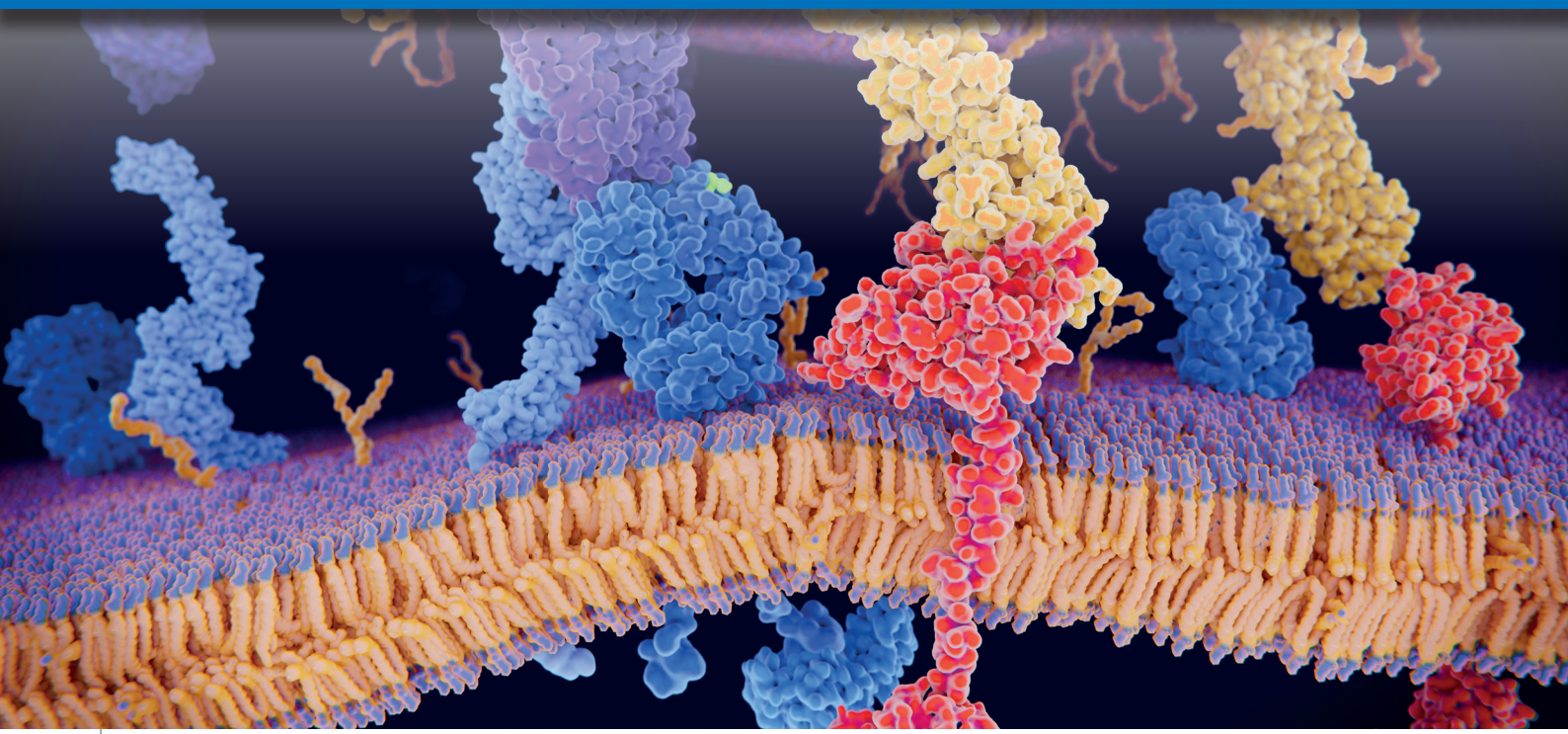

How to influence your ligand density using amine coupling?

