

The Significance of Antibody Orientation in Immunoassay and a Comparison of DSP and Physical Adsorption on Silicon, Chromium, Gold, and Zinc Oxide Surfaces

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Abstract. Antibody immobilization in immunoassays has become more prevalent, due in part to immunosensors and other innovations. Immobilization methods, consequently, have become more varied. As immunoassay experimentation continues, researchers learn what determines the effectiveness of antibody immobilization. According to most sources, orientation plays an important role in the success of antibody immobilization. Various research articles have tested the importance of protein orientation, and their results support this hypothesis. Much of the research of orientation supports the claim that oriented antibodies yield better results, such as higher sensitivity of immunoassays, higher antigen-binding capacity, and more structured properties than randomly oriented antibodies. One of the best ways to achieve a specific antibody orientation is the binding of Proteins A and G. Another technique for immobilization, DSP, is extremely stable on gold surfaces. The experiment described in this report was antibody immobilization using DSP for one portion of antibodies and physical adsorption for the other. This report gives results from research on whether specific antibody orientation improves antigen binding activity in immunoassay, and it shows the results of an experiment on the relative binding affinity of DSP as compared with physical adsorption.

Introduction

Antibody immobilization is an important subject with a variety of purposes such as in diagnostic immunoassays. An immunoassay is the binding of antibodies and antigens in order to measure the amount of either in a sample. One of its applications is the immunosensor, a biosensor made by immobilizing an antibody monolayer onto a transducer, a device that converts energy from one form to another, for use in specified antigen binding, which helps make the sensor more accurate, precise, and reproducible.¹ The study of immunosensors is important to cancer research and the early detection of cancers such as breast cancer and colon cancer; consequently, discovering the effective ways to immobilize antibodies is essential. Two important and popular methods of immobilization are the chemical cross-linker dithiobis(succinimidyl propionate) (DSP) and physical adsorption. Physical adsorption is the molecular binding of antibodies to a solid surface using attraction forces, such as electrostatic force, rather than chemical bonds. The purpose of this report is to describe past research on specific orientation effectiveness and to discover evidence on whether immobilizing antibodies by DSP improves the binding activity of antibodies.

Significance of Orientation in Immobilization

The orientation of the antibodies immobilized on the surface is considered to be a determinant of their effectiveness. An immunoglobulin G (IgG) antibody is considered to be properly oriented and completely active when immobilized on the Fc fragment of the antibody, which has no antigen-binding affinity, rather than the F(ab')₂, which contains antigen-binding sites (Figure 1).²

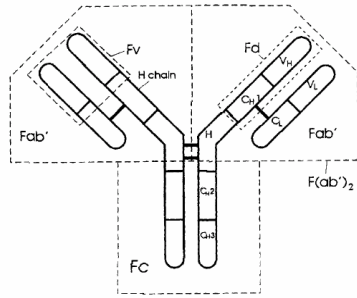


Fig. 1 Schematic diagram of an antibody molecule and its fragments. The molecule can be subdivided into two parts. The Fc fragment contains the antibody effector functions, such as complement activation, cell membrane receptor interaction and transplacental transfer.⁶ The F(ab')₂ fragment contains two identical Fab' fragments, which are held together by the disulfide linkages in the hinge (H) region. The Fab' fragment (antigen-binding site-containing fragment) consists of the heavy (H) and light (L) variable (V) chains (V_H and V_L) and the constant (C_{H1} and C_L) chains. Other segments are the Fv (variable fragment) consisting of the V_H and V_L chains and the Fd fragment which contains the V_H and C_{H1} chains. The carbohydrate moieties locate at the C_{H2} domain and the binding sites for Fc receptors (protein A, protein G and recombinant protein A/G) locate between the domains of C_{H2} and C_{H3}.

