Published in final edited form as:

Electrophoresis. 2015 March ; 36(5): 813–817. doi:10.1002/elps.201400436.

Microchip Immunoaffinity Electrophoresis of Antibody-Thymidine Kinase 1 Complex

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Abstract

Thymidine kinase-1 (TK1) is an important cancer biomarker whose serum levels are elevated in early cancer development. We developed a microchip electrophoresis immunoaffinity assay to measure recombinant purified TK1 (pTK1) using an antibody that binds to human TK1. We fabricated poly(methyl methacrylate) microfluidic devices to test the feasibility of detecting antibody (Ab)-pTK1 immune complexes as a step towards TK1 analysis in clinical serum samples. We were able to separate immune complexes from unbound antibodies using 0.5X phosphate buffer saline (pH 7.4) containing 0.01% Tween-20, with 1% w/v methylcellulose that acts as a dynamic surface coating and sieving matrix. Separation of the antibody and Ab-pTK1 complex was observed within a 5 mm effective separation length. This method of detecting pTK1 is easy to perform, requires only a 10 μ L sample volume, and takes just 1 minute for separation.

Introduction

Molecular diagnostics focuses on the accurate detection of biochemical markers of diseases. Often, blood samples are drawn from the patient and biomarkers are measured to identify the disease state. The usage of biomarkers can be a more effective way of detecting cancer at an early stage compared to tissue biopsy alone.[1] Cancer biomarkers often indicate disruption of the regular cell-signaling pattern resulting in resistance to cell death, uncontrolled proliferation, invasion, metastasis, and activation of angiogenesis.[2]

Thymidine kinase is an important nucleotide salvage pathway enzyme involved specifically in the conversion of thymidine to thymidine monophosphate.[3] There are two types of thymidine kinase in the cell: thymidine kinase-1 (TK1) is found in the cytosol and is cell cycle regulated; the other, thymidine kinase-2, is found in the mitochondria and is constitutively expressed.[4] The mechanism of release of TK1 into the serum is not fully understood, but TK1 concentration in serum is higher in cancer patients than in healthy individuals.[5–7]

Conflict of Interest Statement

One author (K.O.) has patents on the antibodies used in this work and has an interest in TK Bio Tech, which has licensed the technology from BYU.

Typically, radioimmunoassay is used to detect TK1 activity in serum,[8] and enzyme-linked immunoassay (ELISA) is used to determine TK1 concentration.[5] Previous reports indicate that TK1 activity and TK1 concentration are not closely correlated.[6] Another finding showed that TK1 was commonly expressed in its dimeric form and that addition of ATP to the solution resulted in tetramer formation.[9] The tetrameric TK1 also had more catalytic activity than the dimeric form, which is a possible explanation for the discrepancy in TK1 activity and concentration in cancer patients.[6]

A novel antibody that specifically targets TK1 was developed, tested and reported by O'Neill et al.[10] They demonstrated through ELISA, immunohistochemistry and western blot results that the antibody has the ability to detect purified recombinant TK1 (pTK1) and cytosolic TK1 in clinical samples. These classical techniques are sensitive (ng/mL to pg/mL); however, they are time consuming. A possible alternative to detecting TK1 activity (to eliminate the use of radioactive material) is through determining the quantity of each isoform of TK1 since the isoforms correlate with enzymatic activity. Development of an inexpensive, fast, and accurate diagnostic assay for TK1 isoforms and concentration could thus hasten the accurate detection of disease state.

Microchip electrophoresis has been used in separation of different clinically relevant biomolecules because it has many advantages over traditional methods such as use of small sample volumes, fast analysis, low cost, portability and disposability. Most fluorescent tags for on-plate immunoassays can also be used in microchip electrophoresis if appropriate lasers and filters are utilized in the detection system. Importantly, microchip electrophoresis can provide high throughput with multiple lanes, but still maintain low sample and reagent consumption and faster analysis times compared to on-plate immunoassays.[11] Integration of photopolymerized cross-linked polyacrylamide gels in microfluidic devices has been used for separation of immune complexes.[12–13] As an alternative to photopolymerization of gels, buffers with cellulose-based polymers or linear polyacrylamide as dynamic coatings and sieving matrices have been developed.[14–16] Different cellulose sieving matrices were successfully used for genomic and proteomic analysis.[17] The ability of methylcellulose to effectively suppress electroosmotic flow and stabilize the pH gradient allowed efficient isoelectric focusing on a microchip.[18] Electrophoresis in microdevices with hydrophilic polymer sieving matrices and coatings is thus an attractive potential platform for quantifying TK1.

