Mechanism of Bevacinzumab Adsorption with Affinity Ligands And Bioprocess Optimization For Antibody Purification

A THESIS SUBMITTED

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Acknowledgments

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Thank you all

Abstract

Monoclonal antibodies (mAbs) have been found with a wide array of applications as pharmaceutical compounds in the treatment of cancers and diseases such as arthritis, asthma and osteoporosis. In approximate 10 years retrospection, the global market of mAbs experienced a rapid growth, nearly tripling the profit to be approximate US$16.7 billion in 2014. In order to meet the rising demand for mAbs, it is critical for manufacturers to ensure the production efficiency on the premise of product quality assurance. Especially in downstream purification of mAbs, the affinity chromatography as the major capture stage acts crucially in the removal of contaminates including host cell protein (HCP), DNA, antibody variants, viral particles and endotoxin to obtain rapid isolation and high concentration of the target protein. However, drawbacks associated with this technique are the expense of resins for binding mAbs. To reduce the cost, alternative resins have been explored. However, this raises the significance of understanding the mechanism of ligand-mAb binding in terms of binding sites and binding conformational changes for the optimisation of chromatography performance.

To address the aforementioned binding mechanism, the isothermal titration calorimetry (ITC) method was emplolyed for investigation of the thermal dynamic behaviour during free ligand and mAb binding. Two widely used affinity ligands, native Protein A (nSpA) and MabSelect SuRe (MS) ligand, were selected to bind with Bevacinzumab (BmAb). The binding mechanism was determined based on the isothermal parameters such as binding associated coefficient (ka), binding associated enthalpy changes (ΔH) and entropy changes ($T\Delta S$).

Further investigations were carried out by applying BmAb into the affinity columns packed with nSpA or MS ligands to evaluate mAb association and disassociation with immobilized ligands at different operational conditions. It was found that the binding breakthrough curves

are related to the mAb association that reveals distinctive dynamic binding capacities and column binding performance.

Based on above studies, it was found that the binding conformation and binding affinity were different between the native Protein A and the recombinant MabSelected SuRe ligand. The formation of ligand-BmAb binding complex was examine d under various conditions such as pH, temperature and solvent ionic strength. In the end, binding mechanism was understood by the analysis of above conditions in both ITC and Binding breakthrough studies.

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Chapter 1 Introduction

1.1 Introduction

Protein acts as an essential factor that exists in every living organism, and it is responsible for cell signalling, immune responses and other many tasks that are involved in the cell metabolism (Konermann et al., 2011). Monoclonal Antibodies (mAbs), a large component at the protein family, constitute a part of the immune system with the function of identifying and neutralizing foreign objects such as bacteria and viruses. In clinical applications, mAbs have been commercially produced for therapeutic use in treatment of cancer and auto-immune diseases (Konermann et al., 2011). A standard manufacturing process of a mAb is established with two major steps. It starts with cell culture which provides suitable conditions for secretion of the mAb from a host cell, and the following step of protein purification guarantees the safety of the product and also enhances the yield in the manufacturing process (Vazquez-Rey and Lang, 2011).

Protein purification at downstream antibody production is a crucial investment factor. Commercial consideration at the optimization in favour of recovery, capacity or speed ensures a high purity of final products (Healthcare, 2007a). However it poses also a significant obstacle for above improvements to be achieved. This causes the obsessing of higher purity products at global market, and brings the potential prospect for optimization of protein purification techniques (Healthcare, 2007a). Moreover, the core technique in protein purification is chromatography which isolates a specific protein from a crude mixture based on the interaction between the adsorption ligand and the target protein. Therefore, a thorough understanding of protein-ligand interaction becomes the key to help enhancing the efficiency of chromatography in order to increase the purity of final products at mAbs manufacturing.

During the past decade, the inter-protein interaction between mAbs and their corresponding ligands has been studied by model simulation assays. Mathematical modelling such as molecular dynamics (MD) simulation and molecular modelling is introduced (Branco et al., 2012, DeLano et al., 2000, Graille et al., 2000, Starovasnik et al., 1999) which allows mimicking the binding interaction between residues of protein-ligand complexes. Experiments techniques such as nuclear magnetic resonance (NMR) (Kato et al., 1993, Tashiro and Montelione, 1995), circular dichroism (CD) (Frahm et al., 2012, Kelly et al., 2005, Maurer et al., 2011, Yusoff et al., 2009), fluorescence spectroscopy (Dunstan et al., 2009, Frahm et al., 2012, Kun et al., 2009) are proposed for investigation of effects of conformational variation on the binding affinity. However, previous experiments focused only on one type or few types of measurements for probing the binding mechanism of protein adsorption (Brown et al., 1998, DeLano et al., 2000, Gunneriusson et al., 1999, Kato et al., 1993, Pierce et al., 1999). Lack of detection assays also obstructs the study of specific protein interactions. To achieve the optimization of chromatographic performance in industrial mAb purification, in-depth understanding of the inter-protein binding mechanism between mAbs and ligands is required.

1.2 Research Scope

The general aim of this thesis is to experimentally determine the binding mechanism between a monoclonal antibody (mAb) and different types of Staphylococcal protein A (SpA) at various operational conditions. Two major objectives are established to achieve the aim:

1) To experimentally measure the static binding between free ligands, native Staphylococcal protein A (nSpA) and MabSelect SuRe (MS) ligand, and Bevacinzumab (BmAb) by using isothermal titration calorimetry (ITC) technique;
2) To further investigate the binding mechanism by BmAb breakthrough response to two immobilized nSpA and MS ligands in a chromatographic column.

Chapter 2 Literature Review

2.1 Monoclonal antibody

The monoclonal antibody (mAb) is a type of glycoprotein that belongs to the family of immunoglobulins. Unlike the polyclonal antibodies which are secreted by different B cell lineages, the mAbs is generated by the single immune cell lineage that only recognizes an identical epitope of corresponded antigens. Through the history of therapeutic applications, the Mabs revealed in a wide usages at different cancer treatments such as cancers of the colon, breast, lung, neck and brain as well as other diseases such as arthritis, asthma and osteoporosis(Fekete et al., 2013). In approximate 10 years retrospection, the global market of mAbs with about US$ 5.4 billion profit at the beginning of 21st century raised rapidly to approximate US $16.7 billion in 2008 (Reichert and Pavlou, 2004). During this period of time, over 40 novel mAbs have been applied to clinical trial each year, and more than 20 mAbs have attained approvals of authorised public releasing by regulations like the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA)(Reichert and Pavlou, 2004).

The development of Mab production was started in early 1980s with the practical approval of a new cell line technology, Hybridoma technology, by Nobel Prize grantors GeogrgesKöhler, César Melstein, and NeilsKaj Jerne (Cambrosio and Keating, 1992). The hybridoma technology is a fusing technic that merges two types of cells, a specific antibody-producing B cell and a myeloma cell, into a hybrid cell for incubation and mass production. This method ensures a high production rate to satisfy the demand of global market, and guarantees that the identical structure of one type of antibody during the manufacture is maintained(Cambrosio and Keating, 1992).

2.2 Bevacinzumab

Bevacinzumab, shortened as BmAb, is an angiogenesis inhibitor that often applies to the treatments of various cancers including metastatic colorectal cancer (mCRC), advanced nonsquanous non-small cell lung cancer (NSCLC), metastatic kidney cancer (mRCC) and Glioblastoma (GBM). The BmAb belongs to the family of humanized immunoglobulin G 1(hIgG1) targeting specifically to vascular endothelial growth factor (VEGF) to avoid endothelial cell proliferation and subsequent migration(Wragg and Bicknell, 2013). The VEGF acts crucially at the treatment of malignant cancers. When hypoxia appears at where a tumor grows, the angiogenesis would initiate attumor surroundings with new blood vessels forming to restore sufficient nutrients supply for cancer cell amplification. Commence of angiogenesis requires the stimulation of pro-angiogenic factors including VEGF to bind with VEGF receptors (VEGFr) located at Hypoxia region.

The VEGF consists of six subtypes of derivatives (VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and PIGF). The VEGF-A has been discovered that involve mainly in the regulation of increasing vascular permeability, degeneration of the extracellular matrix and cell aggravation. Particularly in cancer cells, when VEGF-A binds to the correlated VEGFr, angiogenesis will be activated to promote cancer cell growth and migration.

The introduction of Bevacinzumab is an antihuman VEGF mAb. It contains great affinity to interact with VEGF-A to form a complex of BmAb-VEGF. This reconstructed VEGF complex may result in the failure of interacting with its correlated receptors. In consequence, the destruction of neoplastic capillaries would occur that reduces tumor growth.

Like most mAbs, Bevacinzumab (~149kDa) consists of 2 heavy chains (~50kDa each) and 2 light chains (~25kDa). In a non-reducible BmAb, 2 heavy chains form a single entity and either side couples with a light chain with disulphide bonds. Together, the formation becomes

a Y-like shape where the top branches of the "Y" is characterised as Fab region and the bottom trunk is Fc region. Each end tip of Fab domains contains the binding sites of six Complementarity Determining Regions (CDRs) that target specifically to VEGF. The hinge region of CH2 and CH3 of Fc fragments are highly conserved by N-glycans that helps to navigate IgG to the Fc receptors from phagocytic cells such as Staphylococcus aureus.

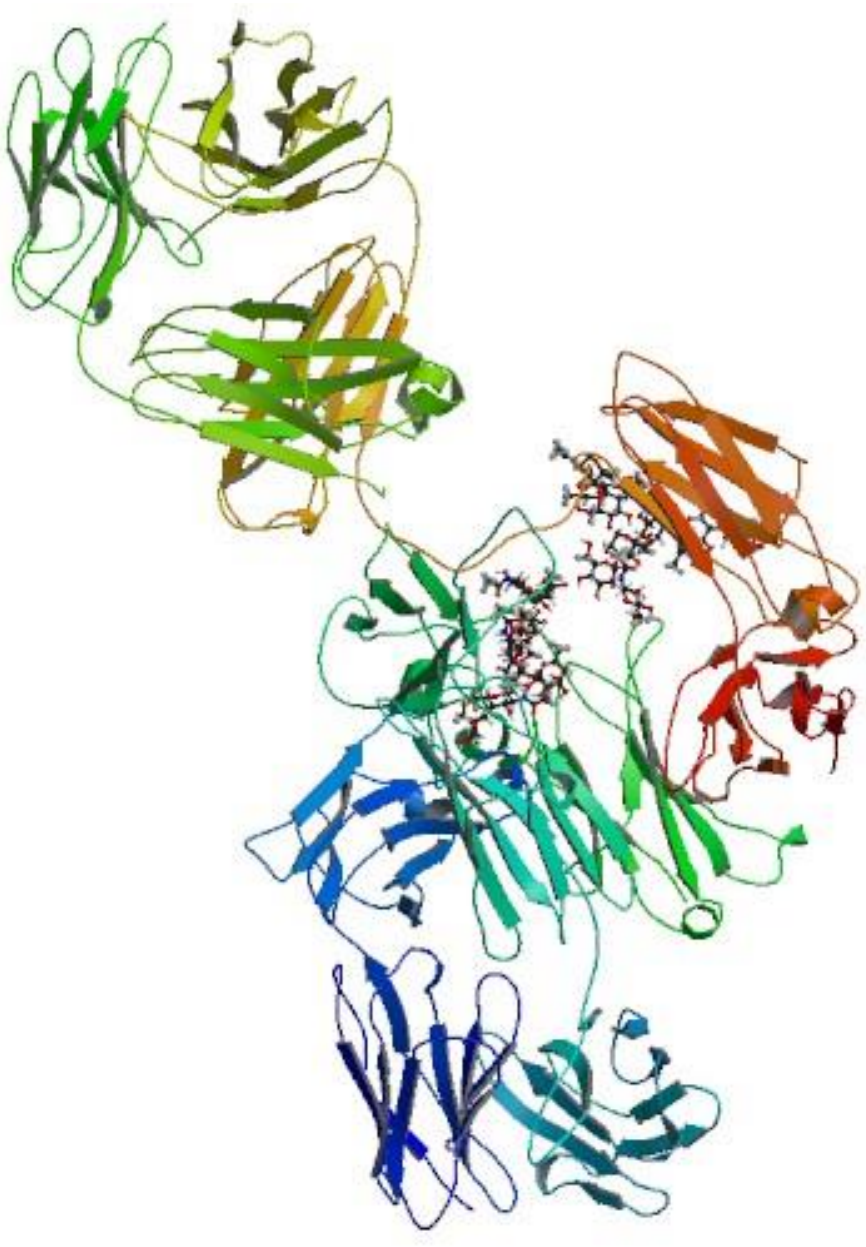


Figure 1 Molecular Simulation structure of Bevacinzumab (Wragg and Bicknell, 2013)

2.3 Downstream monoclonal antibody purification process

The industrial purification of mAbs in downstream manufacture has been well established through decades. From the early 1950s when chromatographic technology has not yet been aware, the mAb purification method was mostly relied on the multiple fractional precipitations combining with low pH treatment and filtrations. However, this method encountered issues such as low efficiency and low possibility of scalable processes which were remained unsolved. Until early 1990s, the improvement at separation media raised the discoveries of different types of chromatographic techniques that brought the protein downstream purification into a new level. Nowadays, a universal platform has been defined for a generic purification process, and this platform is capable of applying to many types of bio-pharmaceutical products (Kelley et al., 2008).

The concept of a development of mAb purification process is based on a three phase purification strategy that involves three major stages of capture, intermediate purification and polishing. The capture is the initial purification of target molecule from crude or clarified source material. At this stage, the target protein is expected to be isolated and also to be stabilised to conserve its activity. Therefore, the key issue is about the speed and volume that the target protein must be captured efficiently to minimise the loss of products. The second stage is the intermediate purification which is the further removal of bulk contaminants after capture. Since the isolated target protein from initial stage of purification has not yet met the applicable criteria of therapeutic usage, other impurities require to be separated including host cell protein (HCP), nucleic acids, endotoxins and viruses. The crucial factor at this stage is still to maintain the product purity and productivity. Usually the speed becomes less important at this stage because most of the protein proteases should have already been removed so that the activity of a protein could be maintained in a long time case. The final

stage of purification is the polishing. The objective of this stage is to remove trace contaminants from previous steps such as leachable ligands, endotoxins and viruses. Moreover, beside the end product requires being at high level of purity, it is also important to ensure the pH, salts or other additives at right conditions for product storage.

The current design of mAb purification process is based on a common sequence of unit operations which has been used by many pharmaceutical companies. At capture stage, the involved main techniques are centrifugation and affinity chromatography. The affinity chromatography in this stage offers great outcomes at capturing as well as purifying mAbs. This technology would help to simplify mAb purification process allowing a high product yield and purity either in capture or intermediate purification. At polishing stage, two types of chromatography are applied, followed by viral removal and finished with UF/DF (Ultrafiltration/Diafiltration).

2.4 Chromatography

As a main purification technique to most biotechnology industries, chromatography has achieved a great success for becoming a large part of downstream process in the manufacturing of many types of bio-pharmaceuticals. The mechanism of chromatography is to separate molecules in a two phase system, one stationary and the other mobile. The molecules with a high tendency to stay in stationary phase obtain higher retention time than those molecules which are more adapted to mobile phase. Therefore, the separation would occur based on the time that is taken by different molecules traveling through the system.

2.4.1 Affinity chromatography

Based on the property of stationary phase, chromatographic methods have been divided into several genres such as affinity chromatography, ion exchange chromatography, gel filtration

and hydrophobic interaction chromatography. Among all of these methods, the affinity chromatography with high selectivity, high resolution and usually high capacity for the proteins of interest attains a great favour in industrial purification of most recombinant proteins.

The mechanism behind is on the basis of a reversible interaction that normally a ligand couples specifically to only one type of proteins. This allows the purification at high efficiency, as well as the purity is also remained at high level. Comparing to other techniques, the affinity chromatography becomes ideal for a capture or intermediated step in a purification protocol(Healthcare, 2007b).