A crucial step within a SPR experiment is the ideal immobilization level to obtain optimal kinetic measurements. This application note provides information about influencing parameters on the final immobilization levels of different example targets using amine coupling. We focused on activation time, sodium ion concentration of the acetate buffer and the EDC (N-Ethyl-N-dimethylaminopropyl carbodiimide hydrochloride) concentration.

Introduction

Johnsson and Löfas [1] described amine coupling as simple and fast immobilization method in 1991. The covalent attachment of proteins to a pre-activated surface, consisting of carboxymethylated dextran was revolutionary for SPR experiments as it broadens the application range of SPR. Nowadays it is a popular method, which we will describe in this application note. Initially carboxyl groups within the dextran-matrix of the sensor surface are transformed into N-hydroxysuccinimide esters due to an reaction of NHS (N-hydroxysuccinimide) and EDC (N-Ethyl-N-dimethylaminopropyl carbodiimide hydrochloride) [1]. Secondly the target of interest is injected in a low pH buffer and

Keywords: Sierra SPR-32, Sierra SPR-24 Pro, surface chemistry, varying target density, improvement of experiments, ease of use

Authors: Sven Malik[1], Cyrill Brunner[1], Paul Ritter[2];
[1] Bruker Daltonics SPR, Hamburg, Germany; [2] Bruker Daltonics, Billerica, MA, US

its primary amines react with the active esters. Finally remaining active esters will be transfored into inactive amides using ethanolamine [1,2,3].

In the present study influencing parameters, like activation time, salt content of the acetate buffer and the EDC concentration are shown to vary the final target density, similar to the publication of Johnson et al. [1].

Material and Methods

Instrumentation

All real-time, label-free (RT-LF) assays were completed using the Sierra SPR-32 system, from Bruker Daltonics SPR (Hamburg, Germany). The Sierra SPR-32 system contains 32 detection spots arranged in a 4x8 array. Eight samples are delivered simultaneously to the detection spots via a continuous flow microfluidic device. A high sensitivity surface plasmon resonance imaging detector, SPR+, is used to measure binding interactions in real time.

Tested Target Proteins and conditions

The proteins used in this study are summarized in Table 1. All proteins have been used at a concentration of 50 µg/mL.

Experiments

All experiments have been conducted using PBS (Phosphate Buffered Saline) buffer containing 0.05% Tween 20, pH 7.4 at 25°C. The target proteins were immobilized on high-capacity-amine sensors (HCA 12pk., Bruker Daltonics SPR, # 1862615) using standard primary amine immobilization chemistry (Amine Coupling Kit I, Bruker Daltonics SPR, # 1862634). During the target protein coupling step acetate buffer pH 4.5 (Bruker Daltonics SPR, # 1862638), pH 5 (Bruker Daltonics SPR, # 1862640) and pH 6 (Bruker Daltonics SPR, # 1862645) were used.

Data analysis:

Data analysis was completed using the Analyser R2 software (Bruker Daltonics SPR) and further evaluated using MS Office.

Table 1: Target proteins and conditions

Target	pH of Acetate Buffer	Supplier
BSA	4.5	Sigma #10814-6
CAII	5	Sigma #C2522-5MG
Human IgG	5	Biozol #009-000-003
Mouse IgG	6	Biozol #015-000-003
Neutravidin	5	Thermo Fischer #31000
Protein A	4.5	Sigma #P6031-1MG
Protein AG	4.5	Thermo Fischer #21186
Rabbit-anti mouse	5	Biozol #315-005-008

General approach

Common SPR sensor surfaces contain a matrix out of carboxymethylated dextran coupled to a thin goldlayer. During the amine coupling approach a portion (~1/3) of these carboxyl groups are transformed (activation) into highly reactive N-hydroxysuccinimide esters due to the injection of a mixture of NHS (N-hydroxysuccinimide) and EDC (N-Ethyl-N-dimethylaminopropyl carbodiimide hydrochloride) in water [1,2].

