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Antibody - Biological warfare agents

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ABSTRACT

An antibody (Ab), also known as immunoglobulin (Ig), is a large Y-shape protein consisting of two heavy chains and two light chains (Figure 1) which form a functionally bivalent monomer that is produced by B cells receptor used by the immune system to identify and neutralize foreign objects such as bacteria and viruses. Current widely used rapid detection systems exploit antibodies for recognition, identification and quantification of target analytes [68]. Abs played an important role in the advancement of diagnostic assays, making these indispensable in diagnostic tests that are commonly used routinely in clinics in classical immunological methods such as ELISA, dot blot immunobinding assays, electro-chemiluminescence, flow cytometry and several microscopic techniques like fluorescence, as well as in the construction of numerous immunosensors. Immunoglobulin M (IgM) usually exists as a pentamer in mammals that predominates in primary immune responses to most antigens, and is the most efficient complement fixing immunoglobulin. It comprises approximately 10% of all normal human serum Ig content. IgM is mainly produced by the immune system for protection against numerous viral, bacterial, fungal and parasitic infections.

Keywords: antibody (Ab), antibody production, immunoglobulin M (IgM), Immunoglobulin A (IgA), Immunoglobulin G (IgG), Polyclonal antibodies (PAb)

1. ANTIBODY: A RECOGNITION ELEMENT IN BIOLOGICAL ASSAYS

An antibody (Ab) also known as immunoglobulin (Ig) is a large Y-shape protein consisting of two heavy chains and two light chains (Figure 1) which form a functionally bivalent monomer that is produced by B cells receptor used by the immune system to identify and neutralize foreign objects such as bacteria and viruses [1-67]. The widely used rapid detection systems exploit antibodies for recognition, identification and quantification of target analytes [68].

Abs played an important role in the advancement of diagnostic assays making it indispensable in diagnostic tests that are currently used routinely in clinics in classical immunological methods such as ELISA, dot blot immunobinding assays, electrochemiluminescence, flow cytometry and several microscopic techniques like fluorescence up to the construction of numerous immunosensors. One of the parameters that should always be considered in immunoassay methods is sensitivity. Sensitivity of the immunoassay is highly dependent on the affinity of the antibodies involved [69].

However, specificity of these antibodies to the target analyte should also be considered. Specificity is dependent not only on the binding property of the antibody but also on the composition of the sample antigen and its matrix, reagent composition, and immunoassay format. The lack of specificity may lead to false positive or negative results. Another parameter that should not be neglected is the cross-reactivity because it plays an important role in the quality of immunoassays.

The ideal antibody exhibits minimal or no cross-reactivity and maximal sensitivity. Cross-reactivity can be defined as an interaction between paratope of antibody and similar epitope or identical epitope presented on different antigens [70].