Introduction of Immunoglobulin affinity binding ligand: Staphylococcus Protein A (SpA)

Staphylococcus protein A (SpA) is a 42-kDa surface-anchoring peptide sequence which was originally discovered in the cell wall of Staphylococcus aureus, a common bacterial pathogen that causes skin infection, respiratory disease and food poisoning in humans (Graille et al., 2000). The application of SpA has been investigated as an immunological tool which binds the most of recombinant antibodies in purification process (Branco et al., 2012). Protein A has shown a great binding affinity to many types of Human Immunoglobulin including most classes of IgG, IgA, and IgM(Tashiro and Montelione, 1995). The structure of SpA was determined as an assembly of three helical bundles (Figure 2) which contains five tandem repeats of homologous immunoglobulin binding domains followed by a C-terminal constituent for cell wall binding and transmembrane (Tashiro and Montelione, 1995). These five domains have been well defined in a peptide sequence, each consists approximately 58 amino acid residues, and the domains were designated as Domain E, D, A, B and C (Figure 2) (Brown et al., 1998, Kato et al., 1993, Starovasnik et al., 1999, Tashiro and Montelione, 1995).

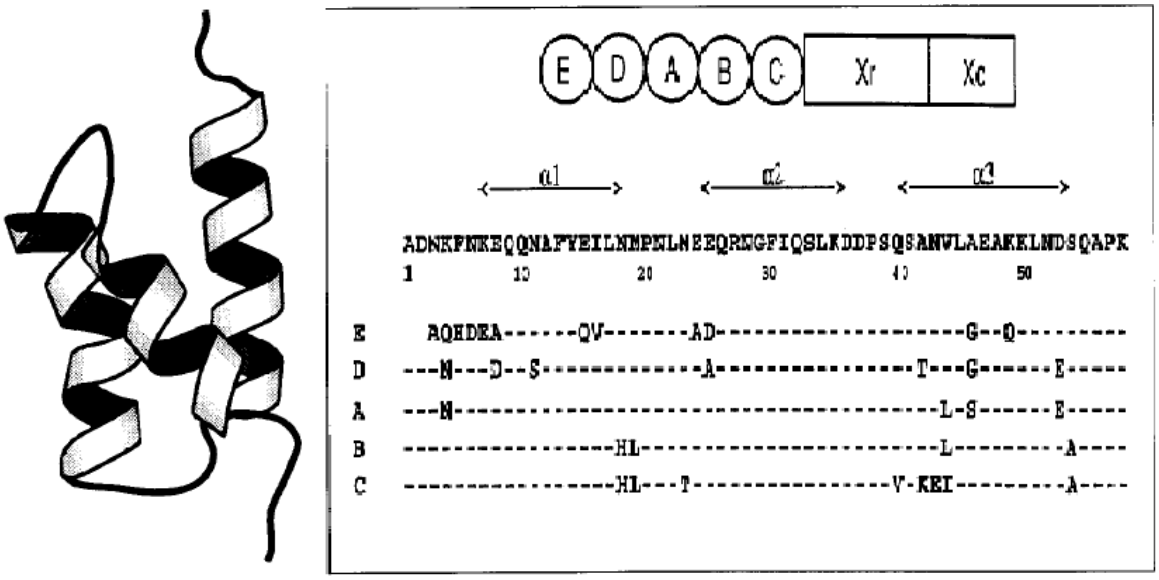


Figure 2 Molscripte ribbon diagram of SpA (left), Domain structure for SpA and sequence alignments of Immunoglobulin binding domains (right) (Tashiro and Montelione, 1995)

2.5 Protein-Ligand adsorption of SpA and Immunoglobulin

Binding of SpA and Immunoglobulin has been studied at macromolecular scale. An early studies by Moks et al. (1986) stated that the interaction between SpA and human Immunoglobulin G (IgG) took place at 2 functional binding sites of IgG and 5 functional binding sites of SpA. They studied the binding based on the five functional binding domains (E, D, A, B, C) of SpA, but associated binding residues at antibodies have not been clearly defined. A further study of Starovasnik et al. (1999) examined the binding isothermal of E domain of SpA in the presence of an excess of TNFR-IgG (containing two IgG1 Fc protein A binding sites) and hu4D5 Fab (containing one VH3 protein A binding domain). The results indicated that the interaction of SpA-IgG occurs at both Fab and Fc region of immunoglobulin. In addition, the affinity of SpA to Fc (with Ka> 10^7 M^{-1}) is significantly

larger than to Fab (with Ka = (2.0± 0.3)*10^5 M^{-1}), and both Fc and Fab involves distinct sets of binding residues that are non-competitive to each other.

2.5.1 Interaction of Immunoglobulin Fab region

The molecular docking structure between B domain of protein A and Fab region of Human IgG was established with the assistance of Molecular Dynamics (MD) simulation and Molecular docking criteria by Branco et al. (2012). By comparing the docking affinity of Domain B and D of SpA, the best binding result is generated between the last two a-Helices from each SpA domains and the hinge region of Fab heavy chains. The binding performance of different domains from SpA was described as resembling each other because of the similar sequence homology (about 77% similarities in peptide sequencing). Another experiment at the interaction between the SpA domain D and the Fab region of the VH3-30/1.9III- encoded 2A2 IgM rheumatoid factor was conducted by Graille et al. (2000). They defined the binding sites of SpA-Fab complex and the major interactions as the salt bridge and hydrogen bonds which contribute most to the formation of the complex. More importantly, their study has suggested that the binding residues of SpA to Fab and Fc region of Immunoglobulin are non-competitive and independent from each other. Their experiments on NMR have indicated that none of the residues that mediate the Fc interaction are involved in Fab binding expect Fln-32 (Graille et al., 2000)(Figure 3).

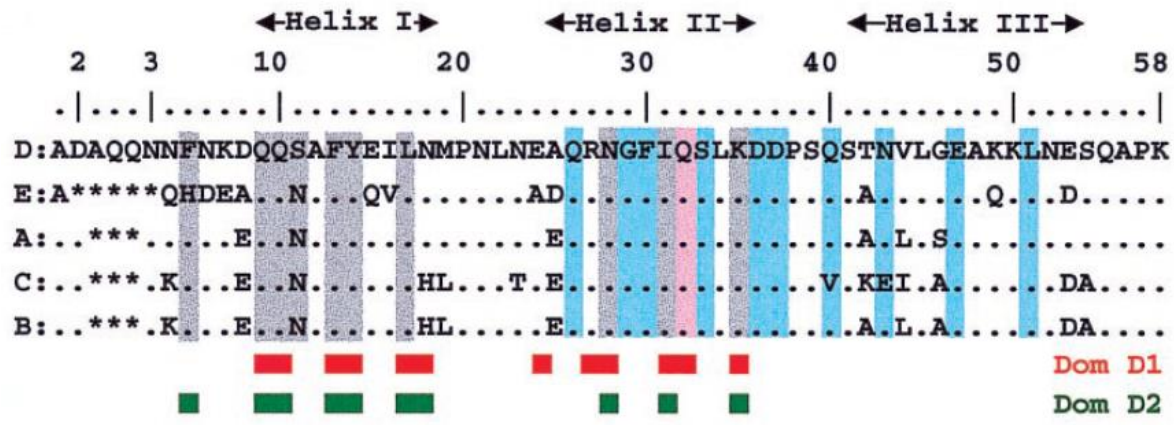


Figure 3 Interaction of individual SpA domains to Fab and Fc, residues involved involved in binding with Fab are highligted in Cyan, and Fc are highlighted in gray, Fln-32 is in pink (Graille et al., 2000)

2.5.2 Interaction of immunoglobulin Fc region

The Fc region of Immunoglobulin plays an important role in biotechnology as the major site of immunochemical adsorption for the study of identification and purification of monoclonal antibodies (Tashiro and Montelione, 1995). Technologies such as NMR and X-ray Crystallography have been applied to the analysis of the binding complex of SpA domain B and Fc (Tashiro and Montelione, 1995). A conformation variation may be involved in the initial binding mechanism and has been detected to be the unwinding or disordering of helix III of domain B according to the isotope shifting at Pro38 (Tashiro and Montelione, 1995). Moreover, the stability of interaction depends on whether Helix I and II stays at accurate orientation to remain the constant docking position for all of the binding residues (Tashiro and Montelione, 1995). Branco et al. (2012)indicated that few of the residues were discovered at the non-polar region closed to the hinge of CH_2 and CH_3 of Fc fragments of IgG, and three possible conformational clusters were characterised with binding energy almost the same(from -7.74 kcal/mol to -8.06 kcal/mol)(Figure 4).

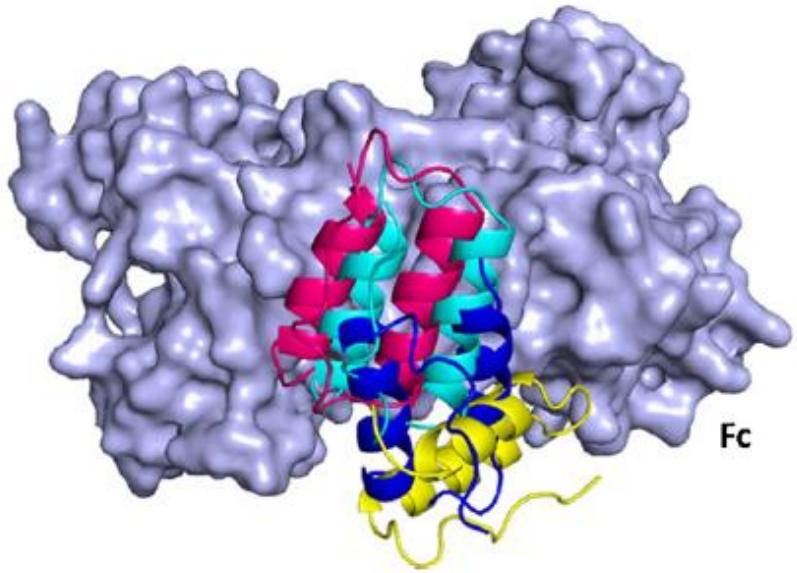


Figure 4 Three possible docking conformational clusters between B domain and Fc of IgG, coloured in magenta, yellow and dark blue respectively (Branco et al., 2012).

The binding region was discovered with the hydrophobic core surrounded and stabilised by a few polar residues (Branco et al., 2012).Another study by DeLano et al. (2000) involved a detailed investigation of the specific binding residues of Immunoglobulin complex. In their article, a vitro analysis was based on the X-ray crystal structure of IgG- Fc, and a consensus binding sites was identified according to the study of binding mechanism between natural IgG-Fc and several Fc peptide targets such as Fc-III, Domain C2 of Protein G, rheumatoid factor and Domain B1 of Protein A. As Figure 5 shows below, the interaction on the consensus binding sites was dominated by hydrophobic interaction, charge-charge interactions can be navigated at both top and bottom and the distribution of hydrogen bonds is at the centre of the hydrophobic core. Comparing to Consensus binding sites, Proein A domain B contains the majority of the common binding regions. Despite lacking salt-brigdes on His433, Arg255, Glu280 and Hydrogen bonds on Ser-254, the driving force still seems to depend on the hydrophobic interaction (Figure 5).

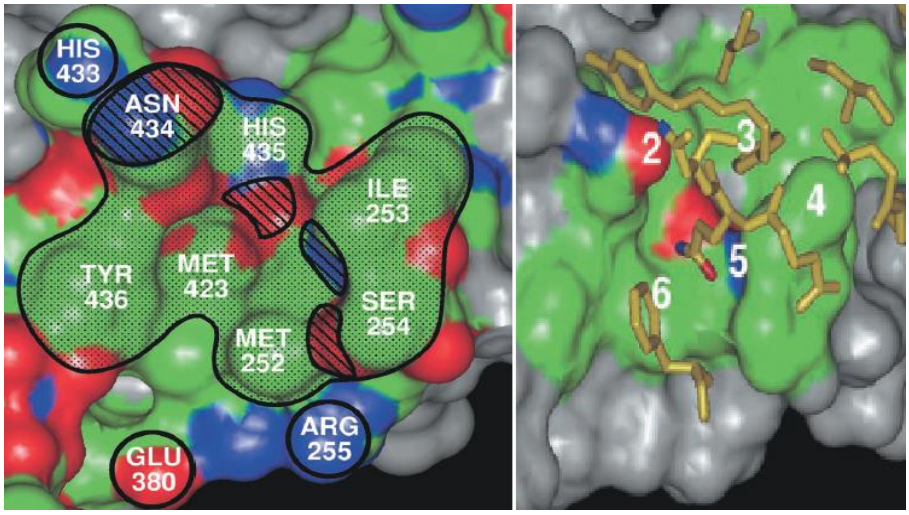


Figure 5 Consensus binding sites to Fc target, diagonal lines indicates the Hydrogen bonding sites, shaded area is for hydrophobic interaction, and circles are salt bridges (left). Protein A domain B binding sites to IgG, (2) (5) hydrogen bonding, (3) (4) (6) hydrophobic interaction (right) (DeLano et al., 2000)

2.6 Combinatorial SpA domain Z

Domian Z is an engineered analogue based on the domain B of SpA. The recombinant DNA technique is engaging to the creation in order to achieve a new DNA fragment which is constituted of a modified Domian B gene sequence (abbreviated Z). This new produced Z peptide is characterised as almost identical in the amino acids sequence as Domain B, except for one substitution at Helix II residue 29, Glycine was replaced by Alanine as shown in Figure 6 (Jansson et al., 1998).

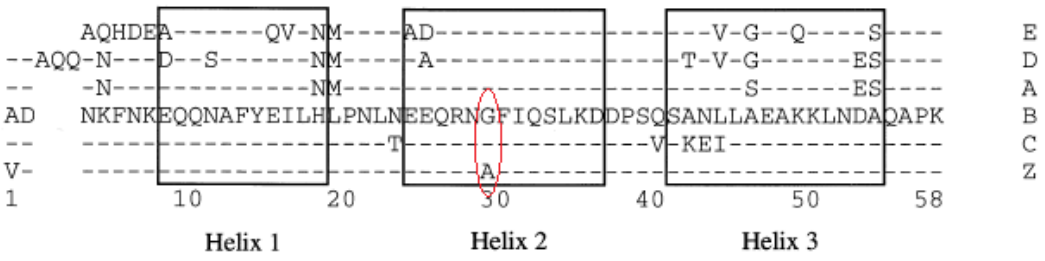


Figure 6 Peptide sequences of natural SpA domains (E, D, A, B, C) and domain Z. A dash (-) means excact amino acid sequence in comparing with B domain, and Red circle indicates the only change between B and Z domain (Jansson et al., 1998)

The application of this new combinatorial domain attempts to obtain specificity binding only to Fc of IgG. Therefore, a study by Jansson et al. (1998)analysed the binding affinity of all individual SpA domains including domain Z by the injections of recombinant human IgG1 Fc fragment (Fc1) and polyclonal $F(ab')^2$ separately (Jansson et al., 1998). This binding experiment was performed by a biosensor which contains the immobilised fusion protein of all SpA domains. The relative binding diagram of this experiment has distinctly revealed that domain Z has a negligible small affinity to the Fab binding other than the natural SpA domains (**Figure** 7).

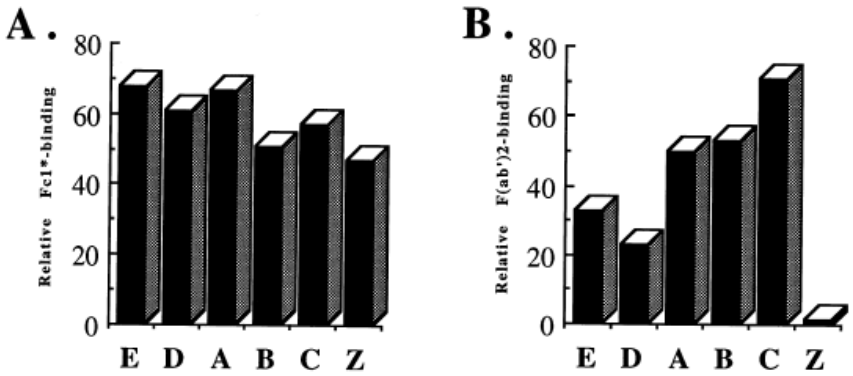


Figure 7 Relative binding activity of six SpA Fc domains (A) and human polyclonal F(ab') (B) (Jansson et al., 1998)

The appearance was deducted by Jansson et al. (1998)who showed that the variation of orientation is at the Helix 1 of domain Z which is suspected to cause a difference in the Fab binding, but the mutation at Position 29 of the Z peptide sequence.

