Method Development for an Easy and Direct Quantitation of Protein Adsorption by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

by

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B.Sc, University of British Columbia, 2011

Thesis Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Science

in the
Department of Chemistry
Faculty of Science

SIMON FRASER

UNIVERSITY Fall 2014

Approval

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Date Defended/Approved: November 20, 2014

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Abstract

The current proteomic approach is to identify and analyze proteins of interest, which can potentially be disease biomarkers. However, these important proteins usually exist in very low concentration, making them vulnerable to sample loss by adsorption during any laboratory manipulations. A simple method, sodium dodecyl sulfate polyacrylamide gel electrophoresis coupled with direct protein adsorption analysis (SDS-PAGE/DPA), is presented here for the quantitation of adsorption-caused protein loss. No complicated steps and expensive equipment are involved; this method for protein study enables measurement of proteins adsorbed on vials at extremely low concentrations (in pg/μl). We used this method to characterize the effects of concentration, time, and volume on adsorption. We also applied this method to discover differential sample loss in protein mixtures and its utility in developing preventive strategies of adsorption. This method is further being employed for studying different aspects of adsorption-caused peptide loss.

Keywords: protein adsorption; SDS-PAGE; proteomics, peptides

To my family and friends

Acknowledgements

I would like to give my sincere gratefulness to my senior supervisor, Dr. Bingyun Sun, for the research opportunities that she has given me and for her guidance, patience, and support throughout the years. Not only do I learn so much chemistry and instrumentation knowledge from her, but also, from the bottom of my heart, highly appreciate her intensive training that improved my research skills tremendously. I also want to thank the undergraduate student, Kinny Wu, for assisting me to carry out the protein digestion experiments.

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List of Acronyms

ACN	Acetonitrile
BSA	Bovine serum albumin
Cy3	Cyanine 3
DPA	direct protein analysis
DTT	Dithiothreitol
ELISA	Enzyme-Linked ImmunoSorbent Assay
F	Phenylalanine
FA	formic acid
FITC	fluorescein isothiocyanate
HCl	hydrochloric acid
HSA	human serum albumin
I	iodide
IAA	Iodoacetamide
IgG	Immunoglobulin G
ITO	Indium tin oxide
K	Lysine
L	Leucine
MB	myoglobin
MS	mass spectrometry
NaOH	Sodium hydroxide
PBS	Phosphate buffered saline
PEG	polyethyleneglycol
R	Arginine
RI	reflective index
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SPE	solid phase extraction
TCEP	tris(2-carboxyethyl)phosphine
TPCK	L-(tosylamido-2-phenyl) ethyl chloromethyl ketone

Chapter 1.

Introduction to Protein/Peptide Adsorption on Solid/Liquid Interfaces

The amphiphatic (being both hydrophilic and hydrophobic) nature of proteins and peptides makes the adsorption of proteins to most solid/liquid interfaces and the main concern is to manage the interfacial adsorption by understanding the behavior of proteins on adsorption [1]; thus, protein adsorption has been immensely studied. The undesired adsorption event is a consequence of inaccurate identification and quantitation of the protein/peptide content from the biological samples [2, 3].

1.1. Objectives of the Thesis

Protein and peptide loss during proteomic studies is a major problem, particularly for detection of hydrophobic peptides. Presaturating a vial surface with an abundant protein is a way in which loss of trace proteins/peptides can be avoided. Therefore, the goal of this thesis was to develop a direct and sensitive method that can be used to quantify the adsorption of bovine serum albumin (BSA), a model protein, to the surface of microcentrifuge tubes made of polypropylene. This type of widely used vial is hydrophobic, and so it is of particular interest to study the adsorption of proteins onto this type of surface. Fundamental knowledge about and the common quantitation techniques for protein adsorption are discussed in chapter 1. The objective of chapter 2 is to provide detailed, experimental procedures for the newly, developed method, by making use of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as a simple and direct method to quantify adsorbed proteins. An accurate and robust routine quantification method for adsorbed protein had to be developed and validated (Chapter 3), which is suitable as a routine examination technique. Moreover, we solidified the method and challenged its sensitivity by investigating those influencing parameters as

well as studying the prevention of peptide adsorption. Last but not least, the newly developed method is subsequently employed to characterize the adsorption of peptide generated by “in-solution” trypsin digestion and future works including revealing the sequence of peptides whose regions would contribute the most for adsorption (chapter 4) are proposed.

This chapter generally provides readers a fundamental understanding of protein adsorption including the reason for protein adsorption on solid-liquid interfaces, the impact of protein adsorption on various biologically related fields, the mechanism the protein adsorption process undergoes, factors controlling protein adsorption event, and the techniques to investigate and quantify the adsorbed protein layer. These issues are essential for making improvements to avoid protein loss by adsorption.

1.2. Protein Adsorption on solid-liquid interfaces

An interface is formed between two different phases when it has a higher Gibbs free energy than the bulk phase [5]. It needs to be thermodynamically stabilized by adsorbing any substances different from solvent molecules [5], and so proteins in solution adsorb almost immediately and spontaneously to any solid surfaces [6]. Protein adsorption is a common but complicated phenomenon since it causes proteins to undergo severe conformational changes, which in turn alter their functions [7-21]. Protein adsorption takes place on all types of solid surfaces. As summarized in a review [4], proteins adsorb to the solid surface, regardless of their sizes, and softness, and surface types, leading to different adsorption quantities, orientation, and thickness of the adsorbed layer. Proteins even adsorb onto surfaces with the same type of charge [22]; hence, the formation of adsorbed protein layers plays a critical role from various points of view, and protein adsorption events are very problematic in many different areas [5, 23], such as: biomedicine [24], the pharmaceutical industry [25], biotechnology [10], analytical science [26], and proteomics [27]. For example, blood in contact with implanted biomaterials frequently induces protein adsorption, and the adsorbed fibrinogens can subsequently cause thrombi being released into blood stream as emboli [28]. Moreover, therapeutic protein adsorption in drug prescription to packaging containers results in loss of drug contents and eventually reduces efficacy [25]. In the

field of analytical science, non-specific protein adsorption on biosensor surfaces, or immunoassay platforms is a serious issue that decreases sensitivity and specificity [29], whereas the loss of low-abundance proteins by adsorption is a major problem in proteomics [30].

1.3. Protein Adsorption Behaviors/Mechanisms at Solid-Liquid interfaces

1.3.1. Driving Force

Proteins in solution are able to self-assemble into a highly ordered secondary and tertiary structure and prefer the folded state to unfolded coil, based on the thermodynamic factors [6]. Protein folding requires that the reduced conformational entropy opposing the folded state must be outweighed by a total of the enthalpic and entropic forces from different chemical interactions (hydrophobic, electrostatic, van der Waals, hydrogen bonding) acting on the protein [6, 28, 31, 32]. Although entropic and enthalpic forces can influence the adsorption, the protein adsorption process is entropically driven: a negative change in free energy of adsorption (ΔG_{ads}) results from the increase in entropy (ΔS_{ads}) upon adsorption, which overrides the enthalpic change and leads to endothermic adsorption [5, 6, 21, 28, 32]. This entropy gain is achieved from release of water molecules from the adsorbent surface, as protein adsorbs and denatures on the surface.

1.3.2. Orientation

Once proteins adsorb onto any solid surfaces, they exhibit different adsorption orientations, which can determine whether a protein monolayer or a multilayer is formed based on its thickness. There are two types of monolayer configurations: one with a short axis (side-on) or another one with a long axis (end-on) perpendicular to the surface [5]. Typically at high protein solution concentration, an “end-on” configuration can be observed, while a low protein concentration usually gives rise to a side-on monolayer; a protein monolayer in its saturation state is thicker in the case of “end-on” than that in the “side-on” case [5, 21, 32, 33].

1.3.3. Conformational Change

It is well-known that proteins will experience a conformational change upon adsorption to a solid surface [7-17, 19-21, 34]. Since the protein-surface interaction induces an increase in free energy, the proteins are inclined to maximize their footprint via structural re-organization by allowing their internal regions to form additional contacts with the surface [5, 21]. Briefly, proteins in solution bulk phase approach the surface in their native state and bind to the vacant sites of the surface. When the conformational re-organization of proteins occurs due to denaturation, they lose their ordered secondary structure as a result of entropy gain. Eventually proteins undergo a relaxation process resulting in a side-on orientation with higher contact surface area with the surface, so lower adsorbed quantity due to smaller adsorption area available on the surface would be observed, subsequently leading to stronger resistance to buffer elution [6, 21].

1.3.4. Protein Layer

Protein adsorption has usually been found to follow a Langmuir isotherm, where surface coverage initially increases with protein concentration or adsorption time, and then reaches a plateau when the surface is saturated [28, 35]. This protein structure can be either a loosely or densely packed monolayer, or even a multilayer. One simple way to distinguish them is by determination of their adsorption quantity. It has been reported that adsorption amounts in between 0.21 and 1.57 $\mu g/cm^2$ suggest the structure to be a close-packed monolayer whereas higher values would indicate the presence of a multilayer [5, 23]. Adsorption kinetics and extent are also dependent on the bulk protein concentration. At low concentration, proteins occupy the surface slowly and their conformational change will result in multivalent site adsorption. In contrast, proteins adsorb very rapidly onto the surface at high concentration, thus relatively impeding their structural change due to limited space available on the surface [21, 35, 36].

1.3.5. Reversibility

Whether protein adsorption onto any solid surfaces is a reversible process is immensely debatable [31, 32, 37-40]. Not only do the protein concentration and adsorption period determine the adsorption strength, but also the protein size and

property, such as the type of interaction between the protein and surface, can make the protein strongly bound to the solid surface. It has been reported that the larger size proteins (>100 kDa) can adsorb strongly to the surface through hydrophobic interaction, but application of sorbent rinsing is able to allow desorption of proteins, especially the loosely bound ones, therefore proving that protein adsorption is not a complete, irreversible process [19, 32, 38, 40, 41]. Furthermore, a longer incubation time allows proteins by a multipoint attachment to be bound more tightly to the surface. Rinsing the protein-adsorbed surface with buffers, solvents, or detergent solutions with sonication can efficiently desorb a significant amount of protein molecules, only leaving a trace amount of protein residue on the surface [21, 32, 41].

1.4. Influence of protein properties on adsorption

1.4.1. Size

The size or molecular weight of a protein can crucially determine the number of adsorbed protein layers formed on the surface and protein adsorption quantity, usually expressed in mass-or-moles per-unit-adsorbent-area [42]. Basic experimental observations show that human blood proteins with higher molecular weight adsorb to a particular fixed adsorbent surface area than smaller proteins, but a higher number of moles of lower molecular weight proteins adsorb than the bigger ones. A review shows [32] that the number of adsorbed protein layers increases with protein molecular weight when the range of the size is up to 1000kDa (IgM); a single layer at surface saturation is obtained by a protein size smaller than 100kDa, whereas bigger proteins such as fibrinogen and IgG needed two layers [40]. A similar effect from larger proteins is also observed for the increase of adsorption amount required to saturate the adsorbent surface, but the increase in adsorption quantity is independent of the increasing bulk protein concentration [40, 42]. Once reaching the saturation of the surface, the proteins with higher molecular weight generally bind more strongly to a surface, since they have a larger contact area, leading to multivalent adsorption. Proteins can even displace smaller, pre-adsorbed proteins while relaxing on the surface [21].

1.4.2. Composition

Proteins are complex biomacromolecules consisting of 20 naturally occurring amino acids as monomeric units in addition to possible post-translational modifications. They contain different domains exhibiting unique properties such as hydrophobic/hydrophilic, polar/non-polar, or charged/uncharged [21, 32], in such a way that if the surface is hydrophobic, the hydrophobic region of the proteins will have a strong affinity for it. It was previously mentioned that hydrophobic interactions are the dominant forces for protein adsorption. In principle, the van der Waals force can account for the hydrophobic interaction which maintains the secondary structure of a protein in folded state; water molecules forming hydrogen bonding with each other are highly oriented around the protein, but no hydrogen bonding can be formed between the protein and water molecules. When the protein in solution collides a solid surface inelastically in the absence of electrostatic repulsion, the protein will denature, undergo a structural change by exposing its hydrophobic region buried inside the protein core to the surface, and result in a large entropy increase that subsequently drives protein adsorption [28]. Therefore, it has been demonstrated that a higher adsorption amount is seen on a hydrophobic surface for the “soft” protein albumin [4]. However, this phenomenon does not always appear to be true that glycoprotein adsorption on the hydrophobic surface is impeded by a high content of the hydrophilic glycans [21, 43]. It has also been reported that lysozyme adsorbs almost equally on both hydrophobic and hydrophilic surface due to its high rigidity that the adsorbed lysozyme is not able to undergo structural and conformational change on the surface [4].

1.5. Influence of external parameters on adsorption

1.5.1. Adsorption Time

Protein adsorption to the surface is a time-dependent process which involves a protein conformational change [10, 16, 32, 36, 44, 45]. In principle, protein adsorbs instantly once contacting the surface, but a longer period of adsorption time results in stronger protein binding strength and higher adsorption amount since proteins denature and unfold during the adsorption process [20, 21, 46]. Contacting surface area between

the protein and the surface increases with adsorption time. Initial studies typically measure the protein adsorbed quantity in the scale of minutes to hours. Regardless to the protein concentration used, there is always an increasing trend in the adsorption quantity on the hydrophobic surface when adsorption time increases [47]. However, this case cannot be applied to the protein adsorption on hydrophilic surfaces for prolonged incubation times. For hydrophilic surfaces it has been shown that the adsorbed amount decreases slightly from 1 to 6 hours [46]. Xu et al. [48] has used atomic force microscopy (AFM) to measure the adhesion force between the protein and surface as a function of time. Protein can adsorb onto a surface within a few seconds, with a small adhesion force; however, as the contact time increases just to about 50 seconds, a sharp increase in the adhesion force is observed particularly in the surface with poor wettability [24]. This phenomenon cannot just be explained by a simple transition from end-on to side-on adsorption, but instead is due to the increase of the molecular footprint.

