

Immobilization of Antibodies on the Attana[®] Carboxyl Sensor Chip Surface

SUMMARY

The Attana[®] Carboxyl Sensor Chip surface has been evaluated in terms of functionality and performance. As follow are the key properties of the Attana Carboxyl Sensor Chip surface

- Provides robust and reproducible immobilization of antibodies
- Surface activation by EDC/sulfo-NHS permits generic conditions to be used for immobilization of IgGs and allows for effective immobilization also at low pH
- Surface density of immobilized IgG can readily be controlled by varying the antibody concentration injected over the activated surface

INTRODUCTION

Attana biosensors are based on the Quartz Crystal Microbalance (QCM) technique, which is an extremely sensitive mass sensor, capable of measuring mass changes in the sub nanogram range¹. The piezoelectric quartz plate that constitutes the sensor can be functionalised by a variety of surface chemistries to obtain surfaces suitable for protein immobilization and bimolecular interaction studies². This technical note describes the functionality and performance of the Attana Carboxyl Sensor Chip surface for immobilization of antibodies.

The Attana Carboxyl Sensor Chip surface is designed for versatile and effective immobilization of proteins on the sensor surface by amine coupling. The objective of this study was to test the surface for immobilization of a range of antibodies; four mouse monoclonal antibodies (mAb A-D) of subclasses IgG₁, IgG_{2a} and IgG_{2b}, two different rabbit polyclonal antibodies (pAb E-F) and one goat polyclonal antibody (pAb G). The dependence of immobilization effectiveness on pH of immobilization buffer and antibody concentration were examined.

METHOD

Attana Carboxyl Sensor Chip surfaces were prepared for immobilization by first inserting them into the sensor systems and allowing them to stabilise in HEPES buffered saline running buffer (10 mM HEPES, 150 mM NaCl, 0.005% Tween[®] 20) at 50 μ L/min. For activation of the surfaces the flow rate was set to 10 μ L/min and the surfaces were normally exposed to a reagent mixture containing 0.2 M 1-ethyl-3-[3-dimethylaminopropyl] carbo-diimide hydrochloride (EDC) and 0.05 M N-hydroxysulfo-succinimide (sulfo-NHS) for a period of 300 sec^{3,4}. The protein to be immobilised was dissolved in an immobilization buffer at a concentration of 50 μ g/mL and was injected over the surface for a contact time of 300 sec. Remaining active groups on the

surface were then deactivated by ethanolamine 1 M at pH 8.5. Examination of activation efficiency with different reagent mixtures was performed by activation of the surfaces by EDC, EDC/NHS, or EDC/sulfo-NHS, respectively. The concentrations for EDC and NHS/sulfo-NHS were consistently 0.2 M and 0.05 M respectively, in the mixtures that were passed over the surface. The experiments were carried out on the Attana[®] 100 and the A100[®] C-Fast biosensor systems.

RESULTS

Activation reagents

Immobilization of monoclonal antibody A (mAb A) was studied after activation of EDC, EDC/NHS or EDC/sulfo-NHS respectively. The results are displayed in Fig. 1.

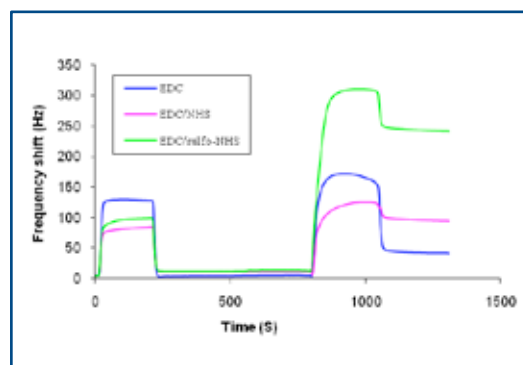


Figure 1: mAb A immobilized on carboxyl surface activated by EDC (blue), EDC/NHS (pink), and EDC/sulfo-NHS (light green), respectively. The time resolved frequency response first shows the sensor response to the activation reagents, which is predominantly reversible with only very small frequency shifts retained after the sample plug has passed the sensor surface. The second injection with antibody in immobilization buffer show a varying degree of retained frequency shifts depending on which activation mixture was used.