Secondly the protein of interest, diluted in a low-ionic strength buffer will be passed over the active surface (target protein coupling). Thereby the buffer in which the protein is dissolved will have a pH lower than the pI (isoelectric point) of the protein itself. Thus the target protein (positively charged) is preconcentrated into the dextran matrix (negatively charged carboxyl-groups) just by electrostatic attraction [1,2]. Once within the matrix the NHS esters react with numerous primary amines, such as the α-amino group, the ε-amino group of lysine, the thiol group of cysteine, and probably to some extent the imidazolium group of histidine and the phenoxy group of tyrosine [1,3].

As a final step (Blocking) the remaining active esters will be transformed into amides due to the injection of 1M ethanolamine at pH 8.5. The high salt content within this blocking agent serves as anti-electrostatic cleaning to wash off electrostatic bound material from the dextran matrix [1,2]. The general principle of amine coupling is illustrated in Figure 1.

The corresponding SPR sensorgram can be seen in Figure 2. Thereby each solution such as activation, target protein and ethanolamine, have been injected for 7 minutes at 10 µL/min.

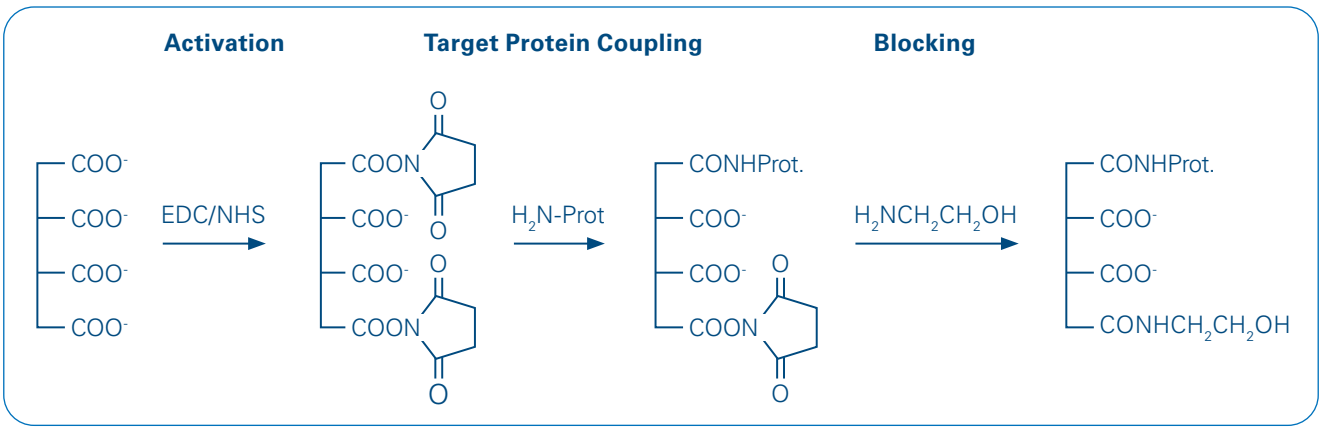

Figure 1: General principle of amine coupling approach [1]

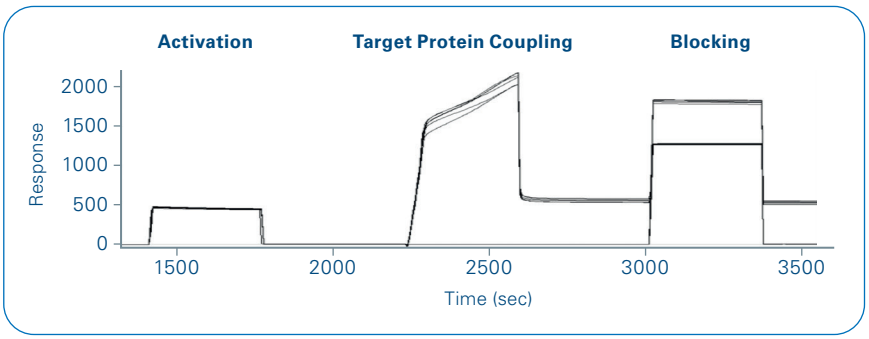

Figure 2: Immobilization of a target protein. After the activation using a mixture of EDC/NHS the target protein is injected, so that its primary amines can react with the reactive esters on the surface. Once the protein is coupled the remaining active esters are blocked due to an injection of 1M ethanolamine