1.5.2. pH

Different pH values of a protein solution can affect the electrostatic state of proteins, ultimately resulting in different adsorbed amounts and binding strength [23]. The net charge is zero when the isoelectric point (pI) of a protein is same as the pH; otherwise, the protein will become negatively charged as the pH is higher than pI, and positively charged when pH is smaller than pI. Thus, a high adsorption rate can be seen when the protein bears opposite charge to the substrate. Studies have shown that maximal adsorption can be achieved when pH is close to pI [12, 46, 49, 50]. For instance, AFM was employed to measure the height of the adsorbed BSA whose pI is about 4.7. At pH 4.5, the highest value of height is observed, corresponding to the largest number of adsorbed molecules per unit area; similar effect is also observed, when pH of the protein solution is at 7, for other proteins such as hemoglobin whose pI is 7.2 [12, 49].

1.6. Surface property effect on protein adsorption

As described earlier, surface properties are as crucial as protein properties for determining the protein-surface interactions. In various fields such as adsorption chromatography, biosensors, or membrane separation, etc. [51], the adsorbents used all have different surface properties such that the current adsorption models are restricted to mimic them for real applications [34]. The surface properties thoroughly depend on surface tension, polarity, charge and morphology and so different techniques can be used for measurement. In general, proteins are reported to adsorb more tightly to hydrophobic than to hydrophilic, to high surface tension and to low surface tension, and to the charged than to uncharged surfaces [21]. Haynes et al. suggested that hydrophobic surfaces destabilize the protein and assist conformational change resulting in stronger protein-surface interactions [28]; however, a rare exception is made on the adsorption behaviors of glycoproteins whose hydrophobic regions are buried inside a shell of glycan, and so they tend to adsorb preferably on hydrophilic surfaces [43].

1.7. Protein desorption by different solutions/buffers

Loosely bound proteins can be partially desorbed by rinsing with buffers [23, 32]. Several solutions such as bases, acids, and detergents have been used extensively for protein desorption, which can yield detailed information on protein-surface interactions and provide an accurate adsorbed amount on the surface [32, 47, 52, 53]. E.S. Leibner et al. have reported that three-time PBS rinsing is sufficient to remove loosely bound proteins [41]. More strongly bound proteins can only be removed by surfactants such as SDS or Tween 20, due to the fact that the binding strength increases with long protein-surface residence time. Different SDS concentrations can vary the percentage of elution and 0.1% SDS was reported to be sufficient to remove the majority of adsorbed protein molecules [47]. However, elutability is strongly dependent on protein affinity to the surface for a given surfactant but not just protein-surface residence time. Besides, Hu et. al. has reported the use of acid, base and water to recover various adsorbed enzymes from cellulosic substrates, and the results suggested that different enzymes desorb uniquely at different pH values [53].

1.8. Reduction and Prevention of Protein Adsorption

To avoid or reduce protein non-specific adsorption, once can block a surface with a reagent such as BSA or casein [13, 19, 29, 54]. One can also modify a surface with polyethylene glycol (PEG) [55, 56] forming a protecting layer. Finally one can spike in detergents or surfactants, such as Tween 20 or Triton X-100 [57-59] to the protein solution samples to induce a competitive adsorption. BSA is commonly used to pre-coat the surface since it is inexpensive and has no interference with quantitation results [19, 27]. BSA pre-adsorption on a PET surface with significantly reduced adsorption of the fibrinogen by fourfold [54], and a high recovery of the protein samples was accomplished [27, 58, 60].

1.9. Introduction to Peptide adsorption

Peptides are a smaller version of proteins and are short chains of amino acid monomers connected by the amide bond. In modern mass spectrometry-based proteomic studies, the identification and quantitation of proteins from biological samples, cells, or tissues largely rely on peptides generated by a sequence of steps including solubilization, protein separation, digestion, affinity capture, and purification [61-66]. However, since there are so many steps involved, peptide adsorption onto not only the sample vial surfaces, but also onto pipet tips, and instrumentation parts induces sample loss and is a big source of error toward quantitative analysis [3, 67-69]. Especially if the peptide concentration in a sample exists in low-abundance, sample loss by adsorption is more severe and therefore lowers the reproducibility of the analysis [68]. The low abundant proteins are mainly hydrophobic and are potential biomarkers of pharmaceutical interest; especially hydrophobic interactions are the dominant force for adsorption, so prevention of protein adsorption is of high priority and precautions are needed to be taken. Perhaps, peptides in solution adsorb dissimilarly to proteins to the solid surfaces as they adopt different orientations, interact with surface differentially and undergo different structural rearrangement [5, 70]. Peptide adsorption in fact heavily depends on its peptide sequence and chain length in addition to the pH, concentration, adsorption time, and size as well as the adsorbent properties as described in previous sections.

To gain a better understanding of protein adsorption mode, adsorption characteristics of peptide fragments would be very informative. In general, if certain protein segments are of great contribution for adsorption onto the solid surface, the peptide fragments correlated to those segments are anticipated to display high affinities for the solid surface. One study has revealed the adsorption profile of BSA and its peptides prepared by lysylendopeptidase onto a stainless steel surface [52]. Remarkably, high BSA adsorption was observed at acidic pH but scarcely at alkaline pH. Regardless to the peptide molecular weight, equivalent results under identical conditions also happened on its peptide fragments comparatively abounded with acidic amino acid residues. Furthermore, the way how peptides adsorb onto a solid surface can substantially impact their biofunctionalities [71]. One good example is the notorious neurodegenerative Alzheimer disease mainly caused by the transition of the alpha-helix to beta-sheet misfolding of the amyloid beta-peptide [34, 72, 73]. The peptide was tested for adsorption on both hydrophilic and hydrophobic surface which attempted to resemble the polar, charged membrane surface and the apolar transmembrane environment, respectively. The authors found that similar adsorption amount was seen on both surfaces at neutral pH, compared to other conditions (acidic and basic environments). However, different structures were observed on two different surfaces: helices on hydrophobic and sheets on hydrophilic surfaces. This is mainly due to the fact that the dehydration process forces the formation of intramolecular H-bonds in the helical structure whose non-polar residues contact the hydrophobic surface; meanwhile, in the hydrophilic surface the neighboring adsorbed molecules may aggregate, promoting intermolecular beta-sheet structures.

1.10. Side Chain, Chain Length, and Sequence Effects on Peptide Adsorption

The adsorption of peptides generated by proteolysis has been widely investigated, but the adsorption of peptides on both hydrophobic and hydrophilic surface can induce different secondary structures including alpha-helix and beta-sheets which associate to form tertiary structures, so the designed, synthetic peptides offering simple model system are also chosen for simplicity for studying protein adsorption[74]. Some

general model peptides, which are composed of lysine (K),arginine (R), alanine (A), leucine (L), and phenylalanine (F), are designed in a specific sequence and length, also being able to induce the formation of alpha-helices and beta-sheets on hydrophobic surfaces [71, 74-77]. LK_{14} is a model peptide that has been extensively studied since it yields an alpha helical structure with leucine side chains oriented towards a hydrophobic surface, whereas lysine side chains oriented towards a hydrophilic surface [77, 78]. When this peptide is tested for adsorption on two different systems, a peptide monolayer dominated by interactions of the methyl group is rapidly formed on the hydrophobic surface while a slow multistep adsorption and structural rearrangements are seen on the hydrophilic surface [77]. It is striking that even if two different peptides, such as LK_{14} and LK_7, contain exactly the same amino acid resides, the LK_7 is barely adsorb onto the hydrophilic surface whereas there is a high adsorption amount detected for LK_{14} on the hydrophobic surface [74]. Moreover, even though hydrophobic interactions undoubtedly dominate the protein/peptide adsorption, one recent study reported that peptide-peptide interaction also plays a critical role in the adsorption at high concentration [75].

1.11. Methods and Techniques for Protein Quantitation

It is important to thoroughly characterize and quantify protein adsorption, in order to have a quantitative description of adsorption [79], to examine whether the biomaterials are successful for applications [80], and to quantify and discover potential biomarkers in proteomics [30]. There are essentially two methods which allow for protein quantitation [25]: the simplest one is an indirect method, also termed solution depletion, which is to measure protein supernatant concentration before and after proteins contacting the adsorbent [5, 23, 32, 38-40, 42, 53, 79]. Another is the direct one that directly measures the adsorbed protein quantity on the solid surfaces; hence, information about the thickness of the protein layer and the protein conformational change can also be yielded [4, 5, 13, 17, 20, 21, 29, 36, 46, 48, 50, 56, 79-81]. Under these two types of quantitation methods, protein labeling and label-free techniques, where pros and cons both exist, have been extensively applied for precise measurements. The discussion below will mainly focus on a wide range of different techniques commonly used for protein quantitation in the last two decades.

1.11.1. Labeling Techniques

Fluorescent Labeling

Some amino acid residues such as tyrosine, tryptophan, and phenylalanine are intrinsically fluorescent in the UV range. Protein labeling can provide assistance for protein quantitation [79]. For the fluorescent labeling technique, the fluorophores that are commonly used are Cyanine 3 (Cy3^{TM}) [25] and fluorescein isothiocyanate (FITC), shown in Figure 1, [57, 79, 82-85]. This technique can essentially be employed in both direct and indirect methods for protein quantitation. Adsorption test of Cy3^{TM}-labeled Fibrinogen (FNG) [25] was performed on pharmaceutical packaging surfaces made of different types of glasses and proved that protein loss by adsorption even takes place in the stopper of the vial. The reported results are highly comparable when protein adsorption was quantified by both direct and indirect assays; plus, when the molar ratio of unlabeled to labeled FNG decreases, the adsorbed protein quantity is as well lowered, signifying the importance of labeling efficiency and the interference of the labeling molecule to protein adsorption [86, 87]. Among all fluorescent labels available such as Nile red, shown in Figure 1, which can possibly leach from the protein molecule, since Nile red is a lipophilic stain and is only attached to the pocket on proteins via hydrophobic interactions [88], labeling covalently by fluorescein via the carbon in the S=C=N bond was proven useful in illustrating the protein conformational change and packaging on the surface. However, when the surface is negatively charged and after reorientation of the adsorbed, fluorescein-labelled proteins, the fluorescein labels would have a tendency to migrate closer to the surface resulting in protonation of the fluorophores. Since fluorescein fluorescence is pH-dependent, protonation subsequently decreases the fluorescence emission intensity [89].

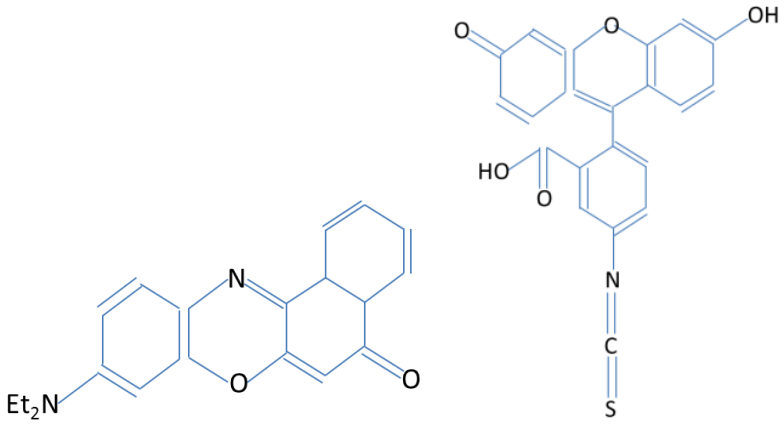

Figure 1: Molecular structure of (a) Nile red (left) and (b) fluorescein (right)

Enzyme-Linked ImmunoSorbent Assay (ELISA)

Due to its high sensitivity and specificity, ELISA is a powerful tool for direct quantitation of proteins and for qualifying their state of activation in complex biological samples, and is mainly exploited in clinical diagnostic, food sampling, and as a microarray in proteomics [90]. The working principle of ELISA is completely relied on the epitope recognition; the analyte is detected by the capture of antibodies that bind to the analyte (epitope) [5, 26, 29, 90]. The antibodies are then detected by secondary reactions, e.g. the activity of an attached enzyme such as alkaline phosphatase (AP). AP liberates a coloured product, upon reaction with an appropriate substrate. The method has even been recently enhanced for multiplex detection that would allow for the determination of 30 individual proteins within a single array [26]. Nevertheless, some drawbacks still exist: the production of monoclonal antibody is very, time-consuming, labour-intensive, and expensive.

Radioactive Labeling

The implementation of radioisotope-labeled molecules for protein quantitation in direct and indirect methods is often due to its high sensitivity in addition to its ability to measure multiple proteins (by multiple labels) adsorbed from complex media [21, 23, 25, 32, 38, 40, 41, 46, 48, 54, 58, 79, 80, 87, 90-92]. The radioisotope of iodine (^{125}I) is frequently chosen due to its chemical reactivity and relatively small size [79]. In brief, $^{125}I^-$

is oxidized to I_2 by using an enzyme called lactoperoxidase in the presence of H_2O_2. Then the electron rich o-position of the tyrosine benzene ring in the protein will nucleophilically attack the iodine [79, 93]. Although the radioisotope labeling method is considered to have minimal influence on the protein properties, its drawbacks are relatively significant. When it is applied to a larger protein (IgG), fragmentation of the protein molecules is consistently observed and an exceptionally high adsorption amount is also detected [91, 94]; BSA aggregation on adsorption is also severe after labeling [94]. Because 125I labelling affects the hydrophilicity of HSA, this method leads to an underestimation of adsorption quantity on the hydrophobic surface [41].

1.11.2. Non-labeling techniques

Ellipsometry (ELM)

Ellipsometry is based on the change in polarization of an electromagnetic wave reflected from a surface and reveals the information about the mass of the adsorbed protein layer and its thickness [17, 21, 46, 81-83, 95-98]. This technique needs planar, reflecting substrates and a strong variation of the reflective index (RI) on protein adsorption [21]. Marsh et. al. has used ELM to monitor the adsorption kinetics of beta-lactoglobulin on both the hydrophilic and hydrophobic surface where a longer adsorption period allows protein to bind more strongly [99]; a closely packed monolayer of molecules is observed and the adsorption amount detected on a hydrophobic surface is significantly much higher than that on the hydrophilic surface. However, one difficulty of ELM is to distinguish adsorbed protein layer from the solvation sphere of the protein layer. The reason for this is that the solvent is often disordered and mixed with water, and so the RI of water and of the protein layer will be very similar [96]. For enhancing the optical contrast, a substrate coated with a thin layer of indium tin oxide (ITO) can be used to facilitate the measurement of the monolayer in the thickness range of 2.6nm, which other optical devices cannot detect [98].

