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Increased sensitivity of SPR assays in plasma through efficient parallel assay optimization

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ABSTRACT

The sensitivity of biosensor assays in complex media such as plasma or serum is often limited by non-specific binding. The degree of binding often varies between individuals and therefore a large number of different plasma samples have to be used during assay development. Some surface plasmon resonance (SPR) biosensors allow for parallel screening of several running buffer compositions, with a number of different immobilization levels for each buffer. These technical possibilities combined with statistical design of experiments (DoE) enable efficient parallel optimization of multiple assay conditions.

In this paper we outline how to increase the sensitivity in SPR-based assays by minimizing background binding and variability from negative control plasma while retaining high signals from positive samples. To mimic immunogenicity studies of biotherapeutics we have used a model assay with anti-rituximab as an anti-drug antibody to be detected in plasma by binding to immobilized rituximab. Immobilization level and sodium chloride concentration were found to be the most important factors to optimize. There were also a number of significant interaction effects and strong non-linearites between the buffer composition/immobilization level and the assay performance, which necessitated DoE based optimization strategies. The applicability of the optimized conditions was verified with several assays (anti-erythropoietin, omalizumab, anti-IgE and anti-myoglobin) in spiked plasma samples resulting in detection levels in the range of 80–170 ng ml^{-1}.

The buffer conditions presented in this paper can be used for other immunogenicity assays on biosensor platforms or as a good starting point for further assay development for new immunogenicity assays.

1. Introduction

Measurement and monitoring of immunogenicity, i.e. the development of anti-drug antibodies (ADAs) in response to treatment with a biotherapeutic drug, is crucial during drug development. Anti-drug antibodies can alter the pharmacokinetics and the efficacy of the drug as well as have negative effect on patient safety. FDA draft guidelines [1] state that testing of immunogenicity should be performed in preclinical and clinical phases of biotherapeutic development. FDA also recommends that an immunogenicity assay should have a sensitivity of 250–500 ng ml^{-1} and be able to detect all isotypes, especially IgM and all IgG isotypes [1]. A multi-tiered approach is usually used for immunogenicity testing. The first step is the screening of patient samples for detection of anti-drug antibodies. The recommendation in the screening phase is to set a cut point that results in 5% false positives in order to minimize the risk of obtaining false negatives [1–3]. The screening step is followed by a confirmatory step to verify screening hits and to remove false positives by a competitive assay. The confirmed positive hits may be characterized further by assessment of isotype (class/subclass), titre, binding stability, epitope specificity and assessment of neutralizing capability.

Surface plasmon resonance (SPR) biosensors are one of the most recently used techniques for immunogenicity studies and show good performance in complex sample matrices, in terms of accuracy, precision and robustness [e.g. 4–7]. The technology has several advantages over other techniques used for immunogenicity testing, such as the ability to detect high and low affinity antibodies, even if they show rapid dissociation [8]. The rapidly dissociating ADAs are often false negatives in assays which includes washing steps, such as ELISA [8]. Biosensors can also detect all human IgG subclasses, including IgG4. IgG4 ADAs have been observed to be second to IgG1 as the major ADA isotype and are associated with high doses and long exposure to therapeutic drugs [9]. IgG4 antibodies can undergo random "half antibody exchange", which can result in bispecific antibodies [10]. Bispecific IgG4 cannot be detected when using techniques such as bridging or homogenous ELISA [11,12]. Another important issue with immunogenicity assays is drug interference.

Abbreviations: ADA, anti-drug antibodies; DOE, design of experiments; LOD, limit of detection.

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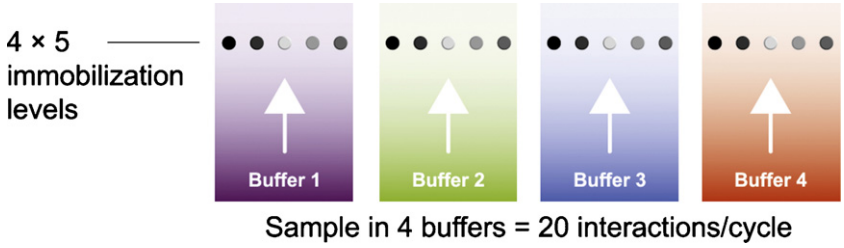

Fig. 1. Schematic picture of the assay setup in Biacore 4000 with four parallel flow cells each containing five detection spots. Four different buffers were analyzed in parallel in combination with four immobilization levels, and one reference.

In Biacore™ T200 acidification and neutralization immediately before measurement can be used to detect ADA also in the presence of drug. Additional benefits of some of the biosensors are the high degree of automation, possibilities for multiplexing and very little hands-on time. However, biosensor assays may be challenging due to non-specific binding from the sample matrix which may reduce overall assay performance and sensitivity. Furthermore, the non-specific binding from plasma and serum can vary extensively between individuals. The cut point in immunogenicity assays is calculated from a large number of negative samples [1–3] and if the variation between these samples is high it results in high cut points and, hence, low assay sensitivity.

A general strategy for optimization of assay parameter settings to increase the sensitivity in plasma assays run on Biacore SPR-instruments is presented in this paper. The strategy is based on parallel experimentation and statistical design of experiments (DoE) [13]. DoE is an efficient way to optimize assay quality by simultaneously varying factor settings (e.g. the composition of buffer and immobilization level) according to a statistical plan and measuring the quality of the assay resulting from the different factor combinations. After all experiments from the plan have been performed a mathematical/statistical model is calculated which estimate the effect each factor and combination of factors (interaction effects) have on assay quality. The parallel configuration of Biacore 4000, which was used in this work, is ideal for assay optimization as it allows for simultaneous screening of four different buffers and up to five different immobilization levels for each buffer condition (Fig. 1). Results are shown on how composition of sample and running buffers, immobilization levels and other running conditions relate to assay quality, using several model systems related to immunogenicity assays.

