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Rapid detection and quantification of specific proteins by immunodepletion and microfluidic separation

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Abstract

Conventional immunoblotting techniques are labor intensive, time consuming and rely on the elution of target protein after depletion. Here we describe a new method for detection and quantification of proteins, independent of washing and elution. In this method, the target protein is first captured by immunodepletion with antibody coated microbeads. In the second step, both the supernatant after immunodepletion and the untreated protein sample are directly analyzed by microfluidic electrophoresis without further processing. Subsequently, the detection and quantification are performed comparing the electropherograms of these two samples. This method was tested using an *Escherichia coli* lysate with a FLAG-tagged protein and anti-FLAG magnetic beads. An incubation of as little as one minute was sufficient for detectable depletion (66%) by microchip electrophoresis. Longer incubation (up to 60 minutes) resulted in more depletion of the target band (82%). Our results show that only 19% of the target is recovered after elution from the beads. By eliminating multiple wash and elution steps, our method is faster, less labor intensive, and highly reproducible. Even in case of non-specific binding at low concentrations, the target protein can be easily identified. This work highlights the advantages of integrating immunodepletion techniques on a microfluidic platform.

Keywords

Immunoprecipitation; microchip separation; microchip immunodepletion; microfluidic protein sizing

Introduction

Separation and quantification of biomarkers are strong tools in biomedical research for a wide range of applications such as disease detection, toxicology assessment, and research diagnostics. Despite significant improvements in the field of separation and instrumentation, there is still an urgent need for fast and sensitive detection of low concentration biomarkers in complex biological fluids. Characterization of these analytes with a high level of sensitivity is challenging and requires new separation techniques. To this end, microfluidic devices have revolutionized the field of separation by providing, high throughput, rapid analysis, and small sample volume. However, the main challenge remains in detection of the analyte due to significant reduction in the volume (less number of target molecules). Among all the methods developed to improve detection sensitivity, the combination of microfluidics

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and immuno-affinity techniques is the most promising. The use of antibodies against the target analyte (antigen) provides robust and highly specific isolation of the analyte, depending on the antibody quality. Combination of this technique with a microfluidic platform ensures sensitive detection of the analyte in miniaturized sample volume. Furthermore, an automated microfluidic platform with minimum preparation steps and the ability of analyzing various analytes simultaneously on a single compact microchip is highly advantageous in rapid point-of-care diagnostics.

Among immuno-affinity methods, immunoprecipitation of proteins from the cell lysate is commonly used for the detection and analysis of target proteins. Surface modified microbeads, against the protein of interest, isolate the target from the lysate under native conditions. The protein is then eluted from the beads, separated by gel electrophoresis and analyzed by immunoblotting [1, 2]. Despite its high sensitivity (sub-nanogram range – depending on the antibody used [3]), immunoblotting is labor intensive and time consuming due to multiple manual steps. Moreover, the precise quantification of proteins is challenged by film or chromogenic detection [4]. Even though protein separation and immunoassays have been performed on a microchip [5–9], a simpler design with minimum manipulations is highly in demand.

All immunoprecipitation techniques using microbeads involve washing and elution steps, which significantly add to analysis time [1, 2, 10–12]. In this paper, we propose a new protein detection and quantification method independent of washing or elution. Our method utilizes an immunodepletion step followed by microfluidic electrophoretic separation. As shown in figure 1(A), immunodepletion is performed to capture the target protein using antibody coated microbeads. Following this step, the supernatant is loaded on a microchip for electrophoretic separation. Simultaneously, the untreated protein sample is loaded on the same microfluidic chip. The two electropherograms are then compared for quantification and detection of the target protein. Microfluidic separation allows for fast quantification of the results (less than an hour) which is performed parallel to detection. The result of this technique is compared with traditional (Western) blotting as well as elution of the target from the beads.

Experimental

Sample preparation

Escherichia coli (BL21DE2) were transfected with the pNO-TAT vector harboring the C-terminal 236 amino acids of the DEAD-box helicase Vasa [13]. Linked in-frame at the C-terminal most amino acid was a FLAG-tag, an octapeptide sequence (N-Asp-Tyr-Lys-Asp-Asp-Asp-Lys-C) for which a monoclonal antibody is available either freely soluble (clone M2, F1804), or linked to magnetic beads (M8823; Sigma-Aldrich). Cells were cultured at 37°C for 8–10 hours and induced with 10mM IPTG for 2 hours. After incubation, cells were lysed by addition of lysozyme (10ug/ml; 10 minutes at room temperature) and repeatedly freeze-thawed between –80° C and 37°C. The cellular lysate was centrifuged at 10,000g for 20minutes and the supernatant was collected and stored at –20°C.

