DEVELOPMENT OF ANTIBODY IMMOBILISATION STRATEGY USING RECOMBINANT HUMAN NEONATAL Fc RECEPTOR

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DEVELOPMENT OF ANTIBODY IMMOBILISATION STRATEGY USING RECOMBINANT HUMAN NEONATAL Fc RECEPTOR

by

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ABTS	2, 2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)
AFM	Atomic force microscope
APTES	(3-aminopropyl)triethoxysilane
Asp	Aspartic acid
β2m	β2-microglobulin
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
CHO	Chinese hamster ovary
CNS	Central nervous system
Cys	Cysteine
DDI	DNA-directed immobilisation
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
Fab	Fragment antigen-binding
Fc	Fragment crystallisable
FcRn	Neonatal Fc receptor
FTIR	Fourier transformation infrared
gIgG-HepB	Goat polyclonal anti-hepatitis B virus surface antigen antibody
Glu	Glutamic acid
Gly	Glycine
GMBS	N-γ-maleimidobutyryl-oxysuccinimide ester
hFcRn	Human neonatal Fc receptor
hFcRn-α	Human neonatal Fc receptor α-chain
HBsAg	Hepatitis B virus surface antigen
His	Histidine
HRP	Horseradish peroxidase
IBA	Indole-3-butyric acid
i.e.	Id est (that is)
IgG	Immunoglobulin G
IMAC	Immobilised metal affinity chromatography
InsFcNP	Insulin-Fc-nanoparticles
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilo base pair
kDa	Kilo Dalton
LB	Luria-Bertani

Leu	leucine
MALDI MS	Matrix-assisted Laser Desorption/Ionisation Mass spectrometry
MALDI-TOF/TOF	Matrix-assisted Laser Desorption/Ionisation-Time of Flight/Time of Flight
MHC	Major histocompatibility complex
MHC-I	Major histocompatibility complex class I
MTS	3-mercaptopropyltrimethoxysilane
MWCO	Molecular weight cut-off
NaCl	Sodium chloride
NHS/EDC	N-hydroxysuccinimide/1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
OD	Optical density
PEAK	Human embryo kidney monolayer epithelial
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline-Tween 20
PCR	Polymerase chain reaction
PL	Photoluminescence
rIgG-HepB	Rabbit polyclonal anti-hepatitis B virus surface antigen antibody
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH	Sulfhydryl group
SKOV-3	Human ovarian cancer cell line
SPR	Surface plasmon resonance
sulfo-KMUS	N-k-maleimidoundecanoyl-oxysulfosuccinimide ester
sulfo-SMCC	Succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxyl
TB	Terrific broth
TBE	Tris-borate-EDTA
TBS	Tris-buffered saline
TBST	Tris-buffered saline-Tween 20
TEMED	Tetramethylethylenediamine
Tm	Melting temperature
Trp	Tryptophan
UV	Ultraviolet
µg/well	Microgram per well
v:v	Volume to volume
w:v	Weight to volume
XPS	X-ray photoelectron spectroscopy

PEMBANGUNAN STRATEGI MENGIMMOBILISASI ANTIBODI DENGAN MENGGUNAKAN REKOMBINAN Fc RESEPTOR NEONATAL MANUSIA

ABSTRAK

Antibodi immobilisasi merupakan asas utama kepada asai yang melibatkan antibodi dalam teknologi biopengesanan. Teknologi tersebut biasa digunakan untuk mengesan and mengukur kebanyakan jenis molekul biologi seperti antigen, biomarker, allergen and bahan-bahan perubatan. Maka, interaksi yang spesifik antara antibodi dengan ligan amat diperlukan untuk menjamin penghasilan semula keputusan dan juga sensitiviti. Komplesiti mengimmobilisasi antibodi adalah faktor utama yang mempengaruhi keputusan sesuatu asai kerana molekul pengecaman, seperti antibodi, mungkin mengalami perubahan dan kehancuran struktur semasa ia dilekatkan pada permukaan pepejal. Untuk memenangi situasi tersebut, cara baharu mengimmobilisasi antibodi amat diperlukan pada masa kini. Kajian ini berusaha untuk meneroka protein pengikat antibodi yang baharu, iaitu Fc reseptor neonatal manusia. Protein ini mempunyai afiniti yang tinggi terhadap bahagian Fc pada antibodi sama ada dalam keadaan *in vivo* ataupun *in vitro*. Ia adalah suatu heterodimer yang dibentuk daripada dua komponen: p51 rantai berat-α (rantai-α) dan β2-microglobulin rantai ringan. Kajian telah melaporkan bahawa semua molekul yang terlibat dalam interaksi dengan antibodi hanya terletak pada rantai-α sahaja. Dengan itu, kajian ini akan menggunakan rantai-α sahaja untuk mengimmobilisasi antibodi. cDNA yang mengekodkan rantai-α (hFcRn-α) telah diklonkan dalam vektor ekspresi bacteria. Banyak usaha telah dilakukan untuk menghasilkan Fc reseptor neonatal manusia dalam bentuk larut di dalam bakteria. Cara tersebut berbeza dengan kaedah biasa yang menghasilkan Fc reseptor neonatal manusia di dalam sel mamalia.

Pertambahan betaine dengan kenaikan suhu yang singkat semasa pertumbuhan bakteria telah berjaya meningkatkan pelarutan protein rekombinan yang dihasilkan. Beberapa asai fungsian termasuk ELISA, analisis pH-bergantungan, dan dot blot imunoasai telah dijalankan untuk mengenal pasti aktiviti asli protein rekombinan tersebut. Keputusan menunjukkan bahawa hFcRn-α yang dihasilkan dengan cara tersebut adalah aktif dan berfungsi. Selain itu, kaedah tersebut tidak memerlukan protokol melipat-semula protein untuk memulihkan aktivitinya. Kajian ini melaporkan suatu kaedah yang senang dan efektif untuk menghasilkan Fc reseptor neonatal manusia. Protein rekombinan tersebut telah digunakan untuk mengimmobilisasi IgG arnab terhadap antigen permukaan virus hepatitis B. Keputusan ELISA menunjukkan bahawa immobilisasi antibodi tersebut adalah bertindak langsung dengan kepekatan hFcRn-α yand terdapat di dalam plat ELISA. Kajian juga dilanjutkan untuk membangunkan asai ELISA sandwich bagi pengesanan antigen permukaan virus hepatitis B degan menggunakan hFcRn-α sebagai protein pengikat antibodi. Keputusan yang dihasilkan telah dibandingkan dengan sistem pengesanan yang menggunakan pelekatan antibodi fizikal. Perbezaan kedua-dua system tersebut telah dikaji dengan ujian-t dua sampel. Keputusan mengimbaskan bahawa antibodi immobilisasi yang melibatkan hFcRn-α dapat menghasilkan isyarat pengesanan yang lebih tinggi berbanding dengan pelekatan antibodi fizikal, terutamanya pada kepekatan antigen permukaan virus hepatitis B yang bernilai 1 μg per isi. Secara keseluruhannya, kajian ini melaporkan calon baharu protein pengikat antibodi yang berpotensi mengimmobilisasikan antibodi dalam orientasi yang betul tanpa sebarangan perubahan struktur antibodi.