In the present study we report the use of a monoclonal anti-TK1 antibody [10] to detect immune complexes with as low as 80 nM TK1 using microchip electrophoresis. We used FITC-labeled anti-TK1 antibodies to monitor the formation of the immune complex. We explored the effect of commonly used buffers on immune complex formation and found a strong buffer dependence. We also studied the effect of buffer viscosity on the separation and peak shape. After identifying an appropriate buffer and sieving matrix we were able to develop an easy to perform microchip electrophoresis assay of Ab-TK1 complexes. This approach could potentially be adapted for detecting TK1 in serum for early diagnosis of cancer or its recurrence.

Experimental Section

Materials and Reagents

Methylcellulose (MC) of different molecular weights (14000, 41000, and 88000 Da) was purchased from Sigma-Aldrich (St Louis, MO). Tween 20 was acquired from Mallinckrodt Baker (Paris KY). Sodium chloride came from Columbus Chemical (Columbus, WI). Tris-base and 10x phosphate buffer saline were from Fisher Scientific (Fair Lawn, NJ). Boric acid, glycine and hydrochloric acid were obtained from EMD Chemical (Gibbstown NJ). Fluorescein isothiocyanate (FITC) and ethylenediaminetetraacetic acid (EDTA) were purchased from Life Technologies (Carlsbad, CA). Centrifugal filters were from Millipore (Billerica, MA). Poly(methyl methacrylate) (PMMA) substrates were purchased from Evonik (Parsippany, NJ). All solutions were made using deionized water, 18.3 M Ω .

Buffer preparation

1% MC solutions were prepared as recommended by the manufacturer. Briefly, MC was first dissolved in deionized water at 80°C, and then the corresponding buffer volume was added to the solution. Cold deionized water (~4°C) was next added up to the required volume and the solution was stored at 4°C for 1 hour. The buffer was then agitated at room temperature for at least 1 hour, resulting in a clear and viscous solution. All buffer solutions had Tween-20 added to 0.01% and were stored at room temperature after preparation.

Device Fabrication

PMMA microchips were fabricated using adaptations of protocols reported previously.[19] Silicon templates were made in the BYU Integrated Microfabrication Lab using standard photolithography techniques to provide elevated channel features (20 μ m tall, 50 μ m wide) in a T design. Microchannel features were hot embossed onto 1.5 mm thick PMMA (2 x 5 cm²) sheets for 30 minutes at 138°C. 3 mm thick PMMA sheets (2 x 5 cm²) with laser-cut reservoirs were thermally bonded as cover plates for 27 minutes at 110°C to enclose the microfluidic channels.

Sample Preparation

Mouse anti-human TK1 monoclonal antibodies [10] were conjugated with FITC as described previously.[20] Excess FITC was removed using Amicon Ultra-0.5 centrifugal filters (30 kDa). Concentrations of the stock antibody were measured to be 1 mg/mL using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies Willmington, DE). FITC-conjugated antibodies were tested for TK1 binding using dot blots on nitrocellulose membranes. Labeled antibodies were stored in 1X PBS at 4°C. Human recombinant purified TK1 (pTK1) was obtained from Genscript (Piscataway, NJ). Full length human TK1 was expressed with a histidine tag, which was used for purification, but then subsequently cleaved.

Contact Angle Measurement

MC (1% w/v) was prepared in 20 mM Tris-HCl buffer. PMMA sheets (2 x 2 cm²) were washed with deionized water and dried with nitrogen. MC solutions (1 mL) were then

placed on one side of a PMMA sheet and incubated for 30 minutes; then excess solution was completely removed by blowing using nitrogen flow. A 10 μ L drop of deionized water was placed on the surface, and the static contact angle was immediately measured using a goniometer (Rame-Hart, Succasunna, NJ). Three measurements were used to measure the standard deviation and average contact angle.

Device Operation

Separations were done with “pinched” injection [20], using +350 V as the intermediate potential and +850 V as the high voltage. A laser-induced fluorescence system coupled with an inverted confocal microscope and a photomultiplier tube was used for detection, as reported previously.[21] The data were collected at 20 Hz using software written in LabView.

Results and Discussion

One of the challenges in using PMMA for microfluidics is its hydrophobicity that affects reproducibility in separation of proteins.[22] Cellulose-based polymer dynamic coatings are effective at inhibiting the nonspecific adsorption of proteins in polymer microfluidic devices by increasing the hydrophilicity of the surface.[23] Three different molecular weight MCs were evaluated as dynamic coatings. As shown in Table 1, treatment of the surface with buffer containing 1% MC of all molecular weights decreased the PMMA contact angle. PMMA treatment with the lowest molecular weight MC (14000 Da) increased the hydrophilicity the most.