2. Experimental

2.1. Instruments and reagents

Biacore 4000 instrument, Series S Sensor Chip CM5, Amine Coupling Kit, HBS-EP+ 10× buffer (100 mM HEPES, 1.5 M NaCl, 30 mM EDTA and 0.5% P20), 10 mM sodium acetate pH 5.0, 10 mM sodium acetate pH 4.0, 10 mM glycine pH 1.5, 10 mM glycine pH 2.0, 10 mM glycine pH 2.5, NSB Reducer (10 mg ml^{-1} CM dextran) were obtained from GE Healthcare, Uppsala, Sweden. Guanidine hydrochloride was purchased from Sigma–Aldrich. Sodium hydroxide was purchased from Labservice. P20 (polysorbate 20), HEPES and sodium cyanoborohydride were purchased from Fluka. Acetic acid and disodium hydrogen phosphate di-hydrate were purchased from Riedel-de Haën. Sodium di-hydrogen phosphate monohydrate was purchased from Prolabo. Mabthera® (rituximab), Xolair® (omalizumab), Aranesp® (darepoetin alpha), Remicade® (infliximab), Herceptin® (traztuzumab), Humulin® (insulin) and Saizen® (somatropin) were purchased from Apoteket Farmaci. Mab X, monoclonal anti-myoglobin and monoclonal anti-IgE are in-house reagents. CKMB was purchased from BioPacific and the monoclonal anti-CKMB antibodies are in-house reagents. Polyclonal anti-erythropoetin was purchased from R&D Systems. Monoclonal anti-rituximab (recognizing the idiotypic determinants of rituximab) was purchased from Abd Serotec and IgE from Calbiochem. All other chemicals were purchased from Merck. Human plasma was purchased from Uppsala University Hospital.

2.2. Methods

2.2.1. Preparation of human plasma samples

Plasma from 20 different donors was thawed and centrifuged at 10,000 × *g* for 10 min and then filtered through a 0.45 μm syringe filter (Millex HA, Millipore).

2.2.2. Preparation of ADA samples

Human plasma samples were spiked with known amounts of anti-drug antibody. The concentration of the antibody varied between 250 and 1000 ng ml^{-1} for different experiments. The volume of added ADA was 1% of the plasma volume. Each plasma sample was then diluted 1 + 1 with 2 mg ml^{-1} NSB Reducer in running buffer (final concentration of NSB Reducer 1 mg ml^{-1}).

2.2.3. Handling of sensor chips and preparation and maintenance of Biacore 4000

After each sensor chip was docked in the BC 4000 instrument, normalization and hydrodynamic addressing procedures were run to ensure optimal running conditions. Analysis temperature and sample compartment temperature were set to 25 °C during maintenance and immobilization. After each immunogenicity assay the instrument was cleaned with the desorb procedure (0.5% SDS followed by 10 mM glycine pH 9.5). The sanitize procedure (~0.7% sodium hypochlorite) was run approximately once every month.

2.2.4. Amine coupling on Sensor Chip CM5

The drug molecules, hereafter named ligands, were diluted in 10 mM acetate with varying pH and immobilized on sensor chip CM5 with standard amine coupling in four spots, the fifth spot was used as reference (Fig. 1). About 2, 4, 6 and 12 kRU rituximab was immobilized for optimization of the anti-rituximab assay. To obtain different immobilization levels, the activation time and the ligand contact time were varied (5–10 min and 1–16 min, respectively). The concentrations of the ligands were 5–30 μg ml^{-1} depending on ligand.

2.2.5. Aldehyde coupling of darepoetin alpha on sensor chip CM5

Due to the high content of sialic acid and the high degree of glycosylation of darepoetin alpha it was not possible to use a regular amine coupling for this protein. Instead the protein was immobilized using standard aldehyde coupling after oxidation with sodium metaperiodate to introduce aldehyde groups. The ligand concentration was 250 μg ml^{-1} and contact time was 3–7 min.

2.3. Assay setup

Three start-up cycles were run at the beginning of each experiment. Control samples were run every 15th cycle. A plasma sample spiked with ADA antibody was used for the start-up cycles and the controls. The samples consisted of negative plasma from 20 different donors. 10 of the negative plasmas were also spiked with ADA antibody and run as positive samples. All samples were run in duplicate in random order. Sample contact time was set to 600 s with a dissociation time of 60 s and a flow rate of 10 μl min^{-1}. Analysis temperature was set to 25 °C and sample compartment temperature was set to 10 °C. The myoglobin surface was regenerated with 60 s 10 mM NaOH followed by 30 s 0.1% SDS. The rituximab surface was regenerated with 120 s 10 mM NaOH followed by 30 s 10 mM glycine pH 2.25. The IgE surface with anti-IgE as analyte was regenerated with 30 s 100 mM NaOH. The IgE surface with omalizumab as analyte was regenerated with 30 s 10 mM glycine pH 1.5 followed by 10 s 10 mM NaOH. The darepoetin alpha surface was regenerated with 60 s 50 mM HCl, 1.5 M guanidine hydrochloride followed by 30 s 10 mM NaOH. In the study of non-specific binding to different drugs the surfaces were regenerated with 120 s 10 mM NaOH.

2.4. Evaluation of data

Evaluation of data was performed with Biacore 4000 Evaluation Software, Microsoft™ Excel®, Tibco® Spotfire® and MODDE™ (Umetrics Inc.). The report point used for evaluation was "Stability early" (SE-report point; 7 s after sample injection end). Data from negative plasma samples was initially analyzed for outliers using Box plot analysis [3]. The outliers were subsequently excluded before calculation of assay quality parameters (e.g. *Z*-value and cut point) [3]. The normality of the data was checked using Anderson–Darling test using Minitab® statistical software.