DEVELOPMENT OF ANTIBODY IMMOBILISATION STRATEGY USING RECOMBINANT HUMAN NEONATAL Fc RECEPTOR

ABSTRACT

Antibody immobilisation is the cornerstone of antibody-based assay in biosensing technology which is predominantly used to detect and measure a wide variety of biological analytes, ranging from antigens, biomarkers, allergens and drug substances. As such, specific interactions between antibody and ligands are therefore needed to guarantee highest reproducibility and sensitivity possible. The complexity of pinning antibodies forms the key factor in interfering the outcome of an assay as the recognition molecule, i.e. the antibody itself, may have undergone structural changes and distortion when it is attached to the solid phase. In view of this, there is a need to identify new methods and novel strategy in immobilising antibody to secure the biosensing system. The present study sought to discover a new candidate of antibody binding protein, the human neonatal Fc receptor. This protein has shown its high affinity specifically towards Fc region of antibody either *in vivo* or *in vitro*. It is a heterodimer constructed of two subunits: p51 α-heavy chain (α-chain) and the β2-microglobulin light chain. Literatures have revealed its binding sites towards antibody and all of the residues involved are solely found in the α-chain. As a result, the α-chain alone was used for antibody immobilisation in the present study. The cDNA encoding for the α-chain of human neonatal Fc receptor (hFcRn-α) was cloned into a bacterial expression vector. Attempts had been made to express the soluble form of human neonatal Fc receptor in bacteria instead of the conventional approach which uses mammalian cells. The addition of betaine with a brief heat shock step during cultivation had successfully enhanced the solubility of

recombinant protein expressed. Several functional assays including ELISA, pH-dependency analysis and dot blot immunoassays were conducted to validate the native activity of the recombinant protein. The findings indicated that the hFcRn-α produced using this approach remained active, functional and more importantly, protein refolding steps were not required to revive its activity. This study presented a simple yet effective approach to express human neonatal Fc receptor. The recombinant protein was employed to immobilise rabbit IgG against hepatitis B virus surface antigen. The ELISA results showed that immobilisation of the particular antibody were directly proportional to the concentration of hFcRn-α coated in the ELISA plate. Study was extended further to develop a sandwich ELISA to detect hepatitis B virus surface antigen using hFcRn-α as antibody binding protein. The results were compared to the detection system developed using physical adsorption of the antibody. The differences in the detection signals generated by these two strategies were validated using independent t-test. The finding highlighted that hFcRn-α directed immobilisation yielded higher signals in antigen detection compared to physical adsorption method, especially at the concentration of hepatitis B virus surface antigen of 1 μg per well. Ultimately, the present study reported a new candidate of antibody binding protein which is capable of immobilising antibody in a proper orientation without additional antibody modification steps.

CHAPTER 1

INTRODUCTION

1.1 Antibody structure and classes

Antibodies, also termed as immunoglobulins, are glycoprotein molecules that bind to target antigens with high specificity. In human, this unique recognition molecule can be classified into five classes based on its structure and biochemical properties: IgG, IgA, IgM, IgD and IgE (Woof and Burton, 2004). Table 1.1 demonstrates the comparison of the different classes of antibodies in human.

All classes of antibodies have its general structure constructed by four polypeptide chain units: two light (L) chains and two heavy (H) chains. Disulfide bonds are the key chemical bonds that cross-link the L-chains and H-chains together to form a functional structure. Both H-chains and L-chains consist of amino-terminal variable (V) regions that involve in antigen recognition and carboxy-terminal constant (C) regions. The C regions of H-chains participate in effector function. All antibodies of same class are similar in the amino acid sequences and numbers in their C regions. However, the V regions are different between antibodies of different types (Wang *et al.,* 2007). More importantly, intrachain disulfide bonds are found in both L-chains and H-chains which create polypeptide loops, also known as domains, in the antibody. These domains are named as V_H, V_L, C_H1, C_H2 and C_H3. The V_H and V_L domains play important tole in forming the antigen-binding site of antibody (Figure 1.1).

Among the five classes of antibodies, IgG is the most abundant and widely used for therapeutic pusrposes. This particular class of antibody has also been thoroughly explored in the antibody immobilisation on solid phase for biosensor development.

Table 1.1 Properties and classes of human antibodies.

	IgG	**IgA**	**IgM**	**IgD**	**IgE**
Physical properties					
Molecular weight, kDa	150	170-420	900	180	190
Molecular weight of H-chain, kDa	50-55	62	65	70	75
Main molecular form	Monomer	Monomer and dimer	Pentamer	Monomer	Monomer
Physiological properties					
Normal adult serum (mg/ml)	8-16	1.4-4.0	0.4-2.0	0.03	< 0.03
Half-life (days)	23	6	5	3	< 3

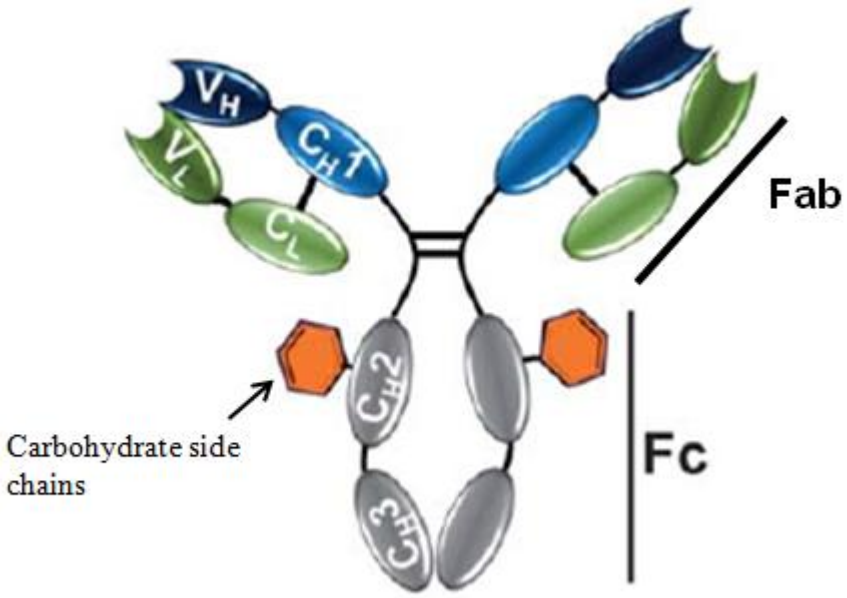


Figure 1.1 Schematic depiction of IgG. All classes of antibodies share similar general structure which constituted of two heavy (H) chains and two light (L) chains. The constant (C) regions and variable (V) regions are shown in the figure (Trilling *et al.*, 2013).

1.2 Concept of antibody immobilisation

Antibody immobilisation is a technique to circumscribe antibodies to a solid phase with retention of its native affinity to target antigens. This technology is of considerable interest in the application of antibody-based assay systems, especially diagnostic platforms. Most of the diagnostic assays involve manipulation of antibodies in the detection and identification of biological molecules in the biological samples, such as the detection of cancer biomarkers and pathogens, measurement of hormones, quantifying drugs in urine and also other miscellaneous fields of interest (Skottrup *et al.*, 2008; Ambrosi *et al.*, 2010; Phan *et al.*, 2012; Pla-Roca *et al.*, 2012; Bertholf *et al.*, 2015; Shi *et al.*, 2015; Pankhurst and McLennan, 2016). Those molecular identification and quantification are critical in forensic investigations, early illness detections and healthcare services involving monitoring of disease progression and treatment options. Consequently, the affinity and sensitivity of the assay system becomes an important key factor as it may shift the result of a diagnosis or a professional decision into different direction when inaccuracy of detection ensued. More importantly, this scenario is subjected to the complexity of the antibody immobilisation strategy as different methods of pinning antibodies will significantly affect the affinity and sensitivity of an assay (Vashist *et al.*, 2011; Trilling *et al.*, 2013).