The choice of buffer affects the formation and stability of immune complexes. Theoretically, the best separation can be achieved by applying high voltage in a buffer having low conductivity. For example Tris-glycine buffer is commonly used for capillary electrophoresis because it has low conductivity and current under high voltage.[24–25] We tested several common electrophoresis buffers for sample flow by positioning the laser at the channel intersection point and measuring the fluorescence over time when voltage was applied (see Table 2). We also observed the current during this process as noted in Table 2, with low currents <30 μ A and high currents greater than this value. Although the current was low, no sample flow was observed in channels when Tris-glycine buffer was used, even at potentials up to +1450 V. This could be due to effects of this buffer and/or MC on the mobility. Moreover, we observed that when 1X PBS was used, the current was high and particles were seen under microscope observation inside the microchannels, perhaps due to Joule heating that partially evaporated the solvent, resulting in buffer salt or MC deposits. Both 0.5X PBS and 1X Tris-borate EDTA (TBE, 90 mM) yielded the desirable combination of low current and sample flow when voltage was applied.

We noted that separation distance affects the protein peak height and shape. Electropherograms of the anti-TK1 antibody by itself showed that increasing the separation distance to the detection point resulted in peak broadening and a decrease in peak height. Background-subtracted peak height increased as separation distance was decreased from 15 to 10 and 5 mm (Figure 1A–C; 0.14, 0.16, and 0.21 units). Peak full width at half maximum also decreased (7.2, 5.5, and 4.7 s) with these same decreasing separation distances.

Previously it has been shown that the choice of buffer can affect immune complex formation.[26] We tested separations in 1X TBE, pH 8.0 with 1% w/v MC-88000. TBE buffer resulted in sharper peaks, but resolution between the anti-TK1 antibody peak and the immune complex peak was not observed (see Figure 2). As in Figure 1, peak broadening was observed with increased separation distance, with full widths at half maximum of 0.15, 0.20, and 0.25 s for 5, 10 and 15 mm separation distances, respectively. Peak height also decreased with increased separation distance (2.4, 0.9, and 0.6 units for 5, 10 and 15 mm, respectively) as was observed in 0.5X PBS (Fig. 1). In Figures 1–2 band broadening with increased separation distance is caused by longitudinal diffusion during the longer separation times. In subsequent immunoaffinity separations, where antibody and antigen-antibody complexes are resolved (e.g. Fig. 3), additional broadening could occur from greater dissociation of formed antigen-antibody complexes with longer separation times or distances, although these conditions were not studied.

We observed that 0.5X PBS buffer containing 1% MC-14000 or MC-41000 failed to resolve free anti-TK1 antibody from immune complex (Figure 3A,B), but buffer with 1% MC-88000 resulted in separation of the unbound anti-TK1 antibody from the immune complex (Figure 3C). These observations agree with other studies where higher molecular weight MC resulted in better separation.[14–15,23] However, with the increased solution viscosity, we had to inject for 60 seconds in MC 41000 and MC 88000 to obtain adequate fluorescence signal, while 30 second injection was enough for buffer with MC 14000. A separation distance of 5 mm was sufficient to resolve the free antibody from the immune complex when 0.5X PBS buffer with 1% MC 88000 was used; moreover, these separations were faster, so we utilized a 5 mm separation distance in subsequent experiments. Increasing the MC concentration above 1% made it difficult to load the solution into the channel and sometimes resulted in delaminated devices because of backpressure during filling.

Using the optimized conditions, we performed microchip electrophoresis of anti-TK1 solutions with increasing pTK1 concentration. Figure 4A shows the electropherograms of anti-TK1 incubated with different pTK1 concentrations; we found that the immune complex peak increased as pTK1 concentration was increased. A plot of peak area of the immune complex correlated with pTK1 concentration (Figure 4B). The immune complex peak was observed at pTK1 concentrations above 2 $\mu\text{g/mL}$ (80 nM); we were not able to observe the complex peak when the concentration of pTK1 was lower.

