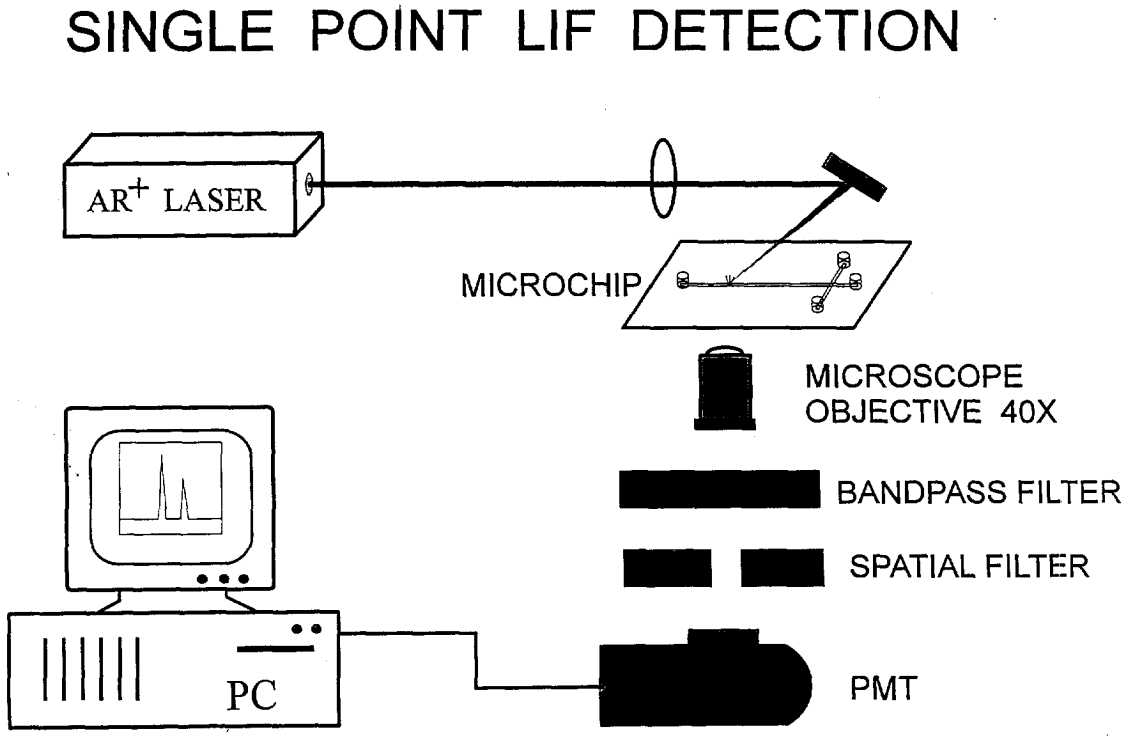

Fig. 3. Schematic drawing of instrumental setup for microchip immunoassay.

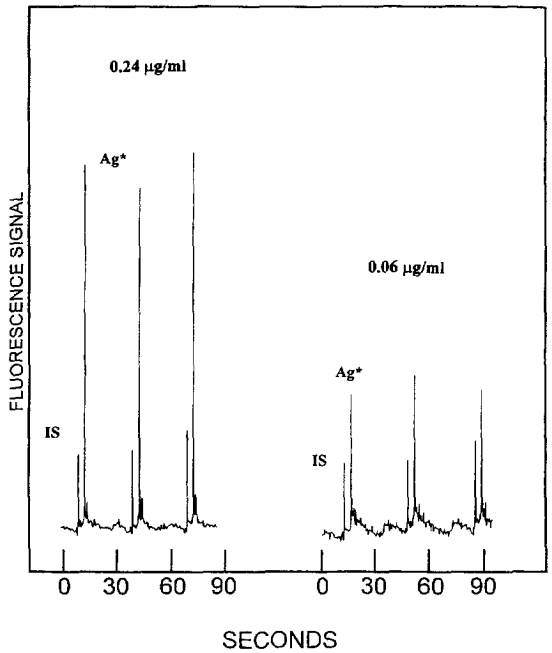

Fig. 4. Microchip immunoassay profile at two levels of sample T4.

shown in Fig. 4. The separations obtained with the chip-based system are much faster than those obtained in capillaries. This is due to the short effective separation length in the microchip (2.2 cm) compared to the capillary (20 cm). This illustrates an advantage of the microchip approach for immunoassays where only a few components of interest are to be separated. While very short capillaries could be used, short separation lengths are more practical on a chip. Resolution is sacrificed with this short separation length when used with the injection volume generated by the offset injector (0.3 nl), but still sufficient in the case studied. Moreover, the trade-off between sensitivity, resolution and analysis time can be adjusted by varying the geometry of the chip with regard to offset size in the injector and the length of the separation channel. The response of the assay is illustrated with two samples having 6 and 24 μg/dl T4, respectively. The somewhat higher baseline noise than observed in our earlier work with cortisol may be a result of capillary surface contamination.

4. Conclusions

We have demonstrated feasibility for an electrophoretic immunoassay for total T4 in serum. Interference from serum components is minimized by the use of the electrophoretic separation. Based on our work with other assays, we believe that the approach used here represents a general scheme that can be applied to a variety of competitive immunoassays and that microfabricated devices show great promise for performing assays of clinical importance.

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