2.7 Effects to the protein-ligand adsorption in chromatography

2.7.1 Ionic strength

The effect of ionic strength is mainly due to the alteration of salt concentration in mobile phase. The addition of salt can either enhance or reduce the binding based on the driven force towards the formation of protein-ligand bound structure(Yan and Huang, 2000). In detail explanation, if the protein-ligand adsorption is dominated by electrostatic interaction, then the binding affinity may be weakened by increase of ionic strength. A relevant study was given by Kondo et al. (1990), the adsorption capacity was measured of applying anti-P1 antibody in Sepharose 4B chromatography at various NaCl concentration (0.05-2.0 mol/kg). The experiment carried out the binding decreases by addition of NaCl. This was deduced that the electrostatic interaction plays a dominant factor in stabilising the bind, thus increasing the ionic strength may lower the affinity of antibody associated with corresponded ligand. In contrast, the binding would be enhanced by increase of ionic strength, if the driven force of adsorption is mainly hydrophobic interaction. This was supported by Zhao et al. (2013), they examined the adsorption of Berberine and immobilized ligand $\beta_2 - AR$ that the occurrence of binding is due to the hydrophobic interaction. By increase of NaCl (10.0 to 500uM) in mobile phase, the polarity of environment was enhanced leading the arisen of binding capacity.

2.7.2 pH

pH is another factor that impacts critically to protein-ligand adsorption. Several studies have pointed out that the pI (isoelectric point) value of either protein or ligand can be explained as the co-factor which directly relates to the pH effect. The Study of Gautam and Loh (2013) analysed human pIgR mimetic peptidic ligand to bind with human IgM by surface plasmon resonance (SPR) assay. The binding condition in this experiment was determined at various pHs (7.4, 8.0, 8.5). Since the isoelectric point of both IgM and ligand is around pH6, increase of pH at alkaline condition may result overall negatively charged IgM and pep14. In this case, the adsorption at higher pH condition decreases according to the force of repulsion of same charged protein and ligand.

2.7.3 Ligand spacer arm

The length of ligand spacer arm is a critical issue that determines the binding performance of any affinity matrix. The length of Spacer arm requires maintaining at certain range where overly short arm may hinder the docking of ligand to target protein and excessively long arm may fold or cross link with each other hence reducing ligand exposure(Fasoli et al., 2013). The spacer arm was also approved to be affected by system flow rate on ligand-protein adsorption. The given research of L-asparaginase interacting with Sepharose 4B at various system flow rates revealed that greater the flow rate applies in system, better the performance of the binding can be. The explanation to this occurrence was that the unfolding of the spacer arm at higher flow rate would enlarge ligand contacting surface and reduce the thickness of stagnant film on particles, thus target proteins could approach ligands more easily to achieve a better binding performance at this point (Martín del Valle and Galán, 2002).

2.7.4 Pore size of pack bed

The pore size of chromatographic matrix has been investigated by McCue et al. (2003) that their study carried out the effects of pore size at various chromatographic parameters including protein uptake, protein permeability and binding capacity. The experiments compared two sized adsorbents, 700 Å and 1000 Å, of protein A chromatography in purification of hIgG. The outcomes indicated that the smaller pore resulted in longer equilibration time, but larger dynamic binding capacity (DBC).

2.8 ITC study in Protein-ligand interaction

Application of Isothermal Titration Calorimetry (ITC) in study of protein-ligand adsorption

The Isothermal Titration Calorimetry (shortened as ITC) is a tool that offers direct measurement to heat related reactions in solution. This technology has been used for studies of the dynamic adsorptions of substances in both miro- and macro-molecular scales.

The ITC apparatus consists of two major components of an injection syringe at top and an enclosed adiabatic chamber at bottom. Inside the base chamber, two identical shaped cells, known as reference cell and sample cell, are built with highly efficient thermal conducing material. The adsorption measurement requires that the syringe containing a ligand is titrated into the cell filling with sample solution. Both of the titrant and solution in sample cell must be at same buffer condition, and this buffer needs to be applied into reference cell as a control. When a titration occurs, the heat generation or absorption inside sample cell would cause temperature variation between two cells. In this case, a feedback power will initiate to bring back the sample cell temperature until it equals to the reference cell. In addition, this differential power (DP) signals appears at large and sharped peaks at beginning of several injections. Since the heat release is proportional to the amount of binding occurs, the DP

signal would eventually fall off to that only the baseline could be observed when the binding reaches to saturation.

Numerous examples of protein interaction have been characterized by ITC measurement. According to the DP signals along the entire titration process, the amount of heat evolved or absorbed on addition of ligand are describe by Pierce et al. (1999)at the formula below.

$$Q = V_0 \Delta H_b [M]_t K_a [L]/(1 + K_a [L])$$

Where Ka is the associated constant, $[M]_t$ is the total concentration of proteins including bound and free fractions, ΔH_b is the enthalpy of binding (in per mole of ligand), V_0 is the cell volume and $[L]$ is the ligand concentration inside the sample cell. In their study, two types of monoclonal antibodies, MAb 2B5 and MAb 2B8, are interacted with the horse heart Cytochrome c (cyt c) in temperature gradient from 270K to 310K. The enthalpy shows a profoundly linear growth at the increasing of temperature (Figure 8), and the protonation/deprotonation was mentioned to be associated with the climbing of enthalpy.

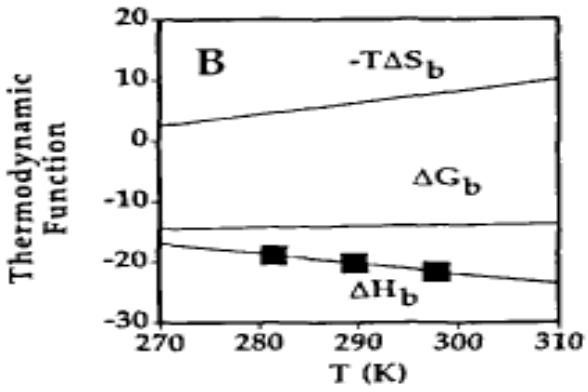


Figure 8 Thermodynamic parameters for the binding of CytC and mAb 5F8 at temperature gradient from 270K to 310K (Pierce et al., 1999)

An enhanced theory was given by Kun et al. (2009). In their study, the desolvation and the conformational change was found to be also relevant to the enthalpy variation besides the

deprotonation/protonation. BSA was applied to their experiments as the only reagent, and the analysis was mainly focused on the energy changes at the different protein concentration and pH. Figure 9 **(a)** below reveals the dissociation degree of BSA which is decreasing by increasing of BSA concentration (dissociation was triggered by titration of NaOH). A deduction was made that the desolvation and conformational change has dominated the alteration to the adsorption enthalpy, since conformational changes are induced by desolvation at the most of time. Figure 9 (b) indicates the net enthalpy of BSA dissociation at pH gradient. Two definite exothermic peaks, which are relevant to the two types of different conformational changes of BSA, could be observed in each curve. These two types of transformation generate the similar values as the change of enthalpy with a closed reading about 25 kJ/mol of all peaks from Figure 9 (b). Therefore, their study assumed the desolvation-conformational change has the major contribution to the alternation of net enthalpy at variable pH and protein concentration rather than the protonation/deprotonation(Kun et al., 2009). In addition, the focus of their experiment was only for the characterising of protein self-assembling, the adsorption among different proteins hasn't been studied much.

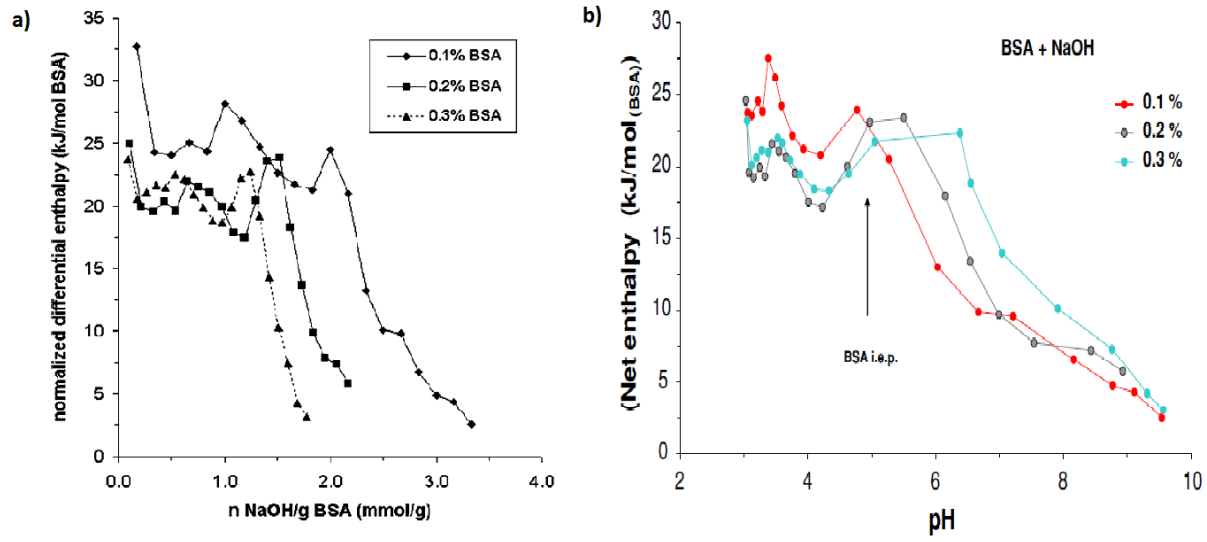


Figure 9 a) The net enthalpy changes of 0.1%, 0.2% and 0.3% BSA at dissociation by the adddition of NaOH, b) the net enthalpy changes at the dissociation as the function of pH (Kun et al., 2009)

Tsai et al. (2002) dissected the adsorption mechanism by study of the interaction between myoglobin and butyl-, octyl-Sepharose hydrophobic adsorbents. ITC was applied to measure the adsorption enthalpies at the increment of salt concentration of $(NH_4)_2SO_4$. In Figure 10 below, the ΔH_{ads} of myoglobin binding to both butyl- and octyl-Sepharose shows a reducing tendency at increment of salt concentration. The heat requirement for dehydration is reduced by increasing of salt concentration. As salt concentration reaching to a certain level (1.0 M $(NH_4)_2SO_4$ on figure), a negative value of ΔH_{ads} is achieved. This indicates the entropy change which may have a strong significance to the adsorption, thus compensates the decreasing of enthalpy at high salt concentration(Tsai et al., 2002).

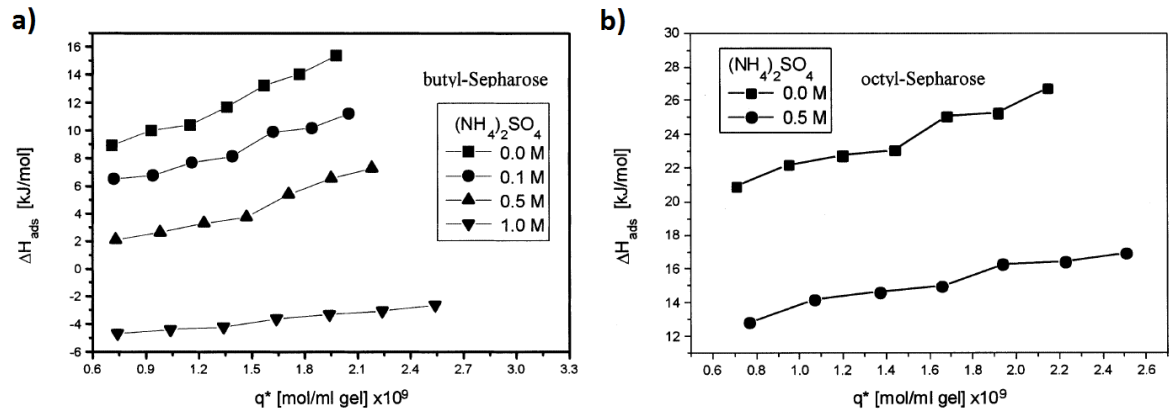


Figure 10 The adsorption of enthalpy (ΔHads) of myoglobin with a) butyl-Sepharose b) octyl-Sepharose at various (NH4)2SO4 concentrations (Tsai et al., 2002)

In addition, a concept of bound protein was introduced to the analysis of adsorption energy change (Tsai et al., 2002). The amount of Bound protein would grow with increment of protein concentration. In this experiment, the ΔH_{ads} has shown a close dependence to the change of protein concentrations. This may due to the growth of the bound protein which enhances the protein-protein interaction. Increasing of the bound protein reduces the distance and triggers the intermolecular interactions among adsorbed molecules, so that the additional energy will be required for overcoming the negative interactions among proteins, such as electrostatic repulsion and steric hindrance, which are unfavourable to the adsorption(Tsai et al., 2002).

Chapter 3 Isothermal Titration Calorimetry Study on BmAb-ligand Interactions

3.1 Introduction

The affinity chromatography is often used as the initial capture stage in industrial purification of monoclonal antibodies (Hober et al., 2007, Schwartz et al., 2001, Sisodiya et al., 2012). The main purpose at this stage is to remove major contaminants including host cell debris, DNA, antibody variants and other minute potential contaminants such as viral particles and endotoxins in order to achieve rapidly isolated and highly concentrated target proteins (Biosciences, 2001, Sisodiya et al., 2012). Affinity chromatography with Staphylococcus Protein A (SpA) based medium, designated as Protein A chromatography, is a traditional method for purification of recombinant monoclonal antibodies (mAbs) due to an early discovery of the specific interaction between Protein A with the immunoglobulin Fc region (Salvalaglio et al., 2009, Xia et al., 2014). Meanwhile, an alternative resin was introduced as a recombinant Protein A ligand, MabSelectSuRe, with greater specificity, binding affinity and stronger alkaline resistance (Hahn et al., 2006, Ishihara et al., 2010). This ligand consists of five identical three-helix bundles, and Z-domains which are the modified domain B of the natural SpA in order to achieve enhanced binding affinity and conformational stability at a high pH condition during protein purification (Xia et al., 2014). However, due to high expense of both the natural and the recombinant Protein A media, it is very imperative to maximize the efficiency of these resins when they are employed for industrial mAbs purification (Pakiman et al., 2012). Other issues such as product stability, dynamic binding capacity (DBC) and processing speed are also concerned at the early stage of purification process (Biosciences, 2001, FDA, 1994).

The binding mechanism of Protein A ligands and mAbs is not fully understood. The interaction is often interpreted as a unique “key and lock” docking process in which the ligand specifically targets certain binding sites of mAbs. Despite the fact that this interaction process is relatively stable and not easy to be disintegrated, there are process aspects, such as solvent ionic strength and environmental temperature, which may affect the binding efficiency. Therefore, investigation of above aspects would allow an in-depth understanding of the mechanism of interactions between ligands and mAbs, thus it assists in the improvement of purification outcomes. In this study, a specific technology, isothermal titration calorimetry (ITC), an assay with a high accuracy in the measurement of energy changes for different types of protein interactions, were employed for the study of mAb - ligand adsorption.

Two types commercial mAb binding affinity ligands, natural Staphylococcus Protein A (nSpA) and MabSelectSuReTM (MS) ligand, were chosen to interact with a monoclonal antibody, Bevacizumab (BmAb) for the isothermal titration study. The study investigates the effect of external operational conditions on the binding affinity, for example, temperature, solvent salt concentration, and pH. The binding mechanism was probed in terms of binding sites and binding conformational changes at the primary and quaternary structure level of the ligand-antibody complexes.

3.2 Material and methods

3.2.1 Chemicals and reagents

The Bevacinezumab (BmAb) was donated by Hospira Inc., Adelaide. The natural Staphylococcus Protein A was obtained from Sigma, and the MabSelectSuRe ligand was from GE Healthcare. The nSpA was lyophilized and kept in a vacuum condition, and the

MabSelect ligand was dissolved in a buffer of 20 mM potassium phosphate, 50 mM sodium chloride and 2 mM EDTA at pH 7.0.