Results and Discussion

Influence of the activation time on target density

Approximately 30-40% of carboxyl groups will be transformed into N-hydroxysuccinimide esters during the activation injection [1], thus it is an important parameter for the final target protein immobilization level.

Chain of causation for the activation time

In order to proof the relation between activation time and final target protein immobilization levels, protein A and neutravidin have been immobilized using four different activation times. For neutravidin the activation times of 8, 6, 4 and 2 minutes were used, whereas for protein A a range from 7.0, 3.5, 1.75 and 0.8 minutes. Clearly the final target protein immobilization level decreases with lower activation time (Figure 4). A special behavior can be observed for neutravidin in this example as even if the activation time is lower (6 minutes) the resulting immobilization level is higher compared to the longest activation used in this study (8 minutes). A potential reason could be the increased amount of electrostatic absorbed protein due to lower degree of converted carboxyl-groups.

Finally it can be said, that the activation time is one parameter in order to vary the final immobilization level. But side effects such as higher electrostatic adsorption due to lower conversion of the carboxyl-groups have to be taken into account.

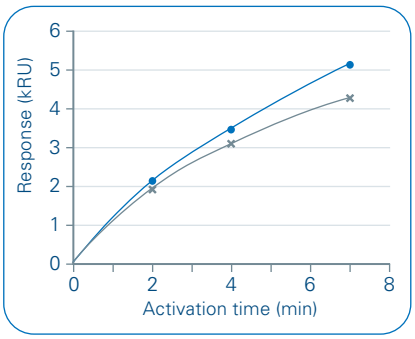

Figure 3: Relation of target protein response over activation time. The lower the activation time, the lower the final target protein level. Example target proteins were protein A and RNase [1]

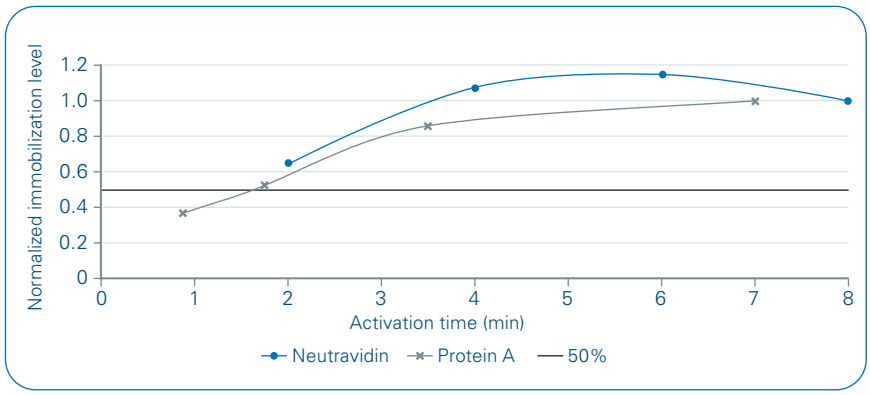

Figure 4: Relation of activation time and final target protein immobilization level. Protein A and neutravidin were immobilized at four different activation times

Table 2: Chain of causation I

Influencing parameter	Result
Lower activation time	lower final target density
	higher amount of non activated COOH groups
	higher protein adsorption (electrostatic) into the matrix

Influence of the salt content of the acetate buffers on the target density

During the injection of the target protein, dissolved in low ionic acetate buffer, a competition appears between the positively charged protein and e.g. positively charged ions within the protein solution for the negatively charged carboxyl groups within the dextran matrix [1,2]. Thus affecting the salt content of the acetate buffer will affect the final immobilization levels.