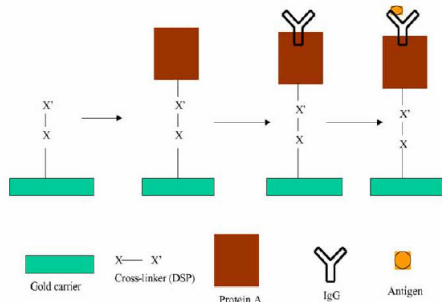
Much of the research done today suggests that binding antibodies on their active Fab fragments prevents antigen binding and, subsequently, antibody activity. If this is accurate, then specific orientation while binding is essential in order to ensure binding site activity. Spitznagel *et al* claims that antibodies that have been covalently bonded have smaller antigen-binding capacities than soluble antibodies because of the random orientation on the support surfaces.³ Lu *et al* supports this idea in another article and later concludes that oriented immobilization methods result in an antigen-binding capacity a factor of 2-8 higher than random binding methods.² The majority of scientific articles on orientation that I have read agree that specific orientation of the antibodies on the binding surface improves antigen-binding affinity, antibody activity, surface density, the sensitivity and range of biosensors, and other functions.^{2,3,4,5,6-10}

Effort has been made, consequently, to immobilize antibodies using methods that cause oriented bonds, in order to maximize function and benefit. In an experiment by Subramanian *et al*, in which monoclonal antibodies were bound by oriented and random immobilization,

the oriented immunosorbents had antigen-binding capacities of 42-48%, whereas the random ones only had capacities of 18-22%.⁶ Lu *et al* also found that, in another experiment, immobilization using Protein A resulted in a sensitivity 10 times larger than that of random immobilization.⁷ Kaku *et al* received similar results: specific orientation was found to improve biosensor sensitivity and range.⁸

Although orientation does play a role in immobilization of whole antibodies, more ostentatious proof of orientation's significance can be found through antibody fragmentation, which requires the separation and immobilization of the Fab' fragments instead. In one article, Lu *et al* concluded that the oriented fragments had a higher surface density and three times more antibody activity than the randomly immobilized antibody fragments.² Rao *et al* suggested that orientation also helped determine the binding abilities of antibodies.⁹ In an article by Lu *et al*, orientation was found to greatly affect the antigen-binding activity of the antibodies, since the antigen-binding activity of the oriented antibody fragments was 2.7 times that of the randomly immobilized ones.¹⁰ Peluso *et al* found that oriented Fab' fragments had the highest surface densities and antibody activity of all random and oriented antibodies, surpassing oriented whole antibodies in antigen binding by 49%.⁴ Shmanai also concluded that random orientation leads to variable antibody activity.⁵

Random orientation is not completely responsible for loss of antibody activity, however. According to Lu *et al*, antibody concentration and antibody orientation played almost equal roles in antibody activity.⁸ Spitznagel *et al* associated the decrease in binding affinity from surface loading with steric hindrance, rather than orientation.³ Peluso *et al* also partially credited steric hindrance for loss of binding activity.⁴



Scheme of site-directed antibodies immobilization on gold substrate

Figure 2

Whether the success is caused by orientation or not, oriented antibodies seem to outperform most typical immobilization methods. Some of these effective binding techniques are Proteins A, G, and their derivatives. Protein A is a part of *Staphylococcus Aureus*'s cell wall and binds only to the Fc segment of an IgG antibody. It was the first of the two proteins to be used in antibody immobilization, and it binds solely with the Fc portion of an IgG antibody of most mammals.² Because of this site-specific binding, immobilizing with Protein A ensures proper orientation on the surface. Schmid *et al* performed an immunoassay experiment in which Protein A was immobilized on a DSP modified gold surface. Protein A suffered no major loss of activity after 8 weeks. In addition, this experiment demonstrated that Protein A could be reused.¹¹

The use of Protein A in immobilization brings many beneficial results. Because the protein binds only to the Fc region, it can achieve full antigen-binding capacity.¹² It is also one of the simpler methods of immobilization, since it only requires two steps. Protein A also causes more active antibodies, although there is a lower surface density.⁴