Z′ is an assay quality parameter that takes into account the difference in signal level between positive and negative samples as well as the variation of positive and negative samples. Plasma spiked with analyte to 250 ng ml^{-1} (500 ng ml^{-1} in the first experiments) was used as positive samples. *Z′* is frequently used in screening assays for low molecular weight compounds. However, in such assays the negative samples are replicates of only one buffer. The negative samples in an immunogenicity assay are plasma samples that originate from different patients. Thus, the variation between negative samples is significantly higher. Therefore, for this study, a modified *Z*-value was used. For calculations of *Z′* the standard deviations are usually multiplied by 3, however here the modified *Z*-value was calculated as follows:

$$Z = 1 - \left(\frac{SD_{pos} + SD_{neg}}{Avg_{pos} - Avg_{neg}} \right)$$

where SD is the standard deviation of positive (pos) and negative (neg) controls, Avg is the average response of positive (pos) and negative (neg) controls

A *Z*-value close to 1 indicates a good separation between positive and negative samples and, consequently, high sensitivity.

To distinguish positive samples from negative samples a cut point was used. The cut point was calculated as follows:

$$\text{Cut point} = Avg_{neg} + SD_{neg} \times 1.729$$

1.729 is a statistical factor for *t*-distribution (1-tailed 95% confidence interval for 20 negative samples). A factor of 1.645 is usually recommended for the calculation of cut point if the number of negative samples ≥50 individuals [3], but since the number of negative samples used was limited ($n = 20$) the factor 1.729 was used. The calculated cut point will result in 5% false positives to increase the likelihood that no sample is identified as a false negative in the screening step [1–3].

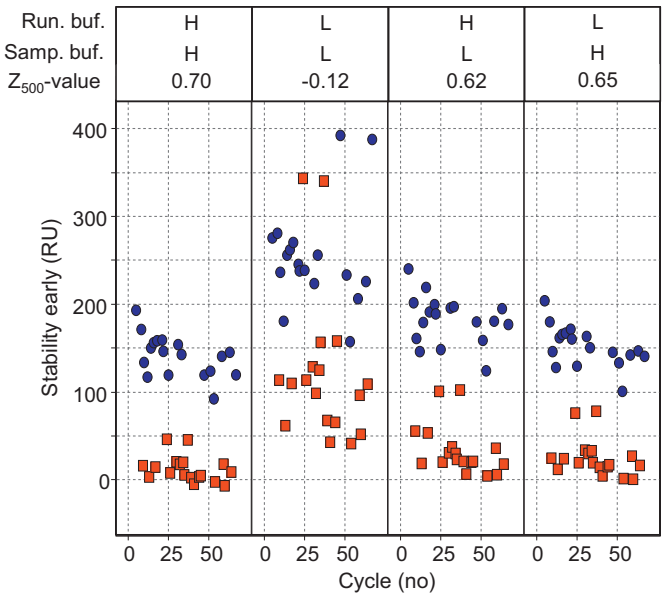

Fig. 2. Interactions between running and sample buffer. The SE-report point of the anti-myoglobin assay in Run 1 plotted vs. cycle. The running (run) and sample (samp) buffers were varied according to the scheme on top. *H* = 100 mM HEPES, 0.5 M NaCl, 0.05% P20 and 5 mM EDTA, pH 8; *L* = 10 mM HEPES, 0.15 M NaCl, 0.05% P20 and 5 mM EDTA, pH 7.0. Red square = unspiked plasma ($n = 10 \times 2$), Blue circle = 500 ng ml^{-1} anti-myoglobin spiked plasma ($n = 10 \times 2$). The figure shows also the *Z*-values for the samples with 500 ng ml^{-1} anti-myoglobin.

If the concentration at cut point was reported as assay sensitivity (LOD = limit of detection) then only 50% of the samples with that concentration would be detected as positives [3]. In this paper LOD was calculated as the lowest concentration that would detect a positive sample above the cut point with 95% consistency [3].

$$\text{LOD} = \text{cut point} + \text{SD} \times 1.833$$

1.833 is a statistical factor for *t*-distribution (1-tailed 95% confidence interval for 10 samples).

The response at LOD is recalculated as concentration by assuming a linear correlation between response and concentration up to 250 ng ml^{-1}.

We used a stepwise DoE optimization approach with several smaller sequential designs to fit the Biacore 4000 runs into a smooth weekly work flow and to minimize noise which could be a result of running longer runs and varying more factors at the same time. Three different statistical design plans (DoE's) [13] were used in the optimization work. The initial scouting experiment was a full 2 level factorial design using two factors (Fig. 2). The full screening experiment was performed using an L9 array design for 4 variables. The optimization experiment was performed using a face centred cubic design for 3 factors. The results were evaluated using multiple linear regression using the factor settings as *x* and the *Z*-value as y.

3. Results

3.1. Strong interaction effect between running and sample buffer composition

The experiments aimed to improve assay quality both by minimizing background binding from negative plasma and variation between different plasmas while retaining a good resolution between ADA spiked and naïve samples. We started by varying buffer concentration, NaCl concentration and pH. Two HEPES

Table 1
Buffer composition in the screening step and assay quality, *Z*-value for the sample with 500 ng ml^{-1} of ADA. No outliers were removed in the calculation of the Z_{500} values. High *Z*-values indicate high assay quality. The results are from surfaces immobilized with 11–13 kRU rituximab and 4–5 kRU myoglobin. The buffers contained 0.275% detergent P20 and 5 mM EDTA in addition to the components listed in the table. The same buffer was used as both running and sample buffer.