The clinical concentration of TK1 in serum of cancer patients varies from 2–50 pM, depending on the type of cancer, while serum TK1 concentrations in healthy individuals are <2 pM,[6,8] so the detection limit has to be improved for this approach to be used in cancer screening. Several factors that could be explored to lower detection limits by improving antigen-antibody binding are ionic strength, temperature, and pH. Neutral pH is commonly used for immunoaffinity assays, although research suggests that immobilized monoclonal antibody to antigen binding capacity may improve as pH decreases to ~3.[27] However, not all immune complexes are stable at low pH; further work would be required to investigate this possibility more fully, which is beyond the scope of this paper. We note that fluorescence quenching occurs at low pH with FITC, but other dyes with high quantum yield in low pH solutions could be used.[28] Serum has a number of abundant proteins such as

immunoglobulins and albumin that may interfere with antigen-antibody binding. Thus, carrying out our assay with serum would benefit from depletion of these abundant proteins prior to microchip electrophoresis (i.e., using a commercial serum depletion kit). Such a depletion step could potentially even be integrated into a microchip design [11] for enhanced automation.

Conclusions

We developed a microchip electrophoresis immunoaffinity assay for measuring thymidine kinase-1 using a monoclonal antibody. We have shown that buffer composition and additives affect sample flow, formation of immune complexes, and separation of unbound antibodies from immune complexes. The separation of Ab and Ab-pTK1 complex was achieved in 0.5X PBS with 1% methylcellulose (88000 Da). Using optimized conditions we were able to separate the immune complex from free antibody within a 5 mm separation length.

We are currently working on using our optimized conditions to detect TK1 in clinical serum samples. Since we monitor the formation of antibody-antigen complex, we may be able to detect the form of TK1, (i.e., dimeric vs tetrameric), based on the electrophoretic mobility of the immune complex. If we can identify TK1 forms in a simple and rapid test, it would be an attractive alternative to radioimmunoassay.

Acknowledgments

We thank Prof. Matthew Linford for letting us use his goniometer and Nitesh Madaan for training on this instrument. Partial funding for this work was provided by the National Institutes of Health through grant R01 EB006124.

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Figure 1.

Effect of separation distance on peak shape. A decrease in peak intensity and increase in peak broadening were observed as separation distance was increased. Separation was in 0.5X PBS with 1% MC 88000, with 25 $\mu\text{g/mL}$ Ab. Injection was at +350 V for 60 s and separation was at +850V; separation distances are (A) 15, (B) 10 and (C) 5 mm. Electropherograms are offset for clarity.

Figure 2.

Attempted separation of Ab-pTK1 complex from free Ab in 1X TBE buffer with different separation distances. (A) 5 mm, (B) 10 mm, and (C) 15 mm. Sample was 25 $\mu\text{g/mL}$ (156 nM) Ab incubated with 25 $\mu\text{g/mL}$ (1 μM) pTK1; injection and separation conditions were the same as in Fig. 1; electropherograms are offset for clarity.

Figure 3. Separation of Ab-pTK1 complex with different 1% w/v MC additives in 0.5x PBS. A) MC-14000, 30 s injection; B) MC-41000, 60 s injection; and C) MC-88000, 60 s injection. Samples were 25 $\mu\text{g/mL}$ (156 nM) Ab incubated with 25 $\mu\text{g/mL}$ (1 μM) pTK1. Separation distance was 5 mm; injection and separation voltages are the same as in Fig. 1; electropherograms are offset for clarity.

Figure 4.

Microchip immunoaffinity electrophoresis of pTK1. (A) Increasing pTK1 concentration results in increasing immune complex peak size, going from 2–25 µg/mL (0.080–1 µM) pTK1. Injection and separation conditions are the same as in Fig. 3. (B) Plot of Ab-pTK1 peak area as a function of pTK1 concentration. Best fit line is $y = 0.123x + 0.26$, $R^2 = 0.94$, $s_m = 0.014$, and $s_b = 0.15$. (C) Peak area ratio of complex to free Ab (which accounts for injection differences). Best line fit is $y = 1.085x - 1.30$, $R^2 = 0.988$, $s_m = 0.053$, and $s_b = 0.60$.

Table 1

Effect of 1% w/v MC treatment on PMMA surface contact angle

MC mol wt. (Da)	Contact Angle
No MC	$71.5 \pm 0^\circ$
14000	$16.6 \pm 2.5^\circ$
41000	$30.5 \pm 0.9^\circ$
88000	$33.2 \pm 0.9^\circ$

Table 2

Current and sample flow for different buffers

Buffer	Sample Flow	Current
1X PBS, pH 7.4	Yes	High
0.5X PBS, pH 7.4	Yes	Low
25 mM Tris-192 mM glycine, pH 8.3	No	Low
90 mM Tris-borate EDTA, pH 8.0	Yes	Low