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APPLICATION OF PULSE WIDTH MODULATION TO A WESTERN BLOTTING DEVICE

A Thesis

Submitted to the Faculty

of

Purdue University

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ThucNhi TruongVo

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ABSTRACT

TruongVo, ThucNhi. M.S.B.M.E., Purdue University, December 2016. Application of Pulse Width Modulation to a Western Blotting Device. Major Professor: Hiroki Yokota.

One of the critical steps in a current Western blot technique is a blotting process, which in general requires one electrophoretic gel for every protein species to be analyzed. In most cases, multiple protein species are analyzed simultaneously and thus it is necessary for a scientist to run multiple gels. In order to make it possible to analyze multiple protein species from a single gel, a novel blotting device, BlotMan, was employed in this study. Designed by Dr. Chien′s group (YC Bioelectric), BlotMan uses pulse width modulation (PWM) for applying a protein size-dependent voltage during a blotting process. In this study, the differential average voltage profile, depending on protein size (e.g. 17 kDa to 140 kDa), was bulit and enabled BlotMan to transfer all protein species in equal efficiency regardless of the protein size. Furthermore, BlotMan consists of a user-friendly, custom-made interface box, which can be remotely controlled by a smart phone. BlotMan′s capability was evaluated using standard protein markers, as well as protein samples that were isolated from chondrosarcoma cells (SW1353) and breast cancer cells (MDA-MB-213). The experimental results revealed that BlotMan was capable of generating 5 blotting membranes from a single gel simultaneously. Protein species such as c-Src, eukaryotic translation initiation factor 2 alpha (eIF2α) and its phosphorylated form (p-eIF2α), lamin B, and β-actin were successfully detected. It is also demonstrated that compared to a regular constant voltage, PWM signals improved transfer efficiency and a signal-to-noise ratio. In conclusion, this study demonstrated that BlotMan was able to facilitate Western blotting analysis by generating multiple blotting membranes from a single gel with an

improved signal-to-noise ratio. Further analysis is recommended for understanding the mechanism of PWM′s action on transfer efficiency and noise reduction.

1. INTRODUCTION

While Western blotting has been a widely used technique to detect and analyze protein expression, its procedure is time consuming and labor intense. One of the bottleneck processes in Western blotting is a protein transfer process (i.e., blotting), from a polyacrylamide gel to a membrane, in which each membrane requires its own gel. The goal of this study is to achieve generation of multiple membranes from a single gel by developing a novel device, BlotMan. In BlotMan, all proteins in a gel are designed to be uniformly transferred to multiple membranes using specifically modulated voltage signals through pulse width modulation (PWM). In order to achieve the intended uniform protein transfers regardless of protein size, a mathematical model for the size-dependent voltage profile for proteins was built [1–3]. Based on the model, a proper voltage gradient with PWM was then predicted and experimentally validated. Using protein samples isolated from two lines of cancer cells, it is demonstrated that BlotMan is capable of generating up to five membranes from a single gel. In those membranes, the expression levels of several protein species were evenly distributed. Besides generating multiple membranes from a single gel, it is observed that the PWM technique improved transfer efficiency and a signal-to-noise ratio.

In this chapter, the background of protein analyses, existing methods for detecting proteins, BlotMan′s design, the PWM, the problems and the proposed solution of this study are described.

1.1 Functions of proteins

Proteins serve crucial roles in many biological processes [4–6]. Proteins function as enzymes that catalyze chemical reactions in metabolic processes of cells. Proteins

can provide structural and mechanical supports for many types of cells and tissues. They are important in other processes as well including the cell signaling, the cell adhesion, and the cell transport. Proteins are responsible for biochemical reactions in living organism such as a gas exchange and transport process, a nutrient absorption, an energy usage and storage, etc. Collagen, fibronectin and laminin are recognized as the most common structural proteins, which give support and provide structure for tissues, make connections or adhesion between tissues and organs. Other proteins are involved in DNA and RNA processes including DNA replication and transcription, and RNA splicing and editing processes. Served as machinery, proteins determine which genes would be expressed and whether mRNAs would be translated into proteins [5, 7, 8]. In the immune system, as antibodies, proteins mainly help remove foreign substances and fight infections. The operation of the immune system is based on the capacity of structural recognition and differentiation between self and non-self types of specialized cells that are able to distinguish and identity foreign substances. The recognition processes usually happen via protein-protein interaction on the surface of the immune system's cells.

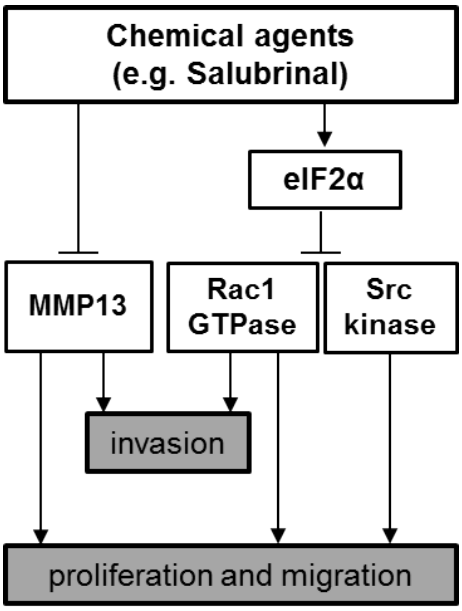

Fig. 1.1. The proposed mechanism inducing the alternation of malignant phenotypes of chondrosarcoma by chemical agents (e.g. Salubrinal) [9].

For instance, Figure 1.1 shows the proposed mechanism of the reduced invasive and migratory behaviors of chondrosarcoma cells (SW1353) by regulating Src, Rac1 GTPase and MMP13 using chemical agents (e.g. Salubrinal) [9, 10]. The activated eIF2α by a chemical agent, Salubrinal in this case, inhibited the Src and Rac1 functions, which led to the suppression in migration and invasion of chrondosarcoma cells. Note that the Src and Rac1 have been known as major contributors in invasion and mobility of many tumor cells [11–14].

Because of these important functions, it is valuable and critical to study proteins, their properties, mechanisms, structures, dynamics and functions. To have answers for diseases such as heart problems, liver and kidney diseases, bone disease, cancer, etc., scientists must master how these molecules assemble into complex forms, how they interact with each other, and how they function and react corresponding the changes of other factors including treatment methods, conditions and dose usages. The conformational changes,the deregulation, or the mutation, for instance, causing the blocking and/or inhibiting interactions between proteins and their binding targets molecules, which induce the disorder in turn on/off cell signal transduction and cell cycle controlling. These changes may lead to cancer or Alzheimer's disease caused by the hyperactive signaling [15, 16]. In fact, the various functions of proteins are based on the different macromolecule structures and shapes of proteins, which consist of different types, numbers and polypeptide amino acids backbone sequences. Therefore, it requires more than one detection and quantitation methods to study proteins structure and mechanism because of their complicated molecular structures, properties, physiochemical and functions. Different information related to protein structures and functions can be provided by different methods [17]. For instance, the macroscopic level information of proteins including the size, molecular weight, the binding affinity and sites, etc. can be obtained by using gel filtration or Western blotting method. The mass spectrometry, ChIP-on-chip assay, or methylated DNA immunoprecipitation (MeDIP) systems can provide information about proteins with several chemical modifications, DNA mutation and methylation, etc.

Therefore, the selection of an appropriate method to study protein highly depends on each research purpose. Different qualities and quantitative informational levels of biological samples can be obtained by different methods.

1.2 Methods of studying proteins

Priority to studies their structures, mechanisms and functions, proteins first must be purified and fractionized by different separation techniques such as centrifugation, liquid chromatography, electrophoresis, etc. [17–19]. The targeted protein may be seized with other proteins or caught in a matrix of proteins and non-protein biological substances. These purification methods will depend on the natural characteristics of both proteins and solutions containing them. All factors such as the nature of mixture, the required volume and quantity of sample, and the research purpose will determine which purification techniques can be used.

Since the target protein isolated from mixture, an appropriate protein detection and analysis method can be further applied. Normally, a protein method involves both experimental methods and computational methods.

1.2.1 Protein purification techniques

Thousands of proteins are purified and isolated in active form based on characteristics such as solubility, size, charge, and specific binding affinity [20, 21]. Normally, any molecule, whether protein mixtures, carbohydrate, or nucleic acid, can be separated from others based on series of separations, which exploit differences among proteins to find out the protein of interest.

To remove surrounded non-target elements including cell membranes and DNA from the interested proteins, protein purification techniques such as centrifugation, filtration, sonication can be used [21,22]. Centrifugation methods are used to separate one type of material from others and measure physical properties of macromolecules such as molecular weight, density, shape, etc. The centrifugation principle is based

on the cells, organelles, or molecules having different masses or densities, which will move down to the bottom of a centrifuge tube at different rates. Centrifugation methods such as differential, isopycnic and ultracentrifugation can be used to separate proteins by expression sites. Chromatography techniques may be used either for preparation purposes providing a purified protein for further analysis purposes, which measure and detect a small proportion of substance in a mixture material. Generally, separation by proteins size, charge and binding affinity can be done by ultrafiltration, gel-filtration chromatography, size exclusion chromatography and preparative native gel electrophoresis, etc [23–25].

Purifying proteins by sizes are separated by using a gel-filtration chromatography technique [26, 27]. Small molecules or proteins have higher penetrative capabilities into the beads and less volume spreading in the solution between beads. The result is that larger molecules have a greater motion through a gel filtration column than smaller molecules do. Other separation methods based on protein′s capable of specific binding to chemical groups, receptors, ligands or other proteins include affinity chromatography using their binding sites, immune-chromatography using immobilized antibodies, or magnetic separation using magnetically tagged antibodies [23–25]. Transcription factors or proteins regulated a target gene expression can also be purified by the affinity chromatography technique. Proteins with a high affinity for specific DNA sequence will bind and be captured while a protein mixture flows through matrix of columns. Then, transcription factors or proteins are effectively recognized after being washed with a high concentration of salt solution. High-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) [28] are two most frequently chromatographic methods for proteins and peptides purification either based on their sizes, charges, and hydrophobicity or based on their similar properties.

Moreover, macromolecules and their fragments such as DNA, RNA and proteins are separated based on their molecular weights, subunit structures, and charges by using gel electrophoresis techniques [29,30]. These techniques are quite simple to use, highly reproducible and most common used to qualitative analyze complex protein

mixtures. With supporting from a sensitive and linear image analysis system, gel electrophoresis becomes one of the highest resolution and popular methods for quantitative and preparative purposes. The denatured proteins are resolved by passing samples through an electrically charged gel matrix, which separate and distinguish polypeptides based on their molecular weights.

1.2.2 Vertical gel electrophoresis

Vertical gel electrophoresis, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS - PAGE), is the most common and easy way to separate many macromolecules proteins and nucleic acids that have different sizes and shape, but have almost the same ratios charge over mass [31–33].

For size fractionating proteins, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS - PAGE) is mostly used. In SDS-PAGE, proteins are bound by SDS, a negatively charged detergent, which denatures proteins by adding negative charges in the same charge-to-mass ratio. During the electrophoresis process, smaller proteins migrate though polyacrylamide pores faster than larger ones [31, 34](Figure 1.2).