Run	Flow cell	Buffer substance	Conc. buffering substance (mM)	Conc. NaCl (mM)	pH	Anti-rituximab Z_{500}	Anti-myoglobin Z_{500}
2	Fc1	HEPES	55	325	7.5	0.49	0.59
	Fc2	Tris	10	325	9	0.40	0.60
	Fc3	Tris	55	500	7	0.59	0.52
	Fc4	Tris	100	150	7.5	0.41	0.23
3	Fc1	HEPES	55	325	7.5	0.43	0.51
	Fc2	Phosphate	10	500	7.5	0.51	0.35
	Fc3	Phosphate	55	150	8	0.07	0.06
	Fc4	Phosphate	100	325	7	0.48	0.34

buffers, one with all factors at low level (L) (10 mM HEPES, 0.15 M NaCl, pH 7) and another with all factors at high level (H) (100 mM HEPES, 0.5 M NaCl, pH 8) were used to ensure that an adequate parameter interval would be chosen for the multivariate studies. In addition, these experiments were run with all four different combinations of running buffer and sample dilution buffer, i.e. H + H (high in both running and sample buffer), H + L (high in running and low in sample buffer), L + H (low in running and high sample buffer) and L + L (low in both running and sample buffer). This was done to evaluate if it was important to have an optimized buffer in the sample or in the running buffer or in both. Two different model assays were used: anti-myoglobin and anti-rituximab in plasma. Both assays gave similar results and the results from the anti-myoglobin assay are shown in Fig. 2. The SE-report point (after 7 s of dissociation) of the raw data combined with the calculated Z_{500}-value (*Z* value for samples with 500 ng ml^{-1} of ADA) highlights several interesting results (Fig. 2). The L + L combination showed the lowest (even negative) *Z*-values and high values were obtained with buffer combinations H + H, H + L and L + H (Fig. 2). The low Z_{500}-value for L + L is caused by a significantly higher variation among plasma samples from the different individuals. This indicates that assay quality will increase when some or all of the buffer components (salt, buffer concentration, pH) are set to high level. Observe that very similar quality was seen with all three combinations of buffer with high concentrations and pH, i.e. H + H, H + L and L + H. This means that there is a strong negative interaction effect between sample and running buffer compositions. Consequently, it is not mandatory to use the optimized buffer both as running and sample dilution buffer. Sensitive samples can be diluted in regular assay buffer if the optimized buffer is used as running buffer or vice versa. If highest possible sensitivity is to be achieved then the H + H buffer combination should be selected.

3.2. Multivariate screening

A multivariate screening was done with a limited number of buffer compositions and immobilization levels in combination. An L9 array design of experiment was used to vary the buffer substance (HEPES, Tris, and phosphate), the buffer substance concentration, NaCl concentration and pH (Table 1). The results from Table 1 and Fig. 2 were used to calculate a regression model between the buffer composition (factors) and the Z_{500}-value. All the varied buffer factors influenced the assay quality significantly (result not shown). The similarity of the results between the two model assays (Table 1) and the strong effect of the immobilization level suggested us to select only one model assay (anti-rituximab) and use 4 immobilization levels for each buffer in the following experiments. From this limited screening the preferred buffer substance was Tris, and Tris was therefore used in the multivariate optimization (Section 3.3 below).

There was a strong quadratic effect for both NaCl and pH which indicate a nonlinear relation between the factors and the assay quality.

The selected screening design (L9-array) did not support the estimation of interaction effects. The significant quadratic effects of the model for the L9-design suggested that we needed a design of higher resolution for the optimization (Section 3.3 below).

3.3. Multivariate optimization

The next step was a multivariate optimization and the composition of each buffer is listed in Table 2. We replicated the centre point experiment in each run to keep track of potential drift in the results. The repeatability of the *Z* values can be seen for the replicate experiments (marked bold in Table 2). The design (face centred cube) included the variation of buffer concentration, salt concentration and pH at 3 levels in 16 different buffers resulting in a total of 20 experiments executed in 5 separate Biacore 4000 runs. For each experiment, data from four immobilization levels were collected resulting in totally 80 rows in the data-file.

The model between buffer composition and Z_{250}-value explained 95% of the variation (R^2 = 95%, Q^2 = 92%). The assay quality was strongly influenced by non-linear terms (quadratic) and interaction effects between the parameters (Fig. 3(a)). For example, the relation with immobilization level was strongly nonlinear (Fig. 3(b)). The interaction effect between pH and immobilization also indicated that the assay quality is not influenced strongly by pH if the immobilization level is kept low (Fig. 3(c)). At high immobilization levels, a low pH results in high *Z*-values. Also salt and buffer concentrations showed an interaction effect; the positive effect of increasing the salt concentration is much more noticeable at low buffer concentrations than at high buffer concentrations (Fig. 3(d)). The effect of increasing salt might be even negative when buffer concentration is at medium level (50 mM). This detailed resolution had not previously been observed and demonstrates the necessity to use a DoE-approach with mathematic modelling in order to obtain the optimal composition of the buffer.

The predicted optimal immobilization level for rituximab was 5500 RU (Figs. 3b and 4), and the predicted optimal buffer (Fig. 4) had the following composition:

75 mM Tris, 450 mM NaCl, 5 mM EDTA, 0.05% P20, pH 7.0.

In other experiments the effect of EDTA (0–10 mM) and different detergents (0.05–0.5% surfactant P20, 0.05% Brij® 35 and 0.05% Pluronic®F-127) were investigated. No significant effect on sensitivity related to EDTA and detergent variation was detected (data not shown). Other assay variables were also varied, such as sample dilution (between 1:1 and 1:20) sample contact time (120–600 s), analysis temperature (13–37 °C) and sensor chip type (CM5 or CM4 with lower negative charge density compared to CM5). The conditions for highest sensitivity in the anti-rituximab assay were found to be sample dilution 1:1, sample contact time 600 s, 25 °C

Table 2
Design of buffer composition optimization step and assay quality results. Four immobilization levels (average 12, 6, 4 and 2 kRU rituximab) were analyzed in each flow cell. Here is only the result from about 4 kRU rituximab surface is shown. The buffer contained 0.05% P20 and 5 mM EDTA in addition to the components listed in the table. The same buffer was used as both running and sample buffer. Replicates are shown in bold. Note that *Z*-values for the 250 ng ml^{-1} anti-rituximab are presented in this table and they cannot directly be compared with the *Z*-values in Table 1 which are for 500 ng ml^{-1}.