Chain of causation for the salt content of the acetate buffer

In order to proof the relation between amount of sodium ions in the acetate buffer and final target protein immobilization levels, protein AG and neutravidin have been immobilized using seven different conditions. An additional amount of 10, 20, 40, 60, 80, 100 and 120 mM of NaCl was used during an immobilization. The final target protein immobilization levels decreases with increasing the amount of sodium ions in the acetate buffer (Figure 6).

It was shown, that the acetate buffer and its sodium ion content can be used as parameter to vary the final immobilization level. But in order to achieve the best exploitation of the protein solution a low ionic strength should be used [1]. Commonly 10 mM buffer concentration is used.

Influence of the activation reagents on the target density

During the activation EDC concentrations >200 mM and NHS concentrations >40 mM will not further impact the final immobilization levels (no further increase). Thus lowering the EDC and NHS concentrations lead to lower immobilization levels and can be used as parameter to vary the final target density [1].

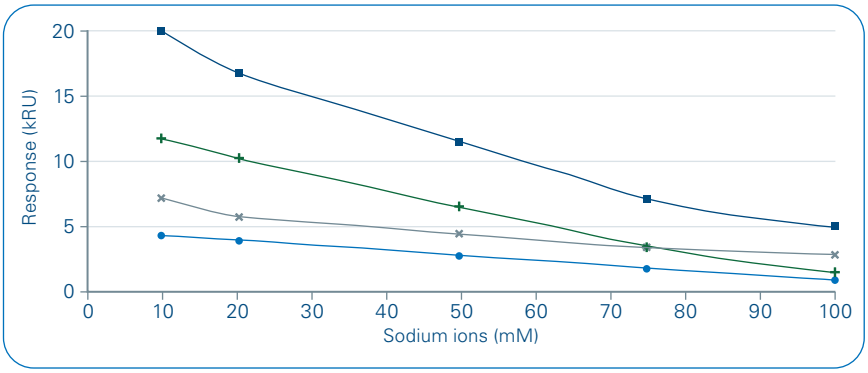

Figure 5: Relation of target protein response over sodium ion content in the acetate buffers. The higher the amount of sodium ions in the acetate buffer, the lower the final response for preconcentrations and immobilization [1]. Response of electrostatically adsorbed (+) and immobilized (•) SpA (300 pg/mL, pH 4.5) and electrostatically adsorbed (■)and immobilized (x) RNase (400 pg/mL, pH 6.0) as a function of sodium ion concentration in the protein solution [1]

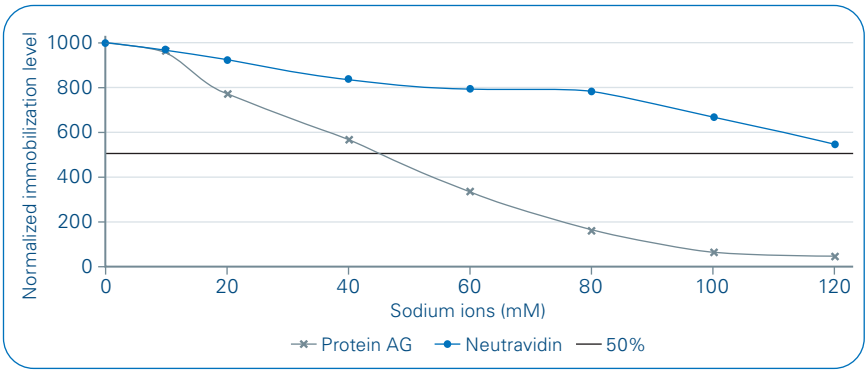

Figure 6: Relation between final target protein immobilization levels over an increase of sodium ions within the acetate buffers. Protein AG and neutravidin were immobilized at seven different conditions