The size-dependent motility of protein molecules can be modeled in the following. Consider a string or rod-like protein as a particle having mass, m, and a total net charge of a molecule, $Q = k_1 m$. The proteins motion can be expressed:

$$m\frac{\partial v}{\partial t} = QE - Kv \tag{1.1}$$

where E (V/cm) = magnitude of an electric field; and K = drag coefficient. Using the initial condition as $v_o = t_o = 0$, the velocity $v(t)$ becomes:

$$v(t) = \frac{QE}{K}\big(1 - e^{\frac{-K}{m}}t\big) \tag{1.2}$$

Using the terminal velocity, $v_{ter} = \frac{QE}{K}$, $v(t)$ can be re-written:

$$v(t) = v_{ter}\big(1 - e^{\frac{-K}{m}}t\big) \tag{1.3}$$

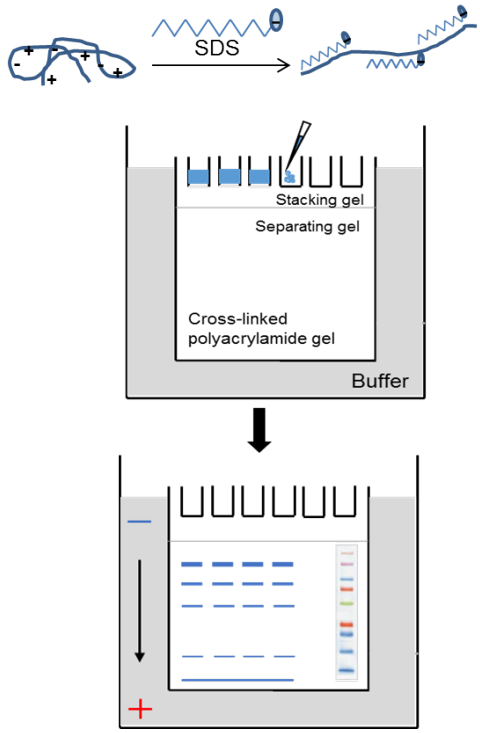

Fig. 1.2. Denatured proteins were fractionized in SDS-PAGE gel electrophoresis and moved towards the positive the anode side.

It is reported by Viney and Feton [35], the drag coefficient, K, is modeled:

$$K \propto \eta l \tag{1.4}$$

where l = the characteristic length of a protein of interest and η = viscosity. Also, it was assumed that $l \propto m^n$, with 0< n< 1 and viscosity $\eta \propto m$

Taken together, the velocity is expressed:

$$v(t) = v_{ter}\left(1 - e^{\frac{-K}{m}}t\right) \approx \frac{E}{m^n}\left(1 - e^{-m^n t}\right) \tag{1.5}$$

$$v_{ter} = \frac{E}{m^n} \tag{1.6}$$

Since n<1, the mobility of proteins depends on their sizes in gel electrophoresis as shown in equation 1.5 and Figure 1.3.

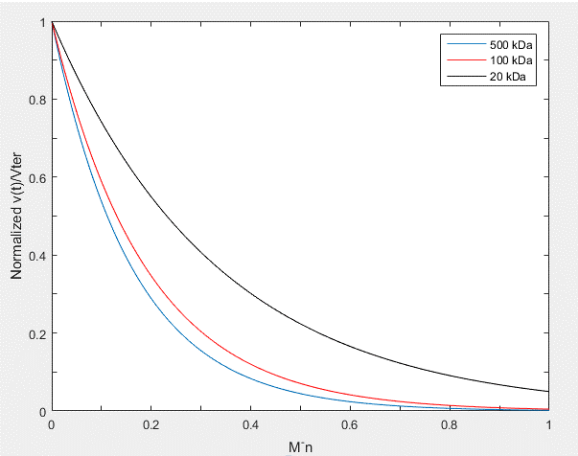

Fig. 1.3. Plot of $v(t)$ versus m^{-n} for different value 0< n< 1 of protein size.

Once separated by electrophoresis, proteins can be further detected either by Western blotting and/or extracted for mass spectrometry analysis. Protein gel electrophoresis is, therefore, a common step in proteomics analysis process.

1.2.3 Protein detection techniques

After being separated and/or purified, protein identification or detection is the next step of studying proteins, their properties, mechanism, structures, dynamics and functions. Several precise and accurate techniques have been developed to detect protein structures, protein-DNA interactions, protein-protein interactions, etc. In this section, some common protein techniques for protein analysis such as X-ray crystallography, polymerase chain reaction (PCR), mass spectrometry, and Western Blot are introduced [36–38].

Methods of studying protein structures

The 3D crystal static and/or dynamic structures, either at atomic resolution level or at molecular level, of macromolecular proteins as well as their chemical bonds and non-covalent interactions are mainly investigated by x-ray crystallography and/or

Nuclear magnetic resonance (NMR) spectroscopy. These two techniques use different calculation and measurement methods to distinguish these macromolecules based on how proteins structure, how they interact with other proteins, how their conformations change, as well as how they act as catalysis. In one hand, the NMR has been used to determined relative positions of all atoms of molecules by measuring interactions of atomic nuclei [39, 40]. The NMR can only be applied for tiny proteins, single domains, fragments etc. In another hand, X-ray crystallography can provide good details for most any size of single proteins, complexes ones, nucleic acids, etc. by scattering electron density of atoms [41]. This method has disadvantages that it can only be used with proteins in crystal forms and for non-hydrogen atoms.

DNA-based detection methods

The proteins - DNA interactions in vivo are studied by a ChIP-on-chip technology which includes the purification method (e.g. chromatin immunoprecipitation (ChIP)) and the DNA microarray (chip) detection method. This technique normally is used for isolating the functional elements in the genomic sites identified by specific DNA-binding proteins. For instance, promoters, enhancers, and repressor elements can be identified by mapping protein markers locations associated with these sites [42–44]. Generally, a ChIP-on-chip experiment can be divided and operated into two major portions: wet-lab and dry-lab. In the wet-lab portion, the target protein is cross-linked with DNA site by formaldehyde fixation. After cell lysis, the DNA is fragmented by sonication and then is subjected to immunoprecipitation (IP) with a specific antibody. The fluorescent tag is normally used to label the single stranded DNA fragments after several steps such as reversed cross-linked, amplification and denaturation. In the next dry-lab portion, double-stranded DNA fragments are formed after labeled the single-stranded DNA fragments are mapped with complementary ones, hybridized and fluorescent signals from array are normalized and used to confirm the correct hybridization of probes as well as the non-specific bounded probes [45].

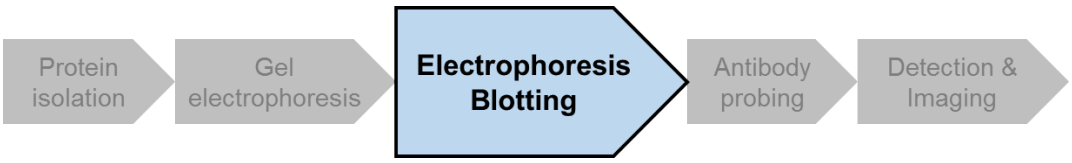

Fig. 1.4. Common steps in Western blot process.

Although ChIP-on-chip is a powerful technique to give high resolution of genome-wide maps in identifying the binding sites of many DNA-based proteins, it is very costly to produce DNA microarrays. Also, it is a major limitation in mammalian genomes application because of highly specific antibodies required to recognize its epitopes.

Method of studying protein-protein interactions

Based on its epitopes recognizable ability following denaturing and separating proteins with SDS and immobilizing on the surface of membrane, Western blot is a powerful technique to detect protein expression levels [36,46–49]. Depending on characteristics of the specific protein as well as the level of information needed, Western Blotting protocols may vary, but they consist common steps such as protein isolation, gel electrophoresis, transfer, antibody probing, and detection (Figure 1.4).

First, the isolated proteins are separated by gel electrophoresis. Protein marker standards are served as molecular weight markers for blot patterns calibration and transfer efficiency monitoring. The migration of proteins in gels depends on their sizes, shapes and charges (as section 1.2.2) [50, 51]. As a driving-force element, voltage is also a parameter affecting the mobility of proteins. The higher applied voltage will make proteins move faster in gels. Then, microfiltration transfer methods such as diffusion, vacuum or capillary blotting could be used to transfer proteins from gels to a membrane. In vacuum transfer, nucleic acids are trapped and immobilized to a membrane by UV light or heat. Proteins migrate up to a membrane, which is placed on the top of the gel-membrane sandwich. However, the horizontal electrophoretic

blotting methods including the tank and semi-dry electrotransfer are more common used in many research lab and clinical sites [52, 53]. These electrophoretic transfers offer quicker, more efficient and high resolution separation of proteins by using the PAGE than others. In an electrophoretic transfer, proteins migrate to the membrane is driven by the electric field strength (V/cm) between electrodes following Ohms law: $V = IXR$, in which I is a current generated by applying a voltage (V) across resistance (R) of materials between electrodes [49, 54, 55]. Besides factors such as the size, shape, net charge of protein, the transfer buffer pH and viscosity, etc. have certain effects, both applied voltage and distance between electrodes also have impacts on proteins elution rates and their resolutions.

After being transferred to a membrane, the interested protein pattern can be detected using a specific detection reagent. Bounding to the first labeled primary antibody, a specific secondary antibody functions as a label agent as well as amplified mechanism of emitted signal process. Then, the emitted signal is captured and measured proportionally to the amount the interested protein. Enzymatic detection methods such as chemiluminescence and chemifluorescence, or fluorescence-based are more popular ways to recognize the target protein. Fluorescence based detection uses a conjugated fluorophore to a secondary antibody, which is excited by a specific wavelength of either a visible or a near infrared light source. This method is used as a means of studying dynamics and locations of proteins in cultures cells. Chemiluminescent, in contrast, is a typically method to analyze gene-expression signals of groups of cells. Conjugating to either horseradish peroxidase (HRP) or alkaline phosphatase (AP), this method is suitable to work in tissues, in cells studies because it can provide a high detection sensitivity and lower background intensity.

Using X-ray film, scanners or a charge-coupled device (CCD) camera-based imager, results of one or more protein bands are further to be quantitatively and/or qualitatively analyzed. Mapping with a known-sized protein marker, the qualitative analysis detects the presence or absence of a specific protein in sample. Together with the significant developments of highly functional reagents, modern technical imaging

method is able to make Western blotting a potential method for analysis proteins quantitatively.

In this thesis, the horizontal electrophoresis blotting process is mainly studied and discussed. BlotMan, a Western Blotting device, using the pulse width modulation (PWM) technique, is central of this study.

1.2.4 BlotMan

A protein transfer unit, a microcontroller unit and a smart phone are mainly components of BlotMan. The transfer unit executes operational commands from the microcontroller unit. The printed 4 mm width strips with 1 mm gaps in between were etched on a plate to make a multi-anode trips board [1–3]. To ensure to get desired size-dependent voltage signals properly, an extra thick blotting paper, a SDS gel, Immobilon-P membrane, and another extra thick blocking paper are placed on the common cathodes and a multi-anode trips board is placed on the top of this stacked sandwich. Connected by a DB15 cable and a ground cable, a microcontroller unit is operational control system. Served as inputs, required commands or information are provided to a computer based controller unit using a keypad/LCD user interface or the Bluetooth technique from an Android smart phone. This required information such as size range of interested proteins, number of copies, membrane types, transfer types, etc. are proceed and then PWM signals of different duty cycles PWM signal and proper average voltage values are generated for each anode of the transfer unit. The BlotMan application on the smart phone sends and monitors experiment commands to the controller unit, and then collects and store experiment data on the smart phone.