Surface Plasmon Resonance (SPR) spectroscopy

Surface Plasmon Resonance (SPR) spectroscopy is a popular surface analysis method based on changes in the optical reflectivity of a thin metal film (typically gold) when molecules adsorb onto the metal surface [11, 21, 45, 80, 100-102]. In principle, a

polarized monochromatic light beam is travelled through a prism and its attached, glass slide coated with gold, and then reflected off the thin gold in contact with the protein solution [45]. The reflectivity is measured based on the reflection angle, which is plotted versus the reflection intensity. Thus, the protein layer thickness and adsorbed amount can be determined [11, 80, 100, 101]. SPR is an analytical platform of interest since it can also perform real-time monitoring of multiple biomarkers with high sensitivity and speed. It has recently been developed to identify protein-antibody interactions by coupling to mass spectrometry (MS), making it suitable for high-content protein microarrays and comprehensive protein analysis for detection of structural protein variants arising from genetic variations and post-expression processing [103]. To-date, SPR-MS is even employed to quantify and identify the lipids and proteins non-specifically bound to the SPR surface [101]. However, a key disadvantage of SPR is the necessity of substrates that must be coated with a metal layer, limiting its versatility.

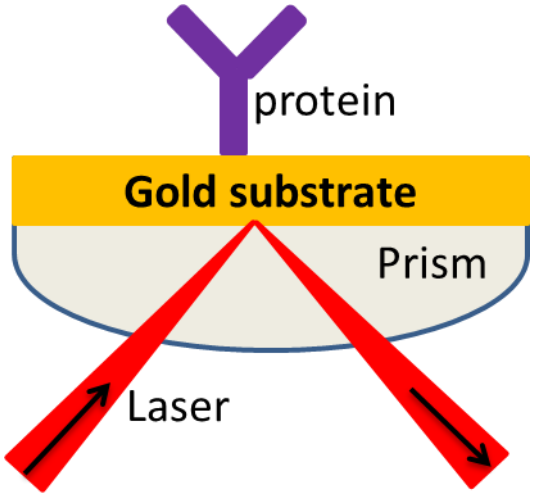

Figure 2: Simple schematic diagram of SPR

X-ray photoelectron (XPS) spectroscopy

In XPS, the sample surface is irradiated with X-rays and this excites electrons in the sample. If the binding energy of the electrons is lower than the x-ray energy, they will be emitted as a photoelectron. Only photoelectrons at the outermost surface leave the sample surface. Once the energy of the photoelectrons is measured, it will provide information on the nature of the atoms. Thus, nitrogen is frequently used for detection compared to sulphur, since nitrogen is very abundant in a protein molecule [70, 81, 104-

107], making XPS a quantitative and highly sensitive technique by revealing the adsorbed protein quantity and the adsorbed layer thickness. Nevertheless, if both the substrate and the protein have similar atomic percentage of nitrogen, no significant difference can be yielded before and after adsorption, and the limit of detection will be worse [109]; another disadvantage is that XPS cannot distinguish between various protein species.

Atomic Force Microscopy (AFM)

AFM consists of a cantilever tip, made of silicon, which probes over the surface of a protein layer. During surface scanning by the tip, laser is shone on the back of the tip and reflected to the photo-detector so that the movement of the cantilever tip will be measured in order to reveal the surface topology of the layer. The distance between the cantilever tip and the adsorbed protein layer generates an adhesion force which can then be plotted as a function of time in order to reveal a change in the protein conformation and orientation [48]. AFM is famously implemented for probing the thickness of protein layers, which can allow for estimation of the adsorption amount, and the surface topology such as the three-dimensional image of the surface, on a variety of substrata and on high-resolution imaging on the protein layer in lateral directions [5, 12, 24, 32, 39, 48, 54, 115-117]. Therefore, this technique is able to quantify protein-surface and protein-protein interactions [118] and to probe the stability of surface tethered protein [119], and so one study has used AFM to examine the conformational change of protein on the CH_3-terminated (hydrophobic) and COOH-terminated (hydrophilic) self-assembled monolayer (SAM) surface and concluded that, in the hydrophilic surface, the HSA molecules do not undergo further conformational change once adsorbed and the adsorption amount is also comparatively smaller than that in the hydrophobic surface [48]. Holmberg et. al. [54] also used AFM to demonstrate that albumin adsorbs in a multilayer fashion on the polyethylene terephthalate (PET) surface and that fibrinogen adsorbs on top of the layer pre-coated with albumin. Nonetheless, one limiting factor in the usage of AFM is that since imaging is carried out with dry surfaces, this condition will affect the folding state of proteins; otherwise, if in-situ measurements are conducted, tip-induced movement of adsorbed proteins is a potential source of error [21]; plus, protein corrugations/aggregations take place when the cantilever tip sweeps across the protein layer [117].

Quartz Crystal Microbalance (QCM)

The QCM technique is based on the monitoring the change in the resonance frequency (f_n) and bandwidths (Γ_n) of the substrate with respect to the mass load. This can be adapted to study protein adsorption and conformational changes based on changing the orientation of the protein side chain and the torsional angle of the protein main chain [21, 56, 80, 120-123]. The mass of a protein will not change after protein conformational change. The adsorbed mass detected by the QCM includes water trapped within the protein adsorbed layer, and so this technique is not suitable for determination of absolute mass [21, 120]. In fact, QCM technique is more appropriate for recording protein adsorption and desorption kinetics when combined with other techniques such as AFM [121, 122] while the QCM–D is an improved, modern technique which can further allow for characterizing the rigidity of the protein layer based on the energy dissipation (D) in such a way that slow and fast dissipations represent rigid and flexible layers respectively [4, 56]. The combined data from the dissipation and frequency changes allow for studying the biphasic adsorption of hemoglobin [49]; the result from this study indicates multilayer protein adsorption on the SAM surface such that a rigid layer is initially formed on the surface, implying protein denaturation and a layer of loosely bound proteins formed on top of the first layer. However, some studies compared the adsorbed mass obtained by different techniques such as ELM, OWLS, radiolabeling, SPR, and QCM-D [80, 97]. It is noticed that QCM-D gave a higher detected adsorption amount on the hydrophobic surface among these techniques since it measured both adsorbed protein amount and its associated water molecules that bind or hydrodynamically couple to the protein layer.

Neutron Reflectometry (NR)

The working principle of NR is based on the neutron beam that enters to and reflects from the protein layer. It is a specular reflection technique, since the angle of the incident beam is equal to that of the reflected beam, and the reflection is plotted as a function of the momentum transfer dependent on the selected wavelength and incident angle [5, 21]. The NR technique is regarded as a powerful tool not only for accurately measuring adsorbed protein quantity and layer thickness, but also for simultaneously investigating the protein conformational change [4, 5, 17, 21, 36, 50, 96, 124-126]. The wavelength of a collimated neutron beam is up to three orders of magnitude smaller

compared to that of UV light which has a lower frequency. According to the De Broglie's equation such that frequency is inversely proportional to wavelength, so a smaller wavelength of a neutron beam offers a very high resolution on the protein layer normal to the surface; This method even allows us to precisely measure the change in adsorbed lysozyme thickness between side-on and end-on orientation every five minutes [127]. Brouette et al. [17] has recently studied the adsorbed protein layer by employing the combination of NR and deuterated myoglobin, which gives a maximum sensitivity for detection in H_2O contrast. More recently, one group has even applied high-pressure sample cell up to 2500 bar to probe the protein layers at the solid/liquid interface with a supreme sensitivity in the range of a few angstroms (Å) [128]. However, this technique is extremely costly, since it needs a neutron source that has limited availability universally.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Glycine-SDS-PAGE is a commonly used SDS electrophoretic technique for testing protein purity, being an analytical method to separating proteins in a solution of protein mixtures; it is also employed to estimate protein molecular weight [140]. The typical protein mass range covered is about 5-150kDa, depending on the total percentage concentration (%T) of the gel that consists of both monomers (acrylamide and the crosslinker, bisacrylamide). Higher %T up to 27% enables lower molecular weight proteins to be resolved [130], since the increase in %T will result in a higher degree of cross-linkage in the gel polymer matrix, and so will decrease the pore size. Some attributions leading SDS-PAGE to success include the introduction of discontinuous buffer systems where the sample and gel running buffers are dissimilar in compositions. The stacking gel on top has a pH 6.7 and a lower percentage concentration of the polymer solution. The resolving gel, at the bottom, has a pH 8.8 and contains a higher percentage concentration. This discontinuous systems permit larger sample volumes to be loaded in the stacking gel (also dependent upon the dimension and thickness of the gel slab) and allow protein samples to be stacked into thin bands in the large pore stacking gel before entering to the resolving gel. This is achieved since the gel running buffer contains glycine; it is neutral at pH 6.7, and negatively charged at pH 8.8, and so the increase in the electric field in the stack gel due to lower ionic strength increases the mobility of the protein. Especially in the glycine SDS-PAGE

system, it separates protein at high pH (pH 8.8) in the resolving gel, minimizing the possibility of protein aggregation and providing clean separation even at heavy protein loads [141].

In principle, SDS is an anionic detergent able to form micelles composed of about 70-80 SDS molecules with the carbon chain in the core and sulphate head group in the shell [141]. In this case, they can strongly intercalate with proteins in a ratio of one DS^- to two amino acid residues (or a constant ratio of 1.4μg SDS per 1μg polypeptide), and so the negative charge per unit mass ratio is similar to all proteins [142]. Once the negatively charged SDS-protein complexes are formed, they migrate towards the positively charged anode at the bottom of the gel during the electrophoretic run and are separated based on the size and the mobility which is affected by the hydrodynamic radius of the proteins. For the SDS-PAGE gel analysis, Coomassie blue and silver staining methods are commonly used, because proteins are generally invisible in the gel. The detection limit of Coomassie blue staining is about 100 ng, while that of silver staining is approximately 100-fold more sensitive but more steps are involved.

SDS-PAGE is a powerful technique for separation of proteins based on their molecular weight [129, 130], so it is typically used for qualitative studies [131]. It is also largely exploited for “in-gel” digestion, which is considered a leading method for conversion of proteins after separation to peptides applicable for mass spectrometry-based proteomic research [61, 64, 68, 101, 132-138], and more recently “out-gel” digestion has also been newly invented [139]. SDS-PAGE is not very common for protein quantitation, but some research groups have still been striving for it mainly by solution depletion method [2, 38-41, 53], and no direct quantitation by SDS-PAGE has been reported. The adsorbent used for protein quantitation requires a high surface area, such as beads, powders, fibers, microspheres, and nanoparticles [2] so that a sufficient change of supernatant concentrations can be clearly observed. For instance, Hu et al. [53] has utilized SDS-PAGE to study the adsorption and desorption behavior of cellulases on microcrystalline celluloses and hardwood pulp. Even though the adsorbed amount cannot be quantified since there is no known quantity of protein included in the gel for quantitation, qualitatively the adsorption/desorption signal observed in the gel are directly proportional to the protein concentration used. One disadvantage of SDS-PAGE

is its low reproducibility due to many steps ranging from sample preparation, to gel casting, and to staining/destaining [129]. Nevertheless, SDS-PAGE for protein quantitation by the solution depletion method has been improved by incorporating a calibration curve in every individual gel to achieve better reproducibility [40]. The data obtained from SDS-PAGE is also reported to be even more reliable than radiometry since the protein structure can be distorted by the radioactive label, which results in a much smaller, measured adsorption quantity [41]**.**

Technique	Ch. 1 section	Basis for detection of protein adsorption	Threshold for detection (ng/cm^2)	Information obtained
Fluorescence	1.11.1.1	Fluorescein-label molecules measured by UV light	~50	adsorption amount
ELISA	1.11.1.2	Epitope recognition by primary antibodies	~100	adsorption amount
Radiometry	1.11.1.3	Radioactivity measured due to radioactive-labelled molecules adsorbed	~20	adsorption amount
ELM	1.11.2.1	Change in the state of polarization light on reflection	0.1	adsorption amount, layer thickness, and adsorption kinetics
SPR	1.11.2.2	Change in the polarized monochromatic light beam upon reflection	0.01	Adsorption kinetics, protein binding interactions, and adsorption amount
XPS	1.11.2.3	Binding energy of the photoelectrons emitted from the sample on the surface	~10	Adsorption amount, and layer thickness
AFM	1.11.2.4	Atomic interaction between surface and the scanning tip	Unavailable	Topology of the adsorbed layer
QCM	1.11.2.5	Change in oscillating frequency of piezoelectric device upon mass loading	0.5	Adsorption kinetics, and adsorption amount
NR	1.11.2.6	Reflectivity of neutron at the interface	<1	Structure of adsorbed protein layer, layer thickness, and adsorption amount
SDS-PAGE	1.11.2.7	Load the adsorbed proteins stripped from surface	<1	Identification of protein species, and adsorption amount

Table 1.1. Summary of different techniques for quantitation of protein adsorption

Chapter 2.

Experimental Section*

2.1. Introduction

Chapter 1 discussed the utilization of SDS-PAGE which has been widely implemented to qualitatively and quantitatively study protein adsorption using the solution-depletion method, which relies on small particles with high surface area on protein adsorption. To be able to extend this SDS-PAGE method for systems with small flatter surfaces, such as regular sample vials, we report here a method coupling it with "rinse-and-stripping" based on direct protein-adsorption analysis (SDS-PAGE/DPA): model proteins directly and tightly adsorbed onto the vial surfaces are quantified by SDS-PAGE. Initially, this coupling was impossible when the small amount of the desorbed protein in a relatively large volume of stripping solution would yield a low concentration of loaded protein that is beyond the limit of most detection methods. We resolve this problem by introducing a condensation step to the stripping solution, such that the stripping solution volume would not affect the final analysis by the capacity limitation of the well. Furthermore, without the aid of any expensive or state-of-art equipment, SDS-PAGE/DPA can simply allow us for estimation of what types of proteins in a complex mixture that would adsorb to the surface of the vial used for sample preparation. Hence, due to the simplicity of the method, we can quickly assess how much protein can be lost by adsorption to the normal microcentrifuge vial surface in daily experiments. Different parameters (protein concentration, adsorption period, surface area, and choices of stripping solutions) that can affect the protein adsorption and

desorption process were also investigated in order to challenge the sensitivity of the method.