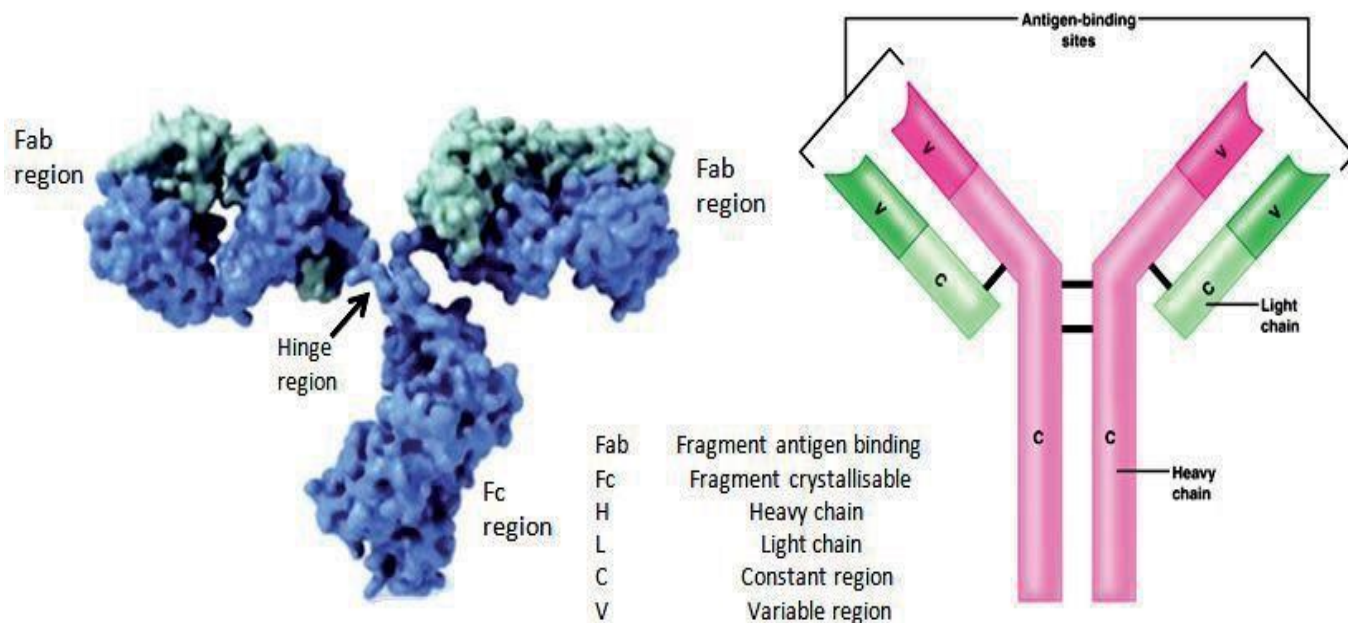


Figure 1. Typical structure of antibody.

1. 1. Structures and classes of immunoglobulins (Ig)

There are five primary classes of Igs: They are IgG, IgM, IgA, IgD and IgE. Each class of Ig are distinguished by the type of heavy chain found in the molecule. The differences in heavy chains polypeptides for each Ig allow them to function differently from each other in specific stages of immune responses [71]. The Polypeptide protein sequences that are responsible for these differences are found in Fc region. While there are five different types of heavy chains, there are only two main types of light chains: kappa (κ) and lambda (λ) [72].

Antibody classes differ in the number of different Y-like units that joins together (Figure 2) to form the complete protein that differentiates them.

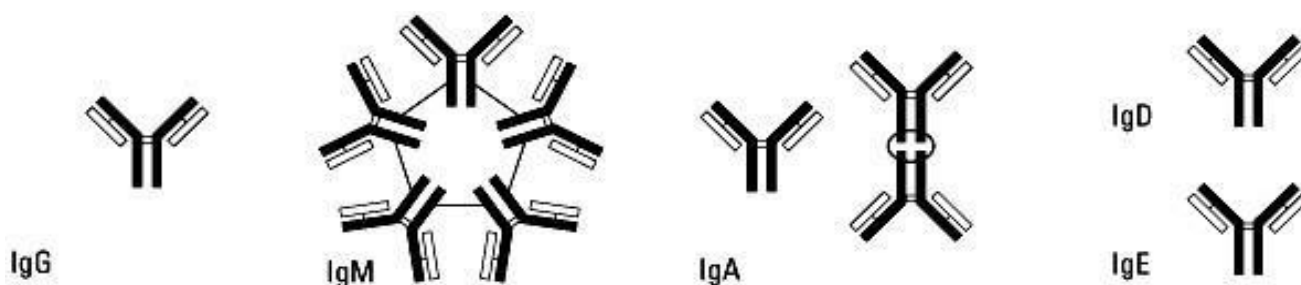


Figure 2. Typical structures of different antibodies.

Immunoglobulin G (IgG), a monomer, is the predominant Ig class present in human serum and is principally responsible for the recognition, neutralization, and elimination of pathogens and toxic antigens [73]. Maternal IgG is the only class of Ig that can transport across the placenta in humans to protect the newborn during the first months of life [74]. Because of abundance and excellent specificity toward antigens, IgG is the principle antibody used in immunological research and clinical diagnostics.

Immunoglobulin M (IgM) usually exists as a pentamer in mammals, which predominates in primary immune responses to most antigens and is the most efficient complement fixing immunoglobulin and comprises approximately 10% of normal human serum Ig content. IgM is mainly produced by the immune system for protection against numerous viral, bacterial, fungal and parasitic infections [75].

Immunoglobulin A (IgA) exists as both monomeric and dimeric forms in serum which comprises approximately 15% of the total serum Ig. Secretory IgA, a dimer, provides frontline defense against pathogens borne in aerosols, the environment, and in the diet because of its abundance in mucosal epithelia (e.g., saliva, tears). Although secretory IgA may not destroy totally the antigen, its principal function is to prevent passage of foreign substances into the circulatory system [76].