An antibody-based assay system is constructed by two main components: the antibody that captures target antigens and the solid phase where the antibodies are attached. The antigen-binding sites are located at the Fab (fragment antigen-binding) regions of the antibody while the Fc (fragment crystallisable) region is the attachment site to the solid surface. There are various types of solid surfaces used,

predominantly glass, gold, polystyrene, nitrocellulose membrane and silicone (Hahn *et al.*, 2008). Figure 1.2 illustrates the basic construction of antibody-based assay.

1.3 Critical consideration in antibody immobilisation

Several crucial factors must be considered during antibody attachment as the immobilisation methods of choice will significantly interfere with the result of an assay. Predominantly, three factors have been identified: the orientation of immobilised antibodies, the stability of immobilised antibodies and the density of immobilised antibodies.

1.3.1 Orientation of immobilised antibodies

In the assay detection, the Fab regions of an antibody contain specific binding sites for its antigens. It is ideal that all of the Fab regions of antibodies are oriented upward, away from the solid surface, to allow maximal capture of antigens. Nevertheless, the interaction between antigens and antibodies will reduce if the antibodies exist in random orientation on the solid phase (Figure 1.3). This scenario happened due to the steric hindrance generated by the solid surface itself or the adjacent antibodies (Kausaite-Minkstimiene *et al.*, 2010; Song *et al.*, 2012). Part of the Fab regions may be blocked and impeded from binding with antigens in the random antibody orientation. Study done by Bonroy *et al.* (2006) showed that the oriented immobilisation produced 2-fold higher in the antigen binding signals compared to the antibodies in random orientation. In addition, a study reported by Tsai and Pai (2009) highlighted that the oriented immobilisation of antibody fragments had demonstrated 50% increase in the antigen binding efficiency in the

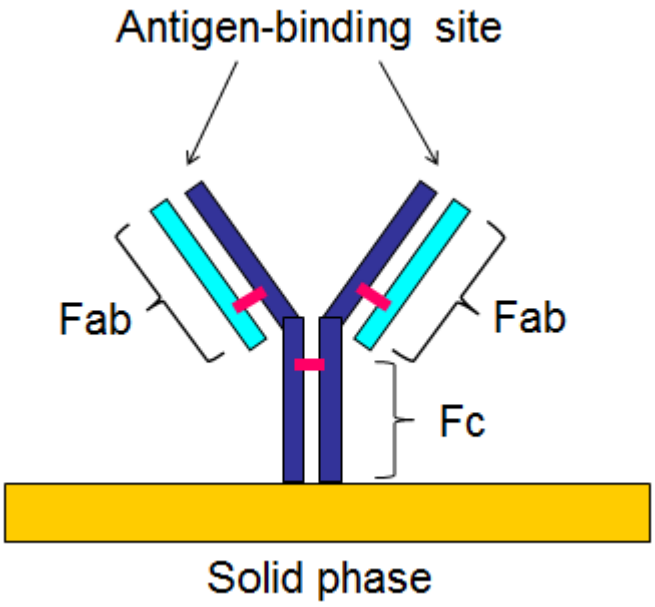


Figure 1.2 The principle of an antibody-based assay. The assay system is constructed of two main components: the antibody and the solid phase where the antibody is pinned.

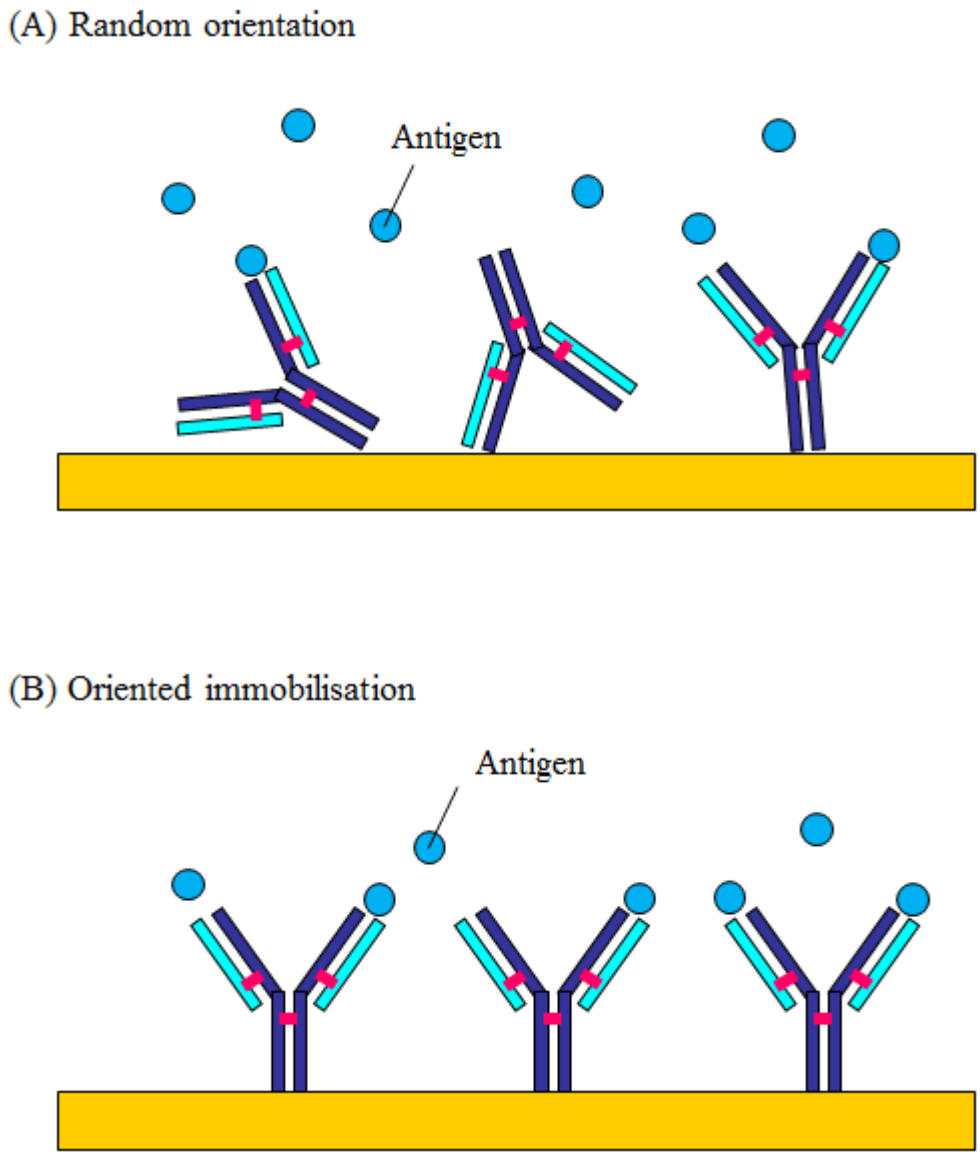


Figure 1.3 The orientation of antibodies on solid surface. (A) Random immobilisation of antibodies generates random orientation and thus it limits the antigen detection in the assay. (B) Oriented antibody immobilisation allows all Fab regions of antibodies facing away from the solid surface. This immobilisation approach maximises antigen-antibody interaction.

detection of *Staphylococcus* enterotoxin B compared to the random antibody immobilisation method.