Table 3: Chain of causation II

Influencing parameter	Result
Increase of ionic strength of acetate buffer	lower final target density
	lower electrostatic protein adsorption into the matrix
	lower amount of protein in the matrix

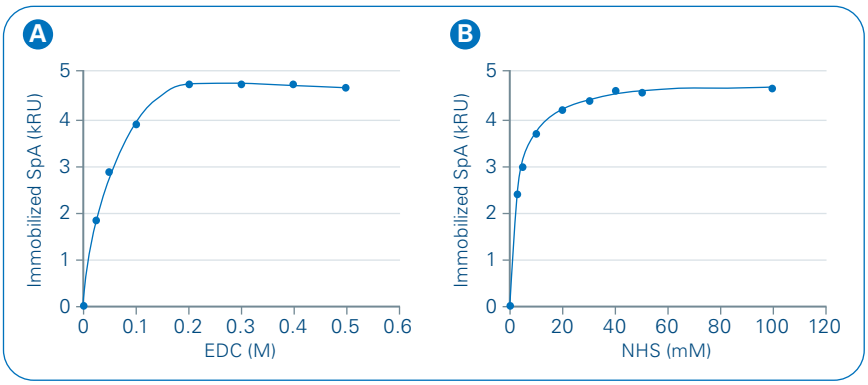

Figure 7: Relation of target protein response over activation reagent concentration (A): EDC dependency; (B): NHS dependency. Up to 200 mM EDC and up to ~25 mM a nearly linear relation can be seen [1]. Response of immobilized (•) SpA (300 pg/mL, pH 4.5)[1]

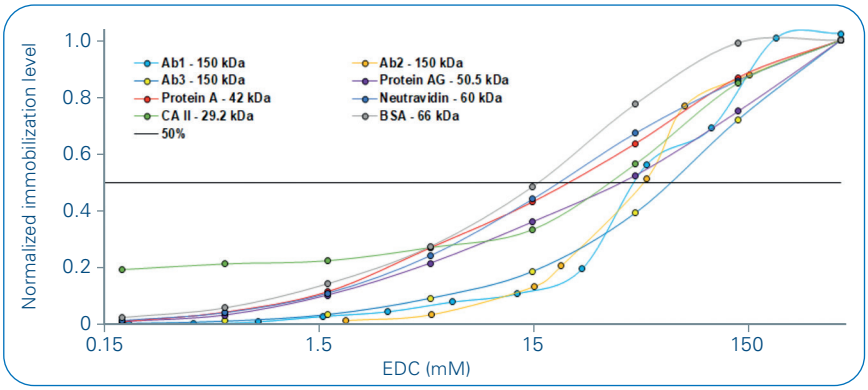

Figure 8: Relation between final target protein immobilization levels over varying EDC concentrations. Protein A, Protein AG, Neutravidin, BSA, CAII, rabbit-anti Mouse antibody, mouse IgG and human IgG have been used at 8 different EDC concentrations

Table 4: Chain of causation III

Influencing parameter	Result
Decrease of activation reagent concentration (EDC, NHS)	lower final target density
	lower degree of reactive N-hydroxysuccinimide esters
	higher protein adsorption (electrostatic) into the matrix