IgD and IgE are found in serum in much smaller quantities than other Igs. Membrane IgD is a receptor for antigen found mostly on mature B-lymphocytes. The biological function of IgD is still a complete mystery since it has been first discovered in 1965 [77]. IgD molecules do not cross the placenta and are not present in body secretions or urine. IgE antibodies are present in external secretions and to bind to basophils and mast cells to

primarily defends against parasitic invasion and is responsible for allergic reactions [78]. Table 1 summarises the properties of each immunoglobulin [72] (<http://www.piercenet.com>).

Table 1. Summary of properties for each immunoglobulin.

Classes	MW of Ig (g/mole)	H-chain type, MW (g/mole)	Total Serum Ig concentration (mg/mL)	(%)	Glycosylation (%weight)	Distribution (vascular)	Function (response)
IgG	150,000	gamma, 50,000	10 – 16 mg/mL	75	3	Intra and extra	Protect against toxic antigens
IgM	900,000	mu, 65,000	0.5 – 2 mg/mL	10	12	Intra	Primary response for toxins and pathogenic species
IgA	320,000	alpha, 55,000	1 – 4 mg/mL	15	10	Intra	protect mucus membranes
IgD	180,000	delta, 70,000	0 – 0.4 mg/mL	0.2	13	lymphocyte surface	Unknown
IgE	200,000	epsilon, 73,000	10 – 400 ng/mL	0.002	12	mast cells in saline and nasal secretions	against parasites

In addition to the five immunoglobulin classes, subclasses of Ig exist in all members of a particular animal species. Antibodies are classified into subclasses based on minor differences in the heavy chain type of each Ig class. In humans there are four subclasses of IgG: IgG1, IgG2, IgG3 and IgG4 (numbered in order of decreasing concentration in serum). Variance among different subclasses is less than the variance among different classes. For example, IgG1 is more closely related to IgG2, 3 or 4 than to IgA, IgM, IgD or IgE. Consequently, antibody-binding proteins (e.g., Protein A or Protein G) and most secondary antibodies used in immunodetection methods crossreact with multiple subclasses but usually not multiple classes of Ig.

1. 2. Antibody production

Immunological assays rely on the use of antibodies as a capturing and labelling molecule for identifying different targets since Ab binds with biological agents with high affinity and specificity. Current immunological assays utilise the sensitivity and specificity of polyclonal and monoclonal antibodies with respect to their bimolecular antibody–antigen interactions [79].

Polyclonal antibodies (PAb) are produced from different B-lymphocyte lines as a mixture of Ig's. Monoclonal antibody (mAb) is the product of one type of B-lymphocyte. Polyclonal antibodies are produced by immunization of a host animal such as a rabbit, mice,

chicken or goat. Several factors have to be considered in the immunization protocol like the quantity of antigen, the route of injection, number and distribution of injection sites, the frequency of antigen injections, the particular adjuvant which enhance the immune response to an antigen, the quantity of adjuvant, and the ratio of antigen to adjuvant. Harvested Igs are always purified from the sera before being used for bio-detection [80, 81].

Monoclonal antibodies are complex and heteromultimeric glycoproteins. They are used as research reagents for diagnosis as well as for therapy for various human diseases and their demand has increased substantially [82]. Köhler and Milstein developed methods for the isolation of mAb's from hybridoma cells in 1975. They demonstrated a cell fusion technique to produce hybrids between myeloma cells and antibody producing cells. The resulting hybrid lines were permanently adapted to grow in tissue culture and were capable of inducing antibody production in mice Rusing Jerne's hemolytic plaque assay, which allows direct visualization of antibody-producing B cells [83]. Monoclonal antibodies that are produced in cultured cells should be in sufficient quantities to allow optimization and further refinement of antibody-based assays and development of the nonradioactive detection technologies that are currently used [68].