Run	Flow cell	Conc. buffering substance (mM)	Conc NaCl (mM)	pH	Anti-rituximab Z_{250}
4	**Fc1**	**55**	**325**	**8**	**0.61**
	Fc2	55	325	7	0.64
	Fc3	10	150	9	0.29
	Fc4	100	150	7	0.48
5	**Fc1**	**55**	**325**	**8**	**0.69**
	Fc2	100	325	8	0.68
	Fc3	10	500	9	0.46
	Fc4	10	325	8	0.66
6	Fc1	10	500	7	0.58
	Fc2	**55**	**325**	**8**	**0.58**
	Fc3	100	500	9	0.40
	Fc4	100	150	9	0.44
7	Fc1	55	325	9	0.62
	Fc2	55	500	8	0.64
	Fc3	**55**	**325**	**8**	**0.61**
	Fc4	100	500	7	0.70
8	Fc1	55	150	8	0.58
	Fc2	10	150	7	−0.01
	Fc3	10	150	7.4	0.11
	Fc4	**55**	**325**	**8**	**0.68**

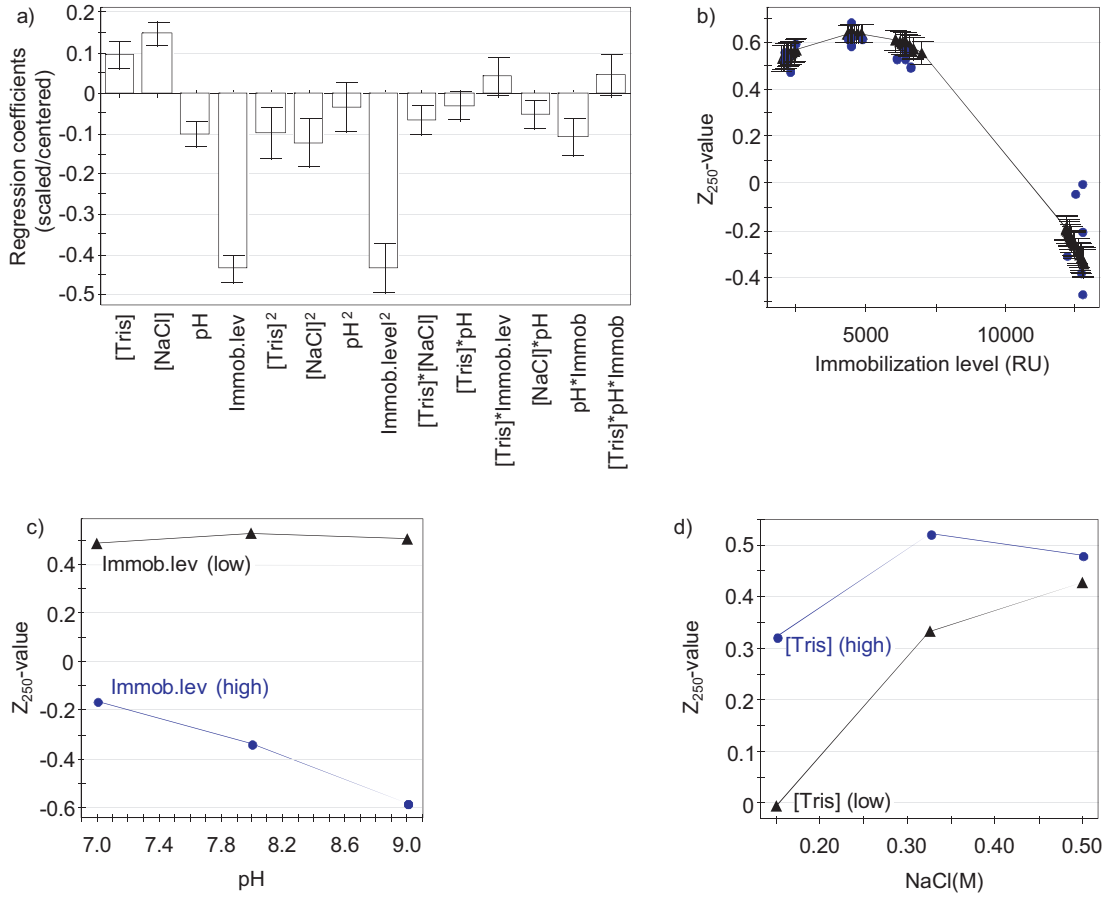

Fig. 3. Evaluation results for Z_{250}-value from the optimization experiment with anti-rituximab. (a) Regression coefficients for Z_{250}-value. The varied factors influenced the assay quality significantly if the confidence intervals (error bars) did not include zero on the *y*-axis. (b) The effect of immobilization levels on the *Z*-value. (c) The interaction effect of pH and immobilization level on the *Z*-value. (d) The interaction effect between buffer (Tris) concentration and NaCl concentration.