3.2.2 Buffer exchange and protein concentration determination

The buffer exchange was carried out to ensure both the ligand and the BmAb at the same solvent condition. In this case, a complete dialysis was required prior to the start of ITC measurements. The lyophilized natural SpA lignad was dissolved in 50 mM potassium phosphate and 100 mM sodium chloride. The concentration of BmAb and the ligand was determined by using the Quick StartTM Bradford protein assay provided by BIO-RAD. The protein solution was initially diluted in the Commassie Brilliant Blue G-250 and its absorbance was read at 280 nm wavelength with a UV-2600 Shimadzu spectrometer.

After measurements of the protein concentration, either the natural SpA ligand or the MabSelectSuRe ligand was mixed with BmAb solution in the same buffer for dialysis. The dialysis took approximately 2 day and the concentration of both ligands and BmAb was measured again to prevent any failure in buffer exchange which may be caused by membrane leakage.

3.2.3 ITC analysis

The isothermal titration calorimetry analysis was performed on a VP-ITC micro-calorimeter from Microcal TM, INC (Northampton, MA). A 1.4 mL sample cell was filled with the BmAb solution diluted to 1.4 uM, and an injection syringe was filled with 250 uL of 7.5 uM natural SpA or MabSelectSuRe ligand solution.

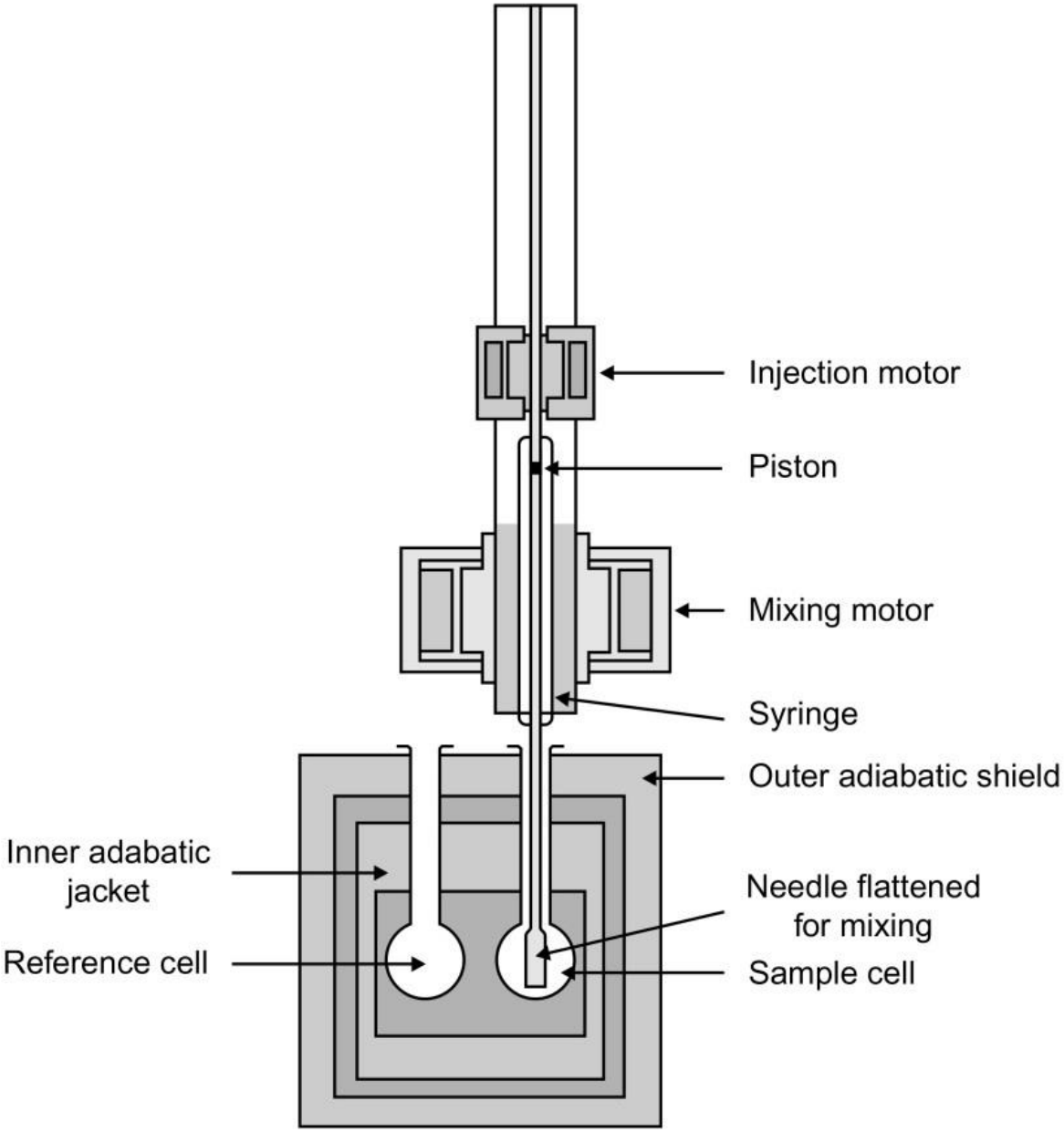


Figure 11 A typical Isothermal Titration Calorimeter (Pierce et al., 1999)

All samples were degassed for 10 min to maintain a steady base line. The initial delay was determined to be 60 s and the stirring speed was set at 307 RPM for continuous mixing. A single titration was performed with 25 injections of 10.0 uL. Each injection lasts 30 s and the duration between two consecutive injections was fixed at 300 s to ensure a complete reaction before next injection. This duration also allows the temperature to maintain at the pre-set

reaction temperature. The temperature control was realised through heating or cooling of the cell jacket until ± 0.5 °C of the desired temperature was reached.

The data analysis was performed at Origin 7® where modules were provided in the default data library to allow the most appropriate linear regression to be fitted for the measured data. The blank titration as a control between the pure solvent and the BmAb solution was subtracted from the experimental data for the titration between the ligand and the BmAb to obtain accurate protein-ligand interaction parameters. The first injection was excluded from data analysis due to a volumetric division from the backlash of the motorized screw in the ITC syringe plunger.

3.3 Results and discussion

3.3.1 The ITC assay

To maintain a consistent and precise titration performance, the solution of the BmAb and the ligand was dialyzed with the same buffer solvent prior to any measurement. This minimizes the extra energy generation caused by solvent differences during titration. When an injection initiates a titration, the heat evolution within the sample cell may generate a negative differential power (DP) signal. The signal reaches its highest absolute value at the beginning of a titration due to the start of the reaction between the antibody and the ligand. When the reaction approaches to completion, the DP signal gradually diminishes to the background level. The DP signal evolution for a typical titration is shown in Figure 12 (a).

The binding thermodynamic parameters were obtained from a titration curve shown in Figure 12 (b), an isothermal plot of the heat generated by each injection against the molar ratio of the ligand to the antibody through the titration process. The titration curve was used for an accurate determination of the reaction stoichiometry. In **Figure** 12, the enthalpy change (ΔH)

is obtained from the heat difference between the initial and the saturated state of a titration process. The associated constant (K_a) is determined from the slope and the binding coefficient (n) is the molar ratio at the half saturated state of the plot. To calculate K_a, a linear regression is performed to fit with the isothermal plot. A one-binding-site model is used for the plot fitting. The K_a is expressed as the equation below:

$$K_a = \frac{\Theta}{(1-\Theta)[X]} \qquad (1)$$

Where Θ is the fraction of sites occupied by the ligand, and [X] is the free concentration of the ligand in the solution.

The overall binding energy (ΔG) and the entropy change (ΔS) for the ligand-mAb adsorption are calculated based on the Van't Hoff equation below.

$$lnK_d = ln(\frac{1}{K_a}) = \frac{\Delta H^0}{RT} - \frac{\Delta S^0}{R} = \frac{\Delta G^0}{RT} \qquad (2)$$

where the K_d is the dissociation constant, R is the gas constant, and T is the temperature in a unit of Kelvin.

Binding energy is associated by Entropy and Enthalpy working together. In this particular situation, Enthalpy is related with electrostatic interaction such as hydrogen bond or Van der Waals interaction. The occurrence of these interaction generates energy which normally results in a negative enthalpy change. However, binding unfolds target protein and ligand leading a negative change in Entropy. Since protein is reluctant to be unfolded, this negative change of Entropy acts against binding. Overall, to achieve spontaneous binding interaction, electrostatic interaction must overcome protein rigidness in order to unfold protein and to expose inner hydrophobic core of protein and ligand for binding.

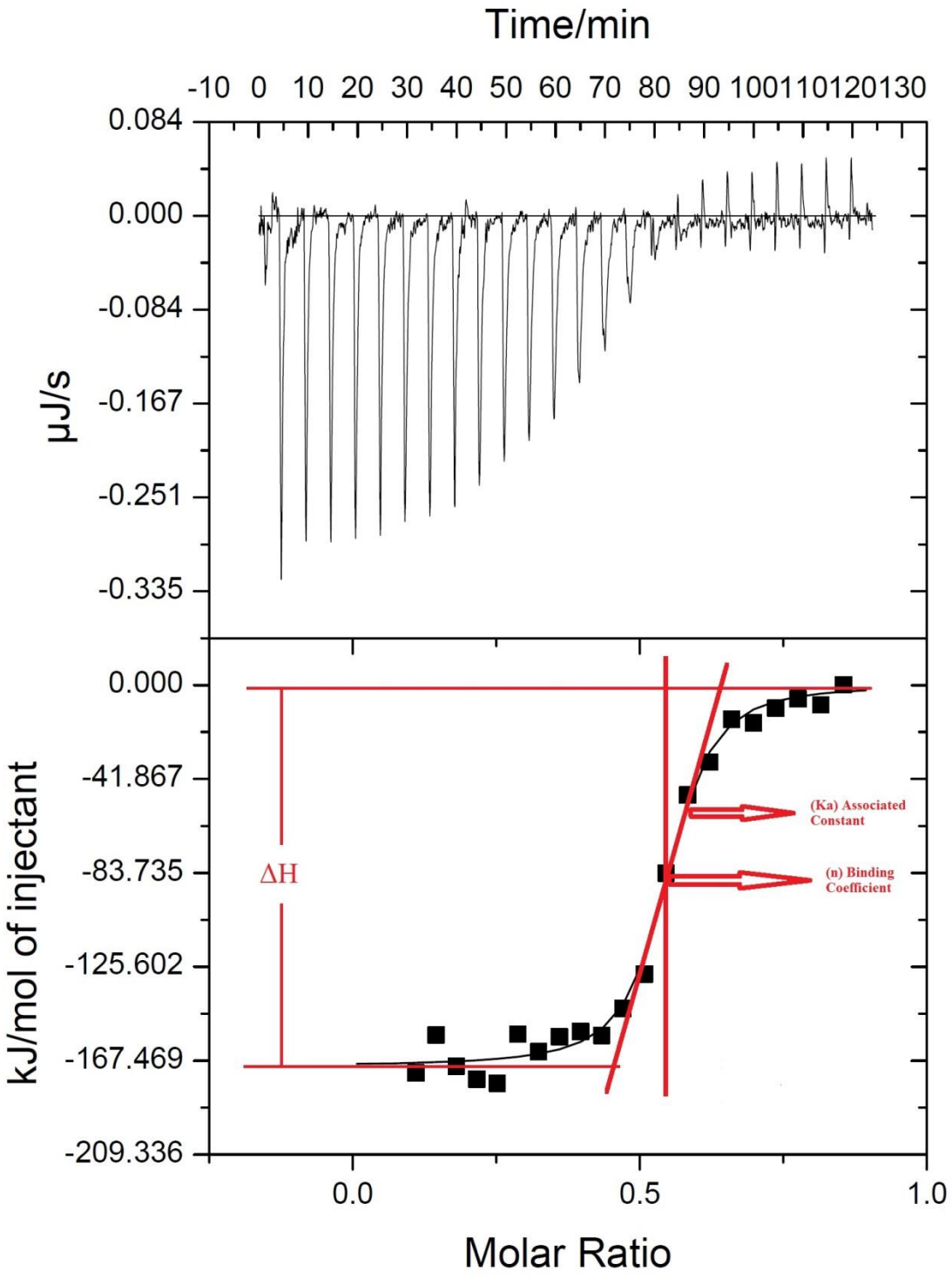


Figure 12 A typical thermogram (top) and the binding isotherm curve (bottom) for the interaction between the native Protein A and Bevacinzumab.

3.3.2 Effect of temperature

Both ligands, natural SpA (nSpA) and MabSelectSuRe (MS), were examined to interact with BmAb by batch isothermal titrations at 15 °C, 20 °C and 25 °C. The key thermodynamic parameters were derived from the isothermal titration curves based on the method in the previous section and they are presented in Figure 13 and 14. It can be seen that the associated constant K_a for the nSpA ligand appears a gradual decrease as the temperature increases. The overall binding energy (ΔG) calculated from ln(K_d) for three temperatures is presented in Figure 13 (b) and all ΔG value are negative at all temperatures. The overall binding energy presents a slight increase with the increment in the temperature. The thermo-parameters of enthalpy and entropy have in a similar tendency as both parameters decrease along the temperature increases as shown in Figure 14 (4). ΔH in this experiment appears in negative values, and the negative enthalpy results from the contribution of electrostatic interactions such as hydrogen bonding and van der waals interaction between the ligand and the mAb. Corresponding to the negative enthalpy, an exothermic reaction occurs with enhanced binding affinity when the environmental temperature is low. The negative entropy presented in Figure 14 (b) is unfavourable towards the formation of the ligand-BmAb binding complex at all temperatures. Since the binding experiences non-spontaneous conformational changes, additional energy is required for the occurrence of binding reaction (Dam et al., 2008). ΔG can also be calculated from entropy and enthalpy from the Eq 2. Although ΔH decreases as the temperature drops, ΔG remains relatively constant due to less negative TΔS which overcomes the loss of ΔH. It can be noticed that the binding occurs spontaneously within the experimental temperature range. In relation to the above interpretations of ΔH and TΔS, the negative binding energy can also be explained in conformational change level. The adsorption is dominated by the electrostatic interaction, and this electrostatic interaction compensates unfavourable conformational changes, resulting in spontaneous formation of the ligand-BmAb binding complex at the experimental temperatures.

The temperature impact on the binding between the MS ligand and BmAb is not significantly, which was evidenced by the change of the binding constant (K_a = 1.36E9 ~ 2.87E9 M^{-1}) within the experimental temperatures. It can be seen from Figure 15 (b) that the binding affinity remains almost constant as the temperature changes, thus the binding affinity of the MS ligand towards BmAb shows a low dependence on the temperature within the experimental range. The overall ΔG decreases as the temperature increases. Changes in enthalpy (ΔH) and entropy (TΔS) are shown in Figure 14 (a) and (b) respectively. Similar to the binding between the nSpA and BmAb, both negative ΔH and TΔS represent favourable and unfavourable interactions of the binding respectively. In order to overcome unfavourable conformational changes during formation of the binding complex, favourable interactions such as electrostatic attraction become dominant to allow spontaneous binding. When temperature drops from 25 °C to 15 °C, both TΔS and ΔH become less negative, which means less conformational changes and weaker electrostatic attractions. a growing favour of hydrophobic interaction also appears to binding so that the binding affinity (Ka) and overall energy (ΔG) can be eventually maintained.