1. 3. Labelling of antibodies

Antibodies are widely used in immunoassays to detect and quantify antigens. The antibody that recognizes the antigen is referred to as the 'primary' antibody and confers specificity to the assay. A 'label' is also incorporated into the assay using one of two methods like indirect or direct detection method to provide measurability. The label in an immunoassay provides either 'direct' or 'indirect' detection of the antigen. With direct detection, the label is attached via a covalent bond to the primary antibody. Alternatively, using indirect detection, the label is covalently attached to a secondary antibody, which is allowed to bind to the target (either antibody or antigen) during the immunoassay forming a sandwich assay format. Some commonly used immunoassay techniques are given in Table 2 along with examples of the types of labels that can be employed.

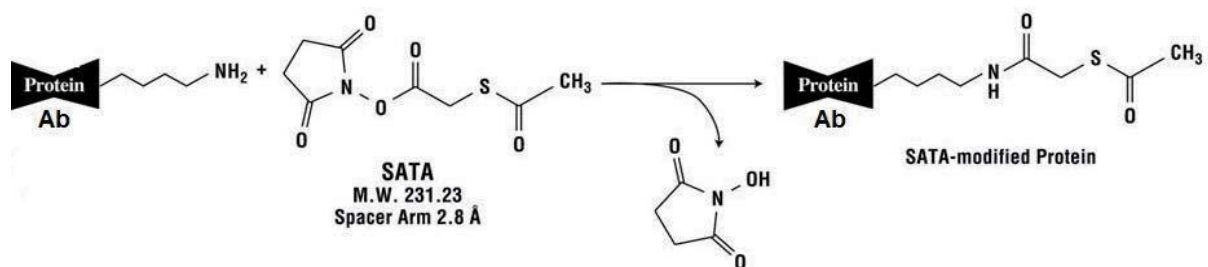
***Table 2.** Types of Immuno-experiments and associated labels.

Immunoassay	Labels
Western Blotting	Enzymes (usually HRP, or alkaline phosphatase)
ELISA	Enzymes, Biotin/Streptavidin
Immunofluorescence	Fluorescent dyes
Flow Cytometry	Fluorescent proteins or dyes, Tandem dyes

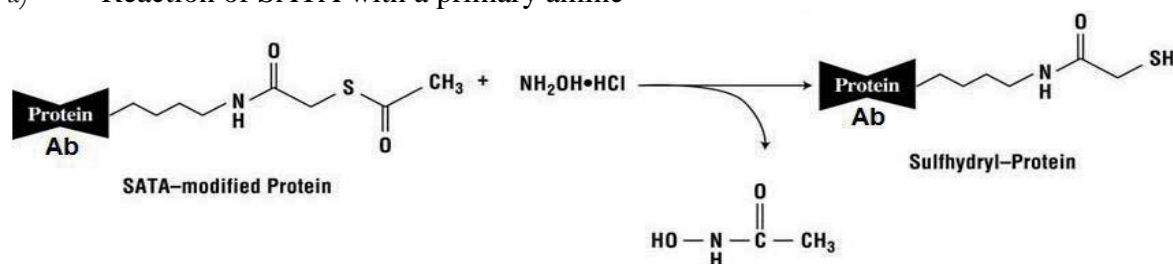
*From <http://www.biomol.de>

Antibodies like all proteins are composed of amino acids, and the side chain of lysine, which terminates in a primary amine (-NH₂), is routinely used to link labels covalently to

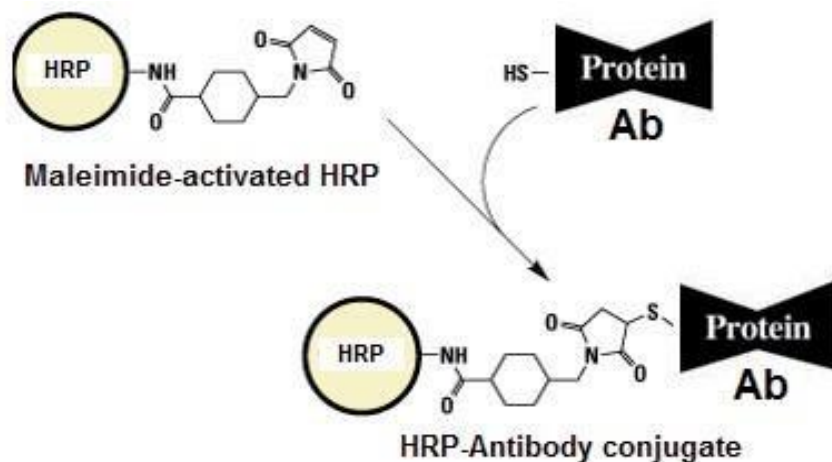
antibody molecules. There are two enzymes that are mostly used for labelling antibodies. They are horseradish peroxidase (HRP) and alkaline phosphatase (ALP).



a) Reaction of SATA with a primary amine



b) Deprotection with hydroxylamine to generate a sulfhydryl



c) Conjugation with maleimide activated HRP

Figure 3. Conjugation process of Ab with Maleimide activated HRP using SATA.