We indeed demonstrated in this study the high sensitivity achieved by silver staining of the SDS-PAGE gel and its easy applications to investigate the competitive and sequential adsorptions, so that protein loss by adsorption can be reduced or prevented for the purpose of proteomic studies. The enhancement of SDS-PAGE/DPA over the “solution-depletion” method by two other techniques (SDS-PAGE and Bradford assay) is also demonstrated. Once the SDS-PAGE/DPA method is validated, we move forward to study the adsorption of peptide generated by trypsin and optimize the condition of BSA-coated surfaces to examine the reduction of peptide adsorption, similarly followed by the characterization with the SDS-PAGE/DPA method.

2.2. Materials

Bovine serum albumin (BSA), equine skeletal-muscle myoglobin (MB), DTT, TCEP, iodoacetamide and Bradford reagent were purchased from Sigma. Micro-centrifuge vials of 0.65-mL volume capacity (cat. 87003-290) were obtained from VWR. Sodium thiosulfate was from BDH Chemicals; silver nitrate and formaldehyde were from Anachemia; sodium carbonate was purchased from Caledon. All other chemicals were obtained from Fisher. The SDS-PAGE apparatus was purchased from Bio-Rad, and Speedvec system was from Thermo Scientific. Urea was purchased from Amersco. TPCK-treated trypsin was from Worthington Biomedical. The C18 Sep-Pak SPE column was from Waters.

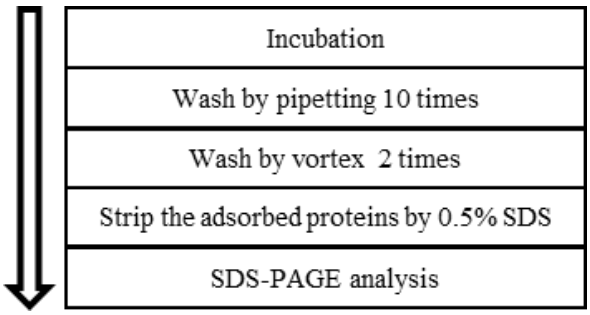

Figure 3: Experimental sequence of the SDS-PAGE/DPA method

2.3. Experimental methods of SDS-PAGE/DPA

2.3.1. Protein adsorption and the “rinse-stripping” method.

Concentrations of protein stock solutions were initially measured by the NanoDrop 2000 spectrometer, and by Bradford assay using the Varian Cary 300 spectrophotometer. Protein stock solution in phosphate-buffered saline (PBS) was diluted directly in 0.65-mL micro-centrifuge vials to 200 uL of final working solution, if not further specified. Figure 3 and 4 respectively shows the experimental steps and the illustration of the method. All the incubations were carried out in upright-position at room temperature without agitation. After defined incubation time, the solution was removed, and the vials were rinsed with PBS first by pipetting the solution up and down 10 times; the rinsing solution was discarded. In the second wash, fresh PBS rinsing was added and 1-min vortex was applied to the vial. Then, the third wash is just the repeat of the second wash. After removal of the final wash solution, the adsorbed protein molecules were stripped off the tube by 200-uL 0.5 wt% SDS in deionized water. All the wash and stripping solutions were collected and dried in Speedvec prior to SDS-PAGE analysis. To validate the SDS-stripping efficiency, additional 200 uL of 0.1-M NaOH or 50% ACN stripping was introduced to the vials after SDS treatment, and analyzed by SDS-PAGE/DPA. All experiments in this study were replicated.

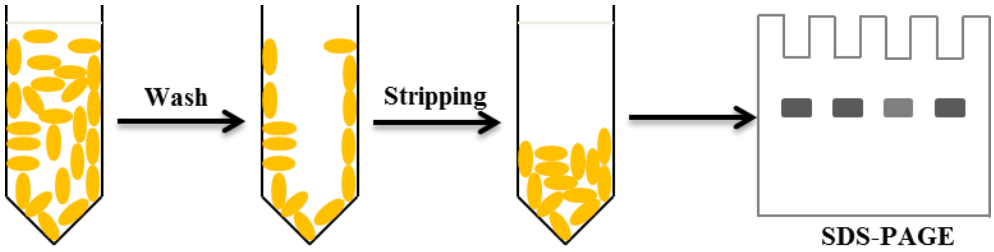

Figure 4: Schematic illustration of the SDS-PAGE/DPA method.

2.3.2. SDS-PAGE protein quantitation.

The SDS-PAGE analyses were carried out using 15% SDS-PAGE and silver staining following established protocols [143-145]. The dried samples were first reconstituted in SDS-PAGE sample buffer (4-fold concentrated buffer comprises 0.25 M

Tris, 0.02% bromophenol blue, 50% glycerol, 10% SDS, and 0.5M DTT), boiled for 10 min at 100 ^{o}C, and then cooled to room temperature before being loaded to the gel. For absolute quantitation, a set of protein standards were applied. Samples containing approximately 25µL solution were loaded into the well, and a voltage of 100V was applied for the electrophoretic run; voltage was stopped when the dye front reached the bottom end of the gel. After the electrophoretic run, the gel was fixed in 10 % acetic acid containing 25% isoproponol (IPA) prior to the silver staining. IPA is short chain alcohols, so it can disrupt any hydrogen bonding holding protein structures together and exposes all hydrophobic portions of the protein core, allowing the disrupted protein chains to associate with each other and form a bigger complex; therefore, proteins are fixed and trapped inside the gel. The gel was then washed for 5 min with distilled water and soaked in water for one hour. It was further soaked in fresh distilled water overnight. The gel was sensitized by 1min incubation in 0.02% sodium thiosulfate so that sulphur ions are deposited on the gel, and it was then rinsed with five changes of distilled water for 1 min each. After rinsing, the gel was submerged in chilled 0.1% silver nitrate solution containing 0.01% formaldehyde and incubated for 20 min at 4°C. Since there are sulphur ions on the gel, they will react with the silver ions which are inhibited to form Ag^{0} on the gel; meanwhile proteins will promote reduction of silver ions. After incubation, the silver nitrate was discarded, and the gel slab was rinsed three times with water for 20 seconds and then developed in 0.02% formalin [0.05% formaldehyde (Anachemia)] in 2% sodium carbonate with intensive shaking. During the development process, the size of the silver grains deposited on the proteins will grow and formaldehyde will be converted to formic acid which can be buffered by the sodium carbonate at pH 10. Once the desired intensity of staining was achieved, the development was terminated by discarding the reagent, followed by washing of the gel slab with 5% acetic acid. The complete staining procedure lasts no longer than 1 h. Developed gels were completely transparent when the sensitization step with sodium thiosulfate was included. The gel image was digitized by LiDE 110 scanner (Canon, Canada Inc.) and converted to intensity profiles using ImageJ (http://rsbweb.nih.gov/ij/). Quantification was based on the total intensity of the protein bands. A calibration curve is generated by plotting the intensity of the protein band as a function of known BSA quantity. Then intensity value of the unknown BSA sample is plugged into the calibration curve equation so that we can compute the corresponding BSA quantity of the unknown samples.

2.3.3. Characterization of the detection sensitivity.

The minimum volume of detection was characterized by using BSA solution of 200 ng/µL concentration at varied volumes for 1-h incubation. The minimum concentration of detection was analyzed by varying the concentration of 200 µL BSA solutions for 1-h incubation. The minimum time for measurable adsorption was also explored by using 200 µL of 1-ng/µL BSA solution. The adsorbed BSA molecules were recovered by the "rinse-stripping" method and quantified by SDS-PAGE as described above.

2.3.4. SDS-stripping efficiency

To validate the stripping efficiency of 0.5% SDS, we also used 200 µL of 0.1M NaOH and 50% ACN respectively to the vials to further remove any remaining protein. The additional stripping solutions were dried, and analyzed by SDS-PAGE/DPA.

2.3.5. Volume effect on protein adsorption

Various volumes (from 20 to 120 µL) of 200 ng/µL BSA in PBS were incubated at room temperature for 1 hour in the 0.65 mL microcentrigufe vial. The adsorbed BSA molecules were recovered by the "rinse-stripping" method and quantified by SDS-PAGE as described.

2.3.6. Adsorption at pg/µL concentration

We characterized the adsorption of BSA at extremely low concentration of 20 and 4 pg/µL. BSA solutions with above concentration at 500 µL volume were incubated for 1 hour at room temperature. The adsorbed BSA molecules were recovered by the "rinse-stripping" method and quantified by SDS-PAGE as described.

2.3.7. Solution-depletion method by SDS-PAGE and Bradford Assay

BSA solutions of 800 ng/µL and 100 ng/µL were incubated overnight at room temperature. Fresh samples were also prepared so that Bradford assay and SDS-PAGE

were both carried out to measure the supernatant concentration of BSA before and after the overnight incubation. In detail, equal volumes (2 µL) of BSA supernatants before and after overnight incubation were loaded on a SDS-PAGE gel and the remaining solutions were used for Bradford assay analysis. Experiments for SDS-PAGE and Bradford assay had replicates and triplicates respectively in this study.

2.3.8. Characterization of competitive and sequential protein adsorption.

Competitive protein adsorption was carried out by applying a mixture of BSA and MB each at 200 ng/µL concentration during 1-h incubation in the micro-centrifuge vials. After incubation, "rinse-stripping" method was applied to collect the adsorbed protein. Sequential protein adsorption was performed by first adding 200 ng/µL BSA to the vials for 1 h. After the PBS rinse indicated above, 200 ng/µL MB was introduced to the BSA-coated vials and incubated for another 1 h. After removal of the MB solution, vials were washed again with PBS and finally stripped by 0.5 wt% SDS for SDS-PAGE analysis.

2.4. "In-solution" Tryptic Digestion

Lyophilized trypsin treated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) was reconstituted in 1 mM HCl at pH 3 to obtain a final stock concentration of 4 µg/µL solution. An aliquot of BSA solution in PBS (100µL corresponding to 350 µg of BSA) was first denatured by adding 10 mM of Tris(2-carboxyethyl)phosphine hydrochloride solution (TCEP) and ultra-high purity urea powder to a final concentration of 8 M, and incubated at 37^{o}C for 1 hour. 0.5 M iodoacetamide (IAA) stock solution was then added to the sample to a final concentration of 15 mM, and the solution was incubated in the dark at room temperature for 30 minutes in order to alkylate the free thiols in the sample. The sample was then treated with 1M dithiothreitol stock solution (DTT) to a final concentration of 10mM and incubated for 15mins to destroy the excessive IAA. Afterwards, approximately 15 µg of undigested proteins was saved. The solution sample was then diluted ten times by 40 mM Tris buffer at pH 8, followed by the addition of trypsin with an enzyme to total protein ratio of 1 to 20, and incubated overnight (~18 hours) at 37 ^{o}C with rotation. Additional trypsin (with an

enzyme to total protein ratio of 1 to 50) was added to the sample and incubation was continued for another 3 hours. Acidification by 12 M HCl to the sample adjusted to pH 1 was carried out to stop the trypsin activity. 15 µg of the resulting tryptic peptides and undigested proteins was subjected to SDS-PAGE for examining the digestion efficiency. Once digestion is completed, supernatant containing tryptic peptides was then washed by C18 solid phase extraction (SPE) cartridge; the clean peptide solution was then dried by speedvac and stored at -80 ^{o}C for further use.

2.5. Peptide adsorption test by "rinse-stripping" method.

Dried, clean peptides were re-dissolved in 0.1% formic acid (FA) for preparing the 1 µg/µL peptide stock solution. This peptide stock concentration is determined based on how much protein is used at the beginning for digestion and based on how much solution we added to re-dissolve the peptide. For instance, if we started with 1 mg protein for digestion, we anticipated to obtain 1 mg of peptide after digestion, followed by the addition of 1 mL solution for re-constitution to obtain 1 mg/mL peptide stock solution. It was subsequently diluted with 0.1% formic acid in the 0.65 mL mircocentrifuge vials to 100 µL of final working solution with desired concentrations. The adsorbed peptide molecules were recovered by the "rinse-stripping" method and quantified by SDS-PAGE as described above.

2.5.1. Characterization of sequential peptide adsorption.

Sequential protein adsorption was performed by first using three different BSA concentrations (200/500/1000-ng/µL) to coat the surface of the vials overnight. Then supernatant was discarded, and 3X PBS rinse was applied as indicated above. Various concentrations of peptides were introduced to the BSA-coated vials and incubated for overnight. After removal of the peptide solution, vials were washed again with PBS and finally stripped by 100µL of 0.5 wt% SDS for SDS-PAGE analysis.

Chapter 3.

Method Development for Direct Quantification of Protein Adsorption by SDS-PAGE*

3.1. Method Development and Validation

The starting point of this research study was to first validate the newly developed direct protein analysis (DPA) method, termed SDS-PAGE/DPA, whose experimental protocol is listed in chapter 2. Validation was then followed by tests of sensitivity and potential application characterized by this method. Briefly, after a defined amount of incubation time followed by the removal of supernatant, three surface rinses by PBS were applied to remove all loosely bound adsorbed BSA molecules. The tightly adsorbed BSA was subsequently stripped by 0.5% wt SDS and characterized by the SDS-PAGE/DPA method. Moreover, a known quantity of BSA was included in each gel to generate a calibration curve to enhance the reproducibility of the silver staining method.