Comparing the binding ability of the MS ligand and the nSpA ligand with BmAb, the K_a value for the nSpA is 10 times less than that of the MS ligand, which indicates that the binding affinity of the MS ligand to BmAb is approximately 10 folds higher than that of the nSpA ligand. The MS ligand as a type of the recombinant Protein A ligand has been successfully optimised to achieve a high selectivity to the selected mAbs. In this experiment, when the temperature arises, the binding affinity K_a for the nSpA moderately decreases while the K_a for the MS ligand remains nearly constant. Therefore, temperature has a negligible impact on the MS ligand for its binding with BmAb. Both electrostatic interactions and unfavourable repulsions due to conformational rigidness, denoted by negative ΔH and TΔS respetively, exist in the formation of the ligand-BmAb complex. While binding is dominated

by favourable electrostatic interactions that counteract unfavourable repulsions. As the temperature rises from 15 °C to 25 °C, both ΔH and TΔS share very similar decreasing slopes for both ligands. Since the binding affnity Ka of the MS ligand is far higher than that of the nSpA ligand, binding between the MS and BmAb is so strong that temperature has a negligible impact on the binding within the experimental temperatures. However, the binding between the nSpA and BmAb is less stable, and is highly dependent on the operating temperature.

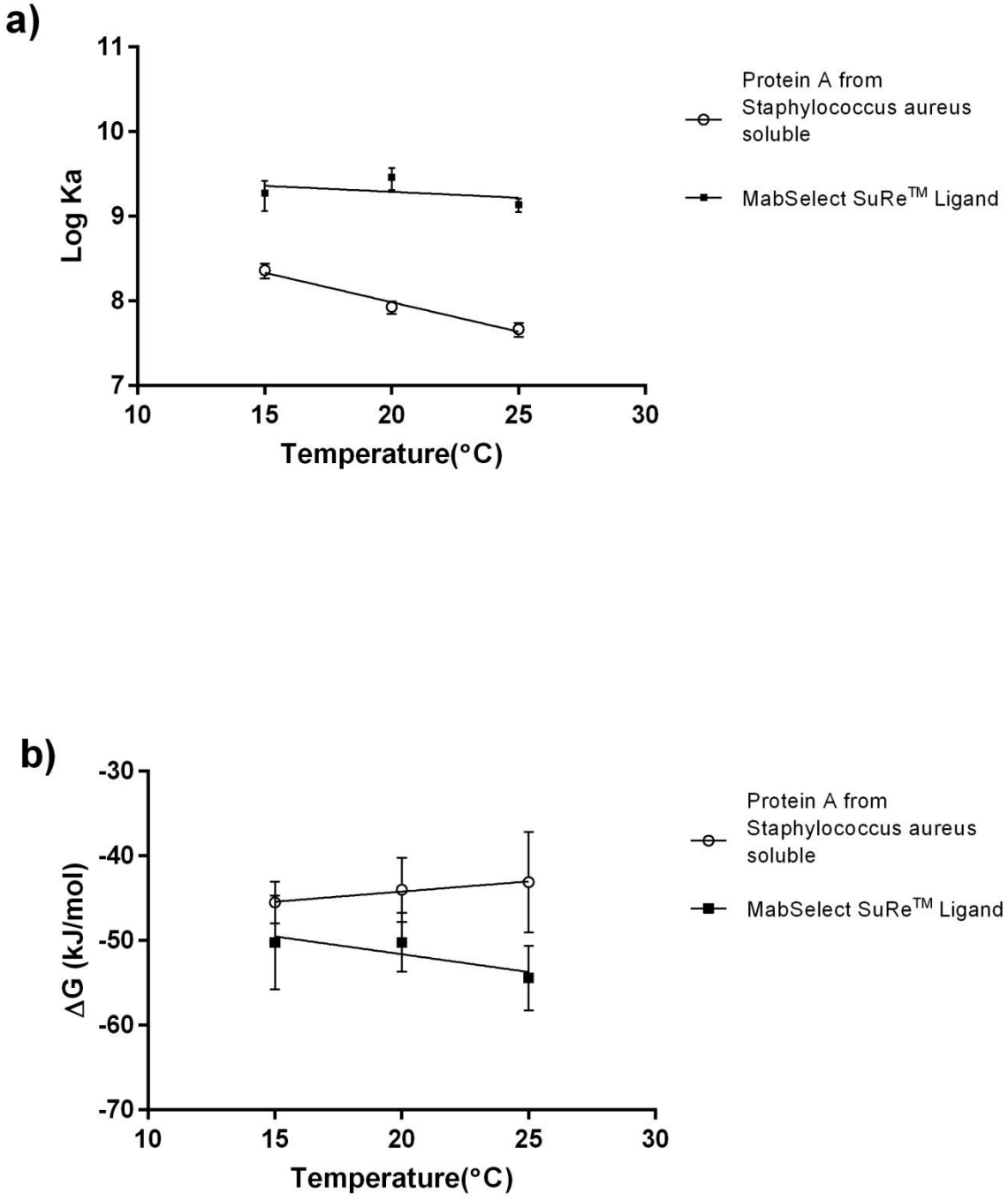


Figure 13 Effect of binding temperature on thermo-parameters of (a) LogKa and (b) ΔG which are derived from the isothermal titration curves of the affinity ligand and BmAb.

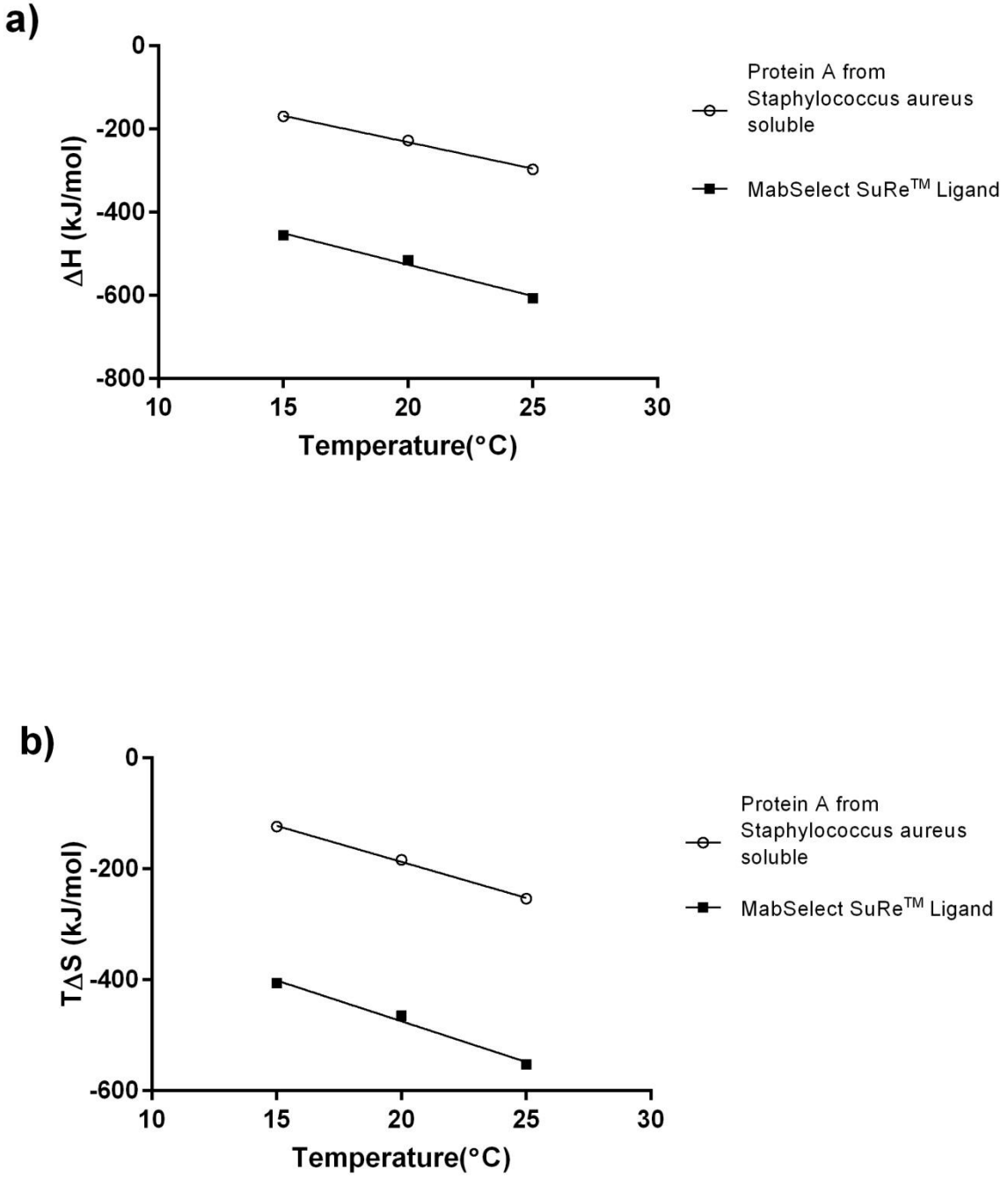


Figure 14 Effect of binding temperature on thermo-parameters of (a) ΔH and (b) TΔS which are derived from the isothermal titration curves of the affinity ligand and BmAb.

3.3.3 Effect of ionic strength

To investigate the solvent effect of solvent on both the intrinsic binding, the binding between ligands and mAbs, and the disolvation of a bounded or unbounded system, the interaction between the proteins and their surrounding buffer (Chervenak and Toone, 1994, Lund et al., 2011), the ionic strength of the solvent was examined by varying the sodium chloride salt concentration in the buffer of the ligand and BmAb mixture. The concentration of sodium chloride was determined to be 100 mM, 500 mM and 1000 mM for the titration. Temperature, pH and the phosphate buffer concentration were kept to be constant during experiments.

The K_a of the nSpA ligand from Figure 15 (a) responds in an incremental trend to a growing salt concentration, and this trend means that the binding affinity of the nSpA to BmAb is highly dependent on the solvent ionic strength. While the overall binding energy ΔG decreases as the salt concentration increases as shown in Figure 15 (b). Both ΔH and $T\Delta S$ are negative and the absolute value for both ΔH and $T\Delta S$ decreases nonlinearly as the NaCl concentration in the buffer solution increases (Figure 16). A reduction in the absolute value of enthalpy with an increase in the salt concentration may result from the loss of hydrogen bonding and/or electrostatic interactions at binding residues, which may be due to occupancy of some charged binding residues between the ligand and BmAb by salt molecules. Meanwhile salt addition decreases in the absolute value of $T\Delta S$, or the unfavourable entropy, indicating that the addition of salt raises the role of the hydrophobic interaction in participating at binding. It was noted that the salt molecule increases the surface tension of the solution in order to expose the inner hydrophobic region of the protein to the surrounding solvent (Chen et al., 2007, Melander et al., 1989, Shibata and Lenhoff, 1992). Therefore, the unfolding of either the ligand or BmAb may be promoted at a higher salt concentration and

the protein conformation change during unfolding process leads to favourable binding between the nSpA and BmAb.

The binding affinity, ΔG, as well as $T\Delta S$ and ΔH for the MS ligand, however, have a minor change during the change in the salt concentration. Therefore, the impact due to salt addition on the electrostatic or hydrophobic interactions during the binding between the MS ligand and BmAb is not significantly. DeLano et al. (2000) reported that the SpA binding domain B contains the highest affinity residues to most mAbs. The MS ligand has a modified head to tail connected SpA domain B, and its binding to mAbs exhibits a much greater affinity than the native SpA ligand. Therefore, the binding between the MS ligand and BmAb is not be greatly altered by salt addition.

In comparison of two ligands, the increase in the solvent ionic strength by salt addition enhances the binding affinity of the nSpA to BmAb. However, when the same solvent condition is applied to the MS ligand, the solvent ionic strength has a negligible impact on its binding to BmAb. The present of salt in the buffer solvent increases the ligand-mAb interaction only if the ligand binding affinity is weak. For a relatively tight binding such as the MS-mAb binding, the ionic strength effect on the binding is negligible.

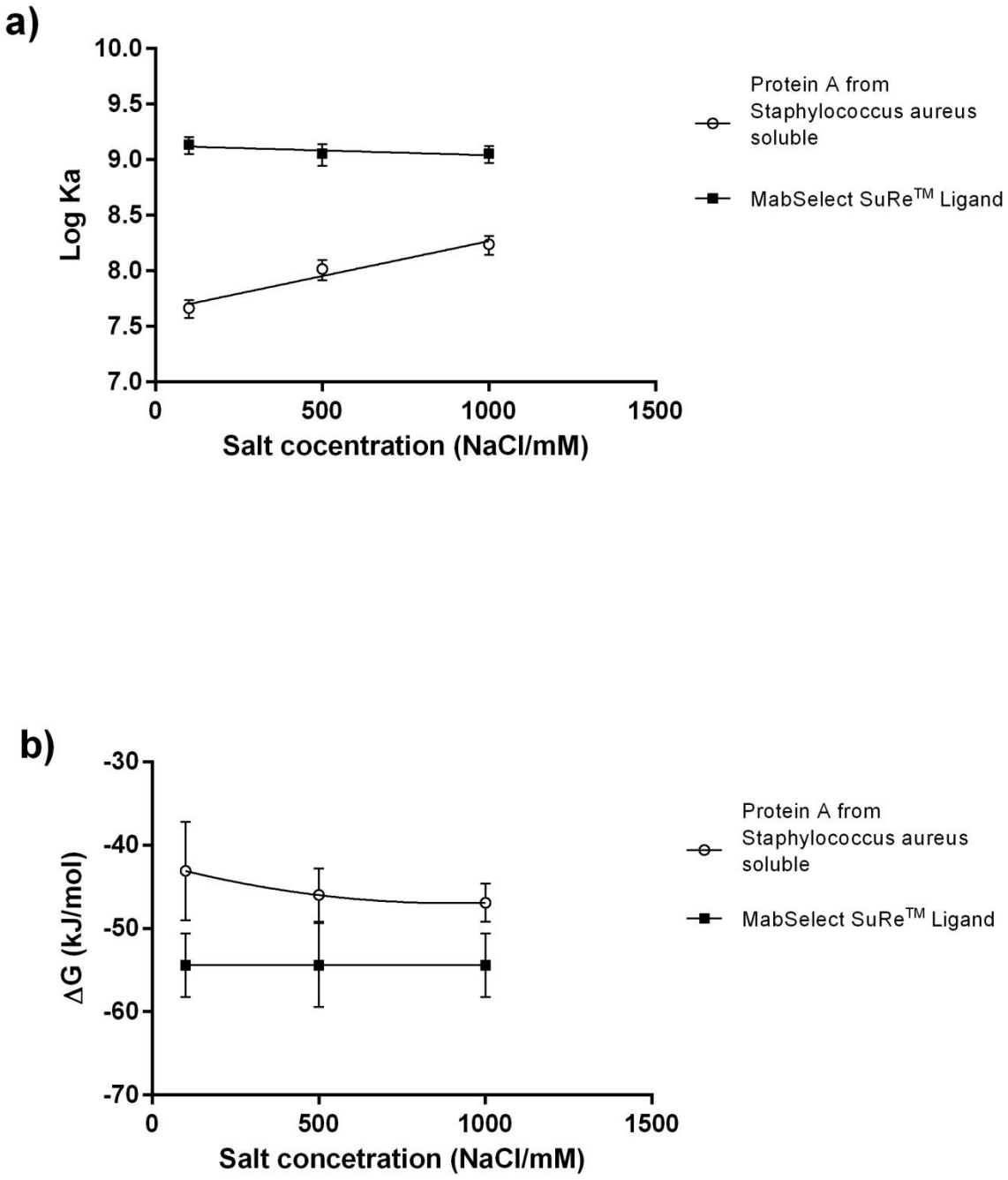
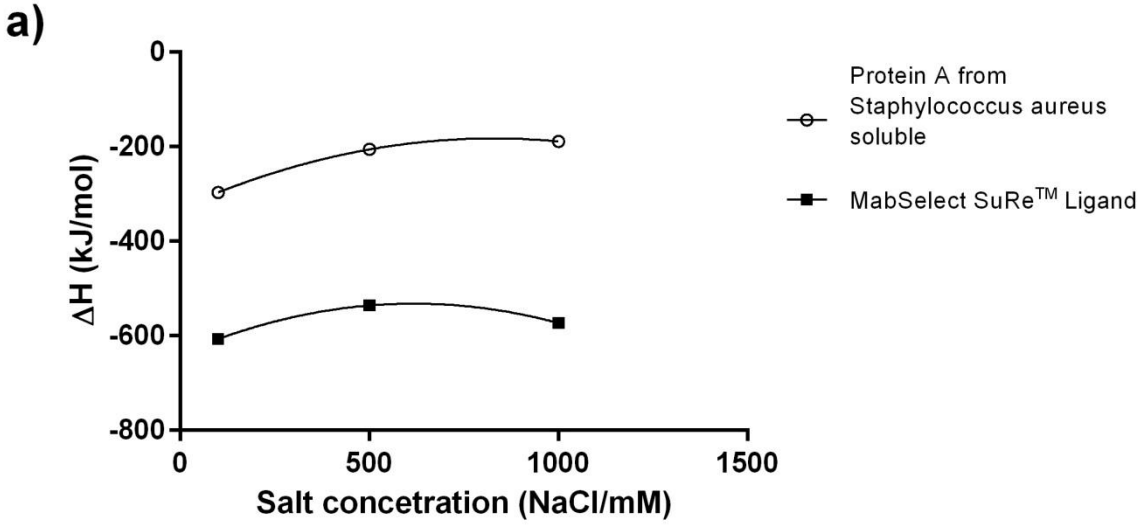