For immunoblotting, 100µg, 70µg, and 35µg in lanes 1, 2, and 3 respectively (Figure 1(B)) of the cellular lysate was first resolved by 4–10% polyacrylamide gel electrophoresis (PAGE). The amounts of protein used, allows for a broad range of mass (~3-fold), with reproducibility within dilution. Once resolved, proteins were transferred to nitrocellulose and processed for immuno-labeling as described in Gustafson et al.[13], Towbin *et al.*[14] and Harlow and Lane [3]. Anti-FLAG antibody M2 (Sigma-Aldrich) was used at 1/5000 dilution in blotto[3] for 4 hrs at room temperature, and the blot was then washed several

times for 5 minutes each with excess blotto. A rabbit anti-mouse secondary antibody conjugated to horseradish peroxidase (Sigma-Aldrich) was diluted 1/5000 and added to the washed blot. After 4 hours incubation at room temperature, the blot was washed as described for the primary antibody and the secondary antibody was detected with an ECL kit (Pierce, Thermo Fisher Scientific) on film following the protocol.

Control experiment

Our method was first tested using a single analyte system containing a biotinylated antibody (mouse anti-human troponin T(cTnT); USBiological) and streptavidin magnetic beads(Dynabeads M-270; Invitrogen). The antibody was diluted to 0.2 $\mu\text{g}/\mu\text{l}$ with phosphate buffered saline (PBS). 10 μl of the magnetic beads was washed with PBS buffer twice and incubated with 5 μl of the diluted antibody at room temperature. The required bead volume was calculated based on the binding capacity of the beads (10 μg antibody per mg of beads). Samples at 1, 5, and 10 minutes incubation time were prepared by mixing on a revolver (Labnet International) except for one minute incubation sample which was manually mixed by pipetting. After each incubation time, the beads were collected by magnetic adsorption and the supernatant was taken for microchip electrophoresis.

FLAG protein detection in a bacterial lysate

A cellular lysate concentration of 7.1 $\mu\text{g}/\mu\text{l}$ was diluted with PBS buffer (0.02M phosphate, 0.15M NaCl) to 1.42 $\mu\text{g}/\mu\text{l}$. The dilution was chosen to accommodate the signal level below the saturation limits of the detector. 20 μl (5 times more than the bead capacity, to ensure complete binding) of the diluted lysate was incubated with 10 μl of anti-FLAG M2 magnetic beads with binding capacity of 0.6 mg of FLAG protein per 1 ml of packed beads. In order to determine minimum incubation time required for depletion, the beads were incubated at 1, 5, 10 and 60 minutes with the lysate at room temperature. The diffusion time for FLAG proteins was approximately estimated to be $t_d \sim \bar{r}^2/2D = (600)^2/2 \times 300 = 600\text{s}$. Here, we assumed that the capture of the target molecules by the surface is faster than the diffusion time, also the average distance between beads was assumes as $l = 600\mu\text{m}$ and diffusivity of FLAG protein $D = 300\mu\text{m}^2/\text{s}$. Here, we assumed that the capture of the target molecules by the surface is faster than the diffusion time. Piyasena et al. (2004) [15] also reported detection of Flag system in microfluidic affinity columns. The experimental data showed high binding rate ($k_{on} \sim 6 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$). Their analysis showed the mass transport limited regime for almost all the experiments. Hence, the incubation times were varied from low (1 minute) to high diffusion times (60 minutes). Additionally, the selected time interval allows for comparing a broad range of conditions (60-fold) at room temperature for immuno-reactivity. The beads were kept in suspension by placing the samples on a revolver. The supernatant was collected following the same procedure as stated above. Elution carried out on the 60 minutes incubated beads with the lysate. For eluting the target, beads were suspended in 20 μl sample buffer (Agilent Protein 80 kit) plus 1 μl of 2-mercaptoethanol (Sigma-Aldrich) after washing with PBS buffer. Following this step, the beads were heated at 95°C for 5 minutes, and the supernatant was taken for analysis. All samples tested here, with the exception of the elution sample, are supernatants following bead incubation. For microchip electrophoresis, samples were prepared according to Agilent kit (Agilent Protein 80) and analyzed three times by 2100 Bioanalyzer (Agilent Technologies).

FLAG protein detection in a target-free protein complex

To further evaluate the dynamic detection range of the target, various concentrations of a FLAG protein(~50kDa) was mixed with four other proteins: lysozyme, soybean trypsin inhibitor, bovine serum albumin(BSA) and ovalbumin (Sigma-Aldrich) each at 200ng/ μl concentration in PBS buffer. The target concentration was varied from 50ng/ μl to 200ng/ μl , providing a target/complex ratio of 0.06 to 0.25. The complex sample was incubated with

anti-FLAG magnetic beads for 10 minutes at room temperature and the supernatant was analyzed by microchip electrophoresis.