In various experiments, Protein A proved to immobilize more effectively than other immobilization methods. In a comparison by Danczyk *et al*, immobilization using Protein A was more functional and oriented than covalent bonding and adsorption.¹³ Babacan *et al* found Protein A to be more stable and reproducible than glutaraldehyde cross-linking.¹⁴ In a

similar experiment, using Protein A resulted in higher sensitivity, more stability, and more reproducibility in a piezoelectric immunosensor than did physical adsorption or glutaraldehyde cross-linking.¹⁵ In contrast, Protein A, along with covalent bonding, did not immobilize as well as physical adsorption when tested by Caruso *et al.*¹⁶ In a study by Ren *et al*, however, Protein A immobilization was found to be more oriented than adsorption, covalent binding, and cross-linking, because these methods immobilize randomly onto surfaces.¹⁷

Although Protein A is a simple and useful immobilization technique, in a variety of studies, B5C1 has been found to be more effective in stability and reusability. B5C1 was derived from the introduction of a cysteine residue with a thiol group to the C-terminus of 5 B-domains from Protein A, and, according to the study by Ren *et al*, it has higher binding activity and more stability than A. In addition, B5C1 has withstood 30 times of reuse without a noticeable decrease in binding activity, a longer reusability than Protein A.¹⁷ In the experiment by Kanno *et al*, B5C1 also caused very oriented antibodies and an antigen-binding activity 4.3 times as large as Protein A.¹²

Protein A is most successful with mammal IgGs but is ineffective in certain animal IgGs, such as goat, sheep, cow, and horse. Taken from the cell wall of *Streptococcus* human pathogenic strains of Lancefield group G, Protein G reacts with more IgGs than Protein A and less with other immunoglobulins. One drawback, however, is that Protein G forms a weaker bond with antibodies that react well with Protein A. In order to counter weaknesses of both A and G, A/G was created. Protein A/G has four binding domains from A and two from G, combining all the aspects of both into an efficient protein.²

Many reports and experiments suggest that antibody orientation plays a significant role in antibody activity and function. The majority of evidence agrees with this hypothesis and supports the use of oriented immobilization methods, rather than random couplings.

DSP Binding in Comparison to Physical Adsorption

I participated in an antibody immobilization experiment in which chips were exposed to each of the binding methods in order to draw conclusions as to whether DSP had a significant advantage in effective immobilization over physical adsorption. I believe that DSP does cause a better binding affinity and that immobilization on the gold surface will display the best relative affinity of all the other metals. DSP has already been considered a stable linker for gold surfaces¹⁸⁻¹⁹ and I believe my experiment will support this hypothesis.

Methods and Materials

In the experiment, four types of chips were used as antibody binding surfaces: silicon, chromium-coated, gold-coated, and zinc oxide-coated respectively. The chips were placed into two 96-well plates: one plate used DSP to bind the antibodies, the other used physical adsorption. The primaries used were Protein G, Prostate Specific Antigen (PSA) antibody, or CA125 antibody in Phosphate-buffered Saline (PBS). The secondary was Horseradish Peroxidase (HRP) labeled monoclonal anti-mouse antibody. The substrate used was tetramethylbenzidine (TMB), which changes the solutions blue.

DSP Plate

In the plate that used DSP, nine of each chip (silicon, chromium, and gold) were used. Only three zinc oxide chips, however, were used. Two of the silicon, chromium, and gold chips, and one of the zinc oxides were used as controls to measure the efficiency of the blocking technique used. Two chips of each type of metal were coated with Protein G. Two

other chips from each metal and the zinc oxide were coated with CEA, except for gold, of which three were coated. Two more of each type of metal chip were coated with CA125. An HRP control without a chip was also incubated in a well to test light sensitivity of HRP and the bias of the blue TMB substrate used in the immobilization method.

Physical Adsorption Plate

In the plate without surface preparation, nine of each metal-coated chip (silicon, chromium, gold) were used. Three of the zinc oxide chips were used. The first two chips of each metal served as controls like the DSP plate samples, whereas one of the zinc oxide was used as a control. Two chips of each metal were coated with protein G. Two more chips of each metal and the zinc oxide were coated with CEA. One well was filled with HRP as a control as in the DSP plate, and CA125 was added to two chips of each metal.