Figure 15 Effect of ionic strength in binding solution to thermo-parameters (a) LogKa and (b) ΔG K and ΔG were derived from the isothermal titration curves of Protein A and BmAb as affinity ligand



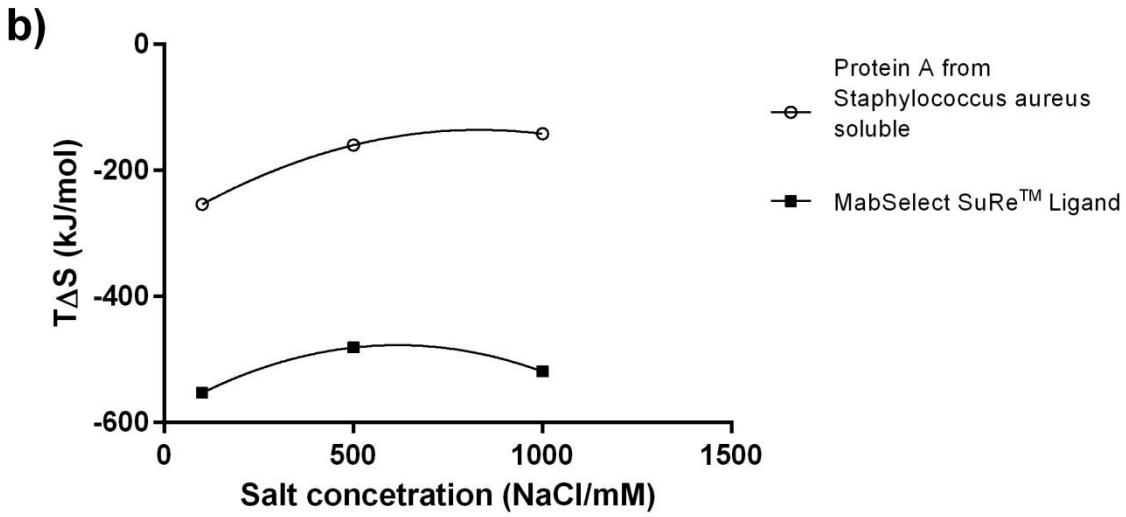


Figure 16 Effect of ionic strength in binding solution to thermo-parameters (a) ΔH and (b) TΔS, ΔH and ΔS were derived from the isothermal titration curves of Protein A and BmAb as affinity ligand

3.3.4 Effect of pH

pH is a crucial factor for the mAb-ligand interaction in affinity chromatography since the gradient alternation of pH in the mobile phase is often applied in the elution stage. The pH effect on the binding was determined at an acidic condition between pH of 4 and 7.

For the nSpA ligand, no response was observed during the titration at pH 4 and the signal for each injection fluctuated around the baseline. This indicates that the binding between the nPsA and BmAb may not occur at pH 4 or very weak binding is formed. Between pH 5 and pH 7, the responses of the K_a value to different pHs appear a convex shape and the value reaches the highest around pH 6 (Figure 17). This reveals that the strongest binding occurs under a slightly acidic condition, not under an exact neutral condition. The changes in enthalpy (ΔH) and entropy ($T\Delta S$) at different pHs are presented in Figure 18 (a) and (b) respectively, the adsorption is mainly the enthalpy dominated. By lowering the pH, the decrease in ΔH in it absolute value could reflect loss of electrostatic interactions during the binding process, or loss of the opposite charged binding residues between BmAb and the nSpA at a the low pH. Since the isoelectric point (pI) of the nSpA and BmAb is 5.1 and 8.1~8.5 respectively (Siegel, 2002, Xia et al., 2014), the overall charge of the nSpA-BmAb complex can be determined at different pH values. At a neutral pH, the ligand and BmAb have the opposite charges and the binding between them can be realised through electrostatic attractions. However, when pH decreases, the net charge of the nSpA becomes less negative and BmAb starts to exhibit a negative charge as well. The electrostatic attraction becomes weakened, which leads to loss of binding affinity. The changes in $T\Delta S$ suggest that the binding is more favourable at a low pH. When the ligand and BmAb denature at a lower pH, the proteins lose their conformational rigidness and start to unfold their structures. Therefore, the loss of conformational rigidness results in the change of entropy becoming much less in

the contribution of protein and ligand association. Since the overall binding free energy almost maintained along pH changes, the binding can be explained moving towards to more enthalpy favourable interaction when pH drops. When the condition becomes too harsh at a pH below 4, the net charge for both the ligand and BmAb becomes positive. Therefore, protein unfolding and no or very weak electrostatic interaction lead to no binding between the nSpA and BmAb at pH of 4 and below.

Similar to the nSpA ligand, the interaction between the MS ligand and BmAb is also highly dependent on the buffer pH where Ka decreases from 1.36×10^{9} M^{-1} to 9.61×10^{6} M^{-1} when the pH drops from 7 to 4 (Figure 17). Likewise, the absolute value of ΔH and $T\Delta S$ display a similar decreasing tendency when pH drops. At a low pH, the polar interaction decreases, and the protein conformation becomes more favourable for binding.

Comparing the binding performance of the nSpA and MS ligand with Bmab at different pHs, one distinctive difference is found at pH 4. No binding is observed between the nSpA ligand and BmAb, while binding is still moderately strong between BmAb and the MS ligand since the Ka for the MS ligand is much larger than the nSpA ligand. For the MS ligand, a harsher elution condition is required to release the BmAb from the MS-BmAb complex, unfortunately, this may lead to denaturisation of BmAb. Furthermore, the highest binding affinity is achieved at pH 6 to 7 for both the MS and nSpA ligand. The thermodynamic parameters for both ligands share the similar trend and they decreases in their absolute values as pH decreases, leading to loss of the specific binding. In addition, the protein denaturation for both ligands and Bmab could result in failure of binding because the denatured protein encounters a conformational alternation, and the structure for the ligand or the mAb is not applicable for the "key and lock" adaption of binding. Branco et al. (2012) applied the molecular dynamics simulation to determine the structure of the IgG Fc fragment and SpA B domain binding complex. When pH shifted from a neutral to an acidic condition, a few hot

binding spots, such as Thr 269 – Arg 146 (Fc domain – SpA domain), Thr 326 – His 137, Asn 303 - His 137, Lys 305 – Asn 140 and Arg 268 – Glu 143, became less stable and consequently weakened the overall binding strength.

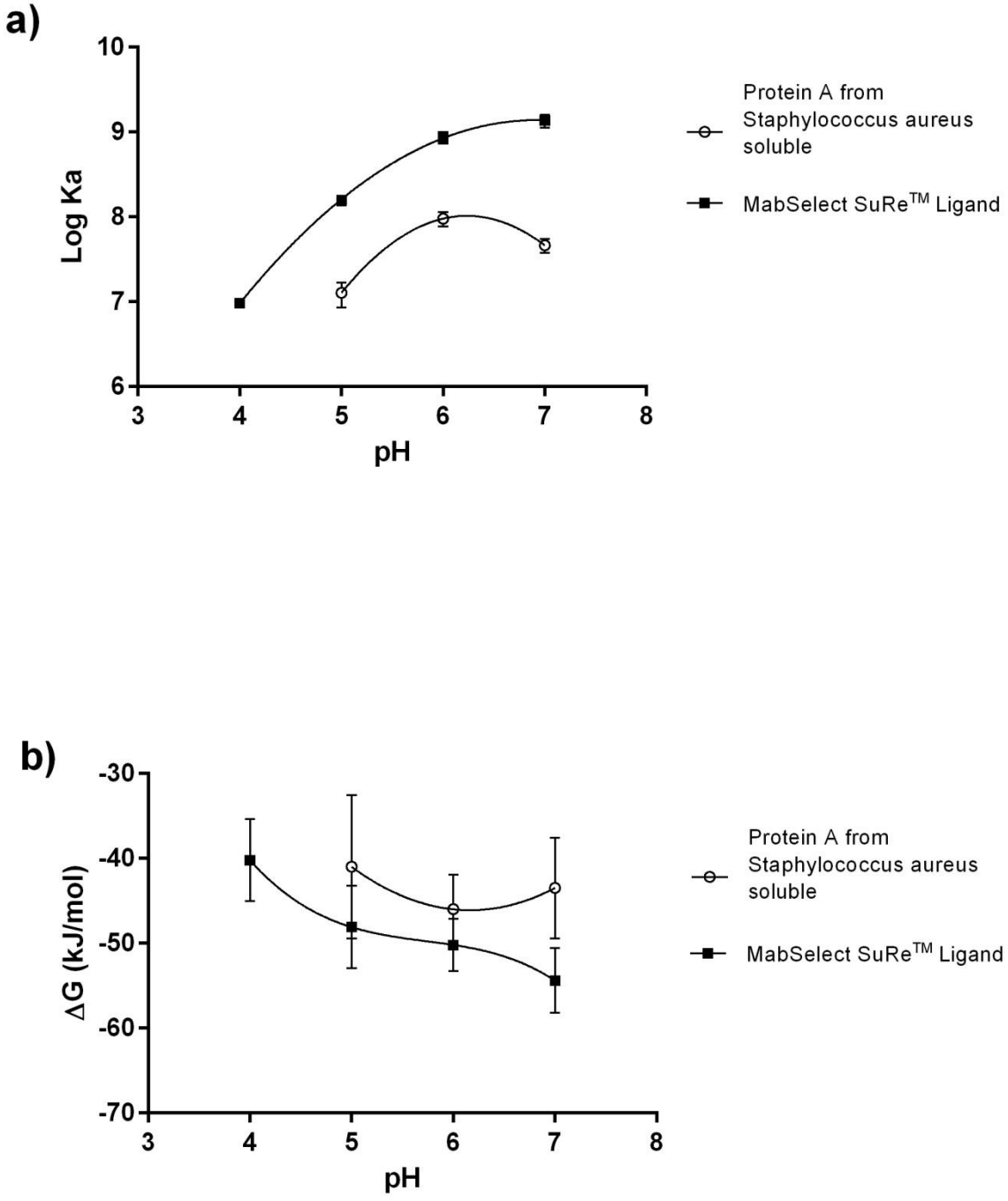


Figure 17 Efffect of pH in binding solution to thermo-parameters (a) LogKa and (b) ΔG,K and ΔG were derived from the isothermal titration curves of Protein A and BmAb as affinity ligand

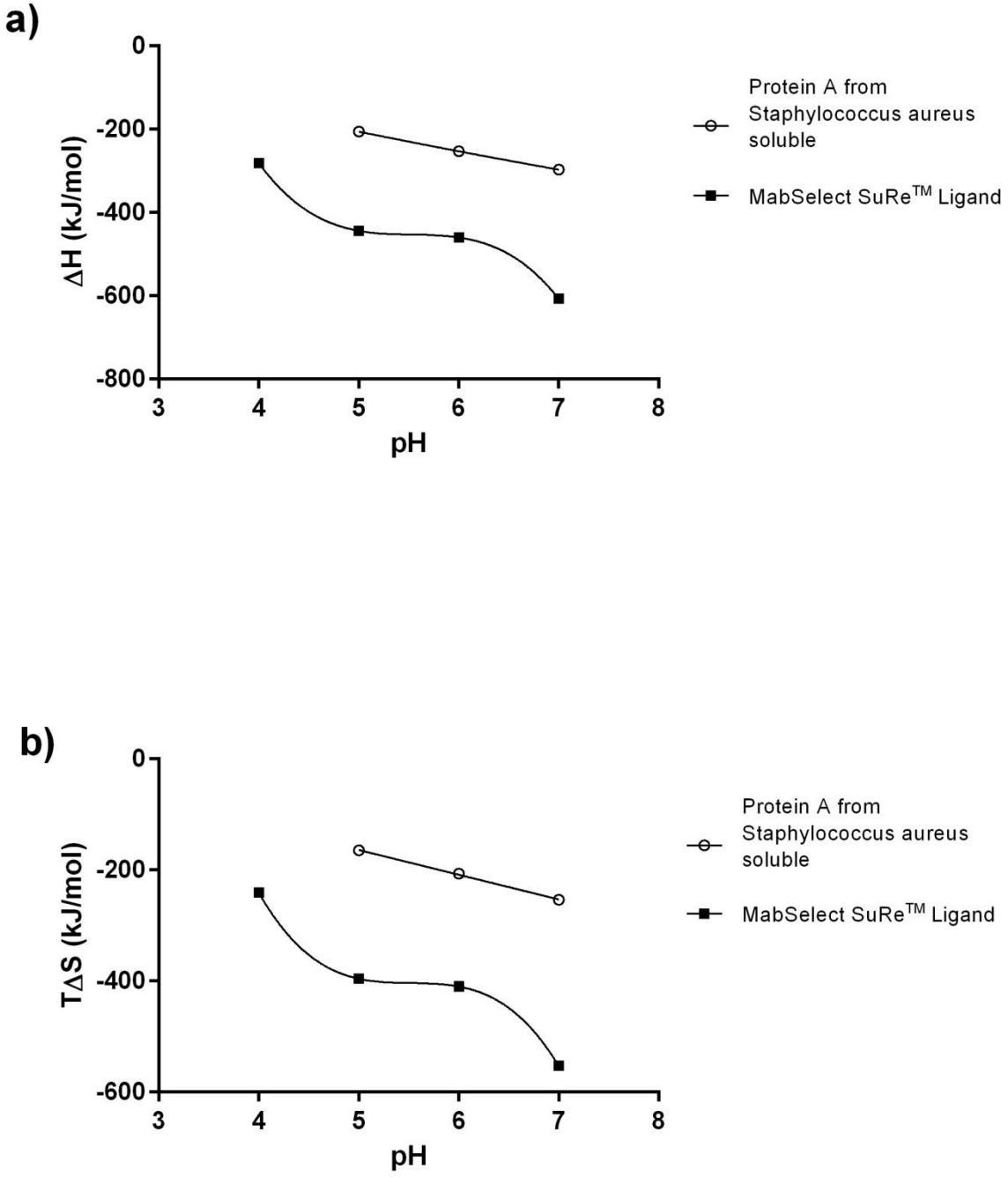


Figure 18 Effect of pH in binding solution to thermo-parameters (a) ΔH and (b) TΔS, ΔH and ΔS were derived from the isothermal titration curves of Protein A and BmAb as affinity ligand

3.4 Conclusion

The binding interactions between BmAb and the nSpA or its recombinant MS ligand were studied by isothermal titration calorimetry analysis under various binding conditions, such as pH, solvent ionic strength and temperature. The binding thermo-parameters based on isothermal titrations were calculated to understand the binding mechanism between the ligand and BmAb. Although the binding may be enhanced by increasing the solvent ionic strength or reducing the ambient temperature, the pH is the dominant factor in the ligand-BmAb interaction. In comparison to the two ligands, the MS ligand shows a much higher binding affinity than the nSpA ligand. There is no detectable interaction between BmAb and the nSpA at pH 4. Therefore, the MS is more likely to be effective in capturing mAbs over a wider range of conditions than the native Protein A.