1.2.5 Pulse Width Modulation (PWM)

Modulating a rectangular pulse wave, Pulse Width Modulation (PWM) technique uses digital signals to control power applications resulting in average voltage gradient

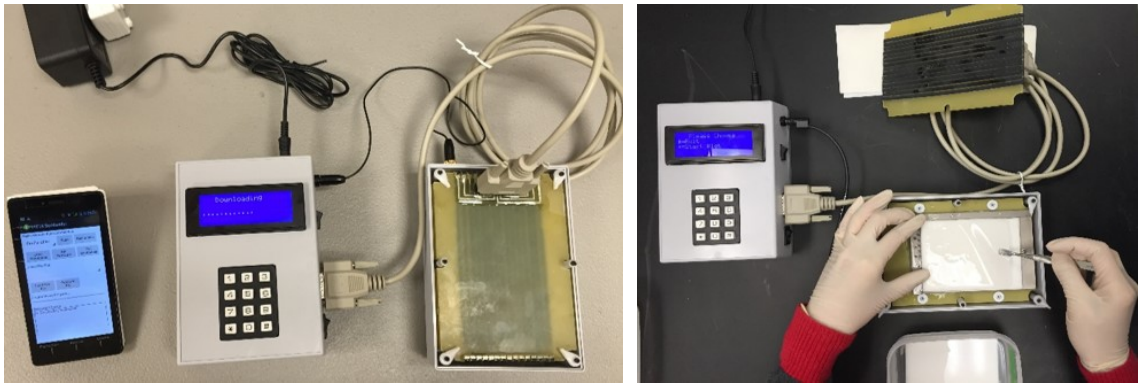

Fig. 1.5. The BlotMan system. (A) BlotMan includes a control box, a protein transfer unit and a smart phone. (B) Procedure for placing a gel on the protein transfer unit.

of the signal, which is applied in blotting process of BlotMan [56–60]. The PWM technique is a very effective and straightforward method to generate different average voltage signal of electrical power between fully on and fully off. The switching on/off time of signals simulate the output voltages in between full on (e.g. 25 Volts) and off (e.g. 0 Volts). The duration of on time is measured in percentage and is called duty cycle. The duty cycles can vary between signal being on/off from 0% to 100% and all ranges in between. Normally, a 50% duty cycle digital signal spends a half of time in the high state and another half in low one. Also, an average signal value of the PWM increases proportional to the increased duty cycle as described in equation 1.7.

$$V_{ave} = \frac{1}{T}\int_0^T V(t)dt = D.V_{max} + (1-D).V_{min} \tag{1.7}$$

where duty cycle $(D) = \frac{Pulsecycle}{period}.100\%$

The Figure 1.6 shows an example of PWM signals with 20%, 40% and 80% duty cycle applying for a specific voltage range (e.g. from 0 V to 25 V). At the same given voltage, the 40% duty cycle signal has shorter pulse duration or time on over its period and smaller average voltage value than those of a 80% duty cycle signal does. The average voltage for 80% duty cycle is 20 V instead of 10 V for 40%. This principle

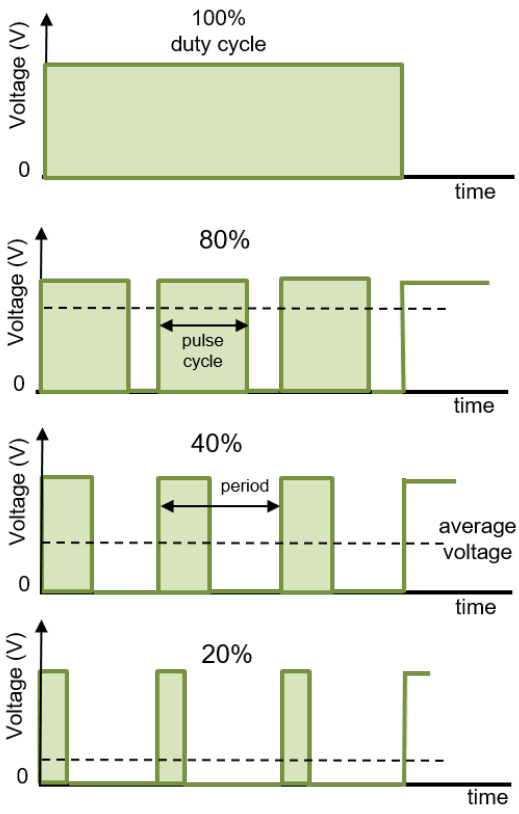

Fig. 1.6. The PWM voltage profiles.

applied in BlotMan to generate the voltage gradient profile for size-dependent proteins later on.

Beside the capability to manipulate power effectively, the PWM technique is able to reduce the energy consumed, simpler and less expensive to fabricate. Also, the signal-to-noise ratio can be improved by the fact that less of the heating is generated because power will get near to zero if either voltage or current is close to zero.

1.2.6 Problem and Proposed solution

Problem

In this study, a new blotting device (i.e., BlotMan) using the PWM technique, which is capable of generating multiple membrane copies from a single gel, was devel-

oped based on the following four reasons. First, generating multiple membranes from a single gel reduces the required amount of protein samples as well as labor and costs. Second, PWM is a straightforward method to generate an effective voltage gradient without using a constant source voltage. In order to generate a voltage gradient for transferring all protein species uniformly to multiple membranes, PWM is one of the best choices. Third, many technical improvements have been implemented in Western blotting devices in recent years, but these improvements are mainly related to the procedure with antibodies. For instance, the systems such as iBlot2, BlotCycler, and iBind provide quick and reproducible antibody reactions through an automated step in blocking, washing and incubation. However, these systems do not provide any improvement in the blotting process to a membrane. Fourth, PWM is a procedure that does not induce extra heat because of intermittent on/off cycling. It is expected that the reduction in heat with PWM contributes to improve a signal to noise ratio.

Proposed solution

Proposed solution: Applying the PWM in BlotMan to generate multiple membranes from a single gel, to improve transfer efficiency as well as protein mobility, and reduce the technical variations among gels.

To achieve this solution, four specific aims should be solved:

- Aim 1: To design the PWM size-dependent voltage profile for proteins.
- Aim 2: To evaluate the effects of alternative choices on the horizontal blotting processes. These effects include gel conditions (e.g. thickness, concentration, and freshness), buffers types (e.g. SDS and non-SDS), and membranes types (e.g. PVDF and nitrocellulose).
- Aim 3: To demonstrate the reproducibility and sensitivity by generating multiple transferred membrane copies from both protein makers and real protein samples and evaluating their signal intensities.

- Aim 4: To apply in cancer study by generating and detecting protein levels from isolated proteins from cancer cells lines.

In order to get solutions for these aims, signal intensities of transferred protein makers into membranes was modeled using the Gaussian distribution. The mathematical model was established and applied to determine the transferred voltage profile, which responded to a non-linear relationship function (e.g $V_t = f(T_m, m_p)$, where T_m = mean transfer time (min) and m_p = protein size (kDa). Using Matlab and Gaussian distribution, all necessary parameters were found for determination the transfer voltage profile, migration distance and blotting time per membrane. In addition, gel concentration, gel thickness, type of membranes, buffers types, etc. were tested to identify the factor variations of alternative options. Then, we examined the advantages of using PWM in both vertical running gel and horizontal blotting. To prove that BlotMan is capable of generating uniformly transferred multiple membranes by conducting experiments with different protein markers and real protein samples from a human chondrosarcoma cell line (SW1353) and a human breast cancer cell line (MDA-MB-231).

2. MATERIALS AND METHODS

2.1 The Blotman-PWM voltage profile

Applying the PWM′s principle of generating size-dependent voltage in BlotMan blotting device, a larger protein received a higher transfer voltage value and vice versa. The voltage signal was generated at maximum 24.0 V for a large protein (e.g. 140 kDa) and at 7.0 V for a small one (e.g. 17 kDa) during the blotting process by manipulating duty cycle values (Figure 2.1). Unlike the current transfer process in which a small protein has higher mobility under a constant voltage, this size-dependent voltage profile of proteins implies that a small protein is expected to receive a smaller voltage signal. This leads to similar transferred signals of proteins can be obtained in multiple membranes.

Besides the size-depended voltage profile, the additional required information includes the protein migration distance and blotting time per membrane. The protein migration distance is needed for voltage assigned at each anode strip. The blotting

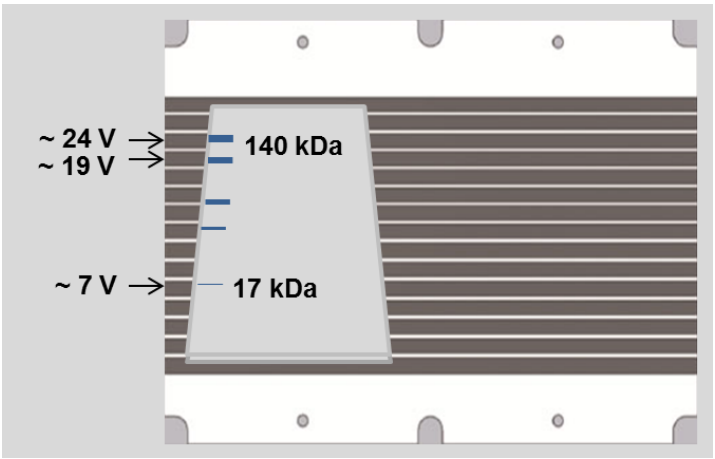

Fig. 2.1. The Multi-anode plate (of BlotMan transfer uint) layout with different voltage value assigned corresponding to size of protein.

time is necessary for generating multiple membrane copies. Taken together, these three necessary information are needed to operate Blotman device.

2.2 The Gaussian distribution

Based on the central limit theorem, the Gaussian or normal distribution is widely used in many areas of statistics as well as engineered fields, especially in signal detection [61–63]. The central limit theorem states that the sample tends to form a Gaussian distribution as the number of samples increases. Compared to other distributions, this distribution often makes statistics straightforward using its mean and standard deviation. The distribution fits well in analyzing noise properties in a linear system. In this study, a signal intensity of blotted proteins, $s(t)$, was modeled using a Gaussian distribution as a function of transferring time (t) and voltage (V). For instance, $s(t) = 1/5$ indicates that 20% of proteins is transferred. The voltage and transfer time are continuous variables, and signal intensity was also assumed as a continuous variable with its mean and standard deviation. Taken together, a Gaussian distribution was used to describe the distributions of proteins signals, which facilitated to estimating the required blotting time with a given voltage signal.

Figure 2.2 shows a transferred protein amount into a membrane is cumulative by time, which can be described as the Gaussian cumulative distribution function (cdf).

2.3 Protein migration formulation

Assuming the same mobility obtained by the same molecular weights of proteins, the migration distance was measured by using the final position of transferred multicolor protein markers onto membranes. Two types of gel thickness (1.0 mm and 1.5 mm) with 10% and 12% of polyacrylamide gels were employed in this study to measure the migration distance. The travelled distances of each protein, in the range of 17 kDa to 140 kDa, were record by ImageJ at different transfer voltages (e.g. at 20 V, 15 V and 10 V) and time points (e.g. 40 min and 60 min). The measured distance

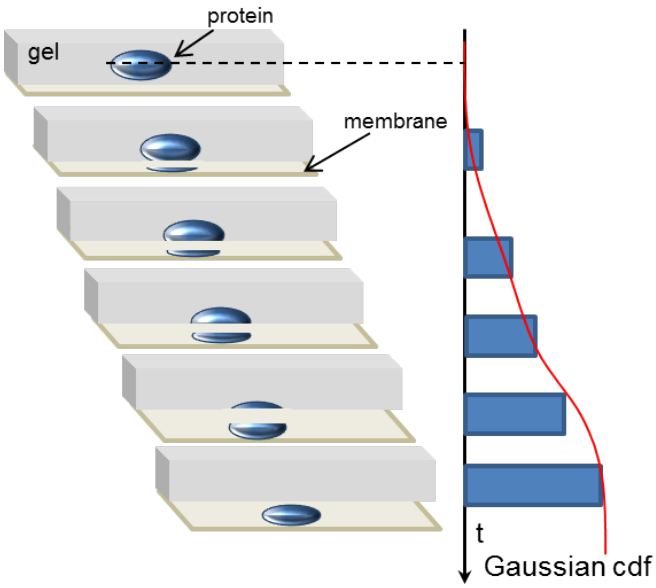

Fig. 2.2. The cumulative signal intensity as function of transferring time (t) and voltage (V).

between smallest and the biggest was set as a unit distance from 0 to 1 and plotted to find the best fit curve for protein positions. The polynomial equation model was used to approximate the migration distances of protein:

$$D = d_1 + d_2 M_{pr} + d_3/\sqrt{M_{pr}} \tag{2.1}$$

where D = migration distance (mm) and M_{pr} = protein size (kDa). Also, d_1, d_2 and d_3 are parameters of polynomial equation. The goodness of fit statistical values for this equation parametric model such as the sum of square due to error (SSE), R square, adjusted R-square and root mean square error (RMSE) were evaluated by matching between the experimental value and the best fit curve generated by Matlab.