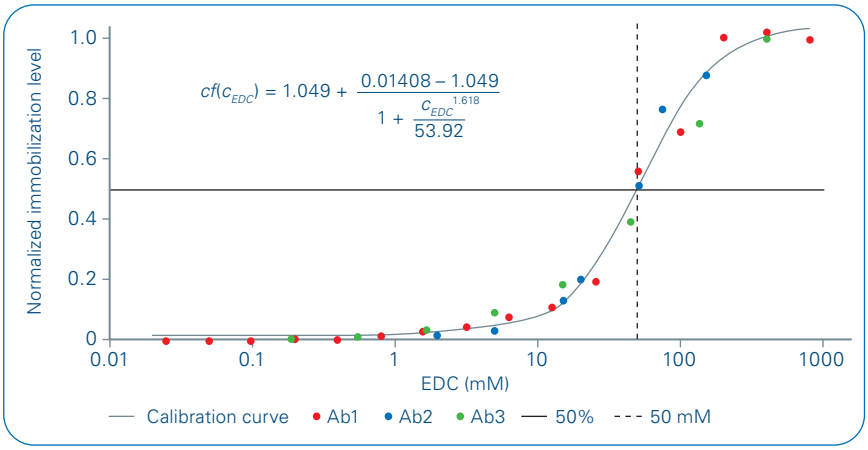

Figure 9: Relation between final target protein immobilization levels over varying EDC concentrations. Ab1: rabbit-anti Mouse antibody, Ab2: mouse IgG and Ab3: human IgG have been used at 8 different EDC concentrations. Approximately 50 mM will lead to 50% of the final target density

Chain of causation for varying activation reagent concentration

During this study we focused just on varying the EDC concentration as NHS prevents the EDC hydrolysis prior protein will reach the sensor surface [1]. The NHS concentration remains constant at 50 mM for all experiments. In order to proof the relation between amount of varying the activation reagent concentration and final target protein immobilization levels seven different proteins have been immobilized using different EDC concentrations. Protein A, Protein AG, Neutravidin, BSA, CAII, rabbit-anti Mouse antibody, mouse IgG and human IgG have been used at 8 different EDC concentrations (Figure 8).

The impact of varying EDC concentration on the final immobilization level was shown for seven different poteins. A detailed evaluation was done for all antibodies used in this study (Figure 9), as all data points achieved from the three antibodies were fitted using a polynom+equation. A final concentration of ~50 mM EDC was calculated to 50% of the final target density.

Thus a rule of thumb could be established, as targets of the same protein class (antibodies) behave similar. For EDC concentration of 50 mM and lower a nearly linear relation can be assumed, see Table 5.

Table 5: Rule of thumb

Immob.level [%]	EDC concentration [mM]
100	400
50	50
25	25
12.5	12.5
...	...

Workflow

- Getting target density using standard reagent conditions
  - EDC: 400 mM
  - NHS: 50 mM
- Protein:
  - use saturating concentration (maximum of electrostatic adsorption)
  - use low ionic strength acetate buffer with optimized pH
- Blocking:
  - 1 M Ethanolamine, pH 8.5
- Calculate the EDC concentration based on Table 5
- Continue immobilization using adopted EDC concentration

This rule of thumb was then used with an unknown protein, which should be immobilized to a density of 1000 RU.

As initial step an immobilization level of 6600±300 RU were achieved under standard conditions (Figure 10). The activation, protein injection and blocking was done on sensor row 1-8D. Following the rule of thumb (Table 5), at 50 mM EDC: 3300 RU and at 25 mM EDC: 1650 RU would be reached. Further immobilizations have been done using 20 mM to achieve ~1000 RU. The final immobilization level are 1060±38 RU (Spot C), 1072±41 RU (Spot B) and 1040±35 RU (Spot A), see Figure 11.