Figure 5 shows the SDS-PAGE results of the BSA resolved in each wash (W) and stripping (T) solutions of three parallel experiments. The BSA standards (Sd.) in Figure 5 were used to build the calibration curve beside the bar chart, which shows the averaged quantity of BSA in each wash and final stripping solutions of the experimental triplicates, as well as the percentage of the adsorbed BSA molecules over the total BSA in the original solution. The desorption amount from 1^{st}, 2^{nd}, and 3^{rd} single wash is 0.4%, 0.1%, and 0.03% of total BSA molecules added respectively. The R^2 of the quantitation standard curve is more than 0.9, and the coefficient of variance (CV) of BSA quantity in

all washes and stripping solutions in Figure 5 are less than 6% [161]. After 0.5 wt% SDS, further stripping using 0.1M NaOH or 50% acetonitrile was conducted and the SDS-PAGE results are shown in Figure 6, and the subsequent quantitation shows a less than 1% additional recovery of BSA [161].

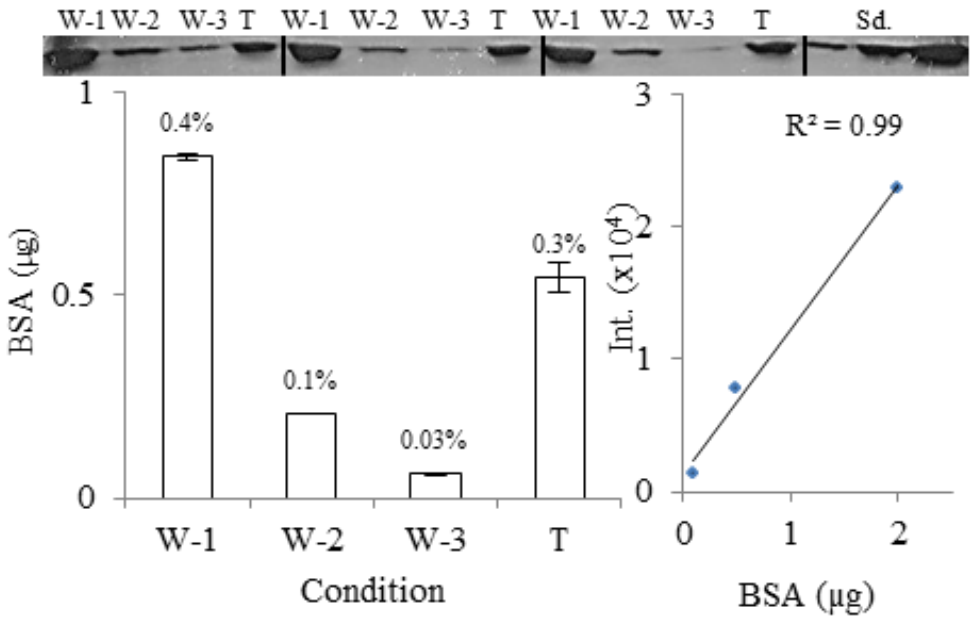

Figure 5: Method Development and Validation Characterization of SDS-PAGE/DPA. (A) Flow chart of SDS-PAGE/DPA. (B) SDS-PAGE image of BSA molecules obtained in three washes (W-1, W-2, and W-3) and the final stripping solution (T) in three repeated experiments. Sd. stands the BSA standard. Below the gel image is the average quantity of the adsorbed BSA molecules in all the solutions and the corresponding standard curve with the R^2 value. The percentage of the resolved BSA relative to the total initial is listed on top of each bar. (Bars represent mean +/- one standard deviation, n=3)

The three consecutive PBS washes are adequate to remove residual BSA in the vial, since by the last wash, only a trace amount of BSA (0.03% of the original BSA) was observed as shown in the bar chart in Figures 3.1. The SDS stripping is sufficient to recover 99% adsorbed BSA as additional basic and organic solvents did not remove any significant amount of protein molecules as shown in Figures 6. Using higher concentration SDS (2 wt%) solutions made no noticeable difference in results from those of 0.5 wt% SDS (data not shown). SDS has been a widely used detergent to desorb proteins, yet its applied concentrations were varied in different studies. Our results

support that the 0.5 wt% SDS concentration is effective. The high linearity of the calibration curve in Figure 3.1 suggests the good accuracy of the SDS-PAGE quantitation, which agrees with the existing knowledge on SDS-PAGE [40] in indirect solution-deletion method. The maximum adsorbed BSA on the polypropylene surface of the microcentrifuge vials we used was 0.3 μg/cm^2 [161], and this result is comparable to previous observations made on BSA adsorbed to hydrophobic plastic surfaces [5, 21, 48, 58, 146-148]

3.2. Sensitivity Test

3.3. Volume, Concentration, and Adsorption time effect

Many parameters such as protein concentration and adsorption time are known for easily influencing the protein adsorption quantity, but only few reports have studied the effect of protein solution volume on adsorption since many adsorption test are usually carried out on a flat surface; it is far from simple to quantify the adsorbed protein using non-labeling method to study the solution volume effect on adsorption. Thus, we characterized the detection sensitivity of SDS-PAGE/DPA starting from volume effect as shown in Figure 7. In Figures 7, the least the measurable volume is 20 μL of BSA at 200 ng/μL concentration, which is equivalent to 0.57cm^2. As seen, the solution volume used decreases with the BSA adsorbed quantity. In Figures 8, the BSA adsorbed quantity also decreases with decreasing BSA concentration used for incubation and the lowest detectable BSA adsorption can be obtained from 100 pg/μL protein solution. When enhancing the silver staining sensitivity by prolonged developing time in Figure 9, adsorption can be quantified from as low as 4 pg/μL protein solutions. The reason for the adsorption percentage quantified to be over 100% (141%) can probably be attributed to the background from the gel due to prolonged staining [161]; regardless of it, complete loss of protein sample can be detected in such an extremely low concentration.

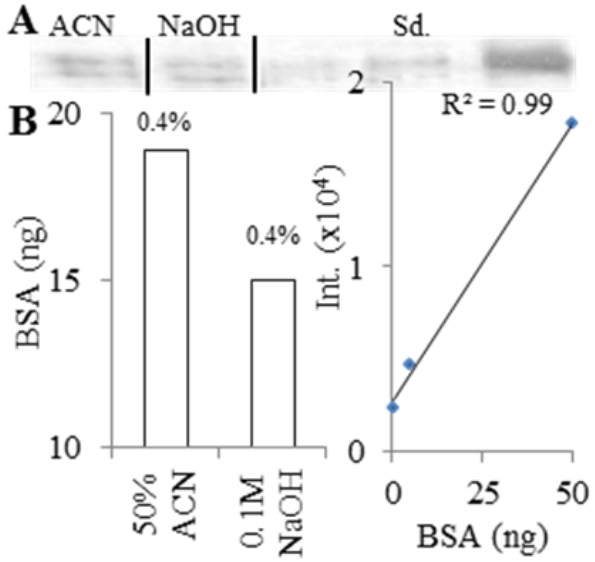

Figure 6: SDS-stripping efficiency. To validate the stripping efficiency of 0.5% SDS, we used 200 uL of 0.1-M NaOH or 50% ACN to the vial after SDS treatment to further remove the potentially adsorbed protein molecules. These additional stripping solutions were dried, and analyzed by SDS-PAGE/DPA.

In the time-cause studies, extended incubation time will enhance protein adsorption and this simple SDS-PAGE/DPA method allows us to demonstrate the immediate protein occupancy of vial surface. Adsorption signal in the gel and adsorption amount are clearly distinguished in the 10-min and 10-sec adsorption, as shown in Figure 10. Incubation time in as short as 10 sec, which is an extremely brief contact that is often seen in the preparation of low-concentration protein samples from the stock solution. Therefore, it is critical to understand and to prevent adsorption-caused sample loss for sensitive and accurate protein studies concerning low concentration conditions, especially in those studies with multiple steps such as single-cell proteomics.

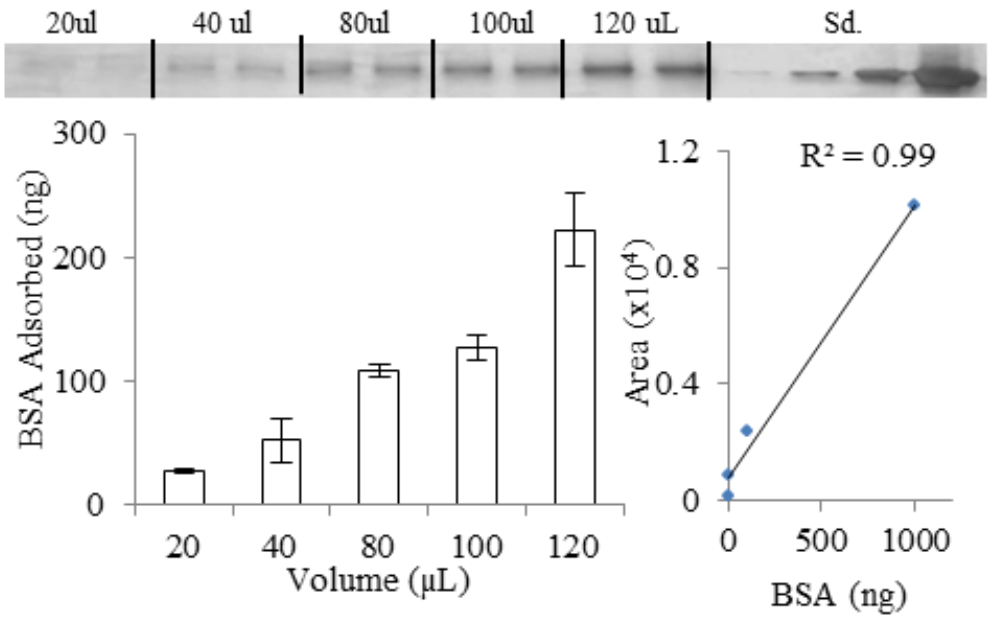

Figure 7: Volume effect to protein adsorption. Various volumes (20 to 120 µL) of 200 ng/µL BSA in PBS were incubated at room temperature for 1 hour in 0.65-mL microcentrifuge vials. The adsorbed BSA molecules were recovered by the “rinse-stripping” method and quantified by SDS-PAGE as described. (Bars represent mean +/- one standard deviation, n=2)

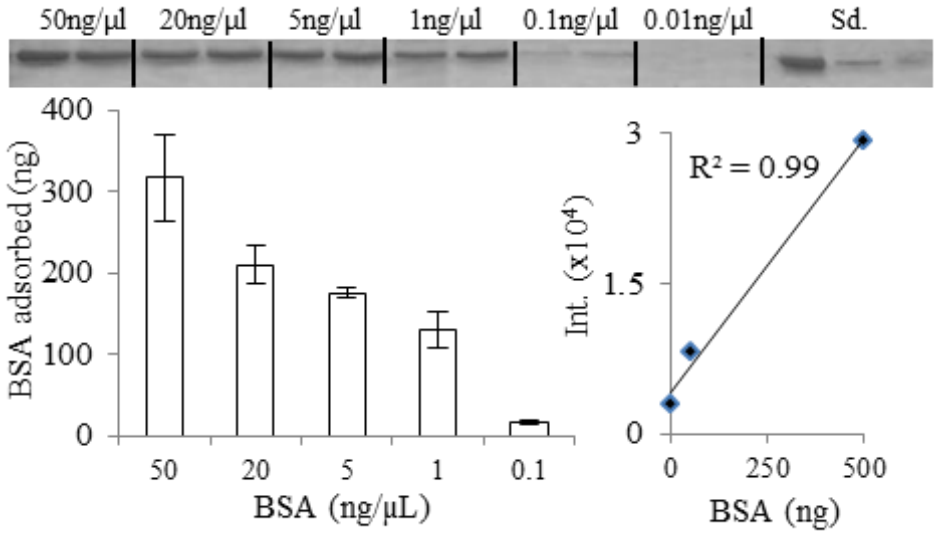

Figure 8: SDS-PAGE image of the adsorbed BSA in six different concentrations with replicates (condition: 200-µL BSA and 1-h incubation). Bar chart of BSA quantity and standard curve is shown below the gel image. (Bars represent mean +/- one standard deviation, n=2)

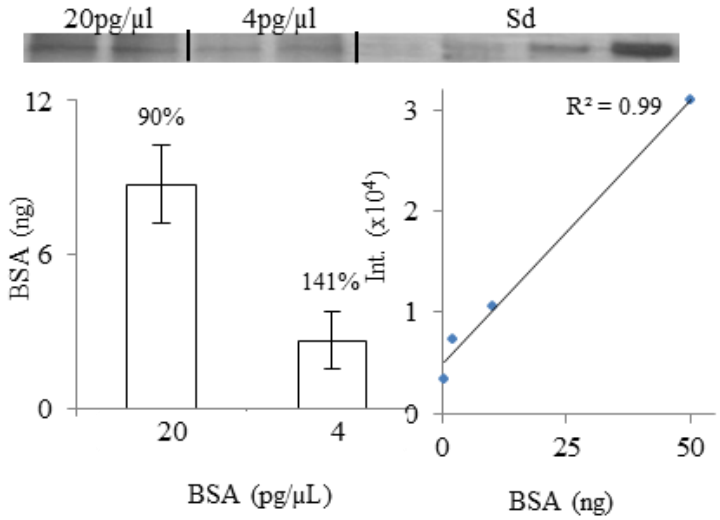

Figure 9: Characterization of BSA adsorption in pg/µL solution. Image of SDS-PAGE results of the adsorbed BSA at 20 and 4 pg/µL concentration. Bar chart of the BSA quantity is below. Adsorption percentage is also marked on top of each bar. (Bars represent mean +/- one standard deviation, n=2)