2.4 Determination of mean transfer time

After transferring onto membrane, signal intensities of proteins were scanned and recorded at different time points (10, 20, 30, 40, 60, 120 and 180 min) for each transfer

voltage (5, 7, 10, 12, 15, and 20 V) (Figure 3.2). Signal intensities of transferred proteins were determined as a function of the transfer time and the transfer voltage. Then, they were modeled using a cumulative distribution function (cdf) of Gaussian distribution. Using the mean (μ) and standard deviation (δ) of cdf, the probability density function (pdf) of each signal intensity was determined. Also, by using the value corresponding to 50% of cdf, mean transfer time for every protein sample at each transfer voltage was determined.

2.5 Formulation of mean transfer time

Mean transfer time of protein, T_{mean}, was predicted by following equation:

$$T_{mean} = a_1 M'_{pr} + a_2 V'_{tf} + a_3 \tag{2.2}$$

where M'_{pr} = modified protein size, V'_{tf}= modified transfer voltage (V). The modified parameters, M'_{pr} and V'_{tf} were defined:

$$M'_{pr} = M^{\alpha}_{pr} \tag{2.3}$$

$$V'_{tf} = V^{\beta}_{tf} \tag{2.4}$$

where M_{pr} = protein size (kDa), V_{tf}= transfer voltage (V), and a_1,a_2,a_3, α and β = five modeling parameters in Eqs. (2.2, (2.3) and (2.4). These parameters were determined to minimize mean square error between the experimental mean transfer time and the predicted transfer time, T_{mean}.

Prediction of transfer voltage as a function of protein size for a given mean transfer time:

When the transfer time is given, the transfer voltage will be calculated as a function of protein size:

$$V^{\beta}_{tf} = \frac{-a_1}{a_2} M^{\alpha}_{pr} + \frac{T_{mean} - a_3}{a_2} \tag{2.5}$$

or

$$V_{tf} = \left(\frac{-a_1}{a_2} M_{pr}^{\alpha} + \frac{T_{mean} - a_3}{a_2}\right)^{1/\beta} \tag{2.6}$$

2.6 SDS-Page gels preparation

Twelve percentage concentration polyacrylamide with 1.0 mm and 1.5 mm gels were prepared using the handmade gel procedure, except 1.0mm and 10% SDS-Page pre-casted gels were purchased from BioRad (1.0 mm, 10% SDS-Page, cat#456-1034). In general, the separating gel solution (1.5 M Tris-HCl pH=8.8, 10% (w/v) SDS, Acrylamide/Bis-acrylamide 30%/0.8% w/v, Multi-Q water, 10% (w/v) ammonium persulfate, TEMED) was added into glass-frame gel casting up to the desired mark line. Water was quickly, but carefully added to minimize the separated gel portion from un-even surface and avoid dry-out. Then, a comb was inserted in after removing water and adding stacking gel solution (0.5 M Tris-HCl pH 6.8, 10% (w/v) SDS, Acrylamide/Bis-acrylamide 30%/0.8% w/v, Multi-Q water, 10% (w/v) ammonium persulfate, TEMED) on the top of solidified separating gel. After gel completely solidified, gels were risen with tap water, rapped by wet paper tower and stored at 4^oC fridge for later use. The materials of handmade SDS-Page gel preparation are given in Table 2.1 and Table 2.2 However, the freshness of the gel is an important factor which affects distance traveled of proteins; therefore, the all handmade gels were made and maintained in the same period of time (e.g. 2 hours) before conducting experiments.

In this study, the 1.5mm thickness and 12% gels were considered as the standard gel condition and were used to conduct almost experiments, except for some specified cases (e.g. 1.0mm thickness and/or 10% concentration).

2.7 Multicolor protein markers

Multicolor protein markers and blue protein standard markers were purchased from Thermo Scientific (Spectra Multicolor Broad Range Protein ladder, cat#26623)

Table 2.1 Required volume for handmade SDS-Page gel preparation.

Item	**Stacking gel**	**Separating gel**
Milli-Q H_20	3.20 mL	2.97 mL
AcrylamideBis-acrylamide (30%, 0.8% w/v) (*)	4.00 mL	0.67 mL
Tris buffer: 1.5M, Tris- HCl, pH 8.8 (*)	2.06 mL	N/A
Tris buffer: 1.5M, Tris- HCl, pH 6.8 (*)	N/A	2.0 mL
10% SDS (*)	100 μL	50 μL
10% (w/v) Ammonium persulfate (AP) (*)	100 μL	50 μL
TEMED (*)	10 μL	5 μL
Total	10 mL	5 mL

(*) From BioRad

Table 2.2 Required volume (mL) for different thickness of gels.

Thickness of the gel	**Stacking gel**	**Separating gel**
1.0 mm	3.0	3.0
1.5 mm	4.0	8.0

and from BioLabs (Blue Protein Standard Broad Range, cat#P7706L). For parameters determination and validation efficiency of the BlotMan device, 15 μL of mixed solution consisting of protein maker and sample buffer (Laemmli SDS- sample buffer, cat# BP-110R) with 1:1 ratio was added into each well of the gel at room temperature.

2.8 Electrophoresis vertical running gel

To determine the size-dependent voltage profile, gels were initially operated at 100 V for 10 min and followed by at 150 V for 45 min. To examine the effects of PWM

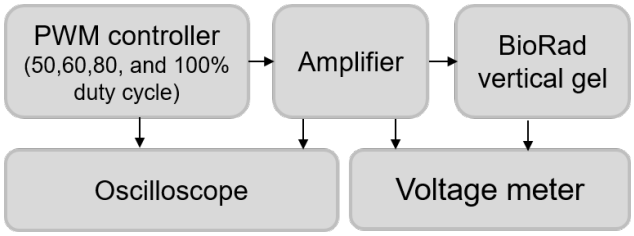

Fig. 2.3. Block diagram of vertical electrophoresis running gels setup

on protein mobility and sharpness, gels were operated at 100 V for 10 min and then run at average voltage of 150 V for 45 min with different duty cycles of 50%, 60%, 80% and 100%. The BioRad electrophoresis system ($Mini - Protein^{®}$ cell) was connected with Device set I (Trek PZD350 amplifier, Elenco Precision-Quad power XP-581) and/or Device set II (Trek PZD350 amplifier, HM350 PWMduty controller) to create different duty cycles. To ensure that desired voltage ranges and correct duty cycles applied, an oscilloscope and a voltage meter were also used to monitor these values during the experiments (Figure 2.3).

Both the 1X SDS-running buffer (BioRad, 25mM Tris, 192 mM glycine, 0.1% SDS, and pH 8.3) and non-SDS running buffer (BioRad, 25mM Tris, 192 mM glycine, and pH 8.3) were used to run gels. The Device set I is capable to generate a constant voltage and 100% duty cycle for the vertical electrophoresis running gels system. Meanwhile, the average voltage and other duty cycle values are supplied by the Device set II. The details of these Device sets are shown in table 2.3.

2.9 BioRad semi-dry transfer

The blotting papers were purchased from BioRad (extra thick blot paper 2.45 mm, cat# 170-3960) and were wetted in standard Towbin buffer (BioRad, 25 mM Tris, 192 mM glycine, pH 8.3, 20% methanol). The Polyvinylidene Diflouride (PVDF) membranes were purchased from Millipore (0.45 m, cat# IPVH00010) and were wetted in 100% methanol and then in Towbin buffer. Also, nitrocellulose membranes were purchased from BioRad (0.45 μm, cat# 162-0114) and were wetted in multi-Q water

Table 2.3 Device sets information.

Items	**Details**
Device set I	Trek PDZ350 amplifier: Input: 0 to± 10 V DC; Output Gain: 0 - 150 V/V) Elenco Precision - Quad power XP- 581: (2-20V DC 2A, 100% duty cycle)
Device set II	Trek PDZ350 amplifier: Input: 0 to± 10 V DC; Output: 0 - 150 V/V) HM350 PWM PWM Generator Module: (1 Hz - 100 kHz, 1% - 99% duty cycle)

and Towbin buffer as well. The sandwich of blotting papers, the gel and membrane was directly placed in contact to electrodes plates of transfer device. In the first part of this study, to determine the mean transfer time and voltage gradient, the BioRad semi-dry transfer system ($Transfer-Blot$® cell) was used to transfer protein makers to a membrane at different transfer voltages from 5 V - 20 V for 10 min - 180 min. In the second part of this study, the BlotMan was used to evaluation and validation of device efficiency such as uniformly transferred of five membrane copies, blotting time for each membrane, the voltage and mean transfer time, and protein analysis applications.

2.10 Evaluation and validation of BlotMan

At first, the experiments with the marker transferred membranes of standard 1.5mm thickness and 12% polyacrylamide gels were conducted to evaluate the function of BlotMan device in term of blotting time, voltage gradient and mean transfer time. Then, two types of cells including a human chondrosarcoma cell line (SW1353) and a human breast cancer cell line (MDA-MB-231) were used to examine the effi-

ciency of BlotMan in this study [9, 64, 65]. The cells were obtained from American Type Culture Collection (ATCC), were grown in DMEM medium containing 10% fetal bovine serum (FBS) and antibiotics (100 units/ml penicillin-streptomycin; Life Technologies) and maintained at a 37^oC and 5% CO2 humidified incubator. The isolated proteins, after 1h and 4h treatment with chemical agents, were collected by using combination lysis consisting radio-immunoprecipitation assay (RIPA) buffer, protease inhibitors (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and phosphatase inhibitors (Calbiochem, Billerica, MA, USA). After vertical electrophoresis running using the BioRad system and transfer processing using Blotman, protein samples were incubated with primary antibody in 1h and then with secondary antibody conjugated with horseradish peroxidase (Cell Signaling, Danvers, MA, USA) in 45 min. The 5 antibodies c-Src, eIF2, p-eIF2, lamin B (Cell Signaling), and β-actin (Sigma) were used to evaluate and quantify protein signals using LAS-3000.

3. RESULTS

The results are presented in four sections corresponding to the four specific aims: the first section includes results of parameters values for vertical migration distance equation, for mean transfer time equation and blotting time for each membrane. These values were used to estimate the size-dependent voltage profile and to operate BlotMan. The second part is about the estimated values of mean transfer time influenced by other factors such as buffer type, membrane type, gel concentration and thickness, etc. From results of the first two parts, the PWM voltage profiles for proteins were determined and applied in BlotMan blotting device. The third part includes validation and evaluation BlotMan using 5 transferred membranes from both protein markers and protein samples from two cancer cell lines. The effects of PWM on both vertical gel and horizontal blotting were also shown by evaluating transfer efficiency and signal intensity. In the fourth part of this chapter, the application of BlotMan in cancer study was shown by detection results protein levels from 5 membrane copies.

3.1 Design the PWM voltage profiles

3.1.1 Vertical migration of protein by electrophoresis (standard condition)

The best fits migration distance equations, as function of the protein size with different gel concentration, were found (Equations 3.1 and 3.2). Measuring distances between the largest and smallest proteins after electrophoresis transfer, the estimated parameters values of Equation 2.1 were normalized to unit length (Table 3.1) and plotted to find the best fit curve for protein positions (Fig. 3.1). The normalized

length unit of migration distance was fit with polynomial equation curve (with 95% confidence bounds):

$$D_{12\%} = -0.6789 - 0.0006055M_{pr} + 6.933/\sqrt{M_{pr}} \tag{3.1}$$

$$D_{10\%} = -0.2101 - 0.001943M_{pr} + 5.325/\sqrt{M_{pr}} \tag{3.2}$$

As results from equations 3.1 and 3.2, the parameter of protein migration distances of 12% gel and 10% gels are slightly different. As expected, the parameters d_1 and d_2 are larger in magnitudes for 10% gels than those for 12% gels which make the migration distance of proteins in lower concentration gel (10%) has relative larger distance ratio than the higher gel concentration (12%) does. In addition, the goodness-of-fit statistical values of these parameters were found with the valid and acceptable ranges. For instance, the statistical values such as SSE, R-square, adjusted R-square and RMSE of D 12% polynomial equation are 0.0009782, 0.9988, 0.9983 and 0.01399 respectively.