1.3.2 Stability of immobilised antibodies

The amounts of active recognition molecules present in a biosensor always draw major attention when designing a diagnostic assay. In some situations, antibodies can be covalently immobilised via self-assembled monolayers of alkanethiol or via specific linkage of biotin-streptavidin (Subramanian *et al.*, 2006; Cho *et al.*, 2007). Some literatures proposed applications of pepsin-digested antibody fragments for assay development (Vallina-Garcia *et al.*, 2007; Nassef *et al.*, 2009). However, those procedures are cumbersome and these immobilisation methods may distort the structure of antibodies, leading to the conformational changes in the antigen-binding sites (Figure 1.4). As a result, the target antigens are unable to interact with antibodies, even though oriented antibody immobilisation is applied (Zhu and Snyder, 2003). This scenario significantly reduces the sensitivity of the assay and false negative results may arise. It is crucial to maintain the intact structure of antibodies and thus, modification of antibody during immobilisation step should be avoided. Study conducted by Vashist (2012) revealed that the modified antibodies, such as biotin conjugation, tetramethylrhodamine isothiocyanate (TRITC)-labelled and Atto 550 fluorescent labelled, has adversely affected the specific binding affinity of antibodies to its analytes in the surface plasmon resonance (SPR) analysis. His finding reported that unmodified antibodies showed higher sensitivity compared to the modified antibodies.

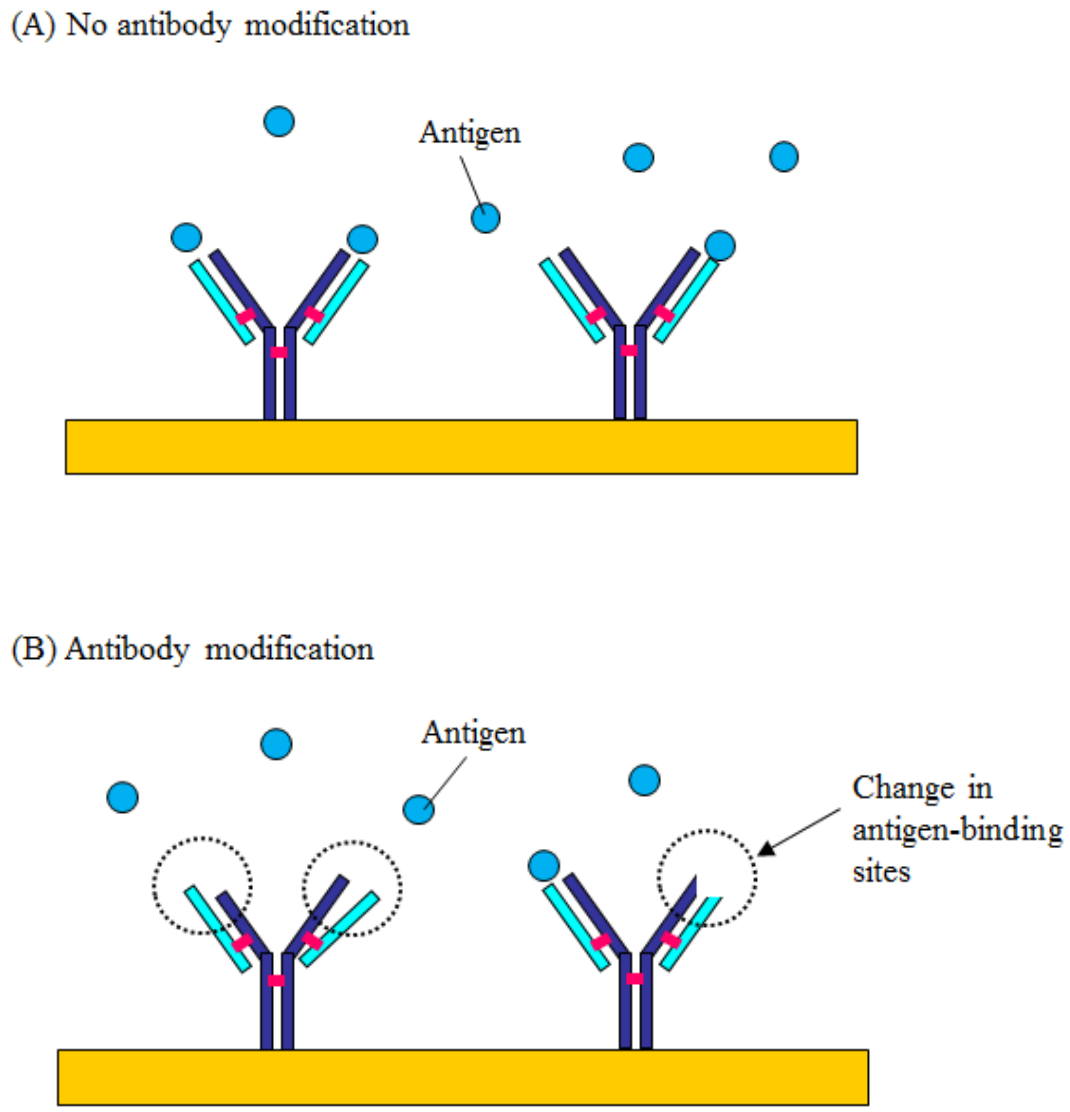


Figure 1.4 Stability of antibodies may be affected by the selected immobilisation strategy. (A) The whole structure of antibodies remain intact after attachment to the solid surface. Antibodies retain its native affinity towards analytes. (B) The antigen-binding sites of antibodies had been changed owing to the immobilisation method and thus, leading to the reduction in the affinity of antibodies.

1.3.3 Density of immobilised antibodies

Attempts had been made to increase the numbers of immobilised antibodies per unit area on the solid surface. This approach is marked as production of high density biosensor (Ruan *et al.*, 2008; Gaster *et al.*, 2011). Contrarily, in low density, there are fewer numbers of immobilised antibodies per unit area (Figure 1.5). The demand for high density biosensors places increasing pleasure on the current analytical technology, predominantly disease detection. At the early stage of infection or tumor proliferation, there is low abundance of biomarkers present in the clinical samples (Harris *et al.*, 2007; Liu and Li, 2012; Duffy, 2013). A high density biosensor with enhanced limit of detection is expected to be able to identify and quantify the target analytes, even though the quantity is minute. Nevertheless, a biosensor with low antibody density may generate false negative result, due to the probability for the antibody to interact with antigen is too low and thus it is insufficient to be translated into a quantifiable signal.