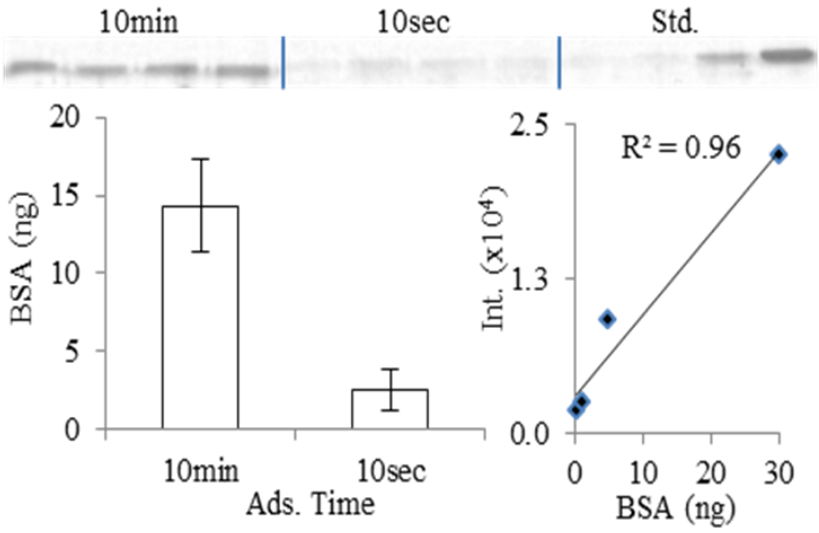

Figure 10: SDS-PAGE image of adsorbed BSA for different incubation times with replicates (condition: 200 µL of 1 ng/µL BSA). Bar chart of BSA quantity shown underneath. (Bars represent mean +/- one standard deviation, n=4)

3.4. Comparison between SDS-PAGE/DPA and the solution-depletion method.

To compare the SDS-PAGE/DPA with the widely used solution-depletion method, we performed solution-depletion analysis on the BSA solutions within the detection limit of SDS-PAGE/DPA. Results are in Figure 11 which also includes the summary of the results. Figure 11A shows SDS-PAGE characterization, while Figure 11B shows the resolved quantity measured by the solution-depletion method. Figure 11C summarizes the Bradford results from the same BSA solution in Figure 11A. No obvious quantity changes were observed between BSA solutions before and after overnight incubation. In general, SDS-PAGE is more sensitive than Bradford assay since Bradford assay relies on the coomassie dyes that bind to proteins via ionic interactions between their sulfonic acid groups and the positively charged amino acids, such as histidine, on the protein;

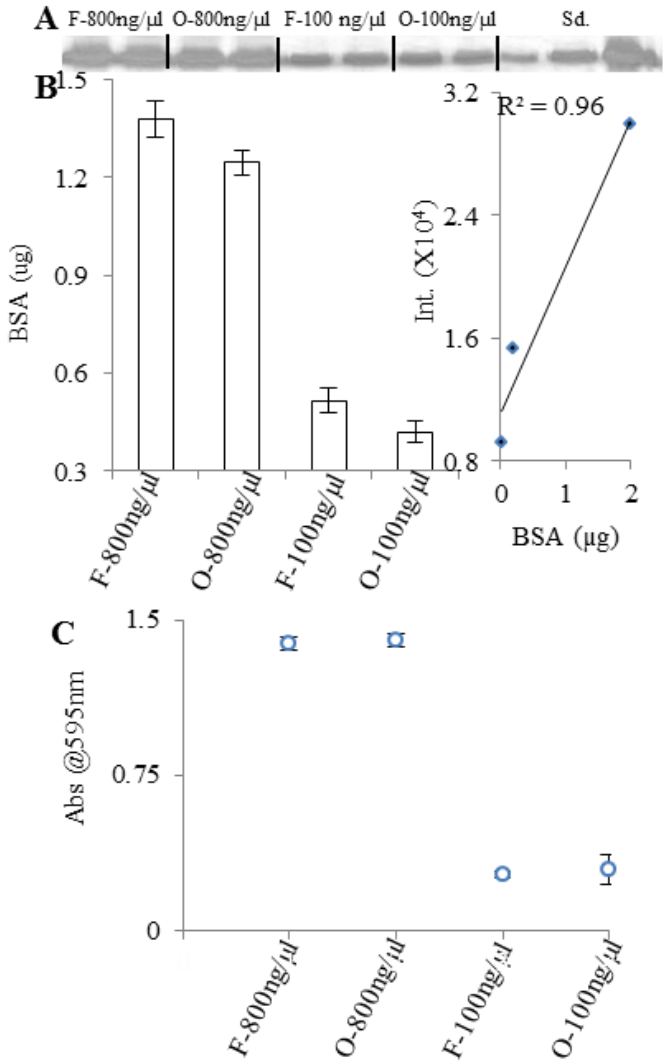

Figure 11: Solution-depletion method by SDS-PAGE (B) and Bradford Assay (C). Comparisons were made based on the freshly prepared (F) and overnight incubated (O) samples. BSA solutions of 800 ng/µL and 100 ng/µL were used. Bradford assay and SDS-PAGE were both carried out, at room temperature, to measure the concentration of BSA before and after the overnight incubation. In detail, equal volumes of BSA solutions before and after overnight incubation were loaded on a SDS-PAGE gel and the remaining solutions were used for Bradford assay. (Bars represent mean +/- one standard deviation, n=2 for SDS-PAGE, and n=3 for Bradford assay)

thus, proteins lack of positively charged amino acids cannot be detected. Yet, in this study, SDS-PAGE/DPA was able to quantify the adsorbed protein molecules from even lower concentration samples than shown in Figure 8. As seen in Figure 11B, the

adsorption amount difference between the freshly preared and overnight incubated samples at two different concentrations (800 and 100 ng/µL) tested is approximately 0.1 µg [161]. However, lower concentrations used in SDS-PAGE/DPA clearly demonstrated the higher adsorption quantity is essentially higher. In comparison to the quantitation results between solution depletion method and SDS-PAGE/DPA shown in Figure 8 where the highest concentration examined is 50 ng/µL, the adsorption amount we quantified is about 0.3 µg. Therefore, SDS-PAGE/DPA is more sensitive than the solution-depletion method in our experimental design, suggesting a more broad, application of SDS-PAGE/DPA than the solution-depletion method. To overcome one difficulty due to the limited capacity of the well in a gel, we have pre-concentrated the stripping solution by drying it prior to SDS-PAGE analysis. Therefore, regardless the volume of stripping solution, all the desorbed proteins can be loaded onto the SDS-PAGE gel. The silver staining method allows SDS-PAGE/DPA readily to detect protein adsorption from as low as pg/µL protein solution adsorption as shown in Figures 9. With drastically increased sensitivity of various analytical techniques and the desire to investigate proteins and cells at individual level to deepen our understanding of the fundamentals of biology, there is a strong demand to fully understand the actual protein concentration in the samples of interest. Accurate measurement of analyte concentration is one of the most critical elements in modeling chemical and physical reactions and in inferring structure, behavior and functions of biological systems. Due to the individuality of protein adsorption mentioned in the introduction, it is important to have a readily available, easy operating, and sensitive tool for individual labs to study their favorite protein(s). The SDS-PAGE/DPA method we developed is to serve this purpose, which integrates several existing techniques and well fills the methodology gap to facilitate researchers in a broad spectrum.

3.5. Characterization of multi-protein competitive and sequential adsorptions.

Because SDS-PAGE is capable of separating protein mixtures while quantifying individual proteins with appropriate standards, we explored the potential of using SDS-PAGE/DPA to study complex multi-protein adsorption events. Figure 12 summarizes two

cases we investigated, i.e. sequential and competitive adsorption of BSA and MB; concentration used for both proteins is 0.2 µg/µL. In the figure, the adsorptions of BSA and MB alone are shown in lanes 1 & 2 and 7 & 8, respectively; the competitive adsorption by BSA and MB mixture (BSA/MB) is shown in lanes 3 & 4; the final result of sequential incubation of BSA first and MB next (BSA-MB) is shown in lanes 5 & 6. In the same gel, standards of both BSA and MB are also included. The adsorbed quantity of BSA and MB is reported side by side in different adsorption events in the bar chart in figure 12 underneath the gel image, as well as including the standard curves with known quantities of MB and BSA.

From the quantitation results, despite no statistically significant difference, the addition of MB slightly decreases the BSA adsorption from 1% to 0.8% and 0.9% in competitive and sequential experiments, respectively. Conversely, the existence of BSA decreases the MB adsorption significantly from 1% to 0.1% in competitive and from 1% to 0.2% sequential experiments. Worth to mention is that the slope of BSA standard curve is about 10-fold less than that of MB suggesting the necessity to have individual standard curve for each target protein [161].

The characterization of multi-protein adsorption is frequently required but impossible to achieve for many methods such as QCM, ellipsometry, and neutron reflectometry; or challenging for others including radioisotope or fluorescence labeling, particularly when the protein mixture is complex. Previously studies have used 2D-PAGE for characterizing protein corona on nanoparticles during the process of in vivo drug delivery. The analysis is effective to separate complex protein mixtures but is inadequate in absolute quantitation. We demonstrated here that with protein standards, SDS-PAGE is capable of determining the quantity of complex protein samples. The separation power of the method allows the use of a mixture of different protein standards, thus no additional lanes are necessary for multi-protein quantitation.

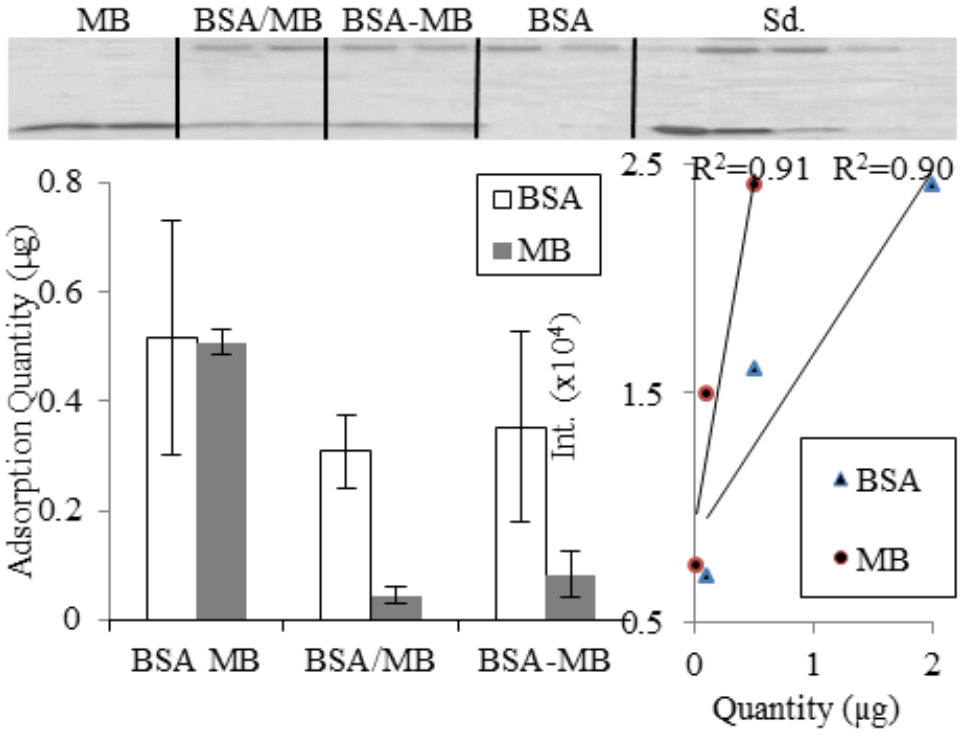

Figure 12: Applications of SDS-PAGE/DPA. SDS-PAGE image of the adsorption from pure BSA and MB solutions, as well as from competitive (BSA/MB) and sequential (BSA-MB) adsorption with replicates. Quantity of the adsorbed BSA and MB is included below the gel image. (Bars represent mean +/- one standard deviation, n=2)

Chapter 4.

Direct Quantitation and Characterization of Peptide Adsorption by SDS-PAGE/DPA*

4.1. Confirmation of Protein Digestion Efficiency

The aim of current proteomics is to exhaustively identify and quantify all the proteins in a cell line, tissue or organism in order to understand biological pathways and processes [66, 149, 150] as well as to profile proteins for research on vaccines and biomedical therapeutics [136, 151].

Two widely available methods have been extensively used in the proteomic approach: “top-down” and “bottom-up” [152, 153]. The former one relies on the study of intact proteins [154], while the later one, also termed shotgun proteomic approach, enables researchers to tackle high-complexity samples for large-scale analysis. The bottom-up approach essentially relies on detection of peptides derived from enzymatic digestion of the protein prior to mass analysis, followed by the identification of corresponding proteins based on the peptides generated [66, 136, 149, 150, 152-156]. Any peptide sample loss by adsorption occurring during laboratory manipulations can ultimately lead to unsuccessful, protein identification and analysis. Hence, preventing protein as well as peptide loss by adsorption is extremely important. Regarding this concern, it is of particular interest to characterize peptide adsorption by the newly developed SDS-PAGE/DPA method and to see whether the BSA coating on the vial surface can offer any positive effect on preventing peptide adsorption as efficiently as myoglobin adsorption.

*1. All the work in this chapter is produced by Gilbert Lee, except for the preparation of peptides and Figure 13 which is assisted by Kinny Wu.