Table 3.1 Normalized protein migration distance (of 1.5 mm thickness gels).

Protein size (kDa)	**Ratio (10%/12%)**	**Normalized migration distance**	
		12%	10%
140	0.00	0.000	0.000
95	1.77	0.096	0.170
72	1.54	0.186	0.287
52	1.35	0.316	0.425
42	1.25	0.416	0.520
34	1.17	0.528	0.619
26	1.09	0.690	0.755
17	1.00	1.000	1.000

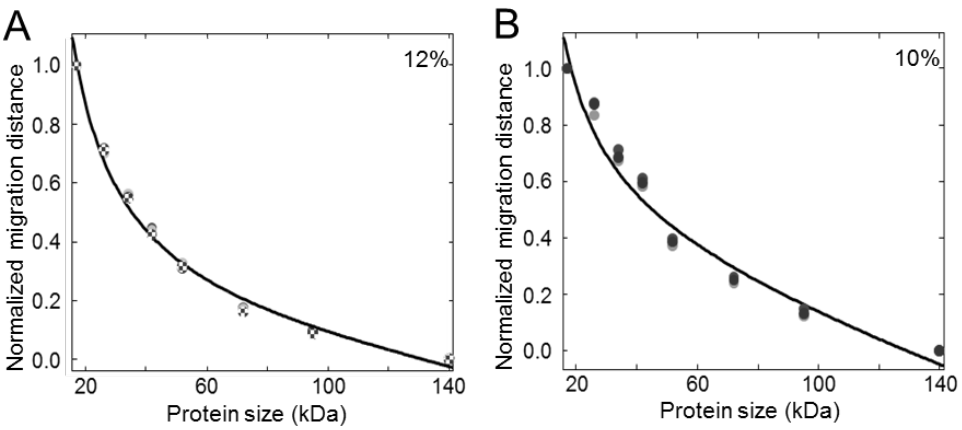

Fig. 3.1. The normalized migration distance profile of a protein. (A) The migration distance profile of 12% concentration and 1.5mm gel. (B) The migration distance profile of 10% concentration and 1.5mm gel.

Figure 3.1 showed that smaller proteins in 10% concentration gel moved farther and closer to 1 than ones in 12% concentration gel did. In general, the distance between bigger proteins sizes (greater than 75 kDa) have larger separations in lower gel concentration (i.e. 10%) than the proteins having smaller sizes (less than 34 kDa) do. Also, it proved that how well of experimental data points (round dots) fitting with the polynomial math models (black curves). Together with the distance 10%/12% ratio on Table 3.1, these polynomial equations are used as basic protein positions determination of BlotMan device.

3.1.2 Signal intensity distribution

Prior to generating the mean transfer time as well as the blotting time for each membrane, the relationship among signal intensities, transfer time and transfer voltage was determined. This relationship was done by conducting series of semi-dry transfer experiments (Transfer-blot cell, BioRad) at a constant voltage of 5, 7, 10, 12, and 15 V for 10, 20, 30, 45, 60, 120 and 180 min. Note that protein bands in each membrane were generated using the identical experimental condition. The figure 3.2

shows transferred proteins, from 17 kDa to 140 kDa, have stronger signal intensities with a longer transfer time and at a higher voltage. For example, more bands and stronger signals of proteins at a constant voltage of 15 V than those at 10 V are shown for the same transfer time of 40 min.

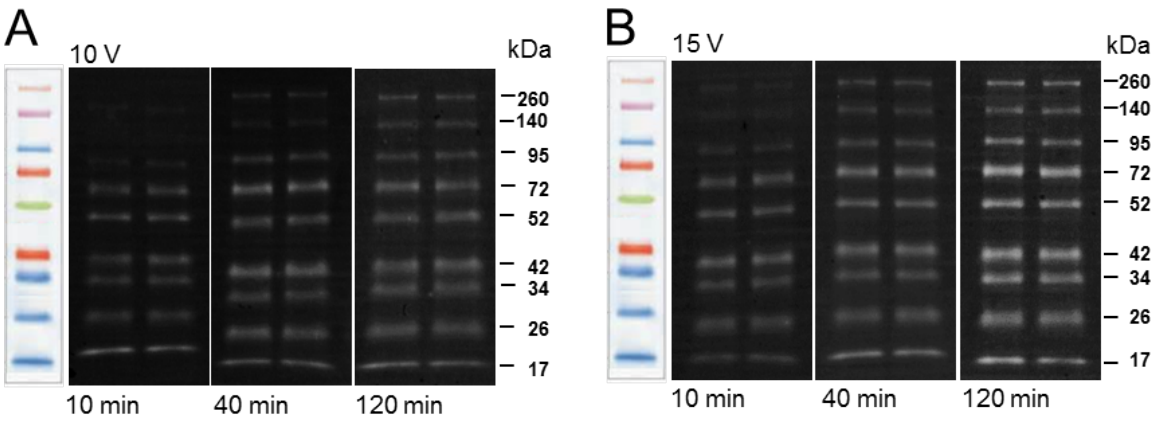

Fig. 3.2. Signal intensity of proteins as function of transfer time and transfer voltage. (A) Signal intensity of protein at 10V in 10, 40 and 120 min. (B) Signal intensity of protein at 15V in 10, 40 and 120 min.

As function of the transfer time and transfer voltage, the signal intensity was normalized and plotted by Gaussian distribution approximation (Fig.3.3). As the smaller proteins were transferred faster and required lower transfer voltages, the distribution of cdf and pdf of a smaller protein size was stiffer and sharper than that of a bigger protein size did. Also, the size of proteins was increased, the cdf and pdf curves moved from the left to the right. For instance, the cdf and pdf curves of 17 kDa, 52 kDa and 140 kDa proteins were stiffer and narrower distributions at transfer voltage of 10 V than those of 15 V. It maybe imply small proteins are more sensitive with the changes of the transfer time as well as the transfer voltage.

These cdf functions are used as a basic principle to determine blotting times of multiple membrane copies. Note that the same blotting time is applied for all membranes if the relationship between signal intensity and transfer time is linear.

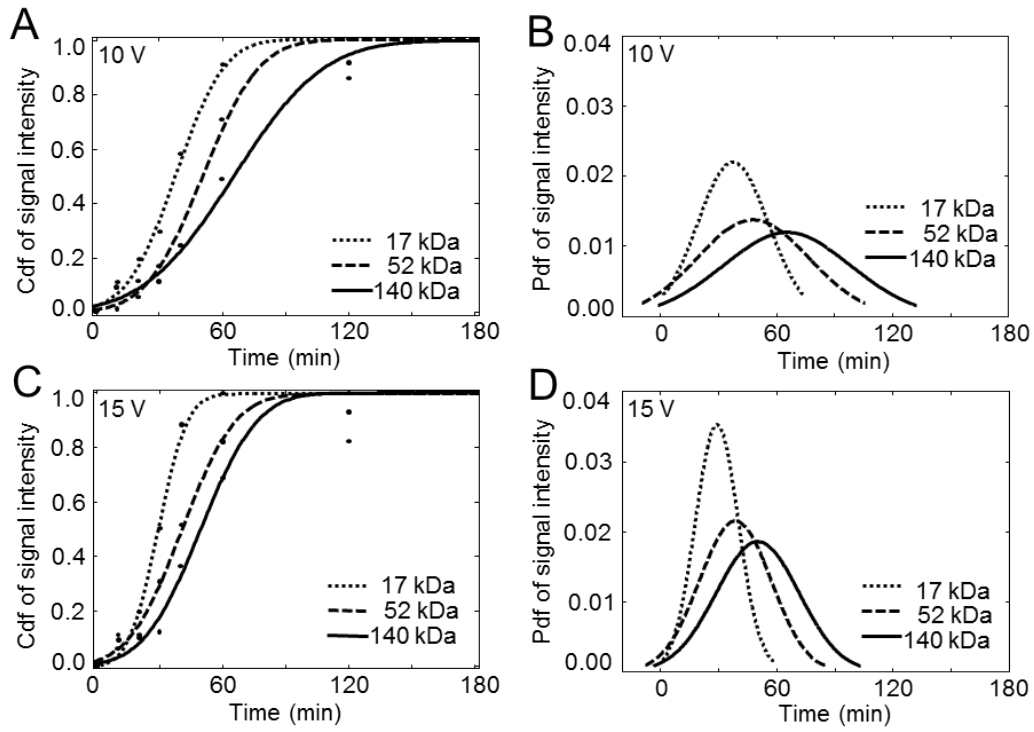

Fig. 3.3. Transfer intensities approximated by Gaussian distribution. (A-B) The cumulative distribution function (cdf) plots for proteins with 17 kDa, 52 kDa, and 140 kDa at 10 V. (C-D)The probability density function (pdf) plots for these proteins at 15 V. Note that 17kDa (dots), 52 kDa (dash), and 140kDa (solid).

However, for 5 membrane copies, the Gaussian distribution function indicates the same transferring duration applied for the 1^{st} and the 5^{th}, or for the2^{nd} and the 4^{th}, but not for the 3^{rd} copy.

3.1.3 Mean transfer time

The experimental mean transfer time as a function of transfer voltage (V) and protein size (kDa) was shown in Fig. 3.4. As expected, it shows that the smaller protein size has smaller transfer time and lower transfer voltage than the bigger size does. For instance, the mean transfer time of protein size at 17 kDa and 140 kDa were about 25 min and 45 min respectively as the same 15 V transfer voltage was applied.

Also, the transfer time was shorten when voltage was increased. As a function of transfer voltage and protein size, the experimental mean transfer time is non-linear function and is more being influenced by transfer voltages than by protein sizes (Fig. 3.5).

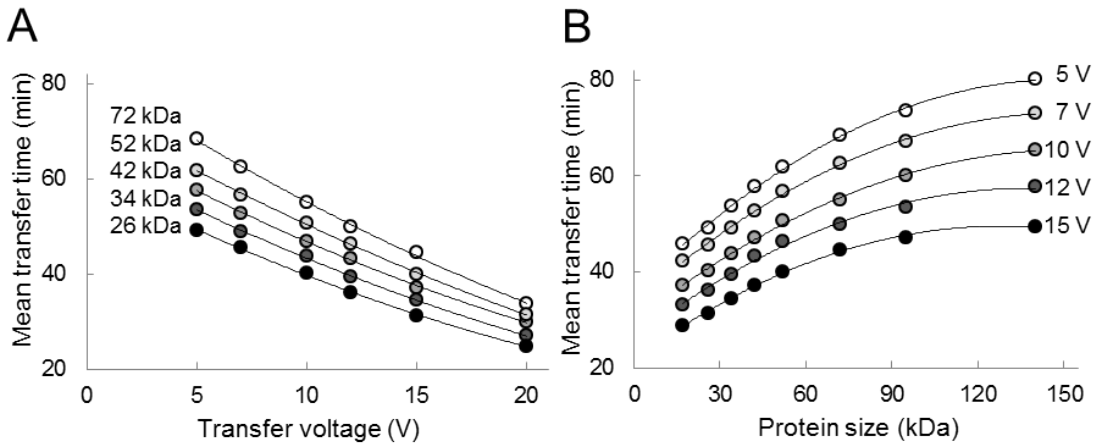

Fig. 3.4. Non-linear relationship graph of three parameters: mean transfer time, protein size, and transfer voltage. (A) Mean transfer time as a function of protein size (26, 34, 42, 52, and 72 kDa). (B) Mean transfer time as a function of transfer voltage (5, 7, 10, 12, and 15 V.