Chapter 4 Breakthrough study of BmAb dynamic binding to immobilised ligands

4.1 Introduction

The consistent demand of monoclonal antibodies for therapeutic treatments of many diseases including cancers require a robust and efficient purification step in their manufacturing process. The approach in which most biological products are purified attains a great preference towards sequenced chromatographic techniques in the industrial downstream process. Among the family of these techniques, the affinity chromatography is claimed and approved to have high selectivity in target protein capture and purification (Biosciences, 2001). Through past few decades, improvements have been made for this chromatographic method in many aspects including ligand modification, alternative resin materials or other types of techniques regarding of multiplying chromatography cycles to minimize the losing target protein (Boi et al., 2009, Gunneriusson et al., 1999, Hahn et al., 2006, Hober et al., 2007). However, the key to the manufacture of biological products especially monoclonal antibodies (mAbs) aims for a cost-saving and efficient process with a minimum number of unit operations in order to avoid the product loss. Among all unit operations, affinity chromatography as the major step for capture and intermediate purification of target protein becomes the bottleneck for the process improvements. The optimisation of affinity chromatography requires a rigorous and thorough study on several process parameters like resin type, loading parameters, and operational conditions.

A special attention has been paid to two types of widely used affinity ligands, the native Protein A (nSpA) ligand and the MabSelectSuRe (MS) ligand. Both ligands have shown to be highly selective for mAb capture, reaching approximately over 95% purity directly from cell

culture media (Bostrom et al., 2012, Hedhammar et al., Hober et al., 2007). The MS is a recombinant form of nSpA with great attributes of alkaline resistance and higher affinity to mAbs but it is more costly than nSpA. In this case, concerns for industrial scaled mAb purification are amounted on the ligand specific binding performance. To maximally reduce the cost and enhance purification outcomes, the parameters that are involved in the manufacturing operations could be essential in affinity chromatographic optimisation. Few latest studies (Boi et al., 2009, Chen et al., 2003, Hedhammar et al., Zhao et al., 2013) have attempted to include parameters like pH, solvent ionic strength and temperature in examination of the Protein A ligand and its mimics. However, the majority of these studies on the binding behaviours are based on molecular modelling other than actual experimental investigations.

In the previous section, Bevacinzumab (BmAb) was used to bind separately to free nSpA and MS ligands. The isothermal titration calorimetry (ITC) technique was applied to determine the binding conformation based on the titrational thermo-parameters such as associated coefficient (Ka), binding associated enthalpy changes (ΔH) and entropy changes ($T\Delta S$). This analysis of the ligand-mAb interaction is in the free solution containing the ligand and the mAb . In this section, analysis of binding breakthrough and dissociation will be carried out by applying BmAb in 1 mL pre-packed chromatographic columns in which nSpA and MS ligands are immobilized on a polystyrene matrix.

4.2 Materials and Methods

4.2.1 Materials

Two types of prepacked affinity columns, HiTrap Protein A HP 1 mL and HiTrap MabSelect SuRe HP 1 mL, were purchased from GE healthcare for laboratory use. A 100 mL of

concentrated recombinant Bevacinezumab (BmAb) with a purity of >98% was provided by Hospira Inc, Adelaide. This Bmab at 4 mg/mL in 0.1 M sodium citrate and 1.0 M Tris-HCl at pH 7.4 was stored around -20 °C.

4.2.2 Determination of protein concentration

Prior to the binding breakthrough experiment, the BmAb was re-suspended in an aqueous form in a solvent consisting of 0.08 M sodium citrate and 0.24 M Tris-HCl at pH 7.4. The solution was applied to a HiPrep 26/10 desalting column to exchange the BmAb sample into a desired buffer. For sample solvent exchange in the prepacked desalting column, a 2 mL loading loop allowed 2 mL BmAb solution applied to the column each time. A period of 15 min at a flow rate of 5mL/min allowed a thorough solvent exchange and also ensured the same protein concentration prior to and after solvent exchange.

The concentration of BmAb was determined by using Quick Start™ Bradford protein assay provided by BIO-RAD. Protein was initially diluted in Coomassie Brilliant Blue G-250 solution and its absorbance was read at 280 nm wavelength with a UV-2600 Shimadzu spectrometer.

4.2.3 BmAbs chromatographic binding breakthrough

The breakthrough analysis was carried out by monitoring ligand occupancy by continuously applying BmAb until saturation in two columns: HiTrap Protein A in 1 mL CV and HiTrap MabSelect SuRe in 1 mL CV. During this process, the UV spectrum responses in an uphill shaped curve were recorded.

Figure 19 AKTA Pure scheme

The AKTA Pure (Figure 19) as a chromatographic platform was used for all chromatographic experiments. The system control software, Unicorn 7, was employed for AKTA Pure operations. The BmAb sample was initially buffer exchanged and centrifuged at 13000xg for 10 min to remove any precipitates. A dilution was then followed to keep the sample concentration at 1.28 mg/mL. After the sample preparation, a 1 mL affinity column was equilibrated with 15 mL the same buffer for BmAb solvent exchange prior to the sample application. 80 mL of the diluted BmAb was pumped into the column at a flow rate of 1 mL/min. For the binding at different operational temperatures, the equilibration buffer and

the BmAb sample were immersed in a water bath in which the temperature was set as the desired one. Experiments for different ionic strengths and pHs had the similar procedure as above.

The binding breakthrough curve was fitted with the Hill-slope specific binding model:

$$Y = B_{max} * \frac{X^h}{{K_d}^h + X^h} \qquad (3)$$

Where X is the ratio of the protein loading mass to the column volume, Y is the percentage of eluent saturation, B_{max} is the maximum specific binding with the same unit as Y, K_d is the binding associated constant and h is the Hill slope. Prism Graphpad as a data analytical tool was used for the curve fitting.

4.3 Experimental Results & Discussion

To study Bmab chromatographic binding breakthrough in the column, the sample should have the equivalent buffer condition in the matrix to probe mAb-ligand interactions in order to avoid possible impacts generated from buffer difference. The BmAb solution prior to experiment requires buffer exchange with the column equilibration buffer to ensure the same solvent when loading BmAb into the column. Buffer exchange was carried out in a pre-packed desalted column.

The breakthrough curve in the HiTrap Protein A column was obtained to assess the binding performance of the immobilized ligands with BmAb at various operational conditions such as temperature, pH and solvent ionic strength. A typical breakthrough curve is shown in Figure 20 by loading BmAb at pH 6 in a HiTrap Protein A 1 mL column.

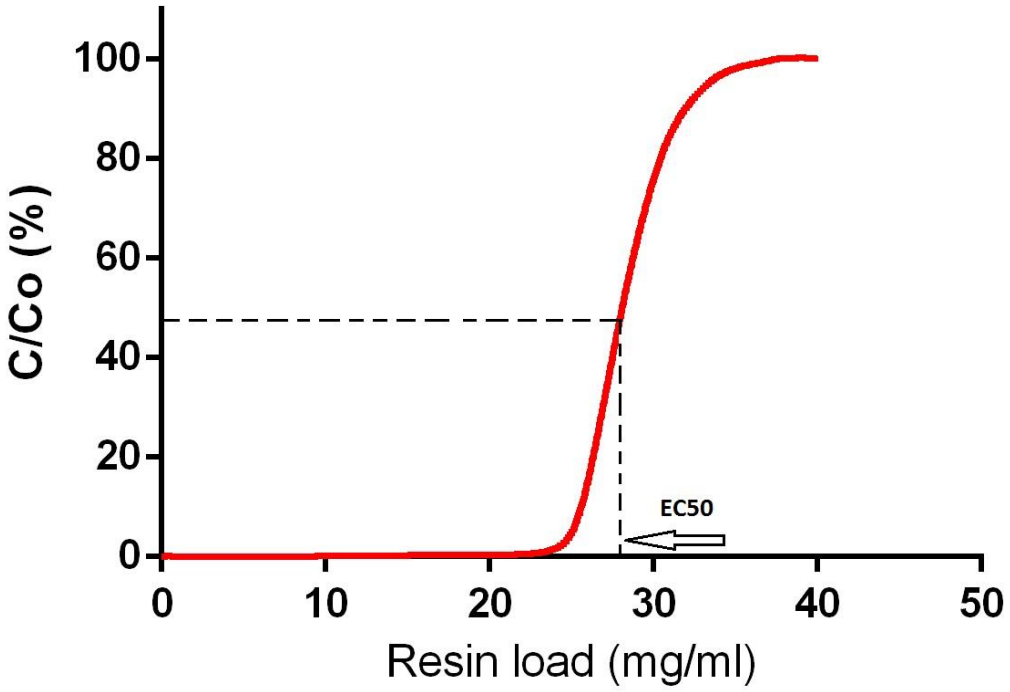


Figure 20 HiTrap Protein A 1 mL breakthrough by loading BmAb at pH 6

C/C_0 in Figure 20, the ratio of the breakthrough BmAb concentration to the initial BmAb concentration, represents the dynamic binding capacity. The X-axis is the resin load which represents the overall amount of protein applied to column with 1ml CV. When loading the sample into the column, there is no protein detected at the small loading volume, and then protein concentration at the exit of the column starts to increase as the more samples are applied. The protein concentration reaches the initial sample concentration when the protein sample volume further increases. From the breakthrough curve, a typical percent for specific binding, termed as a half-maximum binding at equilibrium (EC50), are shown in Figure 20 as marked by an arrow. This factor is crucial in the determination of binding affinity according to the loading of BmAb.

The dynamic binding affinity can be affected by a range of factors including temperature, pH and solvent ionic strength. For each factor, a breakthrough curve was obtained and further

fitted with the Hill-slope specific binding model which describes the binding equilibrium of native SpA ligand to BmAb. Parameters were generated through curve fitting, including the Hill slope (h) and the percentage of specific binding.

4.3.1 Effect of Ionic strength in binding solution

Previous ITC study has demonstrated that the impact of ionic strength on the nSpA ligands is much greater than that on MS ligand when binding with BmAb. It was found that both hydrophilic and hydrophobic interaction were reduced with an increase in salt concentration. However, the environmental polarity was increased due to the addition of salt, and binding affinity was enhanced at a higher salt concentration. The column experimental design was accordant to the previous ITC study by applying different salt concentrations of sodium phosphate buffer at 100 mM, 500 mM and 1000 mM at neutral pH. The BmAb concentration was adjusted to be 1.28 mg/mL and then the BmAb solution was applied to a prepacked column equilibrated by the buffer. The nSpA ligand has a theoretic binding capacity of approximate 30 mg human IgG/mL medium. Figure 21 (a) shows the binding breakthrough curve in three salt concentrations, and Table 1 summarises key parameters captured from the breakthrough curves. The parameter EC50 in the Table 1 reveals the binding affinity is enhanced by an increase in the solution ionic strength. This trend agrees with the ITC data and it can be confirmed that that addition of salt helps binding between BmAb and either the immobilised or the free nSpA ligand.

The same sized prepacked HiTrap MabSelect column was packed with the MS ligand. The binding breakthrough curves in Figure 21 (b) display in a different shape in comparison with the native protein A curve. Such difference can be ascribed to the different binding mechanism between BmAb and above two ligands. Other aspects such as matrix material, resin structures and the space arm length may also be involved in the binding mechanism

resulting in different breakthrough curve shapes. Therefore, the HiTrap MabSelect SURE column packed with the highly cross-linked agarose as the resin material with a bead size of 85 um responses in a tardy incline comparing with the HiTrap Protein A column. By increasing the salt concentration in the mobile phase, the binding breakthrough curves remain unchanged (Figure 21b). The curve shape looks identical for three salt concentrations. In addition, EC50 in this experiment has a minor change in different salt concentration, keeping at. 40 mg BmAb/mL resin. Therefore, the binding affinity in the MS column is not significantly affected by the alternation of the solvent ionic strength. Moreover, the EC50 value for the MS ligand appears 1.5 folds higher than for the nSpA ligand (Table 1). This result confirms the finding in the previous ITC analysis that the binding between the MS ligand and BmAb is too strong to be altered by the salt.

Table 1 Hill slop (H) and EC50 by loading BmAb to HiTrap Protein A and MabSelect SuRe columns at various buffer salt concentrations

HiTrap Protein A column			
	100 mM NaCl	500 mM NaCl	1000 mM NaCl
h	16.98	15.76	16.35
EC50 (mg/ml)	29.85	31.32	32.28
MabSelect SURE column			
h	2.779	2.772	2.734
EC50 (mg/ml)	41.03	40.38	39.14

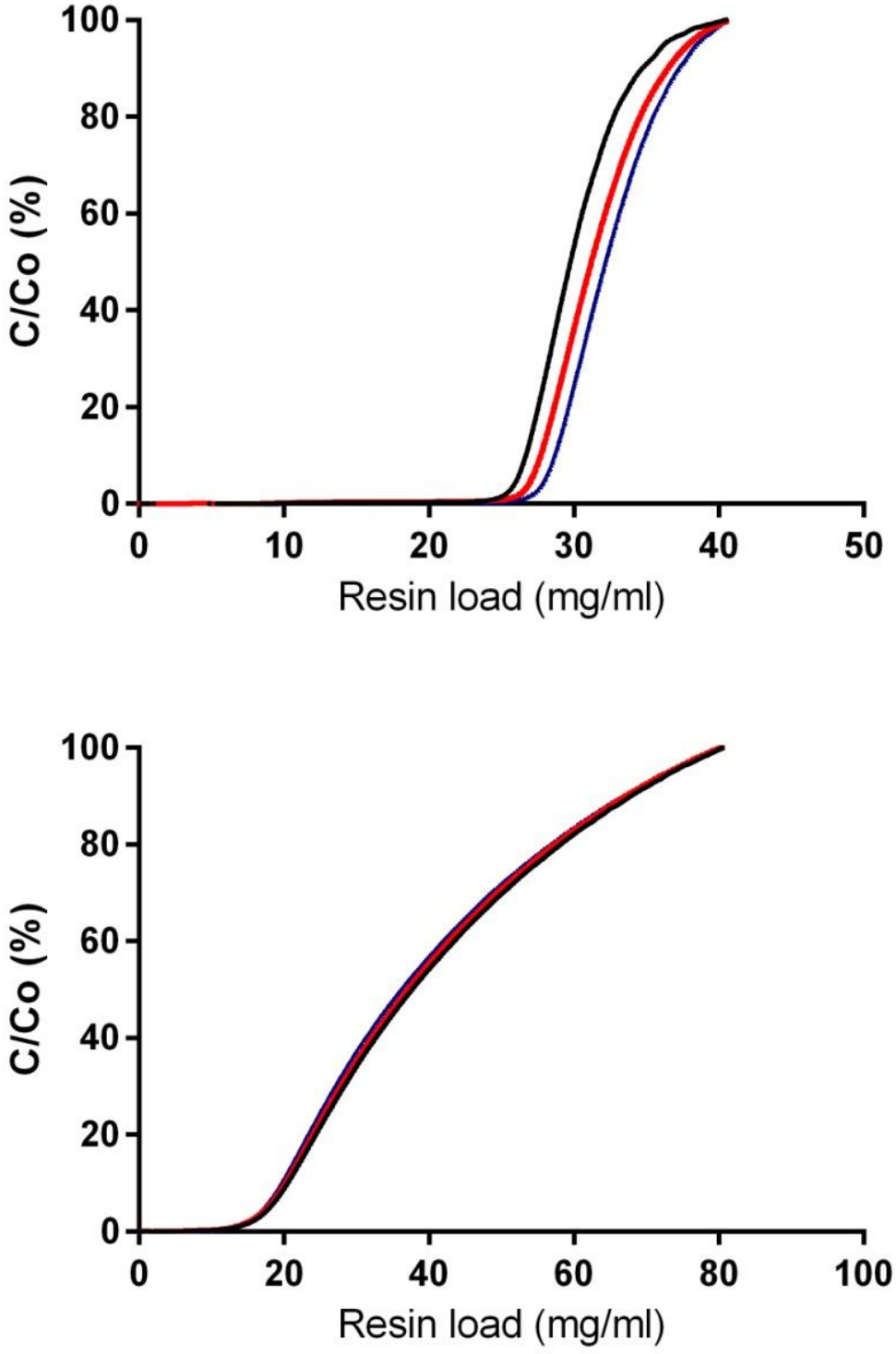


Figure 21 Effect of solvent ionic strength on loading BmAb to a) HiTrap Protein A and b) HiTrap MabSelect SuRe via various NaCl concentration in mobile phase, (Black) 100mM NaCl, (Red) 500mM NaCl and (Blue) 1M NaCl

4.3.2 pH

The affinity interactions between the ligand and the mAb are very sensitive to the variation in pH. From the previous ITC study, binding affinity between BmAb and both ligands reduces significantly by lowering the pH. The protein may be denatured in a low pH which results in the loss of "key and lock" adaption for binding. Therefore, a low pH condition is also employed to elute the binding mAbs out of the immobilised ligands in the packed column in affinity chromatography.