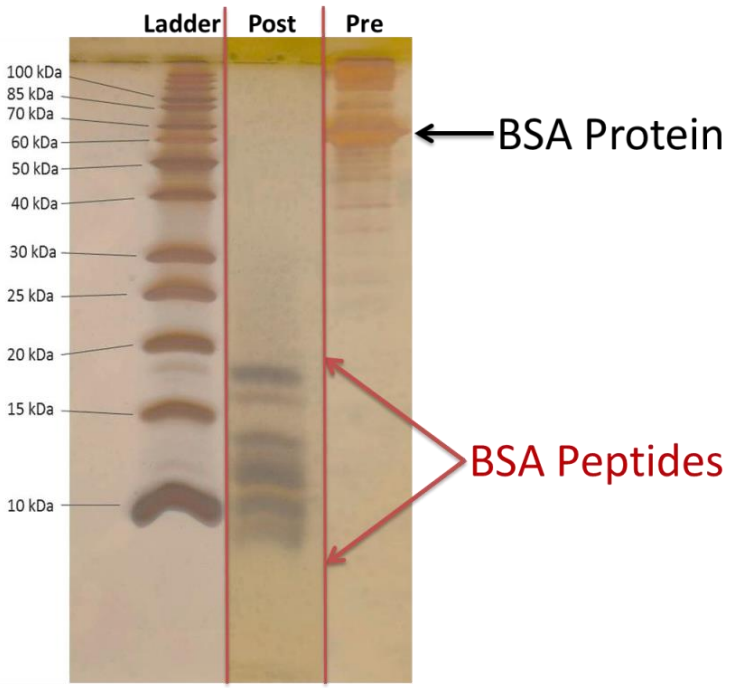

Figure 13: One-dimensional SDS-PAGE gel images of tryptic digestions of BSA

We selected BSA as a model peptide for this study because BSA protein is inexpensive and easily accessible. Thus, generation of peptides for the adsorption test is conducted by the proteolysis experiment such that the BSA protein was digested with trypsin overnight at pH 8. The pattern and efficiency of BSA digestion was monitored using SDS-PAGE as shown in Figure 13 .A clear contrast of the SDS-PAGE bands derived from the native protein bands (Pre) and peptide bands (Post) can be seen. Bands that can be seen on top of the BSA band in the “pre” lane are possibly impurities, since excessive DTT was added in the sample loading buffer; thus, it is undoubtedly that there must be no aggregation of BSA proteins. The protein digestion is successfully completed when the native BSA band at 67k Da is absent in the post-digestion lane and multiple peptide bands are generated at the lower molecular weight region of the SDS-PAGE gel. Furthermore, the image of this gel result is also compared to the literature reference [157] under the same condition to confirm that desired peaks are found in this gel and they are the BSA peptide fragments found in a MW range of around 8k to 20kDa as seen in the “post” column in Figure 13.

4.2. Preliminary study: Assessing the impact of peptide on the BSA layer formed by various concentrations

The SDS-PAGE/DPA method clearly demonstrated that a BSA coating surface of the vial reduces the adsorption of myoglobin (in chapter 3). Previous studies also reported that tryptic peptide fragments are able to adsorb onto the wall of the polypropylene tube [27, 158], so it is interesting to know whether the SDS-PAGE/DPA method can carry out the same characterization by using BSA pre-treated surface to preventing peptide adsorption. From the previous section in the protein study, a concentration of 0.2 µg/µL BSA was found to saturate the vial surface for one hour (results show in appendix A) prior to the addition of myoglobin, so we examined if the same concentration of BSA (0.2 µg/µL) as well as other BSA concentrations is sufficient to reduce the adsorption of the BSA tryptic peptides, as depicted in figure 14.

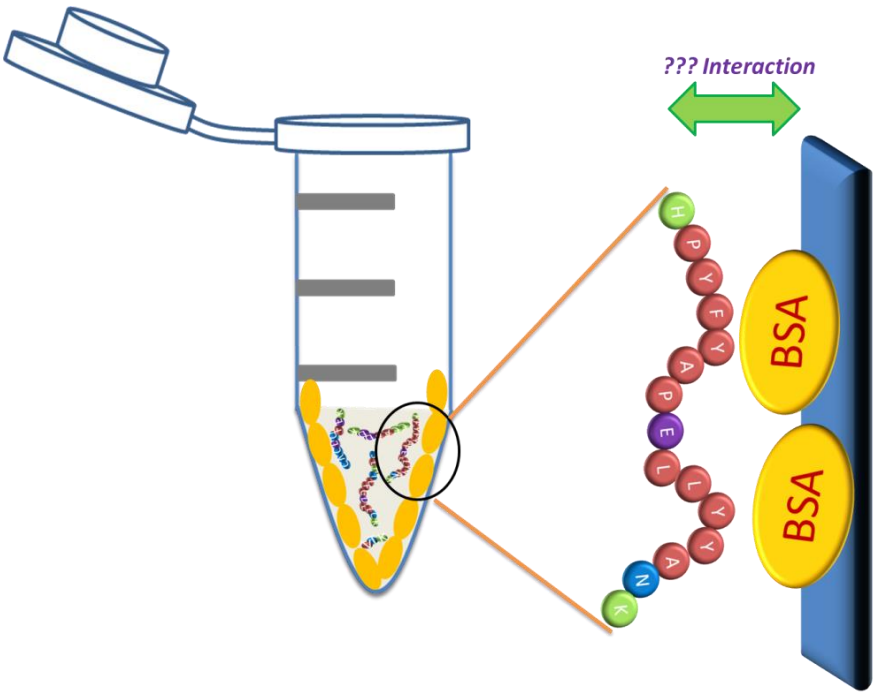

Figure 14: Illustration of peptide adsorption on BSA pre-coated surface of the microcentrifuge tube.

We initiated the adsorption experiment by first forming the BSA layer using various concentrations (1, 0.5, 0.2 µg/µL) on the vial surface; all clean, dried peptides were re-dissolved in 0.1% formic acid (FA) at pH 4 for adsorption tests. The gel image of this test is presented in Figure 15 and the quantitation of peptides is plotted in Figure 16a and b, in addition to the plot of BSA intensity against various conditions in Figure 16c. There is no significant difference in peptide adsorption on BSA-coated surface, compared to that on the bare surface, as shown in Figure 16a and b, and the error bars based on one standard deviation are too large for statistical comparison in the bar chart quantitation.

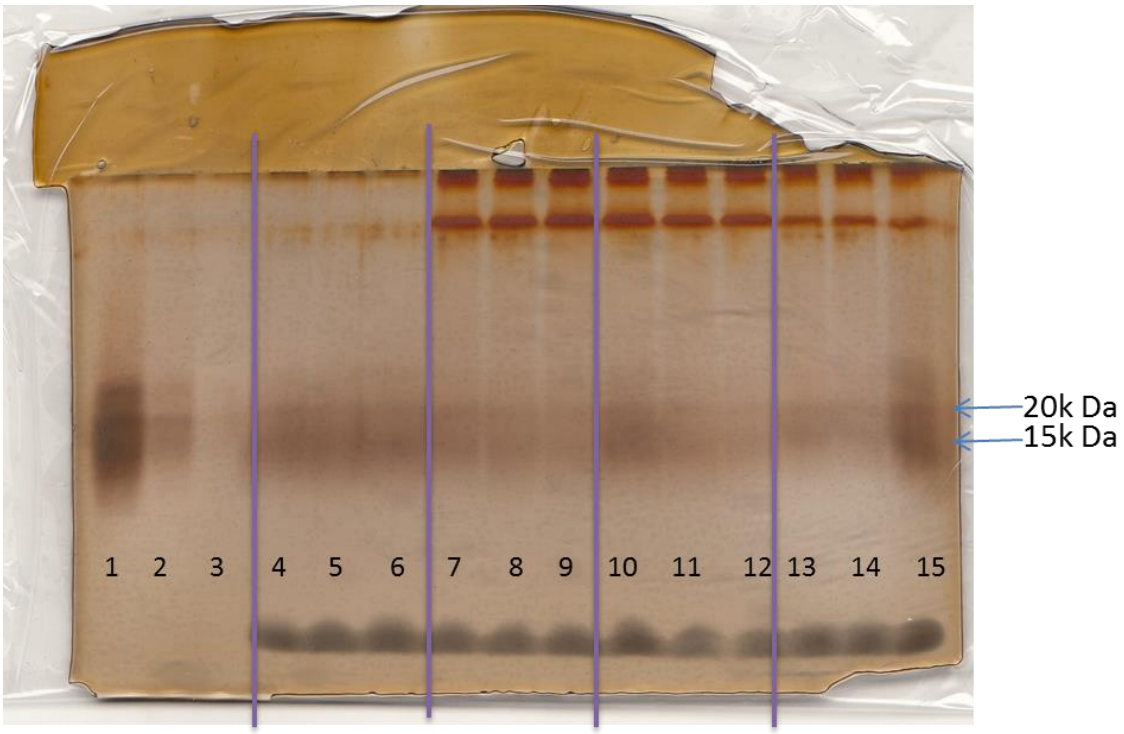

Figure 15: Comparison of adsorption of peptide (1 µg/µL) at various concentrations of BSA coating. All peptide incubations were carried out using a concentration of 1 µg/µL for lane 4-15. Lane 4-6 represent peptide adsorption without any BSA coating, while lane 7-9, 10-12, and 13-15 respectively contain 1µg/µL, 0.5µg/µL, and 0.2µg/µL BSA pre-adsorbed on the surface. Lane 1-3 are the peptide standard of 1000, 200, and 50ng respectively.

One possibility might be that the BSA tryptic peptide fragments might have high affinity for the BSA molecules, since BSA denatured and unfolded after adsorbing onto the surface. It has been reported that a hydrophobic peptide was able to bind to BSA molecules used to pre-saturated the container surface [159], but we cannot see an effective reduction of peptide adsorption. Given that the band signal of peptide alone in the gel image in Figure 15 as well as the quantitation results in Figure 16a and b is similar to that of other samples in BSA-protecting layers, the BSA-coating relatively shows no effect on reducing peptide adsorption. However, we can clearly see a gradual decrease of BSA band intensity after peptides were being applied in Figure 16c. When the BSA layer is formed by 0.2 µg/µL followed by peptide adsorption, its adsorption intensity was half compared to that formed by 0.5 and 1 µg/µL, indicating that approximately twice the amount of adsorbed BSA desorbed from the surface after peptide adsorption for the BSA layer formed by 0.2 µg/µL. The adsorption intensity of the BSA layers formed by 0.5 and 1 µg/µL apparently shows no difference, but there is a significant difference between the adsorption intensity of 0.2 µg/µL and either 0.5 µg/µL, or 1 µg/µL, based on the calculation of one sample t-test. However, since the sample size is small (n=3), so the statistical analysis is not very meaningful In fact, BSA peptide fragments in general are smaller than myoglobin and the property of the adsorbed peptide fragments is not known, it is not possible to draw any conclusion, and more experiments and other characterization methods are required. However, unlike the reduction of MB adsorption, this result proves that 0.2 µg/µL BSA is not the optimal concentration recommended for surface coating to prevent peptide adsorption, since desorption of the BSA layer formed by this concentration (0.2 µg/µL) happened repeatedly.

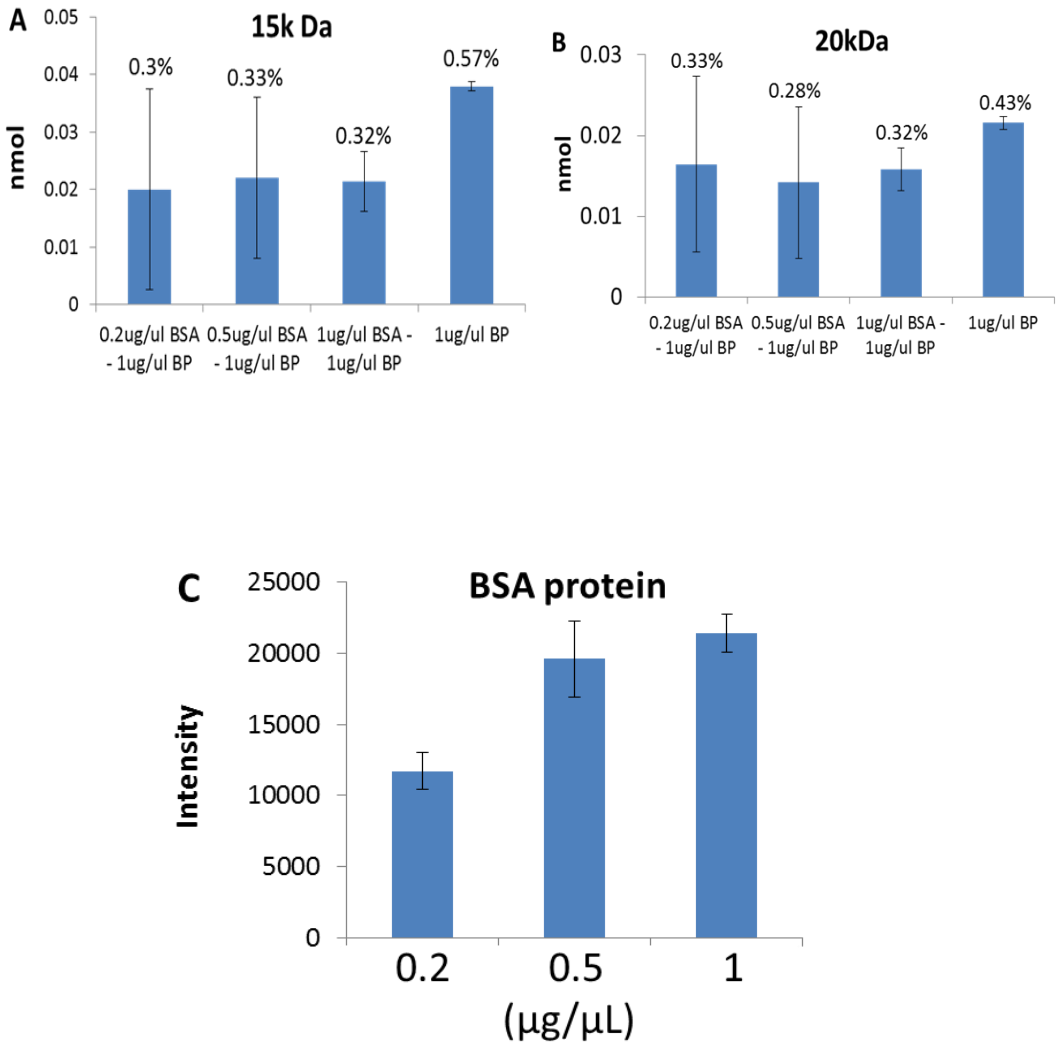

Figure 16: Bar chart analysis for the gel result in figure 15. (a) and (b) respectively are the plot of molar adsorption of 15k and 20k Da peptide fragments on BSA layers formed by different concentrations and the adsorption percentage is located on top of the quantitation bar. (c) Band intensity corresponding to BSA molecules remaining on the surface after peptide adsorption. (Bars represent mean +/- one standard deviation, n=3)

4.3. Buffer effect of peptide adsorption on the BSA-coated surface

In this section, we aim to examine peptide adsorption when peptides are prepared in different solutions: PBS and formic acid. Some previous reports [3, 69, 160]

have demonstrated that selecting buffer choice is crucial to preparing peptide samples; one former study has specifically employed PBS buffer to re-dissolve the dried peptides for testing their adsorption onto the hydrophilic and hydrophobic surface since PBS can alter the secondary structure of the peptide backbone which contributes to adsorption [74]. Therefore, we similarly conducted an intra-gel experiment (repeated samples after adsorption, followed by the experiment in one single gel) for testing the adsorption of peptides in PBS and compared its effect with that of peptides in 0.1% FA onto the BSA-coated surface.