Table 3.2 Parameters values for predicted transfer voltage as the Equation 2.6

Parameter values	a_1	a_2	a_3	α	β
	46.77	- 6.929	- 3.092	0.1516	0.6675

The Table 3.2 shows five parameters of Equation 2.6 with the MSE is 32.85. Note that only one of these parameters has a small scale of changing (e.g. from 0.01% to 0.1%), there will larger scale of effects on estimated transfer voltage and mean transfer time, especially for the small proteins (e.g.$\leq$ 30 kDa). Although all these parameters have certain impacts, the parameter α and β are more sensitive and have higher effects than others.

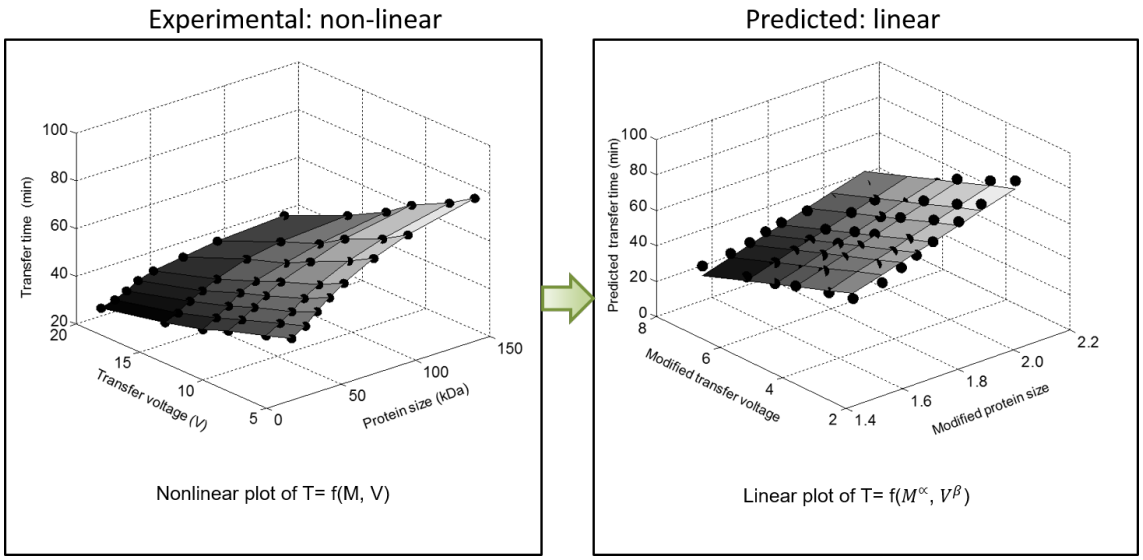

Fig. 3.5. The 3D graph of three parameters: mean transfer time, protein size, and transfer voltage.(L) Experimental non-linear plot of $T_{mean} = F(M_{pr}, V_{tf})$. (R) Predicted linear plot of T_{mean}.

In the Figure 3.5, the predicted transfer time was approximated from the Gaussian distribution and plotted as a function of protein size and transfer voltage (the linear plane). The relationship among predicted transfer time, modified protein sizes, and modified transfer voltage become a linear relationship. To compare with predicted one, the experimental mean transfer time curve (non-linear the black dots) was plotted, also. The parameters of predicted transfer voltage were found and optimized to get the minimum mean squared error (MSE) value. The estimated transfer voltages, as function of the protein size and the mean transfer time, was calculated and shown in Table 3.3. As the maximum of 24 V supplied by power source, the mean transfer time for transferring all proteins (within 17 kDa to 140 kDa range) starts from 38 min.

Table 3.3 Predicted transfer voltage (V) as function of transfer time (min) and protein size (kDa) in standard condition.

Mean transfer time (min)	**Protein size (kDa)**							
	140	95	72	52	42	34	26	17
10	N/A	N/A	N/A	N/A	N/A	N/A	N/A	23.73
12	N/A	N/A	N/A	N/A	N/A	N/A	N/A	23.34
...	N/A	N/A	N/A	N/A	...	...	...	...
...	N/A	N/A	N/A	N/A	...	...	...	...
36	N/A	20.56	18.08	15.44	13.86	12.42	10.74	8.93
38	**23.04**	**19.25**	**16.83**	**14.27**	**12.74**	**11.35**	**9.73**	**7.48**
40	21.67	17.97	15.62	13.14	11.16	10.32	8.76	6.61
...	...	...	...	...	...	...	...	...
...	...	...	...	...	...	...	...	...
54	13.01	10.03	8.18	6.29	5.21	4.24	3.17	1.80
56	11.92	9.05	7.28	5.48	4.45	3.55	2.56	1.32

Note: N/A because its voltage is beyond Blotman power supply

3.1.4 Blotting time for multiple membranes

Based on the relationship between signal intensity and blotting time defined on previous step, the blotting time of each membrane was also estimated by using the unit Gaussian distribution as well. It means blotting time of membranes were ratio distributed between 0 and 1. In this study, we conducted and successful tested blotting time of each membrane which is almost same as predicted one. For instance, 3 membranes will have blotting time ratio 0.402 : 0.196 : 0.402 as shown in Table 3.4.

Table 3.4 Blotting time for each copy of a standard condition gel.

# copies	**Normalized transfer time/each membrane**				
	1^{st} copy	2^{nd} copy	3^{rd} copy	4^{th} copy	5^{th} copy
1	1.000/1.000	N/A	N/A	N/A	N/A
2	0.500/0.500	0.500/0.500	N/A	N/A	N/A
3	0.395/0.402	0.210/0.196	0.395/0.402	N/A	N/A
4	0.340/0.342	0.160/0.158	0.160/0.158	0.340/0.342	N/A
5	0.290/0.302	0.143/0.139	0.117/0.118	0.143/0.139	0.290/0.302

Note: The ratio is the expected/ the experimental values

3.2 Evaluation the effects of alternative choices on the estimated transfer voltage

Effects of gel concentration (10% vs. 12%): Using 10% concentration gel as an alternative option, gels were transferred at different transfer voltages (e.g. 15 V and 10 V) to determine the effect of gel concentration factor on mean transfer time. The results show that effects of gel concentration is not a big significant difference by 6% (Table 3.5).

Effects of gel thickness (1.0 mm vs. 1.5 mm): the mean transfer time was significant lower as the thickness of gel decreased from 1.5mm to 1.0mm. The effects of gel thickness factor is 48% (Table 3.5).

Effect of membrane type (PVDF vs. nitrocellulose): The same testing conditions (1.5 mm, 12% SDS-Page gel, transfer voltage 20 V, SDS-running buffer) were applied to test the different transferring effects between the PVDF membrane and the nitrocellulose one. As the result, the mean transfer time values of two type of membranes are not significant different (Table 3.5).

Table 3.5 The estimated transfer voltage ratios of alternative conditions.

Parameters	**Standard choice**	**Alternative choice**	**Ratios (Standard choice/ Alternative choice**
thickness factor	1.5 mm	1.0mm	1/1.48
% gel factor	12%	10%	1/1.06
membrane type factor	PVDF	nitrocellulose	1/1.03

3.3 Demonstration the reproducibility and sensitivity of BlotMan

Uniformity of multiple membranes

As the relationship among mean transfer time, transfer voltage and protein sizes defined in section 3.1.3, all proteins can get the same transfer efficiency by applying voltage gradient for size-voltage dependent proteins. With the size-dependent voltage profile generating by WM signal, BlotMan is capable to generate multiple membranes from a single gel and relatively the same transferred signal intensity. To achieve the same transfer signal efficiency for all protein sizes in range of 17 kDa to 140 kDa, a higher voltage value was applied to a large protein at 50% duty cycle. In this study, multiple membranes (e.g. 2, 3 and 5 membranes) were generated from a single transferred protein marker gel (Figure 3.6).

Protein species have similar signal intensities for each case of 2, 3 and 5 membrane copies as shown in Figure 3.6. Note that the total transfer time for each case of number of copy is the same and the blotting time of each copy in each case is calculated as in Table 3.4.

In the case of 5 membranes, the normalized signal intensity was further analyzed by computing variation among each protein size and each copy. The signal intensities of 5 blotted membranes are almost identical distributed with the mean variation of 5.2%.

3.4 Application of generating multiple membranes on the cancer study

Two cancer cell lines including SW1353 chondrosarcoma and MDA-MB-231 human breast cancer cells were employed and conducted with experiments with 5 membrane copies to valid the capability and effectiveness of BlotMan device.

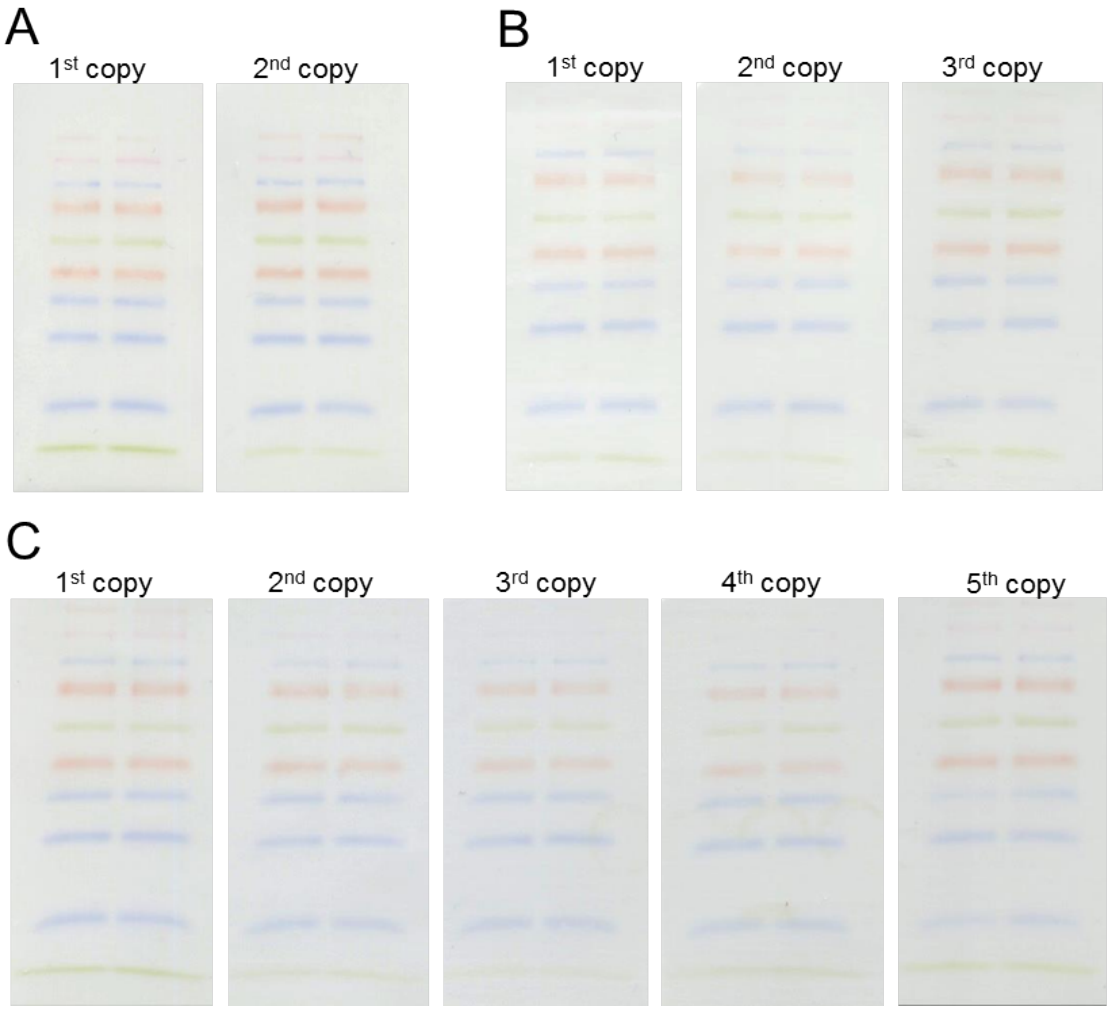

Fig. 3.6. Uniform multiple membranes of protein marker from a single polyacrylamide gel. (A) Generation of two membranes from a single gel. (B) Generation of three membranes from a single gel. (C) Generation of five membranes from a single gel.