Procedure

First DSP was applied for 1 hour and 30 min to one plate. Then the primary was applied to each plate: Protein G, PSA or CA125 antibody in PBS. Then the plates were incubated at 37.8°C for 1.5 hours. Later, unused binding sites were blocked by washing two times with PBS and adding 0.1% BSA in PBS and incubating the plates at 37.8°C for 30 minutes. Next, the secondary (Horseradish Peroxidase (HRP) labeled anti-mouse antibody) was added by washing the chips twice with PBS-TWEEN® solution, applying the secondary antibody into the PBS-TWEEN® solution and incubating at 37.8°C for 1 hour. Next, the chips were washed thoroughly with PBS-TWEEN® solution and moved to an unused well before adding the TMB substrate. The HRP catalyzed in the presence of TMB. The absorbance was measured at 650nm using a standard plate reader.

RESULTS

For the plate using DSP, one of the gold chips coated with Protein G had the highest absorbency of all the metals. In the second set of Protein G chips, silicon had the highest absorbency. All three of gold's CEA-coated chips had the highest absorbency of all the other CEA-coated metals. For the first set of CA125 chips, silicon had the highest absorbency. In the second set, gold's absorbency was highest. (Table 1)

Table of absorbencies for silicon, chromium, gold, and zinc oxide using DSP

	Control	Control	Protein G	Protein G	CEA	CEA	Nothing C- CEA	CA-125	CA-125	HRP control
Si	0.074	0.103	0.505	0.626	0.960	1.166	0.039	0.942	0.494	0.041
Cr	0.080	0.157	0.494	0.446	0.882	0.434	0.038	0.322	0.270	0.054
Au	0.220	0.354	1.002	0.484	1.008	1.221	1.245	0.602	0.708	0.036
ZnO	0.066				0.751	0.415				

In the physical adsorption plate, gold had the highest overall absorbency rate. Although silicon had a higher rate for the first set of Protein G-coated chips, gold had the highest absorbency for the other set of Protein G, for CEA, and for CA125. (Table 2)

Table of absorbencies for silicon, chromium, gold, and zinc oxide using physical adsorption

	Control	Control	Protein G	Protein G	CEA	CEA	HRP control	CA125	CA125
Si	0.051	0.176	3.260	0.351	0.563	0.578	0.050	0.380	0.405
Cr	0.155	0.061	0.544	0.419	0.375	0.438	0.043	0.326	0.247
Au	0.084	0.095	0.663	0.873	1.058	0.930	0.060	0.799	0.575
ZnO	0.056				0.783	0.775			

Discussion

Overall, the results of the two tables were quite variable. Many of the control and empty wells showed absorbencies of 0.1 and higher, which seemed too high for controls, and the absorbencies between the plates were very inconsistent. Though gold did remarkably better in absorption than the other metals, the DSP table's results varied in too many areas, such as

in Protein G and CEA's absorbency rates, to be taken at face value. The table that used physical adsorption, did not vary as greatly as the DSP table. It still varied in similar ways, however. The variance of both tables can probably be attributed to the challenge of the chips flipping upside down as they were positioned in the wells and cleaned. In addition, because the tool used to apply the solutions was not the proper size, a difference in volume of the solution among the wells could have also caused the variance and misleading absorbency figures.

Conclusion

An experiment was done to test the relative binding affinity of silicon, chromium, gold, and zinc oxide using DSP or physical adsorption. Although the data showed that the gold surfaces used had the highest relative binding affinity, the results were too variable and inaccurate to make any strong conclusions in this area. More experiments will have to be done that produce a more coherent trend before DSP's higher binding ability over physical adsorption can be determined. In addition, in order to test the effectiveness of specific orientation, the experiments should be done using a tagged antigen rather than a secondary antibody. Although the experiment did not give the results needed to draw conclusions about orientation or DSP, I have learned more about the process of immunoassay and how oriented binding affects antibodies from this research experience.