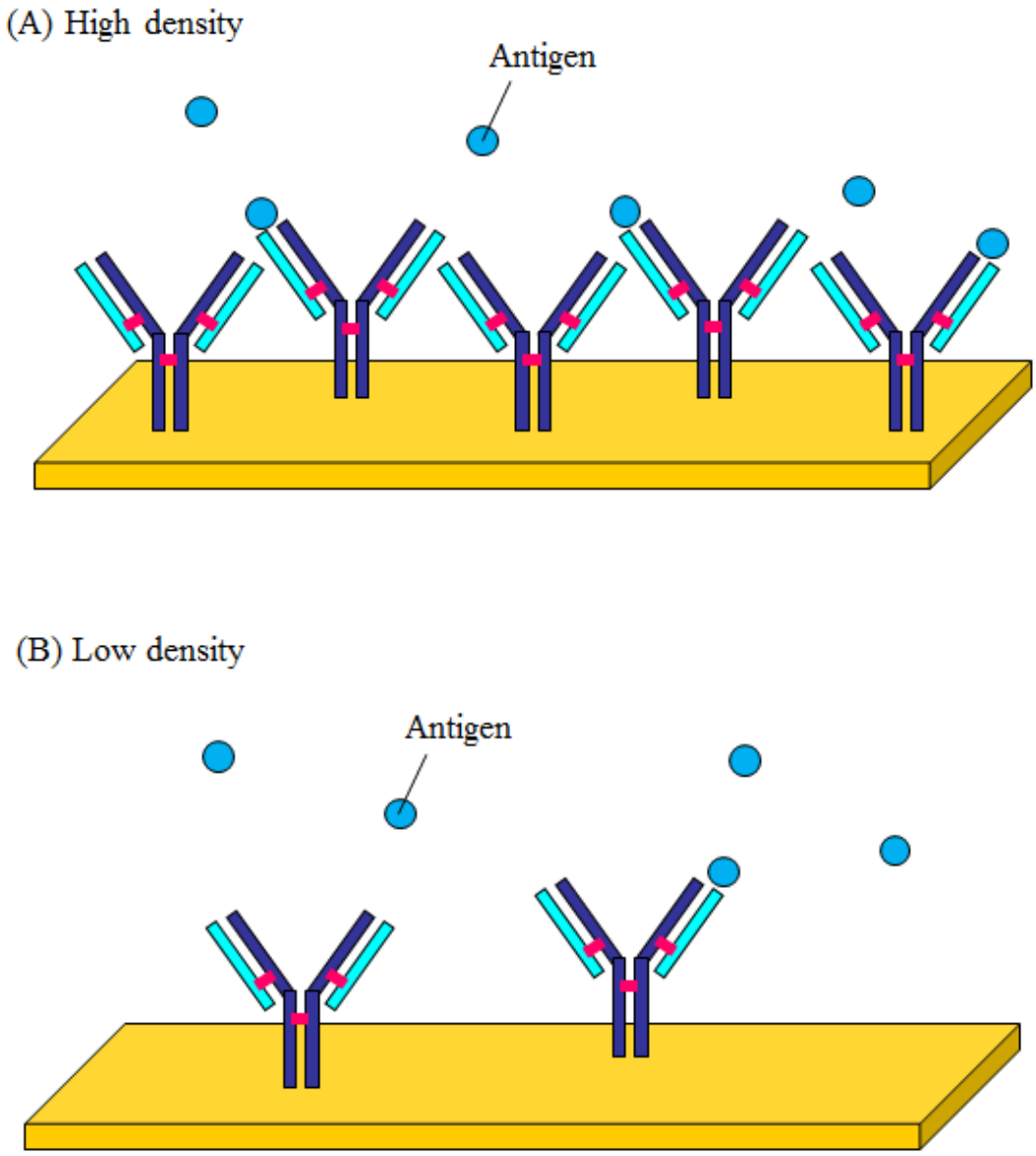


Figure 1.5 Density of immobilised antibodies will interfere the sensitivity of an assay. (A) At high density, there are large numbers of antibodies attached to the solid surface per unit area. (B) At low density, only a few antibodies are attached to the solid surface per unit area.

1.4 Strategies of antibody immobilisation

Advance methods of antibody immobilisation had been reported up to date, all these approaches can be classified into three categories: physical adsorption, covalent coupling and modification, and antibody binding proteins.

1.4.1 Physical adsorption

Antibody can be directly immobilised to a solid phase through non-specific interactions, such as hydrophobic and hydrophilic interactions. This method is marked as physical adsorption (Figure 1.6). This approach does not involve covalent bonding and thus omits the sophisticated pre-treatment procedures of solid phase (Butler *et al.*, 1992; Qian *et al.*, 2000; Pavlickova *et al.*, 2004). In view of this, physical adsorption of antibody can be carried out easily in laboratories with basic equipment and materials. The minimal antibody-surface handling procedure and cost efficiency have added major advantages for this strategy. The solid phase used for antibody immobilisation can be a plastic surface or nitrocellulose membrane. Nevertheless, the drawbacks created by the physical adsorption method have overshadowed its advantages. Due to the formation of weak interactions between antibodies and the solid surface, the adsorbed antibodies may slowly leach from the surface itself. This limits the possibility of long-term storage for future detection purposes. Furthermore, the density of antibody generated using this method is not always very high. Besides, it is noteworthy that only 20 – 30% of antibodies immobilised using this strategy appear in the proper oriented manner (Angenendt *et al.*, 2002; Taussig and Landegren, 2003). This makes the detection difficult when the analytes are in microscale or nanoscale concentration. Physical adsorption of antibody will promote conformational change in the structure due to direct contact

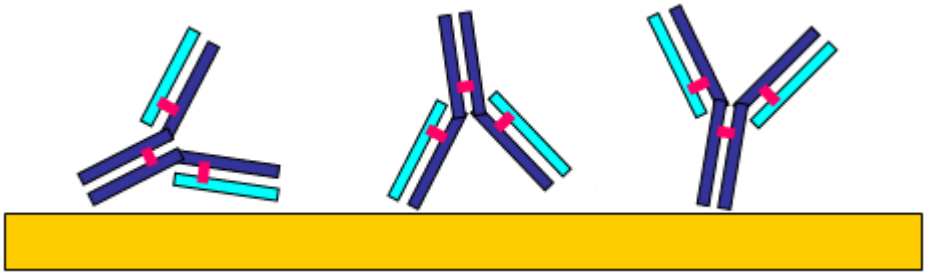


Figure 1.6 Antibody immobilisation through physical adsorption. This strategy relies on the non-specific interaction of antibody to the solid phase. As a result, the antibodies immobilised will exhibit in random orientation manner.

with the surface and thus leading to antibody denaturation (Butler *et al.*, 1993). All these situations will lower the limit of detection by the assay system.