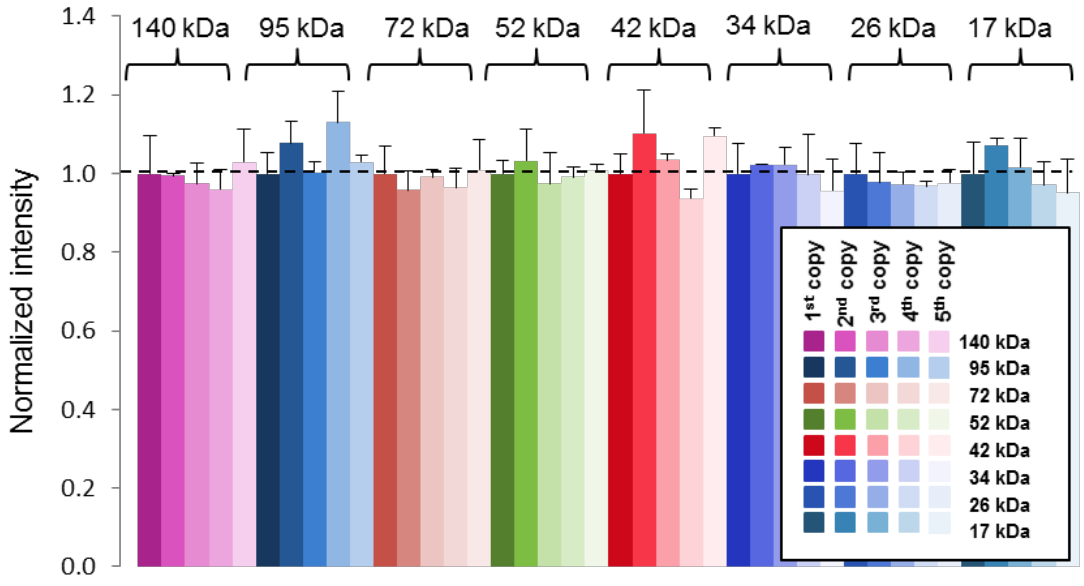

Fig. 3.7. Variations of blotting intensities among five membranes from a single gel.

The first example to valid the capacity of BlotMan device is the detection β-actin activity of 5 membranes from SW1353 chondrosarcoma protein samples, including a non-treated served as a control group and an agent (e.g. Agent a) treated group. The normalized signal intensities shown in figure 3.8B verify that the variation among these membranes is not significant by only 5.2%.

The second example to examine the efficiency of BlotMan is the detection different proteins such as c-Src ($\sim$ 60 kDa), eIF2α, p-eIF2α ($\sim$38 kDa), lamin B ($\sim$ 70 kDa) and β-actin ($\sim$46 kDa) for each of 5 membranes (Figure 3.9). These protein samples collected from MDA-MD-231 human breast cancer cells in presence and absence of chemical x and y during 1 hour and 4 hours treatments . Although the elevation proteins levels are different, both agent x and y upregulated p-eIF2α, which is used to detect the decrease proliferation of cancer cells (e.g. MDA-MB-231 cells). The c-Src protein expression level doesn$'$t seem to be altered by agent x while agent y down regulated slightly of c-Src after 4 hrs. The protein levels of eIF2α, lamin B and β-actin seemed unchanged. Note that a proto-oncogene kinase c-Src is known to

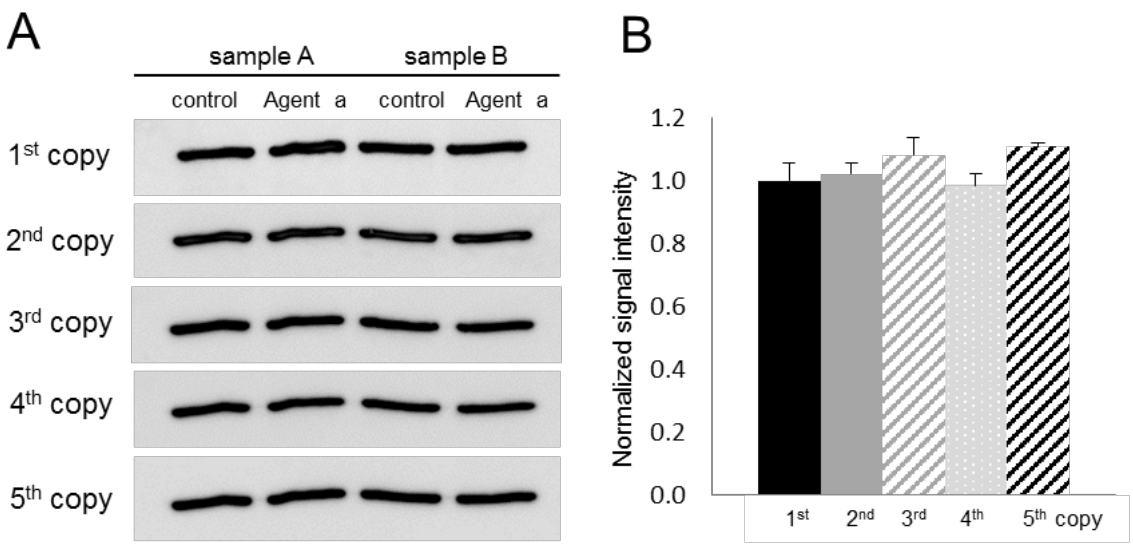

Fig. 3.8. Detection of protein levels in 5 membranes from a single gel with $\beta - actin$. (A) Five membranes of $\beta - actin$ using proteins isolated from SW1353 chondrosarcoma cells. (B) Variation of the normalized signal intensity among 5 membrane copies.

connect to cancer growth, lamin B is used to detect a certain component in a nuclear envelop, and β-actin functions as a control band.

3.5 Unexpected results from using PWM

Improved vertical migration of protein in gel electrophoresis: From collected data of travelled distance of proteins under the identical experiment conditions (e.g. standard 1.5 mm and 12% gel, average at 150 V applied and 1 Hz frequency maintained), it shows that the vertical migration distance of proteins is greater by using the PWM signal. As the effects of time delay motion behaviors (at least for a short period time at beginning of the time-off period) and size-dependent voltage on proteins, the migration distance decreased when the duty cycle of PWM signal for vertical electrophoresis gel was increased from 50% to 60%, 80% and 100% (Figure 3.10 A).

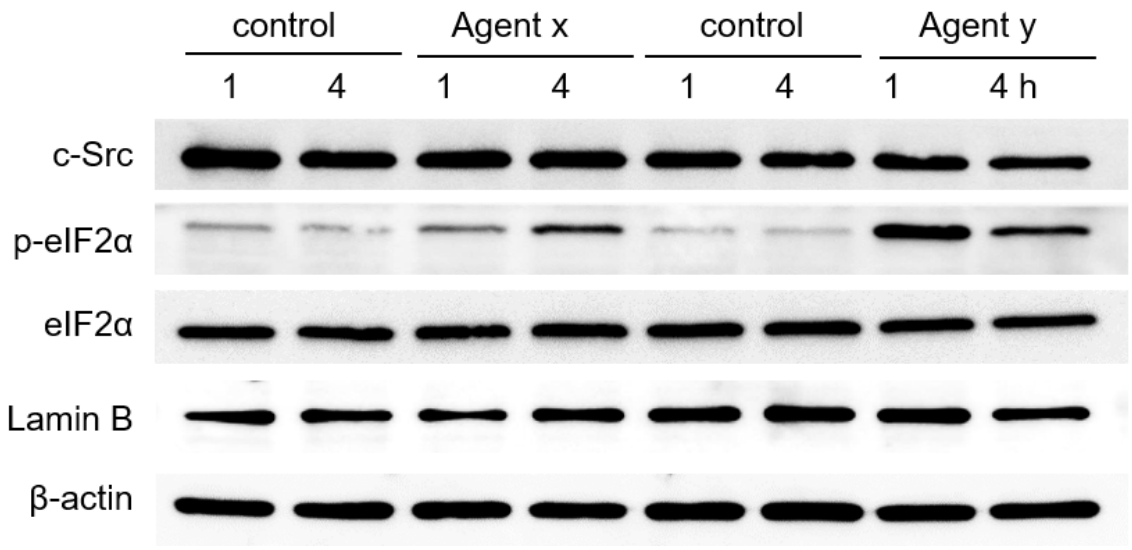

Fig. 3.9. Detection of c-Src, p-eIF2α, eIF2α, lamin B, and β-actin using protein samples isolated from MDA-MB-231 breast cancer cells.

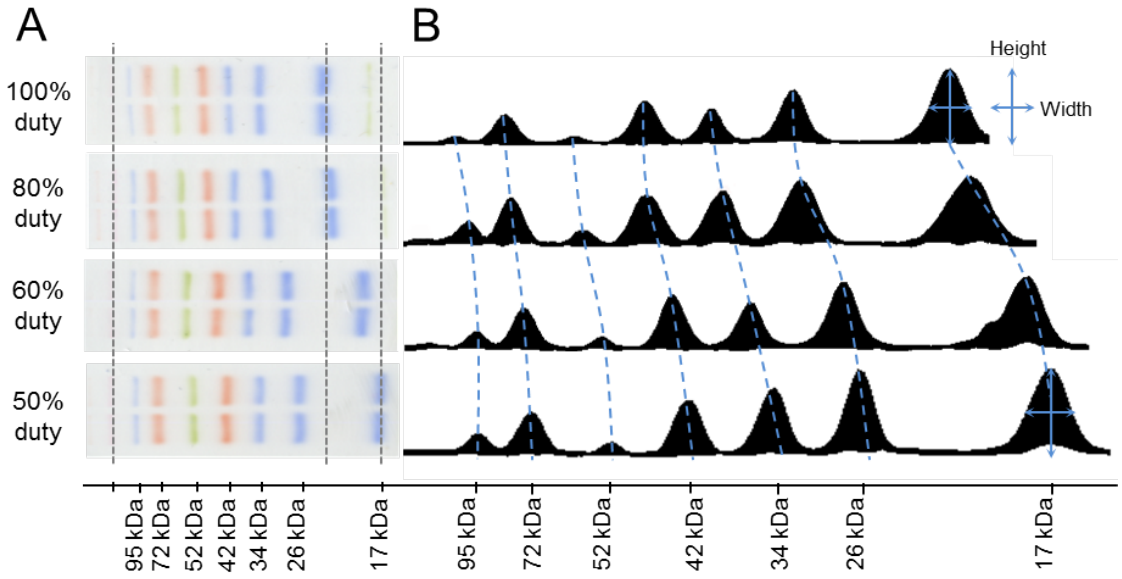

Fig. 3.10. Effects of PWM on migration distance and signal intensity. (A) Migration distance of 7 protein species in a vertical gel in response to 50, 60, 80, and 100% duty cycles.

Besides other factors such as gel concentration, buffers, and pH, the magnitude of voltage applied, the duty cycle and the protein size have some significant impacts on the migration distance of proteins.

Enhanced signal intensity by PWM signal: Signal intensity of transferred proteins is influenced both by the electrophoresis vertical running gels process and the blotting process with BlotMan. As shown in Figure 3.11, the signal intensities of 7 protein species was measured by ratio of peak height to the width at $\frac{1}{2}$ peak height. The normalized signal intensities are estimated 38.97% in average higher for smaller duty cycles at 50%, 60% and 80% than that at a constant 100% duty cycle.