The gel image of the adsorption result is shown in Figure 17; plots of the peptide adsorption intensity and the remaining BSA-adsorbed molecules on the surface against different adsorption conditions are presented in Figure 18a and b respectively. For lanes 1 to 3 that represents the known peptide standard in Figure 17, only the 1000 ng peptide standard sample in lane 1 is observed, while the 100 and 10 ng peptide standard in lane 2 and 3 respectively cannot be seen, so we are not able to construct the standard curve for quantification of the adsorbed peptides; however, we measured the band intensity of the peptide signal in the gel to qualitatively study the peptide adsorption intensity (lane 6-13). The qualitative result in Figure 18a demonstrated that when peptides are in PBS, the intensity of their adsorption onto the empty surface (lane 8-10) is approximately 1 fold more than that onto the BSA-coated surface (lane 11-13). On the other hand, when the adsorption was tested on the BSA-coated surface, peptides in 0.1% FA (lane 6-7) appear to adsorb slightly less than those in PBS (lane 11-13). The BSA-coated surface does not appear to reduce the adsorption of peptide in 0.1% formic acid as seen in the previous section (4.2) in the quantitative results in Figure 16a and b, but we can obviously observe a different adsorption result for peptides in PBS onto both the empty and BSA-coated surface in this section in Figure 18a. In Figure 18a, the y-axis represents the relative adsorption intensity of the 10kDa peptide fragment. We examine how much peptide adsorbs when they are prepared in PBS and 0.1% formic acid while in Figure 18b, we examine how much BSA remains on the vial surface after peptide adsorption and 6 times of surface rinsing as a control.

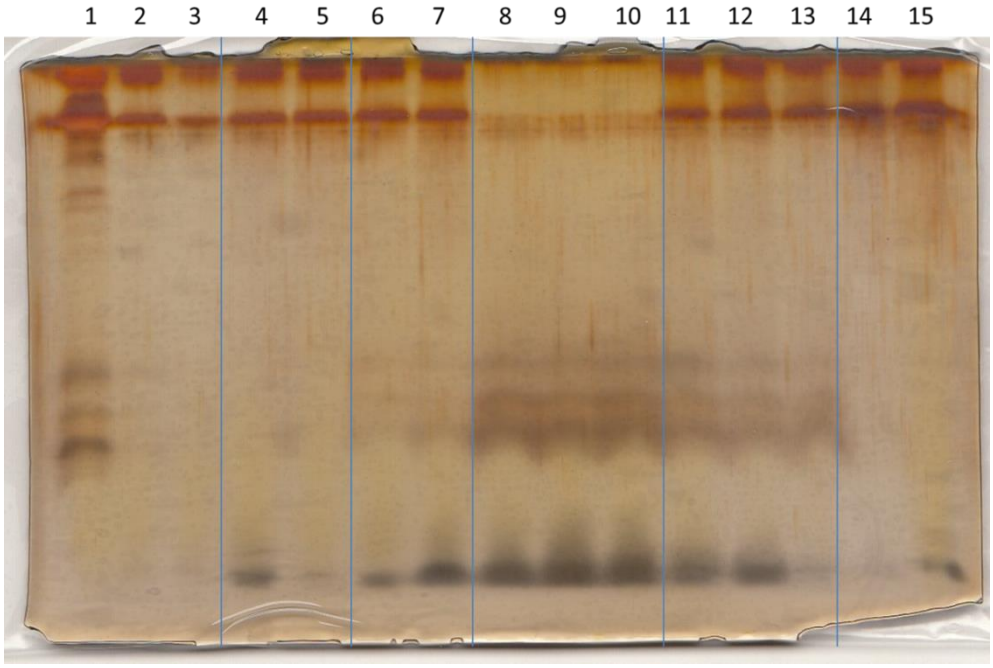

Figure 17: Solvent effect on peptide adsorption. Lane 1-3: known BSA and peptide standards. Lane 4-5: 0.1% FA incubated on BSA surface. Lane 6-7: adsorption of peptides in 0.1% FA onto BSA-coated surface. Lane 8-10: adsorption of peptides in PBS onto empty surface. Lane 11-13: adsorption of peptides in PBS onto BSA-coated surface. Lane 14-15: BSA control with 6 washes. Concentration used for all BSA coatings is 0.2 µg/µL and 1 µg/µL peptide solution is used for adsorption.

Reports in general showed that BSA as a blocking reagent can effectively reduce peptide adsorption [27, 160], but rarely did they address the influence of buffer/solvent on peptide adsorption onto BSA-coated surface. Thus, we determined to evaluate if formic acid would yield any detrimental effects on the BSA-coated surface (0.2 µg/µL) by comparing the 6-wash effect (lane 14-15) and incubation of 0.1% FA (lane 4-5) as a control on the BSA-coated surface. The quantitative result in Figure 18b showed that the remaining BSA molecules on the surface after 0.1% FA incubation is about 2-fold less than those on the surface after 6 washes. Meanwhile, we are also able to perceive a decrease in the adsorbed-BSA molecules after peptide adsorption regardless of any solvent/solution used, in comparison to the BSA-coated surface after 6 washes. It is indeed interesting to point out that formic acid is able to remove the adsorbed-BSA molecules from the surface, but the adsorption of peptides in 0.1% formic acid onto either bare or BSA-coated surface did not exhibit much difference based on the result from section 4.2 in Figure 16a and b. Therefore, in this project, it indicates the reason for

the popular use of formic acid in peptide sample preparation in mass spectrometry analysis. However, more experiments are required to repeat and conduct to further solidify our current preliminary findings.

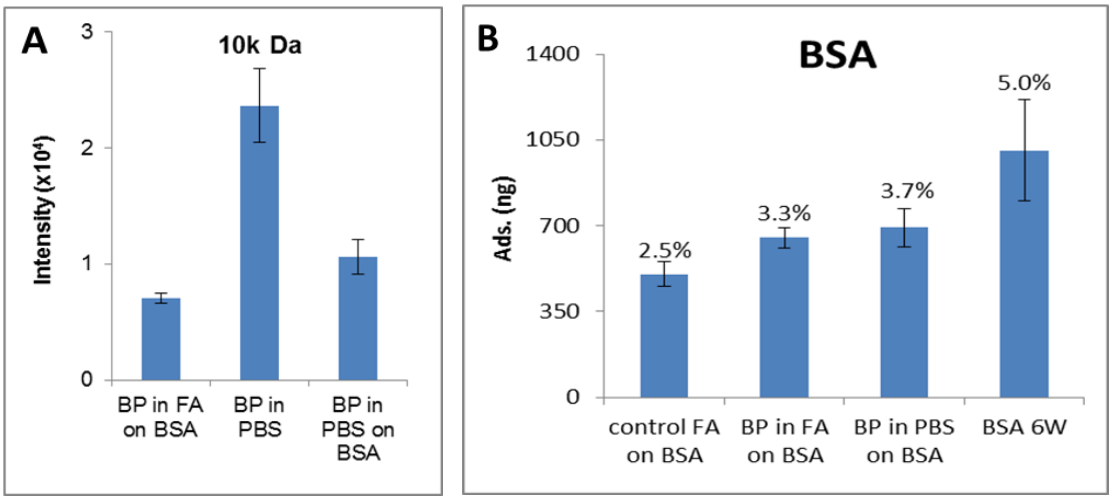

Figure 18: Bar chart analysis for the gel image in figure 17. BP denotes BSA peptides. (a) Plot of the 10k Da peptide fragment adsorption intensity as a function of different adsorption conditions. (b) Adsorption quantity of BSA remaining on the surface based on different conditions and the adsorption percentage is shown on top of the quantitation bar. 0.2 µg/µL BSA solution was used for surface coating and 1µg/µL peptide solution was used for adsorption.

Chapter 5.

Conclusions and Future Work

5.1. Conclusion

A simple and direct adsorbed protein quantitation method by using SDS-PAGE has been developed. The method was initially verified by the surface-rinsing scheme and demonstrated reproducibility from three repeated experiments. No expensive equipment and complicated steps are involved; this method is capable of measuring proteins, that silver-stains well, adsorbed on sample vials at extremely low concentrations (4pg/µL), which corresponds to an adsorbed mass of 2.5ng BSA. We also achieved a high sensitivity for this method by characterizing the effects on time, and sample volume on adsorption. We eventually applied this method to discover differential sample loss in a protein mixture and its application in developing preventive strategies of adsorption. In this study, the maximium blocking efficiency of BSA to MB is approximately 85% (which is achieved in the competitive adsorption). Regarding to the section 1.8 which mentions the reduction of fibrinogen adsorption by 4-folded in a previous study, it is worth to mention that bigger proteins usually adsorb more such that fibrinogen is approximately 20 times bigger than myoglobin, so the fact that we obtained a higher blocking efficiency by BSA is dependent on the protein used for any adsorption study. Having had the developed method available, we analogously applied this method to examine the peptide adsorption effect. Peptides, successfully generated by tryptic digestion of BSA protein, adsorb differently onto the vial surface when they are incubated in different solutions, suggesting that it is critical to choose an appropriate solution to re-constitute peptides in order to avoid adsorption. Since current findings are still preliminary, more experiments are required for us to better understand peptide adsorption.

5.2. Future Work

We are striving to develop bioanalytical methods for proteins/peptides since one main area for future study is the identification and quantitation of undiscovered proteins of therapeutically use via advanced proteomic approaches. However, protein/peptide sample loss by adsorption during sample preparation is an inevitably severe problem, so preventive strategies are of urgent need. Proteins from biological samples of interest are often hydrophobic and occur in low concentrations, so they bind to surfaces of materials used in sample preparations and analytical instruments. Therefore, we devised this simple method for protein quantitation as a starting pointing to determine how much sample would be lost by adsorption in routine experiments.

For continuation, the study of peptides will be expanded. The determination of lowest detectable concentration of the peptide by adsorption is a priority, so we can know at which peptide concentration level the threshold for entire sample loss by adsorption is, which is highly beneficial to our knowledge to discover low-concentration proteins. Next, it is desirable to test the adsorption of peptide prepared in solvents other than PBS and FA. The solubility issue of peptides is serious. All bioanalytical assays suffer from low sensitivity, inaccurate measurements, and high non-reproducibility due to incomplete solubilization. Furthermore, the amino acid residues in the peptide sequence is possible to undergo structural change in different solvents, so some commonly use solvents in the proteomic approach, such as ammonium bicarbonate and tris-buffer, are worthy of investigation. We can also vary the pH of the buffer to examine whether it will affect the peptide adsorption quantity. Once knowing the peptide concentration that can saturate the vial surface, we can optimize the BSA concentration to form a protecting layer for preventing the adsorption of peptide. Moreover, when peptide is re-dissolved in PBS, it is interesting to spike PBS with BSA, promoting a competitive adsorption between BSA and peptides on the BSA-coated surface. Finally, it is necessary to determine which peptide sequences would have a higher affinity for the sample vial surface, so we can strip the adsorbed peptide from the surface and directly have them characterized by HPLC-MS/MS. Comparison can be made based on peptide adsorption onto bare and BSA-coated surface; the amino acid sequence of the adsorbed peptides can be revealed and further evaluated.

From the above discussion, we have learnt that peptides adsorb onto surface of the sample vials, indicating that peptide loss by adsorption occurs in all proteomic approaches. Therefore, if the above suggestions can be successfully implemented, not only can we reduce peptide adsorption but we can also apply other tryptic peptides from various proteins of interest to the method. Proteins from cell lysate are good candidates since cells contain many proteins, and so it will allow us to know which type of proteins or peptides have higher affinity for the surface of sample vials used in routine experiments for the proteomics.

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Appendix.

Supplementary Materials

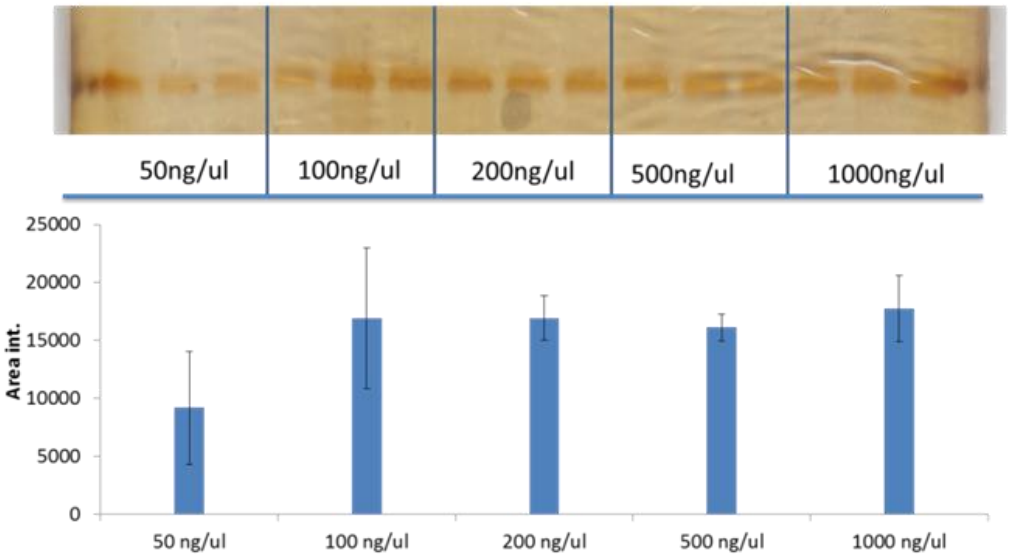

Figure 19: Examination of surface saturation by various concentrations of BSA ranging from 50 to 1000 ng/µL at 200 µL for 1 hour incubation. The gel image result is located on top and underneath is the plot of relative adsorption intensity as a function of different BSA concentrations. (Bars represent mean +/- one standard deviation, n=3)