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References

- ¹ You, H. X., Disley, D. M., Cullen, D. C., Lowe, C. R. 1995. A scanning tunnelling microscopic study of covalent immobilization of immunoglobulin G on gold: effect of the bias voltage on topography. *Micron*. **26**: 121-132.
- ² Lu, B., Smyth, M. R. and O’Kennedy, R. 1996. Oriented immobilization of antibodies and its applications in immunoassays and immunosensors. *Analyst*. **121**:29R-32R.
- ³ Spitznagel, T.M., Clark, D.S. 1993. Surface-density and orientation effects on immobilized antibodies and antibody fragments. *Bio/Technology*. **11**:825-829.
- ⁴ Peluso, P., Wilson, D.S., Do, D., Tran, H., Venkatasubbaiah, M., Quincy, D., Heidecker, B., Poindexter, K., Tolani, N., Phelan, M., Witte, K., Jung, L.S., Wagner, P. and Nock, S. 2003. Optimizing antibody immobilization strategies for the construction of protein microarrays. *Anal Biochem*. **312**:113-124.
- ⁵ Shmanai, V.V., Nikolayeva, T.A., Vinokurova, L.G. and Litoshka, A.A. 2001. Oriented antibody immobilization to polystyrene macrocarriers for immunoassay modified with hydrazide derivatives of poly(meth)acrylic acid. *BMC Biotechnol*. **1**:article 4.
- ⁶ Subramanian, A. and Velander, W.H. 1996. Effect of antibody orientation on immunosorbent performance. *J Mol Recognit*. **9**:528-535.
- ⁷ Lu, B., Smyth, M.R. and O’Kennedy, R. 1996. Immunological activities of IgG antibody on pre-coated Fc receptor surfaces. *Anal Chim Acta*. **331**:97-102.
- ⁸ Kaku, S., Nakanishi, S. and Horiguchi, K. 1989. Enzyme immunoelectrode for insulin incorporating a membrane partially treated with water vapor plasma. *Anal Chim Acta*. **225**:283-292.
- ⁹ Rao, S.V., Anderson, K.W. and Bachas, L.G. 1998. Oriented immobilization of proteins. *Mikrochim Acta*. **128**:127-143.
- ¹⁰ Lu, B., Xie, J., Lu, C., Wu, C. and Wei, Y. 1995. Oriented immobilization of Fab’ fragments on silica surfaces. *Anal Chem*. **67**:83-87.
- ¹¹ Schmid, A.S., Stanca, S., Thakur, M.S., Thampi, K.R., Suri, C.R. 2005. Site-directed antibody immobilization on gold substrate for surface plasmon resonance sensors. *Sensor Actuat B-Chem*. (In Press).
- ¹² Kanno, S., Yanagida, Y., Haruyama, T., Kobatake, E. and Aizawa, M. 2000. Assembling of engineered IgG binding protein on gold surface for highly oriented antibody immobilization. *J Biotechnol*. **76**:207-214.
- ¹³ Danczyk, R., Krieder, B., North, A., Webster, T., HoganEsch, H. and Rundell, A. 2003. Comparison of antibody functionality using different immobilization methods. *Biotechno Bioeng*. **84**:215-223.
- ¹⁴ Babacan, S., Pivarnik, P., Letcher, S. and Rand, A.G. 2000. Evaluation of antibody immobilization methods for piezoelectric biosensor application. *Biosens Bioelectron*. **15**:615-621.
- ¹⁵ Pei, R., Hu, J., Hu, Y. and Zeng, Y. 1998. A piezoelectric immunosensor for complement C4 using Protein A oriented immobilization of the antibody. *J. Chem Technol Biotechnol*. **73**:59-63. Lu, B., Smyth, M.R. and O’Kennedy, R. 1996.
- ¹⁶ Caruso, F., Rodda, E., Furlong, D. N. 1995. Orientational aspects of antibody immobilization. *J Colloid Interf Sci*. **178**:104-115.
- ¹⁷ Ren, X., Kobatake, E., Aizawa, M. 2000. A new type of reusable piezoimmunosensor fabricated by a recombinant IgG protein. *Analyst*. **125**:669-671.
- ¹⁸ Katz, E.Y. 1990. A chemically modified electrode capable of a spontaneous immobilization of amino compounds due to its functionalization with succinimidyl groups. *J. Electroanal. Chem*. **291**:257-260.
- ¹⁹ Hermanson, G. T., Mallia, A.K. and Smith, P.K. 1992. Immobilized Affinity Ligand Techniques, Academic Press Inc. pg 59.