The results clearly show that the most efficient immobilization is obtained on carboxyl surfaces activated by EDC/sulfo-NHS, whereas less efficient coupling responses are achieved with surfaces activated by EDC or EDC/NHS. This is probably due to the dissimilar modification to the surface charge that is the result of activation with the different mixtures. Activation by EDC replaces the negative charges of the carboxyl groups with a positive charge, whereas activation by the EDC/NHS mixture results in a neutralization of the negative charge. Activation by EDC/sulfo-NHS replaces the negatively charged carboxyl with a

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more negatively charged sulfo-group. The results indicate that, (1) the electrostatic attraction between the surface and the protein is essential for successful immobilization, and (2) activation with the EDC/sulfo-NHS reagent mixture provides an effective method to accomplish this.

IMMOBILIZATION PH

The optimal pH for antibody immobilization was investigated for two monoclonal mouse antibodies and one polyclonal rabbit antibody. Immobilizations over a wide pH-range were performed using different immobilization buffers, and the results are summarized in **Fig. 2**. It was found that mAb B, which has a pI of 7.1 – 7.9, could be efficiently immobilized over the whole pH range that was tested. For mAb A, however, which has a pI of around 5.1 the immobilization is effective only below pH 5.0. Above pH 5.0 the immobilization is highly sensitive to small increases in pH and already at 5.5 the immobilization has been decreased to one third of the maximum level. The polyclonal antibody E can be efficiently immobilized over a broad pH range, which is reasonable in view of its polyclonal nature.

These results support data previously reported, stating the difficulty to immobilize a monoclonal antibody using an immobilization solution with a pH higher than the antibody pI⁵. The general belief is that antibodies with a negative charge are repulsed by the negatively charged surface under these conditions. Interestingly, efficient immobilization can still be obtained at a pH down to 2.5, due to the extremely low pK_a of the sulfo-NHS ester which retains its negative charge also at low pH. Consequently, the charge conditions before and after activation by EDC/sulfo-NHS are different. There is a higher attraction between the activated surface and the antibody at lower pH, since the antibody is more positively charged at lower pH.

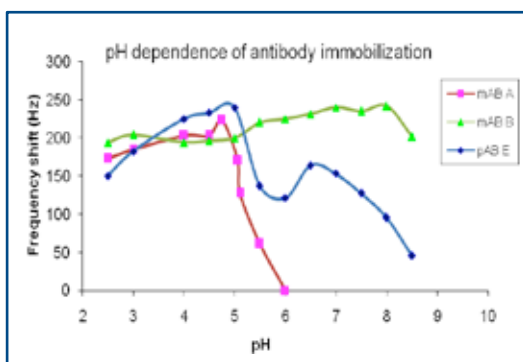


Figure 2: The pH dependence of antibody immobilization for three different antibodies.

It is evident from **Fig. 2** that the electrostatic charge attraction between antibodies and surfaces is a key factor for a successful coupling reaction. It is also interesting to note that a high level of immobilization is still retained, despite the fact that the reaction rate of the coupling reaction normally decreases at low pH. Immobilization down to pH 2.5 has been proven viable.

METHOD VERSATILITY AND REPRODUCIBILITY

To examine the versatility of the surface, four mouse monoclonal antibodies (mAb A-D), two different rabbit polyclonal antibodies (pAb E-F) and one goat polyclonal antibody (pAb G) were immobilised on Attana Carboxyl sensor surfaces. Immobilization was conducted at pH 4.5 in 10 mM sodium acetate buffer for all antibodies except for mAb C which was immobilized at pH 7. The results are presented in **Fig. 3**. Immobilization reproducibility was examined by running several repeats of especially mAbs A-C. Specifically, all the immobilizations of mAb A resulted in 230 Hz ($\pm 10\%$) of immobilized antibody. The average coefficient of variation (CV) in immobilization on Attana Carboxyl Sensor Chip surfaces run on several different biosensor instruments for these antibodies was 4%.

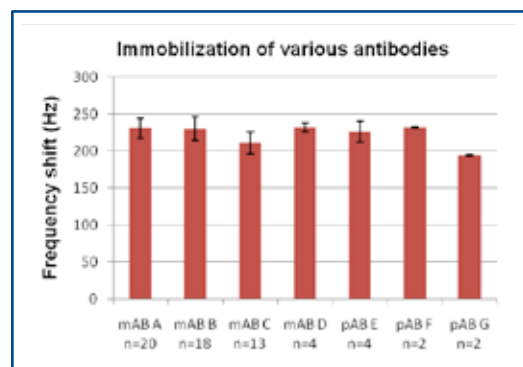


Figure 3: Immobilization of various antibodies on the Attana Carboxyl Sensor Chip surface. Error bars signifies one standard deviation and the number of samples run are indicated below the bars.