Results

The control experiment performed on a biotinylated antibody, as a proof of concept, demonstrates the high efficiency of this method for depleting the target analyte. The amount of adsorption is calculated by comparing the target peak area of each sample with the untreated antibody (Figure S1). One minute incubation results in 95% reduction of the target band. At 5 minutes and above, 99% depletion was observed. After establishing the high efficiency of our method, the performance of this technique was tested in a more complex system of bacteria lysate (as used in blotting experiment). Cells expressing Vasa-FLAG protein have several major protein bands, including one at approximately 36 kDa, which is the predicted mobility of the DNA-encoded construct. By performing the traditional (Western) immunoblotting, it was verified that the 36kDa band is the FLAG-tag protein (Figure 1(B): lane 1–3 under immune-blot). An alternative approach for this assay is to use a protein chip following elution of proteins from beads that had been pre-incubated with the lysate. This approach was recently demonstrated [12] but still required several hours to be accomplished with multiple manual steps. In the case of the queried bacterial bands here within whole cell lysates, a pre-absorption approach might be sufficient to rapidly detect the band of interest using minimum reagents as demonstrated in our method.

Samples were analyzed on an electrophoresis gel. Figure 1(C) (lanes 2–5) clearly shows the depletion of two bands at 12.4 kDa and 36.3 kDa for four incubation times. In comparing the intensities of the two bands in lanes 2–5 with the lysate, the target band is detected by the significant depletion of the 36.3 kDa band. The presence of the 36.3 kDa band in the elution sample (lane 6) not only confirms the presence of the target, but also the effective adsorption of the FLAG proteins on the beads. Even at one minute incubation, a significant depletion of the target protein is clearly observed. This suggests that the time scales for diffusion t_d and reaction t_r for transport and adsorption of FLAG protein were in the range of minutes.

For detection and quantification of the depleted FLAG protein the electropherograms of lysate, 1 and 60 minutes samples are compared in Figure 2(A). Even though one minute incubation is sufficient for the detection of target band on the gel (Figure 1(C)), the depletion continues at longer incubation times. However, the rate of depletion decreases after 10 minutes of incubation time (embedded graph in Figure 2(A)). This is evident as rate of diffusion transport decreases with decrease in concentration gradient between the bulk and the surface.

In order to compare our method with the elution technique, the electropherograms of supernatant and eluted protein after 60 minutes incubation are compared in Figure 2(B). The presence of a distinct peak at 36.3 kDa, overlaying the 60 minutes target peak, confirms the eluted protein as the FLAG protein. Quantification of depleted and eluted FLAG protein was performed using the peak area (including the non-specific adsorption) and is listed in Table 1.

$$\%adsorbed = \frac{\text{area under the curve(lysate)} - \text{area under the curve(sample)}}{\text{area under the curve(lysate)}} \times 100$$

Table 1 (column 5) shows more than 66% adsorption of FLAG protein in the first minute of incubation and that the adsorption of FLAG protein gradually increases, from 66% to 82% after 59 minutes of incubation. The non-specific adsorption of 12.4 kDa band is also shown

(column 3) with approximately constant adsorption (43%) in all of the samples. Since the 12.4 kDa peak appears far away from the 36.3 kDa FLAG protein peak, the non-specific binding does not affect the quantification of the target protein. However, the sensitivity of the assay would be limited in cases of interference of the target peak with other protein peaks in the sample. It is important to note that peak interference is a challenge in all the current methods available. More investigations are needed to improve resolution of the peaks by choosing different gel properties [16], or changing the script of the system. Release of only 19% of the FLAG protein from the beads shows that even though the target can be detected after elution, and further improvements are possible by optimizing the elution process, an accurate quantification is not possible by this method.

Finally, the dynamic detection range of the target (~50kDa FLAG) was determined in a protein complex at different concentrations. The complex sample consists of four other proteins, each at 200ng/ μ l. The target electropherogram peak is shown in figure 2(C) for one of the sample runs. All the peaks can be easily identified arriving at 35.5 seconds. As shown in figure 2(C)(inset), the fluorescent intensity is linearly proportional to the target concentration.

The effect of non-specific binding of other proteins on the detection of the target was also investigated by electropherogram peak analysis. These electropherograms are shown in a supplementary figure S2. Figure 2(D) shows the percentage adsorption of each protein in the complex sample. It is important to note that, even though the most significant depletion (95%–100%) was observed in the target band, other proteins in the complex were also depleted (less than 40%) through non-specific adsorption. Our results suggest that even at very low concentration of target (50ng/ μ l) and in case of non-specific binding of other sample components, the rapid and high specific detection of the target can be achieved.