In order to be consistent with the ITC study, pH ranging from 4 to 7 was used for binding breakthrough study for both ligands. BmAb as the target protein was diluted at 1.28 mg/mL with a buffer prepared at pH 4, 5, 6 and 7 respectively. The same buffer condition was also used for equilibrating the column prior to BmAb loading. The breakthrough curves at different pH are shown in Figure 22 (a) for the nSpA ligand and key parameters are summarised in Table 2. Binding in the neutral pH appears to have the highest affinity and its EC50 value is distinctively greater than other pHs. When the buffer condition becomes acidic, the binding breakthrough curve shifts to the left with less units of resin load, and the resulting EC50 value displays a significant drop at a lower pH. This agrees with the previous ITC finding that binding between the nSpA ligand and BmAb is highly pH dependent. The ITC analysis suggested that the minimum binding is found at pH 4 and no signal is captured even when the nSpA ligand is saturated to BmAb. According to the breakthrough curve, the occurrence of binding at pH 4 was shown in a dramatic drop to other pHs, trending towards to the state of elution.

The binding breakthrough curves for the MS ligand shown in Figure 22 (b) display a distinctive curve shape from the native Protein A ligand. The breakthrough curves have a

much smaller gradient than those curves in Figure 22 (a). The binding capacity of the MS ligand is 2 folds greater than that of nSpA. Therefore, the binding between the MS ligand and BmAb shows a much higher affinity. When the same pH condition is applied to the column, the binding breakthrough shifts much smaller unit of resin load to the left than the nSpA ligand. This shift in the breakthrough curve due to pH changes is still quite significant comparing to the ionic strength effect. The binding is still relatively strong with a high capacity at pH 4.0. In this case, the elution for the MS-BmAb binding complex therefore would require a harsher condition, requiring a much lower pH condition to release BmAb out of the binding complex in the column.

Table 2 Hill slop (H) and EC50 by loading BmAb to HiTrap Protein A and MabSelect SuRe columns at various buffer pHs

HiTrap Protein A column				
	pH 4	pH 5	pH 6	pH 7
h	19.64	21.52	19.77	17.08
EC50 (mg/ml)	18.99	25.70	28.20	29.84
MabSelect SURE column				
h	2.376	3.113	3.021	3.765
EC50 (mg/ml)	37.29	39.11	38.70	43.97

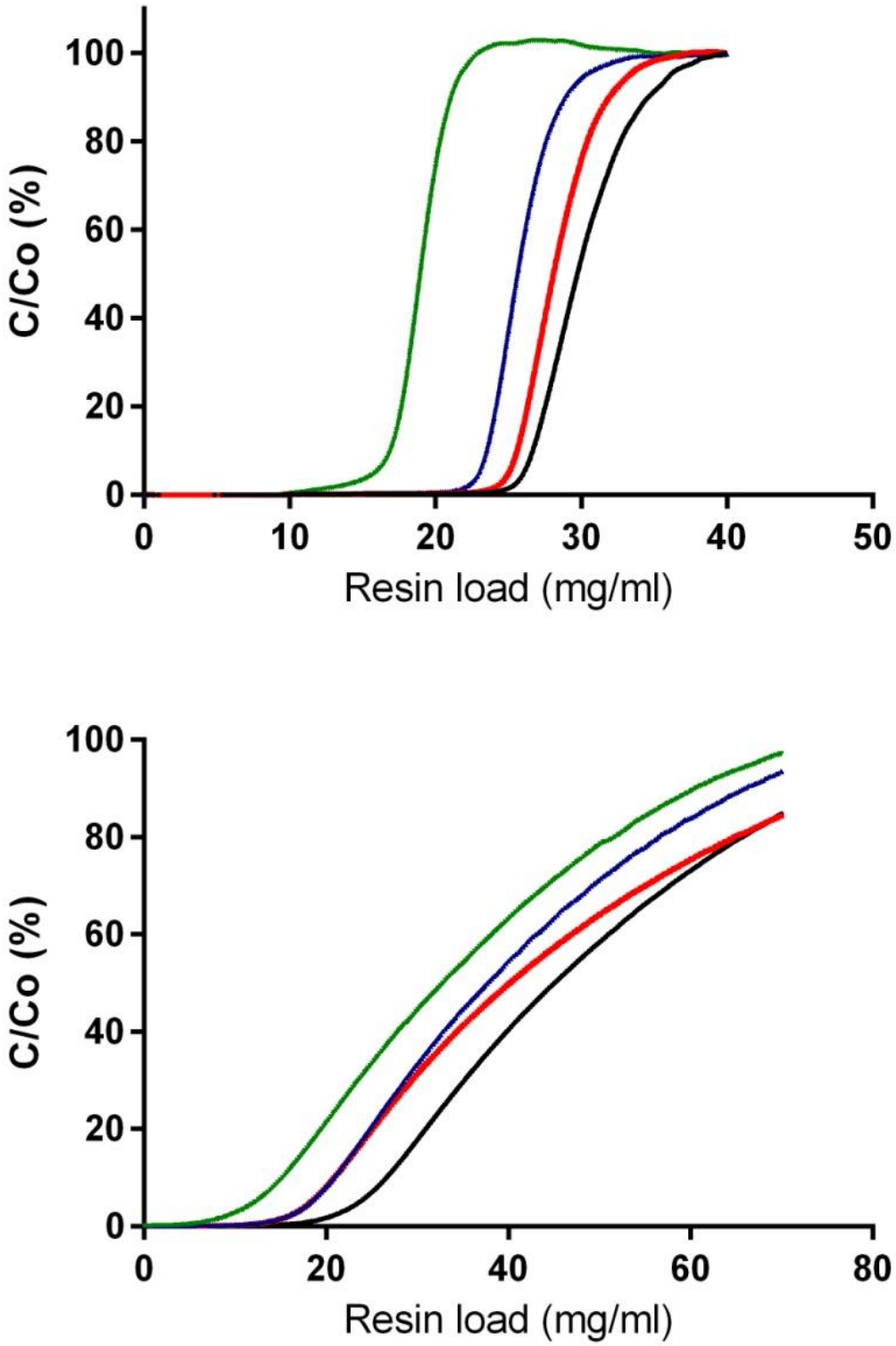


Figure 22 Effect of pH on loading BmAb to a)HiTrap Protein A and b) MabSelect SuRe via various pHs in mobile phase, (Black) pH 7, (Red) pH 6, (Blue) pH 5, and (Green) pH 4

4.3.3 Temperature

The experimental design of the temperature effect on binding breakthrough study is relatively challenging than ionic strength and pH. Temperature control in this experiment was not performed at the temperature of 15 °C, 20 °C and 25 °C as set in the previous ITC study, since the 5 °C gap between each temperature is hard to be maintained through the BmAb loading process. Therefore, the experiment was conducted at 4 °C and 25 °C respectively. To maintain at a low temperature of 4 °C, both the BmAb sample and the buffer were stored in an ice bath during the experiment, and the column was equilibrated by the cold buffer for at least 30 min prior to the loading experiment to ensure the column resin at the same temperature.

A small shift to the right is observed for the breakthrough curves from 25 °C to 4 °C for the nSpA lignad. The corresponding binding parameter EC50 has a minor change when the temperature drops. A small increase in EC50 represents a minor enhancement of binding at a lower temperature. This finding confirms our previous ITC analysis that a decrease in operation temperature enhances the binding since the affinity coefficient (K_a) increases as the temperature increases. Therefore, temperature changes have an impact on the binding between the nSpA and BmAb, but the impact is much less significant than one due to changes in pH and ionic strength.

A HiTrap MS column packed with the MS ligand was operated at the same temperature as the HiTrap nSPA column. The mAb breakthrough curves at 25 °C and 4 °C overlap with each other as shown in Figure 22 (b). The curves and their derived EC50 values in Table 3 indicate that the temperature has no impact on the binding. The same conclusion is also drawn from

the ITC data which suggest the affinity coefficient (K_a) keeps almost constant for temperatures ranging from 15 to 25 °C. Therefore, binding between the MS ligand and BmAb is quite independent of the operational temperature.

Table 3 Hill slop (H) and EC50 by loading BmAb to HiTrap Protein A and MabSelect SuRe columns at various temperatures

HiTrap Protein A column		
	25°C	4°C
h	16.98	18.06
EC50 (mg/ml)	29.85	31.13
MabSelect SURE column		
h	2.793	2.705
EC50 (mg/ml)	40.89	40.47

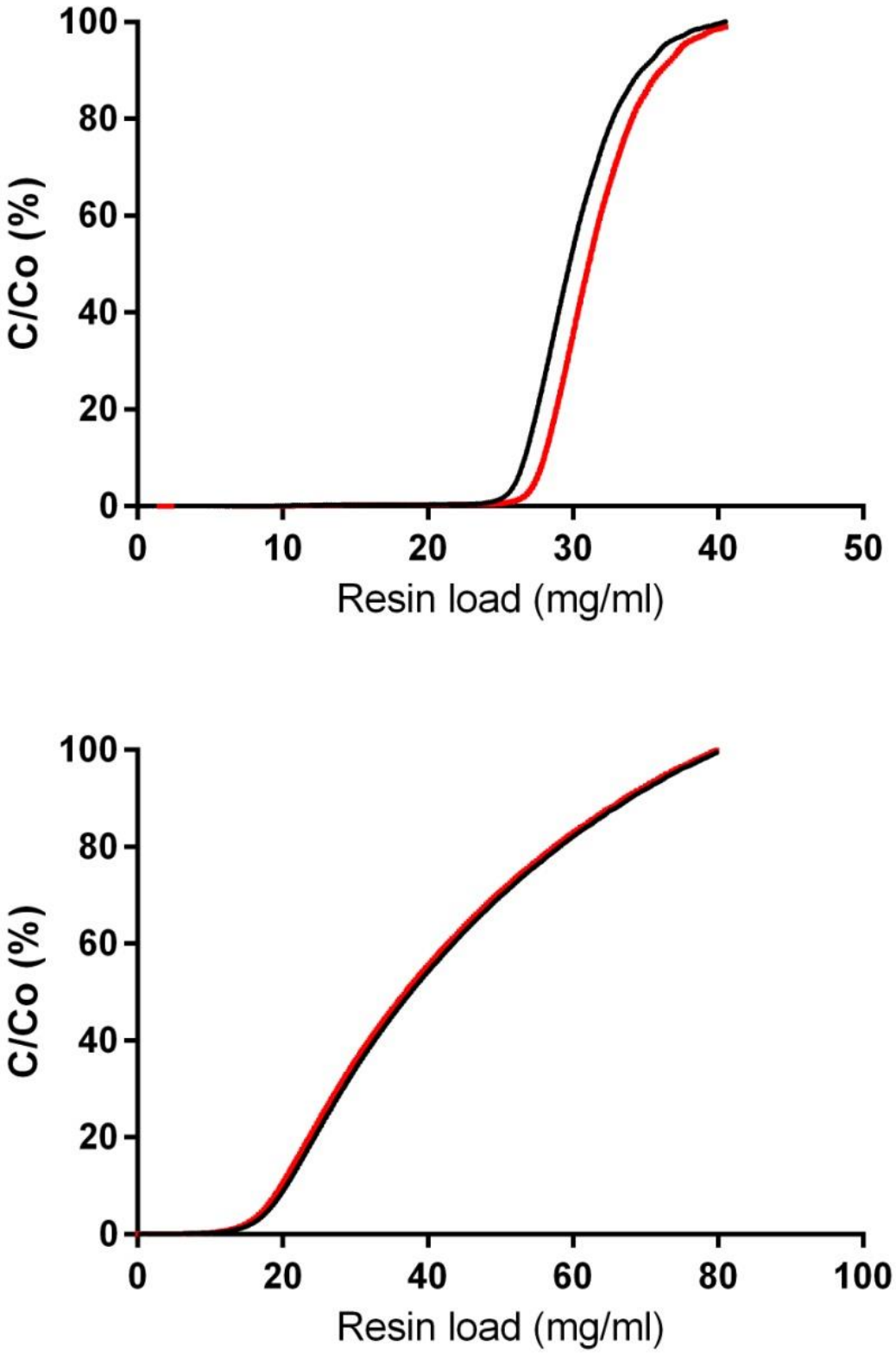


Figure 23 Effect of temperature on Loading BmAb to a)HiTrap Protein A and b) MabSelect SuRe at various temperatures, (Black) 25°C and (Red) 4°C

4.4 Conclusion

The binding of BmAb with two immobilised ligands was investigated in two prepacked columns, a HiTrap Protein A HP 1 mL and a HiTrap MabSelect SuRe 1 mL. The mobile phase pH, ionic strength, and temperature were varied during the mAb capture process. The temperature and ionic strength show a minor influence on the binding performance in the HiTrap Protein A HP 1 mL column regarding the breakthrough curve and it EC50 value, but no impact on the binding in the HiTrap MabSelect SuRe 1 mL column. However, pH is a crucial factor in the alternation of binding between BmAb and two ligands. A dramatic loss of the binding capacity is observed for both columns as pH drops. In agreement with the ITC study, the immobilised MS ligand shows active binding with BmAb at pH of 4 which suggests a harsher elution condition with a pH should be less than 4 would be required to achieve complete elution of mAbs in the industrial chromatographic applications.

Chapter 5 Conclusions and Recommendations

5.1 Conclusions

The aim of this study is to experimentally measure the thermodynamic parameters of the binding interaction between affinity ligands and monoclonal antibodies (mAbs), which assist in understanding of the binding mechanism between the mAb and different types of Staphylococcal protein A (SpA). The isothermal titration calorimetry (ITC) analysis was carried out for examination of the binding between the BmAb and two free affinity ligands: the native Protein A (nSpA) and the MabSelect SuRe (MS) ligand. The dynamic binding between the mAb with two immobilised ligands packed inside a column was also examined. Both studies were conducted with a range of temperatures, pHs and solvent ionic strengths.

The ITC results reveal that for both ligands, the free energy of the binding reaction decreases with a reduction of pH from 7 to 4. Similar decreases in both enthalpy and entropy associated with the interaction are also observed during the pH drop. There is no binding of the nSpA ligand with BmAb at pH 4. The MS ligand displays a relatively less sensitive to the changes in the salt concentration and temperature than the nSpA ligand. These findings suggest that chromatography resins based on the MS ligands are more likely to be effective over a wider range of conditions than the resins based on nSpA.

The similar binding results in pre-packed columns confirm with the ITC results that the temperature and solvent ionic strength have negligible impacts on the mAb breakthrough curve and its derived EC50 value for the immobilized MS ligand, and very minor impacts for the immobilized nSpA ligand. The mobile phase pH influences the column dynamic binding for both immobilized ligands and loss of the binding capacity is observed for both columns as

pH drops. However, the binding between the MS ligand and BmAb is much stronger than that between the nSpA ligand and BmAb at the same pH condition. Complete elution of mAb out of the column would require a much acidic mobile phase, which may denature the mAb in the industrial chromatographic operations

5.2 Recommendations

pH as a major impact factor has been studied in the prepacked column in the range between 4 to 7. The elution condition for releasing BmAb in the affinity chromatographic column will be investigated to allow capture of the majority of BmAb. As mAbs may permanently lose their activity in a low pH, it is recommended that the mAb refold study should be conducted at pH of 4 and below in order to determine the lowest pH condition in which the mAb can maintain its activity.

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