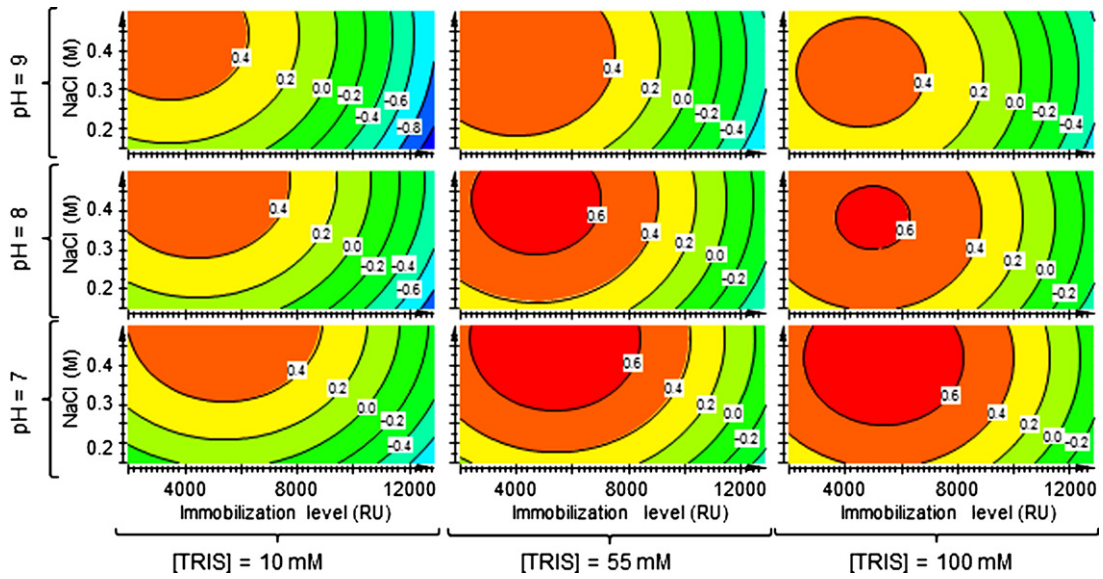

Fig. 4. Contour plot of Z_{250}-value for the anti-rituximab assay. The NaCl concentration is plotted vs. immobilization level for 9 different combinations of pH and buffer concentration level. High *Z*-values indicate high assay quality.

analysis temperature and Sensor Chip CM5 in combination with the optimized buffer and optimized immobilization level.

A comparison was done between the commonly used HBS-EP+ buffer and the optimized buffer at different immobilization levels for the anti-rituximab assay (Fig. 5). High immobilization level and HBS-EP+ buffer resulted in high amounts of non-specific binding from the plasma samples and a large variation between the negative plasma samples (red square symbols in Fig. 5a). This led to a high cut point and thus a low sensitivity. By decreasing the immobilization level (and still using HBS-EP+) the sensitivity increased slightly (result not shown). The use of the optimized buffer on a surface with high level of immobilized rituximab decreased the non-specific binding but the variation between the different negative samples was still high resulting in a rather low sensitivity (result not shown). The combination of an optimal immobilization level and the optimized buffer (Fig. 5(b)) showed a 5-fold improvement of assay sensitivity compared to the conditions shown in Fig. 5(a). Note the low non-specific binding and low variation between different negative plasma samples in Fig. 5(b). In order to obtain high sensitivity it is thus important to run the assay with a combination of optimal immobilization level and optimal buffer.

3.4. Low non-specific binding with the optimized buffer

Five different biotherapeutic antibodies and two protein-based drugs were immobilized on Sensor Chip CM5 to further study the non-specific binding from plasma samples to drug molecules. The optimized Tris buffer with high salt concentration was compared with the commonly used HBS-EP+ buffer. The nonspecific binding and variation from the different plasma samples was significantly lower with the optimized buffer to all examined drugs (Fig. 6).

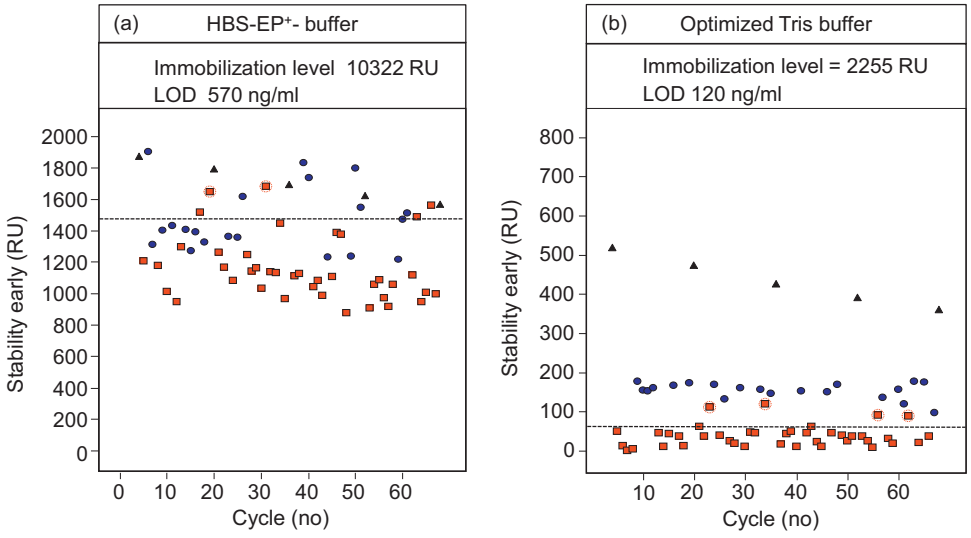

Fig. 5. Anti-rituximab assay with different immobilization levels and different buffers. SE-report point vs. cycle number for 1000 ng ml^{-1} anti-rituximab (black triangle) in one plasma and 250 ng ml^{-1} (blue circle) anti-rituximab in 10 different plasmas (in duplicates). Twenty different plasmas in duplicates (without anti-rituximab) are shown as red squares. The cut points are shown with a dashed line. Note the different scales on the *y*-axis.