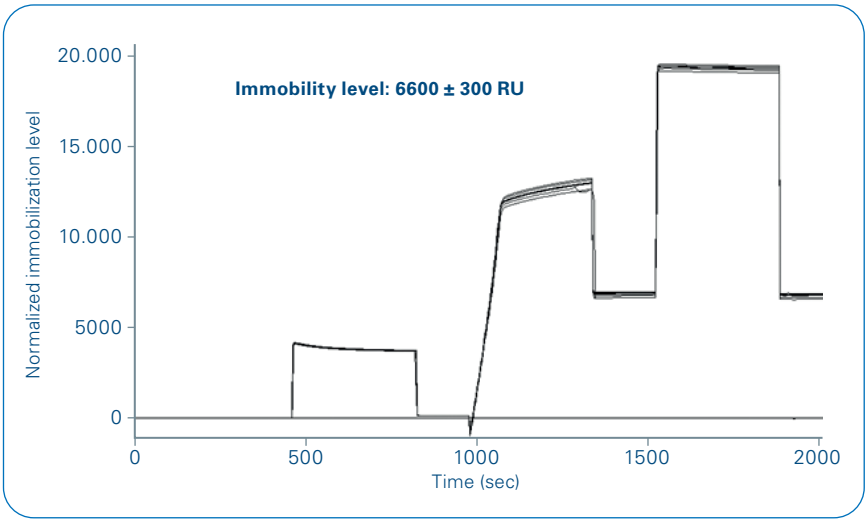

Figure 10: Initial target protein density under standard conditions was 6600 RU on 1-8D

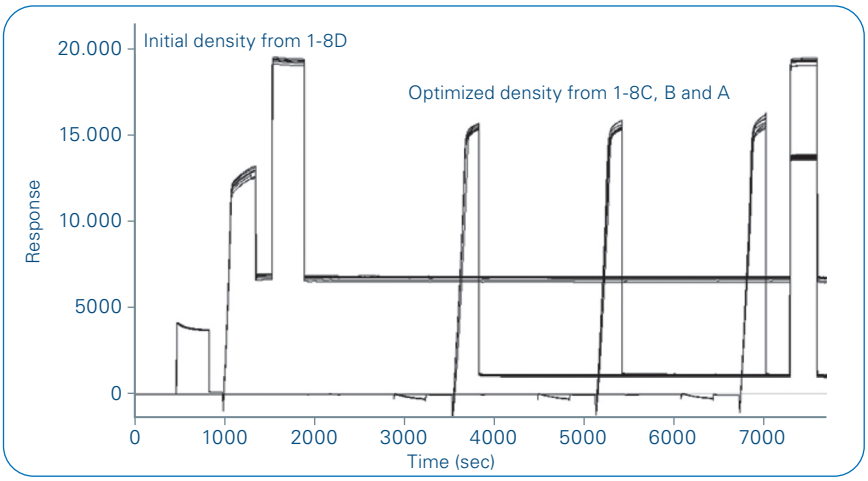

Figure 11: Optimized target protein density using 20 mM EDC leads to ~1000 RU on Spot 1-8 C, B and A

Conclusion

- This study underlines the options of actively influencing the protein target density on the sensor surface using amine coupling. It was demonstrated, that the concentration of the activation reagents, the sodium ion concentration in the acetate buffer, but also the activation time could be used to vary the target density.
- Furthermore a rule of thumb should help to initialize the optimization within experiments, where a certain target level is needed or multiple densities should be investigated.
- Rule of thumb (to be used as approximation): 50 mM EDC will lead to 50% of the immobilization level generated under standard conditions:
  - 400 mM EDC
  - 50 mM NHS
  - Saturating protein concentration

Learn More

You are looking for further Information?
Check out the link or scan the QR code for more details.

www.bruker.com/spr

References

[1] Johnsson et al., 1991; *Immobilization of Proteins to a Carboxymethyldextran-modified Gold surface for biospecific Interaction Analysis in surface plasmon resonance sensors*

[2] Mol et al., 2010, *Surface Plasmon Resonance, Methods and Protocols*, Springer Protocols

[3] Cullen et all., 1987/88 *Detection of immuno-complex formation via surface plasmon resonance on gold-coated diffraction gratings*

For Research Use Only. Not for Use in Clinical Diagnostic Procedures.

Bruker Daltonics GmbH & Co. KG

Bremen · Germany
Phone +49 (0)421-2205-0

Bruker Scientific LLC

Billerica, MA · USA
Phone +1 (978) 663-3660

ms.sales.bdal@bruker.com – www.bruker.com