Recent studies have suggested the employment of porous silicon as solid phase for physical adsorption of antibody (Jane *et al.*, 2009). One benefit of using porous silicon attributed to its large surface area to volume ratio. This allows loading of large amounts of biological molecules in a minuscule volume. Naddaf and Al-Mariri (2011) presented a procedure to immobilise antibody against human interleukin-6 in porous silicon. The efficiency of porous silicon in pinning antibody was studied with several spectroscopy techniques includes Fourier transformation infrared (FTIR) absorption spectroscopy, X-ray photoelectron spectroscopy (XPS) and photoluminescence (PL) spectroscopy Although the results implied the potential use of porous silicon in antibody immobilisation through physical adsorption, however, there were no functional assays conducted to validate the native affinity of the antibody. Furthermore, in their study, the antibody coating period required was 48 hours at 4°C. It is longer than the conventional physical adsorption of antibody performed nowadays. For example, in enzyme-linked immunosorbent assay (ELISA) with a conventional polystyrene surface, only an overnight incubation (approximately 16 hours) at 4°C is needed. On the other hand, Yan *et al.* (2011) reported a modified method of physical adsorption of antibody on porous silicon which required shorter antibody coating time, approximately 30 – 60 minutes. Angiotensin I antibody was loaded onto porous silicon surface followed by detection of angiotensin I in the samples. Matrix-assisted Laser Desorption/Ionisation Mass spectrometry (MALDI MS) was used to analyse the result of detection. Their findings pinpointed the advantages of physical adsorption of antibody onto porous silicon as the results yielded lower background signals with addition of high antibody

density. Nevertheless, the use of porous silicon for physical adsorption of antibody necessitates additional treatment step to stabilise the matrix used. This makes the approach become cumbersome and also lacking of cost efficiency. Studies should concentrate on improving the outcome of antibody immobilisation with minimal sophisticated result interpretation.

1.4.2 Covalent coupling and modification

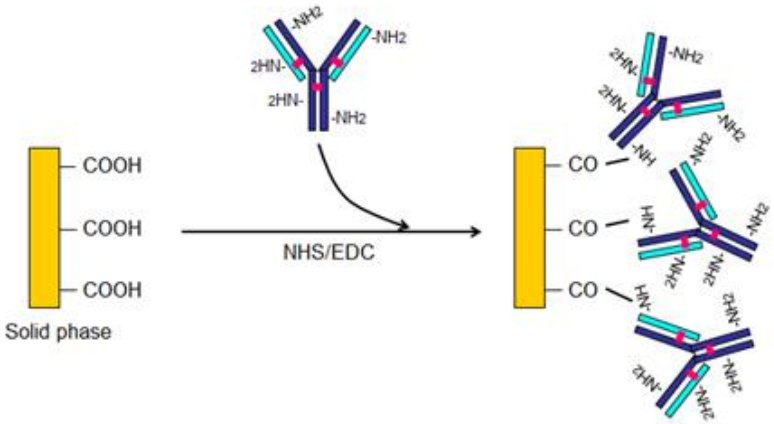
Antibody immobilisation via covalent coupling becomes a preference in the past decades. This approach relies on chemical treatment on the solid phase in order to be able to form covalent bonding with the specific functional groups in antibody. One common example is via amino groups in antibody. The solid surface is chemically treated to generate free carboxyl groups. Soluble activator, such as N-hydroxysuccinimide/1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (NHS/EDC), is introduced to initiate cross-linking between the free amino groups of antibody and the carboxyl groups on solid surface (Figure 1.7A). A stable covalent bonding is formed and allowed antibody to be immobilised on the solid phase (Danczyk *et al.*, 2003; Kumada, 2014). This approach is known as amine coupling which has commonly used in SPR analysis. Nevertheless, due to the presence of multiple free amino groups in an antibody, this method will create random antibody orientation on the solid phase and, potentially, a reduction in assay sensitivity.

The trend of covalent coupling of antibody has evolved to the use of bifunctional cross-linkers to attach antibody to solid surface (Wang and Jin, 2004; Cao *et al.*, 2007; Raj *et al.*, 2009). Bifunctional cross-linker acts as a bridge to connect two adjacent molecules which possess either the same functional groups (homobifunctional cross-linkers) or different functional groups (heterobifunctional cross-linkers). The adoption of bifunctional cross-linker in antibody immobilisation relies on its unique feature of long spacer arm. This characteristic minimises the steric hindrance generated by the solid phase itself and makes the antigen-binding sites more accessible to the target antigens. One commonly used homobifunctional cross-linker is glutaraldehyde, which forms cross-linking between amino groups of two adjacent molecules (Batalla *et al.*, 2008; Huy *et al.*, 2011). In a research

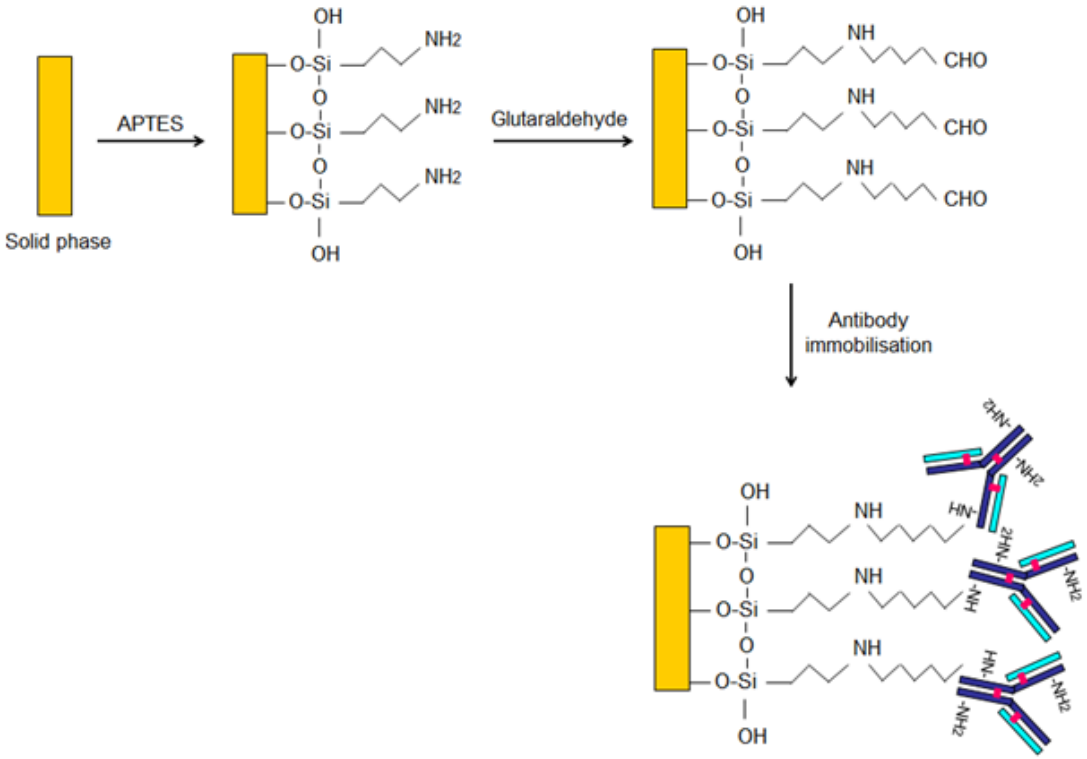
conducted by Gunda *et al.* (2014), the silicon surface was priorly treated with (3-aminopropyl)triethoxysilane (APTES) to generate amino groups on the surface (Figure 1.7B). It was followed by a reaction with glutaraldehyde reagents to produce glutaraldehyde-activated surface. Antibodies were then immobilised through its free amino groups in the structure. A uniform and homogenous surface with high density of antibody was observed through surface analytical techniques such as atomic force microscopy. On the other hand, antibody immobilisation through heterobifunctional cross-linker, such as N-γ-maleimidobutyryl-oxysuccinimide ester (GMBS), had been demonstrated by Corso *et al.* (2008). In their study, zinc oxide was selected as the solid phase for attachment of antibody. 3-mercaptopropyltrimethoxysilane (MTS) was treated on the zinc oxide surface to generate free sulfhydryl groups. GMBS, a heterobifunctional cross-linker which targets on sulfhydryl group and amino group, was reacted to the modified surface. Antibody with its free amino groups was then interacted with GMBS to immobilise on the solid surface (Figure 1.7C). Functional assay was conducted and the result reflected that the antibody maintained its native activity.