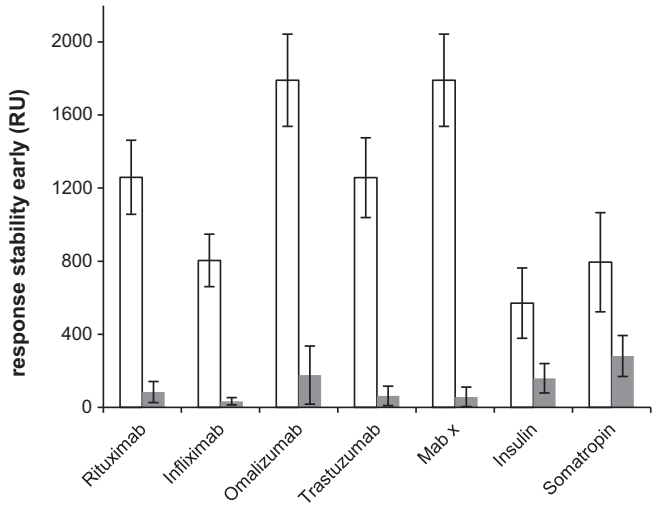

Fig. 6. Average non-specific binding from 20 different plasma samples to seven biotherapeutic proteins. The unfilled bars show the results with regular HBS-EP+ buffer and the grey bars show the result with the optimized buffer. Error bars show the standard deviation. The immobilization level for the biotherapeutic antibodies varied between 4 and 6 kRU. The immobilization level of insulin was 1.2 kRU and the growth hormone somatropin was immobilized to 9 kRU.

3.5. Verification of sensitivity with other assays

Experiments were done to verify that the new buffer conditions did not only give lower non-specific binding, but also improved sensitivities for more assays than the anti-rituximab assay. A sensitivity of 120 ng ml^{-1} has been calculated for the anti-rituximab assay with the new conditions (Fig. 5(b)). Here the sensitivities were analyzed for anti-myoglobin, anti-IgE, omalizumab and anti-erythropoietin. A comparison was made between HBS-EP+ and the new optimized buffer. The setup of each experiment was identical with the anti-rituximab experiments with 20 negative plasmas and 10 plasmas spiked with positive control antibodies. Shankar et al. [3] recommended the use of a pooled matrix for determination of sensitivities. However in this study plasma from different donor was used which means that the sensitivity values include the biological variation and not only the analytical variation and would thus reflect the situation in an immunogenicity screening assay.

The results showed that the sensitivity improved 2–5 times for all immunogenicity assays included in the study with the use of the optimized buffer. All samples with 250 ng ml^{-1} ADA in the assays investigated were correctly classified as positives (i.e. were above the cut point). Calculated sensitivities were 140 ng ml^{-1} for anti-myoglobin, 170 ng ml^{-1} for anti-IgE, 80 ng ml^{-1} for omalizumab, and 80 ng ml^{-1} for anti-erythropoietin. The assays with the new buffer conditions have sensitivities well below the recommendation of 250–500 ng ml^{-1} from FDA guidance for immunogenicity.

3.6. The effect of high salt concentration on rapidly dissociating and low affinity ADA

One of the previously mentioned benefits of using the Biacore technology for immunogenicity testing is the ability to detect fast dissociating ADAs. Studies were therefore performed to ensure that the new buffer conditions did not impede the detection of fast dissociating antibodies. Eight different monoclonal mouse anti-CKMB antibodies with different association and dissociation rates for binding to human CKMB were used in the study.

The optimized buffer did not change the dissociation of any of the studied interactions compared to HBS-EP+. However, the association was increased for two of the antibodies and decreased for two other antibodies. The decreased association rate made it difficult to detect the two antibodies when optimized buffer was used as both running and sample dilution buffer. However, by using optimized buffer only as running buffer and HBS-EP+ as sample dilution buffer, the response levels and the association rate increased to detectable levels.

For all antibodies tested, the assay sensitivity in plasma increased when the optimized buffer was used instead of HBS-EP+ buffer. Even though the response from 2 of the monoclonal anti-CKMB antibodies decreased, assay sensitivity was still improved due to higher decrease in background binding and variation of the negative plasma samples.

3.7. Adoption of assay to an easily prepared buffer

The assays were optimized towards high sensitivity, measured as high *Z*-values. Fig. 7(a) illustrates the correlation between the *Z*-values and the LOD in ng ml^{-1} for all experiments from the optimization step (Table 2) with 80 combinations of immobilization levels and buffer compositions. As can be seen all experiments with the highest immobilization levels (12 kRU red, group 1 in Fig. 7(a)) and almost all experiments with the lowest NaCl concentration (150 mM triangle, group 2 in Fig. 7(a)) have the lowest assay sensitivity irrespectively of pH and Tris concentration. Regression models were calculated using only immobilization level and NaCl concentration (including interaction and quadratic effects) as factors and they could explain more than 85% of the variation in Z_{250}-value and LOD. The conclusion that immobilization level and NaCl concentration dominated assay quality led us to try a shortcut in which the NaCl concentration of a commercially available HEPES buffer was increased, in combination with optimized immobilization level of rituximab. In this experiment extra NaCl was added directly to a ready-made 10× HBS-EP+ buffer, which was when diluted it to 1× (final concentration of NaCl 500 mM,10 mM HEPES, 3 mM EDTA, 0.05% P20). The signals of outliers were significantly higher in this HEPES buffer, but after outlier elimination, assay sensitivity was comparable with the optimized Tris buffer. This was verified with several assays (anti-rituximab, anti-erythropoietin, omalizumab and anti-myoglobin).

It is also interesting that LOD values below 200 ng ml^{-1} anti-rituximab can be found in a few experiments with 0.15 M NaCl Fig. 7(b). In these cases (2 triangles with LOD < 200 ng ml^{-1}) the concentration of Tris was 55 mM and pH 8, i.e. an effect of the interaction effect between buffer and NaCl concentration. Thus, if it is not suitable to use high NaCl concentration in an assay a moderate to high concentration (50–100 mM) of the buffering substance might decrease background binding and increase assay quality.