There are four main chemical approaches for antibody labelling: (i) NHS esters, (ii) heterobifunctional reagents, (iii) carbodiimide and (iv) sodium periodate. The antibody labeling procedure is complicated by the fact that the antibody and label have multiple amines. In this situation it is usual to modify some of the lysines on one molecule (e.g. the

antibody) to create a new reactive group (X) and lysines on the label to create another reactive group (Y). A 'heterobifunctional reagent' is used to introduce the Y groups, which subsequently react with X groups when the antibody and label are mixed, thus creating heterodimeric conjugates. A common procedure of this method is the use of succinimidyl acetylthioacetate (SATA). The SATA reacts with the primary amine of the antibody to form a SATA-modified protein. Formation of thiolated protein can be achieved by deprotecting the sulfhydryl group of the SATA-modified protein. The conjugate can now be reacted with the label that has been also modified with maleimide (Y). Figure 3 summarises the chemical reaction of the conjugation process.

In the carbodiimide conjugation method, 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC or EDAC) is a common crosslinking agent used to create covalent links between amine- and carboxyl-containing molecules. Carbodiimides activate carboxyl groups, and the activated intermediate is then attacked by a primary amine present in the antibody or protein as depicted in Figure 4. Carbodiimides are commonly used to conjugate antibodies to carboxylated particles (e.g. latex particles, magnetic beads), and to other carboxylated surfaces, such as microwell plates or chip surfaces. In the case of sodium periodate, this chemical cannot be employed with the vast majority of labels but is quite an important reagent in that it is applicable to HRP, which is the most popular diagnostic enzyme aside from ALP. Periodate activates carbohydrate chains on the HRP molecule to create aldehyde groups, which are capable of reacting with lysines on antibody molecules. Since HRP itself has very few lysines it is relatively easy to create antibody-HRP conjugates without significant HRP polymerization [84].

1.4. Immobilisation of antibodies

Numerous methods are available for the immobilisation of macromolecules that are suitable for the construction of biosensors. Some methods will not be described due to large number of techniques that exceed the scope of this work and only techniques that bear importance to the work presented will be discussed. One of the main issues in the development of an immunosensor is maintaining the immunorecognition capability of the antibody after it has been immobilized on the sensing surface [85]. There are three well-known available methods for attaching antibodies and antigens onto solid surfaces based on interactions [86]: (i) adsorption, i.e. physical-chemical adsorption, (ii) covalent attachment and (iii) affinity binding. Covalent methods improve uniformity and reproducibility of the immobilised proteins, i.e. antibodies or antigens, onto different solid substrate surfaces by using defined linkages or strong gold (Au)-sulfur (S) bonds to form self-assembled monolayers (SAM) [87]. However, these methods may result in randomly immobilised protein and the binding sites might be partially blocked and this could lead to a decreased binding activity and selectivity of the antibodies resulting in heterogeneous output and false negatives.

Much effort has recently been put in the development of more highly efficient antibody immobilisation methods with regioselectivity in a uniform orientation making it more sensitive than those with random orientations for immunobiosensors [88]. Among the strategies reported is the use of immobilised proteins from *Staphylococcus aureus* (protein A), *Streptococcus C40* (protein G), and *Peptostreptococcus magnus* (protein L) to bind with the antibodies to the surface. Proteins A and G bind to the Fc region in the heavy chains, while protein L binds to κ -light chains outside of the antigen-binding site. They bind in a wide variety of antibodies with different affinities as presented in Table 3.

Table 3. Binding characteristics of Protein A, Protein G, and Protein L.