On the other hand, oriented antibody immobilisation through covalent coupling can be achieved by targeting specific molecules or functional groups which present at the Fc region of antibody. Several studies had shown that the carbohydrate side chains present at C_H2 domains of antibody are potential candidates for oriented covalent coupling (Vijayendran and Leckband, 2001; Franco *et al.*, 2006). Peluso *et al.* (2003) demonstrated an approach of antibody immobilisation through biotinylated carbohydrate side chains of Fc fragment (Figure 1.8). In their study, the carbohydrate side chains presented at C_H2 domains were oxidised to produce aldehyde groups.

(A) Through amino groups



(B) Through homobifunctional cross-linker (Glutaraldehyde)



(C) Through heterobifunctional cross-linker (GMBS)

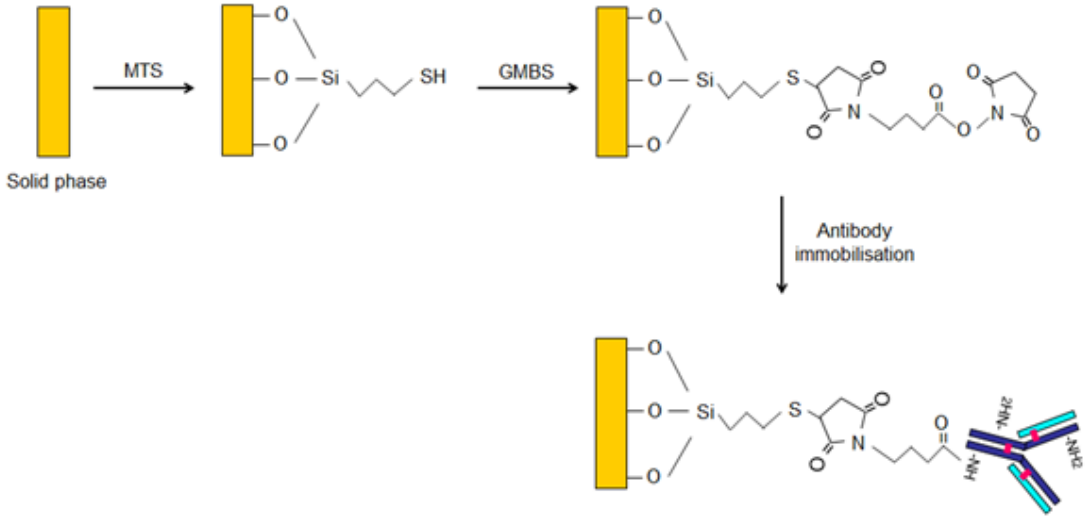


Figure 1.7 Covalent coupling of antibody to solid phase. Several approaches are demonstrated either through (A) amino groups; (B) homobifunctional cross-linker; (C) heterobifunctional cross-linker.

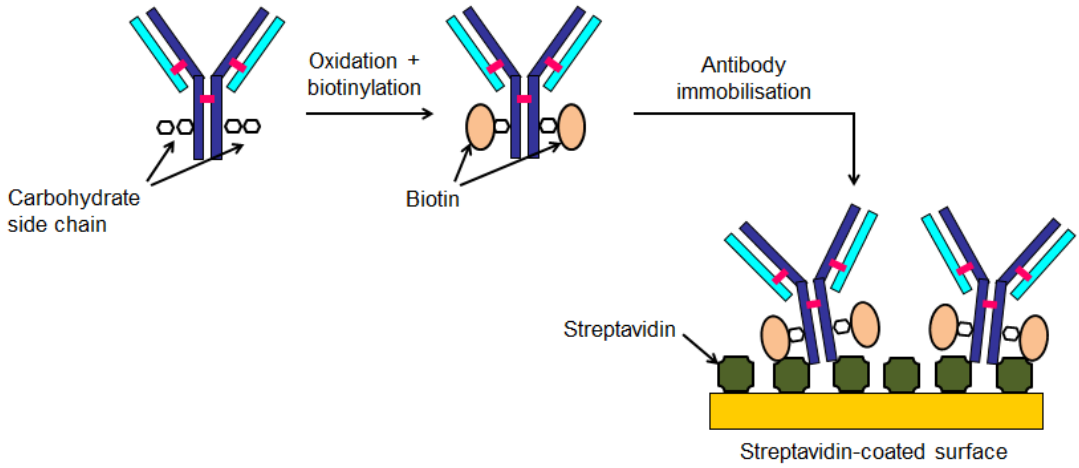


Figure 1.8 Antibody immobilisation through the specific interaction between biotin-streptavidin.

This made the structure susceptible for biotinylation by biotin reagent. The site-specific biotinylated antibody was then immobilised onto a streptavidin coated surface. This method utilised the specific interaction between biotin and streptavidin to form a stable covalent coupling. The finding reported an increase in approximate of 33% in the antigen binding activity compared to random biotinylated antibody.

Alves *et al.* (2013) had described a new strategy of oriented covalent coupling targeted on the nucleotide binding site of antibody. Nucleotide binding site is a highly conserved region found in the Fab of antibody in all isotypes from various species (Rajagopalan *et al.*, 1996). This binding pocket is constituted of four amino acid residues: two residues of tyrosine in the variable region of light chain; one tyrosine and one tryptophan in the variable region of heavy chain. These amino acids carry aromatic side chain in their structure. Importantly, exposing the aromatic side chains to UV light (254 nm) triggers formation of new covalent bond with another aromatic ring in close proximity. This feature was utilised in the study of Alves and colleagues for photocross-linking of the nucleotide binding site to an aromatic ligand namely indole-3-butyric acid (IBA). IBA was prior conjugated with biotin reagent to generate IBA-EG_{11}-biotin. Antibodies were incubated with IBA- EG_{11}-biotin followed by UV irradiation (Figure 1.9). This step allowed covalent linking between the nucleotide binding site of Fab and the aromatic ring in the IBA. The IBA-EG_{11}-biotin functionalised antibodies were then immobilised onto a streptavidin coated surface. The sensing system produced was validated through detection of prostate specific antigen. The result showed that antibody immobilisation through this approach generated higher sensitivity for more than 3- fold in the detection of prostate specific antigen compared to the amine coupling immobilisation.