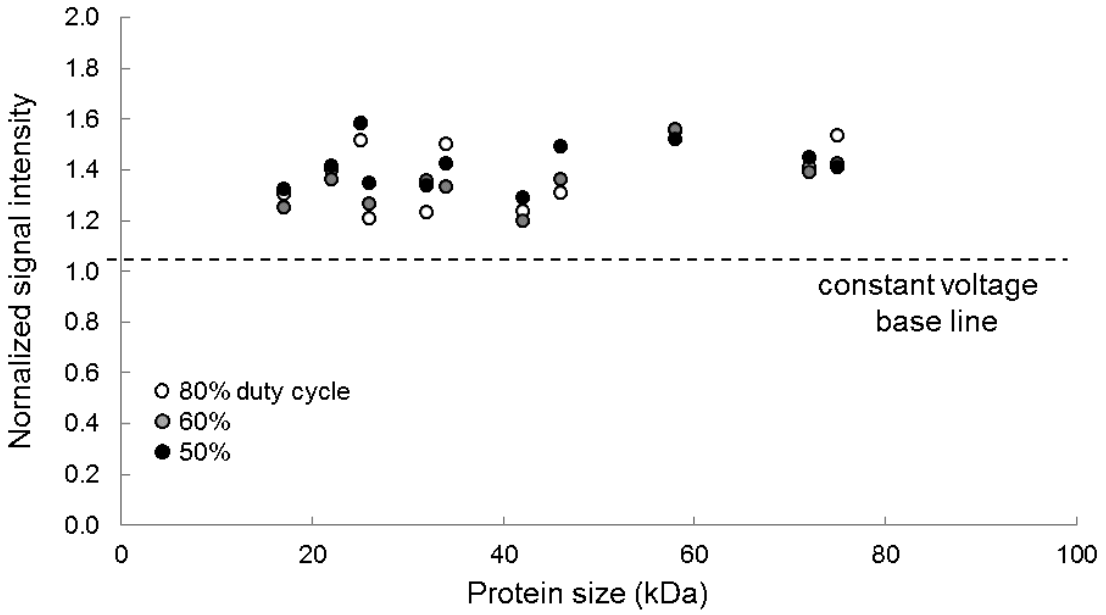

Fig. 3.11. Normalized signal intensity of varying protein species corresponding to the case with 100% duty cycle.

4. DISCUSSION

Although, both the PWM signal and voltage profile show significant advantages in the blotting process, major concerns have been raised during this study including the mechanisms behind the effects of PWM on protein mobility and signal-to noise ratio as well as the use of the Gaussian distribution.

4.1 Enhanced a protein mobility by PWM

Regarding the enhancement of protein mobility under PWM signals, a few mechanisms have been proposed [66–71]. One mechanism, based on a biased reptation model under dielectrophoresis, describes that the molecular orientation along the electric field alters during the on/off cycle and this alteration in alignment enhances protein mobility. It is also reported [72] that the mobility of rod-like molecules is affected by their alignment under the pulsed electric field. Namely, the mobility increases due to the reduced drag force when molecules align its major axis along the pulsed electric field. The mobility is also shown to be dependent on PWM duty cycles, frequencies, and field strengths. In the vertical electrophoretic gel for BlotMan, the electric field strength was approximately 10 V/m and the frequency was 1 Hz. It is not clear, however, whether this PWM condition for BlotMan is able to induce alterations in protein alignment along the direction of electric fields.

Another potential mechanism of the enhanced protein mobility with PWM is based on potential molecular motions (time delay motion) during an off duty period. The current voltage for PWM is determined with an assumption that no molecular motions take place during this off period. However, it is reasonable to consider that it takes a finite time duration for any molecules to make their velocity zero. Thus, proteins retain non-zero velocity at least, in the beginning of this off duty period and

proteins gain extra mobility in the off period besides the on period. Collectively, it is recommended that appropriate mathematical models should be built and the experimental result regarding the enhancement of protein mobility should be quantitatively evaluated.

4.2 Sharpness of a protein improved by PWM

The mechanism behind PWM-driven enhancement of the signal-to-noise ratio with BlotMan has to be investigated. It is reported by Slater and Noolandi [73–75] that a non-uniform electric field, whose field strength decreases along the direction of migration, may improve the signal-to-noise ratio. Under this particular electric field, proteins of a given species may reduce their motility as they proceed to the front of the band, while they elevate their motility as they trail. In BlotMan, PWM was applied to generate a gradient of electric fields in two processes: vertical gel electrophoresis and blotting. In vertical electrophoresis, no gradient of electric fields was generated and PWM was used to achieve an average effective voltage with variable duty cycles. In blotting, the gradient of electric fields was generated perpendicular to the direction of molecular migration. Thus, BlotMan does not apparently generate the non-uniform electric field along the direction of migration as in the study by Slater and Noolandi.

One potential cause of the non-uniform electric field might be un-intended non-square waveforms in PWM instead of the intended square waveform. The command signal instructs BlotMan to generate a square waveform in PWM. In reality, however, the actual waveform may not be a perfect square and its corners might be slightly rounded. If this roundness of the leading edge is not the same to that of the trailing edges, there is a possibility to induce a non-uniform electric field along the direction of migration. It is recommended that the mechanism of the enhanced signal-to-noise ratio should be further investigated, for instance, using different waveforms that may clearly induce a gradient of electric fields along the direction of migration.

The sharper and stronger transferred signal intensity can be obtained from taking a little advantage of faster migration rates of proteins is size-dependent feature. Although it causes no or a little problem if a small amount of proteins diffused out the surrounding environment (e.g. running buffer) during a longer transferring process, it matters if loading amount of protein samples is small and blotted bands is broader on membranes. With shorter transferring time, the bands are sharper and signals are stronger due to less amount of loading samples diffused.

4.3 Concerns of using Gaussian distribution

Although a Gaussian distribution may provide an approximate distribution of signal intensity, a concern is its endless tails as well as its symmetric profile. With its endlessly extended tails, a Gaussian distribution cannot accurately represent actual signal data, which are in a finite range. Furthermore, the signal profile may not be necessarily symmetric in terms of transfer time and this potential deviation seemed stronger for smaller proteins with a shorter transfer time (Figure 3.3). In this study, the modified Gaussian distribution, which removed two extending tails (i.e., containing 95% of data within ± 2 standard deviations), was employed. To improve the prediction of voltage and transfer time, it is recommended to evaluate application of other distribution functions such as a binomial distribution, which may not have endlessly extended tails with asymmetric profiles.

4.4 The effects of the alternative choices

In this study, it is shown that PWM voltage signal provides at least 3 advantages: proteins travel farther in vertical running gel, signal intensities of proteins are improved and uniformity of multiple membrane copies are generated by a single gel. In a standard gel (1.5 mm thickness and 12% concentration), at average 150 V and 1 Hz of testing condition, the migration distance of protein as applied different duty cycles of 50%, 60%, and 80% was increased by 22%, 16% and 4%, respectively. The mobility

of proteins was affected both by voltage magnitude controlled by manipulating duty cycle (on/off duty) time and their sizes (Equation 1.5 and 1.7).

In addition, the migration distances of proteins were depended both on the gel concentrations (12% vs 10%). The migration distance of protein markers profiles is normalized and have non-linear relationship. The larger protein moved with higher ratio migration distance than the smaller proteins in lower gel concentration (Table 3.1). In addition, the buffer condition contributed to the problem that the protein markers had unexpected positions. The observed data showed that the motility of larger proteins in no-SDS buffer became faster and then they had larger traveled distances than that of the middle and smaller size of proteins (data was not shown). Therefore, the buffer with SDS is recommended to use in order to get better results. However, more experiments need to be conducted and the mechanism should be studied.

Moreover, the measured signal intensities of these transferred membranes were improved in comparison to the constant voltage case (100% duty cycle). For instance, the sharpness of bands was increased by 43± 9% (50% duty cycle), 38 ± 12% (60% duty cycle), and 39 ± 13% (80% duty cycle) among all protein marker sizes (Fig. 3.11). As expressed from equation 1.5, the mobility of proteins depends on their sizes and electric field strength. As the size of protein is getting larger and larger, the migration velocity of protein will reach the terminal velocity is no longer depended on the size (as shown in equation 1.6). In addition, protein mobility also depends on the electric field strength, which is altered by duty cycles. The lower duty cycle applied in PWM will have more impacts on the velocity than the high duty cycle does (Figure 3.10).

4.5 Others

We also demonstrated that the transfer time as a function of protein sizes and transfer voltage has nonlinear relationships (Fig.3.4). The protein sizes show that

their effect on the change of the mean transfer time profiles is more sensitive than that of the transfer voltage itself. The estimated transfer voltages were calculated based on the mean transfer time model, with modified transfer voltage and modified protein sizes (Fig. 3.5 and Table 3.2). For instance, 1.5 mm thickness and 12% gel and mean transfer time from 38 minutes, the predicted values of transfer voltage were within the range from 23.04 V to 7.48 V applied for a large protein (140 kDa) to a small protein (17 kDa), respectively. However, the transfer voltage values for small proteins (e.g. less than 30 kDa) were smaller than predicted transfer voltages. Therefore, it is possible that a fraction, representing relationship between protein size and transfer voltage, would be introduced in order to adjust the transfer voltage values of small proteins. It means the main source contributing to the error of the mean transfer time model might was from these small proteins.

The effects of other conditions such as gel dimensions and buffer contents on transfer time were studied. The predicted mean transfer time of proteins was affected by gel thickness, gel concentration, running buffer as well as type of membranes. In comparisons between results of mean transfer time profile for the standard condition (1.5 mm thickness and 12% gel) and the mean transfer time profiles of other conditions, the effect of gel thickness was significantly higher than the effect of gel concentration. In this study, only certain conditions (e.g. gel types, buffer, and membranes) were examined. Other conditions such as blocking paper thickness or other different gel concentrations (e.g. 4% -8%) and thickness (e.g. 0.75 mm) could be further studied as well. Moreover, the protein range, other than from 17 kDa to 140 kDa, could be an interested target to study.

5. CONCLUSIONS AND FUTURE DIRECTIONS

Compared to the current and conventional blotting methods, BlotMan with PWM was capable of providing multiple membranes from a single gel with high transfer efficiency and superior signal intensities. To determine the PWM voltage profiles for proteins in various sizes, the mathematical model was developed and the effects of gel dimensions, and types of the buffers and membranes were evaluated. Sensitivity and reproducibility of BlotMan was validated using multi-color protein markers as well as protein samples that were isolated from two human cancer cell lines. Using 5 membranes from a single gel, the concentrations of 5 protein species such as c-Src, eukaryotic translation initiation factor 2 alpha (eIF2α), phosphorylated eIF2α (p-eIF2α), lamin B, and β-actin were successfully detected. These proteins except for β-actin were linked to proliferation and migration of tumor cells, while β-actin was used as a control. The signal variations among these membranes were 5.2% for the multicolor protein marker and 5.1% for β-actin.

Prior to concluding this study, two recommendations for future studies are presented. First, the mechanism of PWMs action on protein mobility and a signal-to-noise ratio needs further investigation. Compared to a uniform voltage signal, PWM signals with the same average voltage to the uniform signal improved protein mobility and sharpened protein bands. To understand the mechanism, it is necessary to investigate a dynamic alteration in protein conformation under a rapidly changing on/off electric field. This study focused on mathematical model building and characterization of BlotMans capability with PWM, but the mechanistic study was not conducted. It is likely that protein behaviors are dependent on PWM waveforms and frequencies, which were fixed to the square wave and at 1 Hz in this study. Second, BlotMan has a user-friendly interface, but it is possible to further improve BlotMans usefulness. Two specific directions are: (a) a smart phone based operation using Bluetooth

technology, and (b) implementation of an automatic membrane attaching/detaching process using a rolling membrane system.

In conclusion, it was demonstrated in this study that BlotMan was a novel protein blotting device that enabled the reduction in the required amount of protein samples, as well as labor and costs, by generating multiple membranes (up to 5 copies) from a single gel. Voltage signals with PWM improved transfer speed and signal intensity, and the device was validated using multi-color protein markers and protein samples harvested from human cancer cell lines. It is expected that BlotMan significantly contributes to improving protein analysis works with Western blotting.

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