CONTROLLING IMMOBILIZATION LEVEL

For some applications of biosensor analysis it is appropriate to reduce the amount of protein that is immobilised on the surface in order to avoid issues with, for instance mass transport limitations and avidity effects^{6,7}. Two different approaches for controlling the surface capacity were introduced and evaluated. First, the influence of antibody concentration on immobilization level

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was examined. By choosing the appropriate antibody concentration, the surface capacity could be carefully engineered. The results displayed in **Fig. 4** show that the immobilization is indeed sensitive to the antibody concentration. At a concentration between 1 $\mu\text{g}/\text{mL}$ and 5 $\mu\text{g}/\text{mL}$ the immobilization is highly dependent on the concentration, whereas for concentrations higher than 20 $\mu\text{g}/\text{mL}$ the increase in immobilization is marginal. The conclusion can be drawn that the best concentration range for control of the surface capacity is 1-5 $\mu\text{g}/\text{mL}$. Unfortunately, the strong interdependence between the antibody immobilization level and the antibody concentration in this range also makes it more difficult to obtain defined and reproducible surface capacities due to the influence of, for instance, dilution errors.

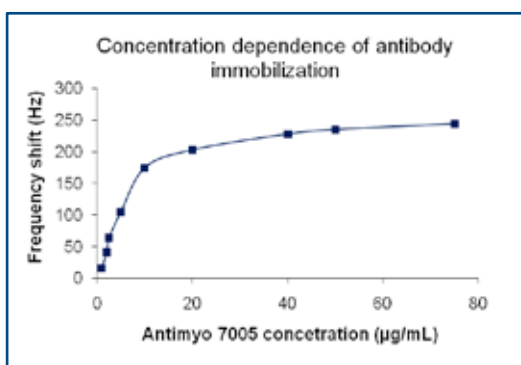


Figure 4: mAb A immobilization over a range of antibody concentrations.

The possibility to further improve the control of immobilization density by a titration approach was tested. The titration approach involves making repetitive manual injections at low concentrations of antibody until a target immobilization level has been reached.

The suitable target immobilization level is determined by the requirements set by the application in order to for instance avoid mass transport distortion of kinetic data. Since the ethanolamine deactivation normally removes around 10 – 20 Hz of material from the surface, the actual number injections, and corresponding frequency response, should be compensated for this. Here, two different target levels were chosen; 50 Hz and 100 Hz of immobilised AB, respectively. By running repetitive injections of 1.3 $\mu\text{g}/\text{mL}$ and 2 $\mu\text{g}/\text{mL}$ immobilization levels of 67 Hz and 92 Hz, respectively, were obtained (**Fig. 5**).

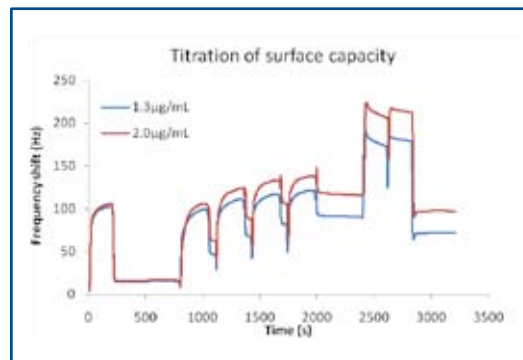


Figure 5: mAb A immobilization by a titration method.

CONCLUSIONS

The Attana[®] Carboxyl Sensor Chip surface has been proven suitable for immobilization of several different antibodies. Generic conditions for antibody immobilization were determined which involve the use of an EDC/sulfo-NHS activation mixture and immobilization at pH 4.5 in 10 mM sodium acetate buffer. The surface was also tested for immobilization over a wide pH range and showed surprising efficiency even at a pH as low as 2.5. Immobilization reproducibility was shown to be very robust for several antibodies tested. The average coefficient of variation was determined to 4%. Two methods for controlled immobilization were tested and proved to be suitable for preparation of low surface capacity sensors.

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