Species	Antibody Class	Protein A	Protein G	*Protein L
Human	Total IgG	S		S
	IgG ₁ ,	S	S	S
	IgG ₂	W	S	S
	IgG ₃	S	S	S
	IgG ₄	W	S	S
	IgM	NB	NB	S
	IgD	W	NB	S
	IgA IgA ₁ , IgA ₂	W	NB	S
	IgE	M	NB NB	S
	Fab	W	W NB	S
	ScFv	W		S
Mouse	Total IgG, IgM	S	S	S
	IgG ₁	NB	NB	S
	IgG _{2a} , IgG _{2b} ,	W	M	S
	IgG ₃	S	S	S
Goat	Total IgG S6	W	S	NB
	IgG ₁	W	S	NB
	IgG ₂	S	S	NB
Rabbit	Total IgG	S	S	W
Rat	Total IgG	W	M	S
	IgG ₁	W	M	S
	IgG _{2a}	NB	S	S
	IgG _{2b}	NB	W	S
	IgG _{2c}	S	W	S
Pig	Total IgG	S	W	S

W = weak binding, M = medium binding, S = strong binding, NB = no binding-means information not available, *Binding will only occur if the appropriate kappa light chains are present.

The binding affinity only refers to species and subclasses with the correct kappa light chains. Lambda light chains and some kappa light chains will not bind (From Pierce Biotechnology TECH TIP #34 <http://www.piercenet.com>).

Another method that has gained attention is the direct immobilisation of the antibody itself through its fragment region and thiol modified antibodies. The F(ab') fragment, which is about 50 kDa, can be produced from the reduction of F(ab')₂ fragments by using cysteine [87]. The Fab' fragment contains a free sulfhydryl group that may be alkylated or used in conjugation with an enzyme. In contrast, F(ab')₂ fragment antibodies are generated by pepsin, papain, ficin or bromelain digestion of whole IgG antibodies to remove most of the Fc region

while leaving intact some of the hinge region [89]. F(ab')₂ fragments have two antigenbinding F(ab') portions linked together by disulfide bonds, and therefore are divalent with a molecular weight of about 110 kDa. To date, the use of F(ab') fragment in the field of biosensing has been widely explored such as graphene field-effect transistor immunosensor [90], SPR-based immunosensors for human growth hormone detection [91] and bovine leukaemia virus [92] and quartz crystal microbalance for the detection of pathogenic Escherichia coli O157:H7 [93-142]. The antigenbinding activities of immobilized F(ab') fragments gained improved sensitivity of developed biosensors as compared to conventional use of whole antibody having random orientations when immobilised [87].

Figure 4 shows a scheme of typical immobilisation of proteins and antibodies by selfassembled monolayer (SAM) of thiolated functional group through carbodiimide conjugation chemistry and direct chemisorption of fragments.

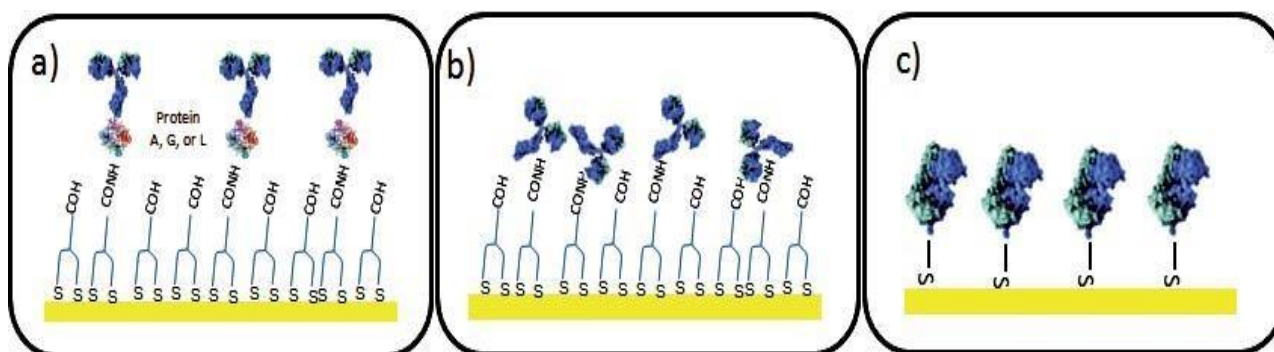


Figure 4. Typical scheme of immobilising immunoglobulins using SAM of a) protein A, G, or L, b) whole Ab and c) fragment Ab (F(ab)). The metal layer was modified by SAM bipodal through carbodiimide conjugation chemistry for a and b.

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