4. Discussion

The combination of statistical design of experiments and the parallelism of Biacore 4000 (four flow cells with five spots each) offers an efficient technological platform to perform assay optimization. We optimized the conditions for an anti-rituximab assay and verified that the selected buffer significantly reduced background binding from plasma with 6 other drugs. It can be argued that by using a buffer with a high ionic strength the risk of generating false negatives will increase. However, the reduction in plasma background binding and variability out-weighs that risk and instead the risk of generating false negatives is minimized as the sensitivity is much better using the optimized buffer. It was also shown that it is possible to use a milder sample dilution buffer in combination with a buffer with high ionic strength as running buffer without any dramatic effect on the assay quality. The strong negative interaction effect between NaCl concentration and buffer

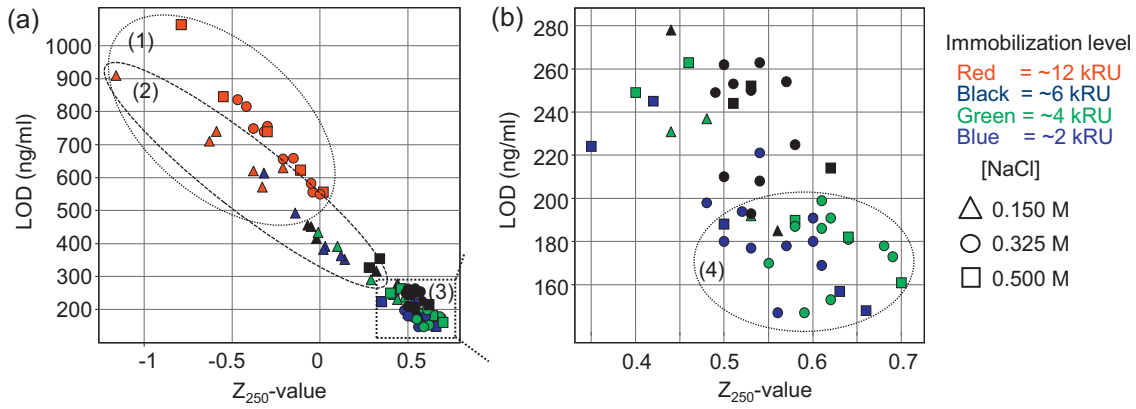

Fig. 7. Scatter plots of level of detection (LOD) vs. Z_{250}-value. Each point in the plots represents an experiment from the buffer optimization step with different levels of immobilization, NaCl concentration, Tris concentration and pH. (a) Group 1 with highest immobilization level (red, ~12 kRU) and lowest NaCl concentration (group 2, triangle, 0.15 M NaCl) have low sensitivity. (b) A close up of group 3 in Fig. 7(a) shows that a vast majority of samples with the highest Z_{250}-value and lowest LOD have either an immobilization level of 2 kRU (blue) or 4 kRU (green) in group 4.

substance concentration indicates that these are at least partly interchangeable. Thus, if buffer substance concentration (e.g. Tris or HEPES) is kept high (50–100 mM), the NaCl concentration can be kept relatively low while the sensitivity of the assay remains high.

The SE-report point, measured 7 s after the end of the injection, was used to measure binding and as base for our calculations of *Z*-value and cut points. Initially other report points (1 and 2 min after the end of injection) were tested. These gave slightly better statistics as the rapidly dissociating non-specific binders had time to dissociate. However, we decided to use the early report point to minimize the risk of getting false negatives from rapidly dissociating ADAs. It is important to remember that rapid dissociation do not explicitly mean that the interaction is of low affinity. For example an antibody with an on-rate of $5 \times 10^6\ M^{-1}s^{-1}$ and an off-rate of $10^{-2}\ s^{-1}$ has an affinity of 5 nM and cannot be considered as a weak binder. Still there is a significant risk that these rapidly dissociating high affinity antibodies will be missed in assays with washing step(s) included such as ELISA.

The immobilization level correlated very strongly with assay quality and for both the rituximab and the myoglobin systems, the optimal levels were quite low (2–6 kRU and 3–4 kRU, respectively). When we used the combination of *Z*-value and LOD to visualize assay quality it became clear that immobilization levels of 2, 4 and 6 kRU gave better results than the12 kRU for the anti-rituximab assay (Fig. 7). Higher immobilization levels gave in many cases a significantly increased difference between positive and negative plasmas, but at the same time the variability between different plasmas increased. The increased noise outweighed the increase in signal. However, for the omalizumab assay it was found that the non-specific binding from plasma to immobilized IgE was very low and in that case a higher immobilization level resulted in higher assay quality. Thus, the optimal surface density is drug dependent and should be optimized for each individual model system during assay development.

In most published immunogenicity assays with Biacore short sample injection times (30 s-2 min) and high sample dilution (1:10–1:100) have been used. Most studies have also been done with high immobilization levels and HEPES buffer with physiological ionic strength. There are however some assays that have been performed with higher ionic strength in the buffer. Antibodies against erythropoiesis-stimulating agents were detected in an assay with the buffer 25 mM Tris, 325 mM NaCl, 3 mM EDTA and 0.05% Tween 20, pH 9 [14], that is conditions very similar to the conditions that were found in this optimization. The assay for anti-tocilizumab was run with a buffer with 300 mM NaCl [15] and a buffer with 500 mM NaCl was used to reduce unspecific binding from bovine serum [16]. The optimized buffer presented in this work is generic and can be used as a starting point for optimization of other biosensor assays run in plasma. This in combination with 5–10 min sample injection, sample diluted 1:1 and optimized immobilization levels has the potential to increase sensitivities in immunogenicity assays run on biosensors.

5. Conclusion

Parallel experiments using 4 immobilization levels and DoE – based buffer optimization strategy rapidly found that immobilization level and the salt concentration were factors that influenced the sensitivity most. A good quantitative and graphical overview of the main effects, strong non-linearities and interaction effects could be identified, interpreted and utilized. A strategy to obtain high sensitivity in an immunogenicity assay with biosensors would be to optimize the immobilization level and add extra salt (final concentration ~500 mM) to the commonly used HBS-EP+ buffer. The buffer conditions and the optimization strategy presented can most probably be extended into all types of biosensor assays run in plasma and related sample matrices.

Conflict of interest

All the authors are employees at GE Healthcare.

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