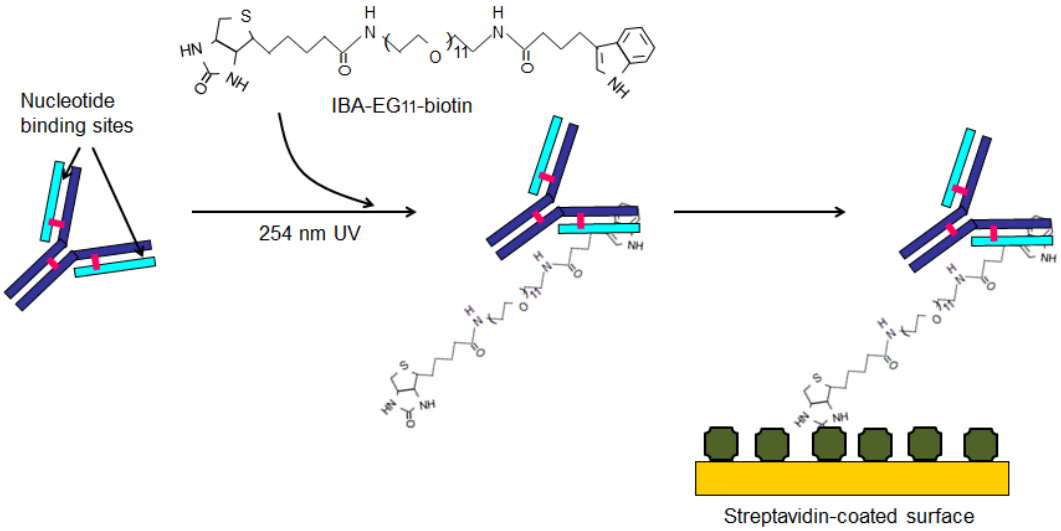


Figure 1.9 Covalent coupling of antibody by targeting the nucleotide binding sites at Fab regions.

Modification of antibody structure is a common approach in the covalent coupling of antibody. The disulfide bonds between two heavy chains of antibody can be split using reducing agent such as 2-mercaptoethylamine into two half-immunoglobulin G (half-IgG) fragments. This strategy enables antibody attachment to the gold surface through the free thiol (-SH) groups yielded (Figure 1.10A). Studies claimed that this method of antibody modification did not interfere with the antigen-binging sites of antibody yet producing oriented half-IgG on the solid phase (Karyakin *et al.*, 2000; Wang *et al.*, 2005). Billah *et al.* (2010) presented a method to attached anti-myoglobin half-IgG fragments employing the cross-linking function of heterobifunctional cross-linker, succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxyl (sulfo-SMCC). This cross-linker was used to couple the amino-terminated gold surface and the free thiol groups in half-immunoglobulin fragments. Myoglobin detection in serum was carried out using the sensing system developed. The result reported an enhancement in the limit of detection, with range of concentration from 10^{-13} M to 10^{-6} M, compared to the immobilisation of whole anti-myoglobin antibody through biotin/avidin strategy which showed detection range of concentration from 10^{-12} M to 10^{-6} M (Billah *et al.*, 2007).

Some researches had favoured the use of smaller antibody fragments for development of immunoassays. A popular method of preparing antibody fragment involved pepsin digestion of antibody to form $F(ab')_2$ fragment and numerous smaller peptides of Fc. The $F(ab')_2$ fragment was subjected to reduction process by 2-mercaptoethylamine to produce Fab' fragments (Brogan *et al.*, 2003). This approach produced free thiol group in opposition to the antigen-binding site of Fab' fragments. The free thiol group formed in Fab' fragments facilitated immobilisation on gold surface (Figure 1.10B). Functional assays, such as detection of C-reactive

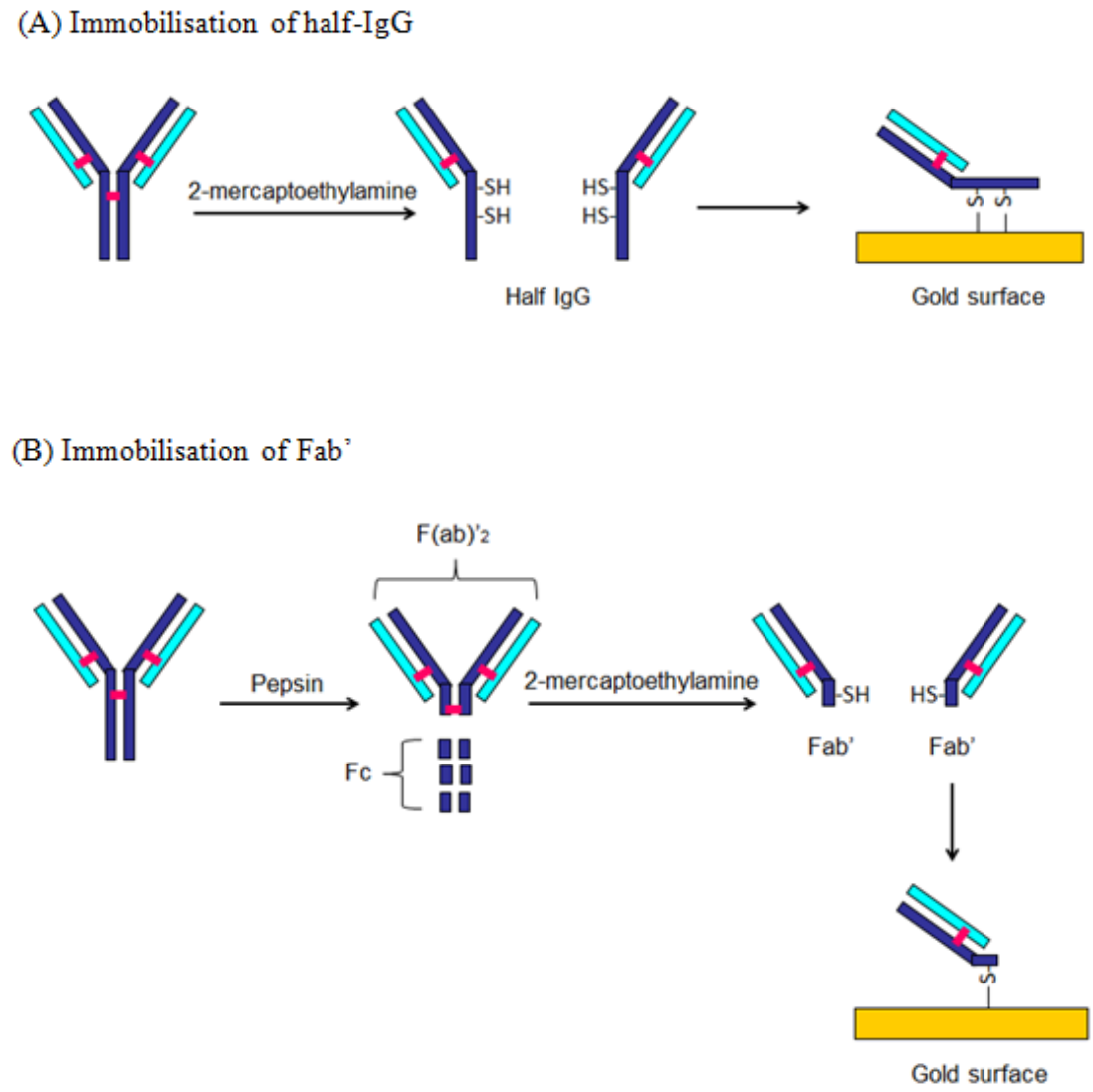


Figure 1.10 Antibody immobilisation through production of antibody fragments.