Discussion

Speed, simplicity and sensitivity are key factors in designing a diagnostic device. Whether this device is used in the research laboratory or the clinical diagnostic lab, the combination of these criteria ultimately determines its application. Our proposed method is optimally suited in low complexity protein mixtures where each of the bands may be seen individually, or in a whole cell lysate in which some of the bands of interest are in significant relative abundance. Under these conditions, our detection technique is ideal for rapid verification of a target protein (minutes). The efficiency of this method is minimized where the concentration of the target protein is significantly less than other proteins in the mixture, such as HSA (Human serum albumin) in the serum. Here the depleted band would not be amply detected; although a decrease in multiplex heights are suggestive of target protein presence. In more complex systems with multiple peaks, the detection performance can be optimized using different gel matrices, antibodies, sample buffers and bead properties. In addition, the incubation time and temperature can also be optimized for maximum efficiency.

Considering the binding capacity of the beads (0.6mg FLAG protein per 1 ml of beads), 10 μ l of beads used in the experiments results in binding of 6 μ g protein to the beads. For the above experiments, the beads were incubated with lysate at almost 5 times the binding capacity (20 μ l lysate=28.4 μ g protein), to ensure maximum adsorption. However, even with the excess amount of protein (more than bead capacity), the specific binding affinity of the beads provides sufficient depletion of the target for quantification purposes. The sensitivity of this method depends on the detection limit of the microchip sizing assay which is ~50ng/ μ l before loading on the gel.

Conclusion

By eliminating multiple wash and elution steps, our modified immunodepletion method not only minimizes the analysis time, but also provides a robust and more reliable quantification platform. Even in cases of non-specific binding and at low concentrations, the target protein is significantly depleted and can be identified. Although, the target can be detected by the current bead based immunodepletion techniques, the quantification is limited due to the low recovery of the target after elution from the beads.

We emphasize that this new technology is not dependent on an antibody. Lectin, or other specific affinity methods are equally valid. The binding matrix could be a lectin used to study various glycosylated proteins, interacting proteins for which they are already available and thereby selectively adsorb even isoforms of protein family members, small molecules attached to the beads to interrogate the starting protein mixture, and even cis-elements of a DNA promoter region. Further, the target can be detected in any population of protein mixture, including cellular lysate, serum, or body fluids. The only requirement of this system is that they be soluble during the depletion procedure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

cTnT	Cardiac Troponin T
PBS	Phosphate Buffered Saline
BSA	Bovine Serum Albumin
PAGE	Poly-Acrylamide Gel Electrophoresis
HSA	Human Serum Albumin

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Figure 1.

Schematic of proposed immunodepletion method (A). Immunoblot using a conventional Western method (B). Lanes 1, 2 and 3 correspond to 100, 70 and 35 micrograms of protein loaded per lane that was then stained with Coomassie or labeled by immunoblotting with anti-FLAG antibodies. The asterisk indicates the position of the FLAG-tagged protein species. Agilent (protein 80) pseudo gel of lysate, elution and incubated samples (C). All samples, except the elution, are supernatant after incubation with anti-FLAG beads.

Figure 2.

Detection of FLAG protein (A), comparison of two incubation times in depleting the target (1.42 μ g/ μ l after depletion with anti-FLAG magnetic beads. The arrow indicates the target peak. The adsorption kinetic of FLAG protein is shown in the inset. Comparison of eluted and incubated samples after 60 minutes (B). The 60 min sample is the supernatant of the lysate (1.42 μ g/ μ l after 60 minutes depletion with magnetic anti-FLAG beads. The elution sample is the eluted FLAG protein from the beads after 60 minutes incubation. The arrow shows the target peak. Detection of FLAG protein in a target-free complex sample (C). The concentration of each non-FLAG protein is constant at 200 ng/ μ l. The inset shows the linear detection range of the target with respect to concentration. Depletion of FLAG protein in a protein complex at different concentrations (D). The concentration of each non-FLAG protein was kept constant at 200 ng/ μ l while changing the target concentration. Samples were incubated with anti-FLAG magnetic beads for 10 minutes. Error bars are the standard deviation obtained from three independent studies.

Table 1

Quantification of the immunodepleted FLAG protein. The listed values are the average of three independent experiments (mean $\pm$ standard deviation).

Sample	12.4kDa peak area	% adsorbed	36.3kDa peak area	% adsorbed	%released
Lysate	244.1 $\pm$ 4.8		107.6 $\pm$ 4		
1 min	133 $\pm$ 6.8	45.6	36.5 $\pm$ 1.2	66.1	
5 min	128.3 $\pm$ 4.2	47.5	29.8 $\pm$ 2.1	72.3	
10 min	146 $\pm$ 0.6	40.3	25 $\pm$ 1.3	76.8	
60 min	147.6 $\pm$ 3.4	39.6	19.2 $\pm$ 1.5	82.2	
Elution			20.7 $\pm$ 5.1	80.8	19.2