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RESEARCH ARTICLE

Monitoring bioactive and total antibody concentrations for continuous process control by surface plasmon resonance spectroscopy

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Monoclonal antibodies have become an increasingly important part of fundamental research and medical applications. To meet the high market demand for monoclonal antibodies in the biopharmaceutical sector, industrial manufacturing needs to be achieved by large scale, highly productive and consistent production processes. These are subject to international guidelines and have to be monitored intensely due to high safety standards for medical applications. Surface plasmon resonance spectroscopy — a fast, real-time, and label-free bio-sensing method — represents an interesting alternative to the quantification of monoclonal antibody concentrations by enzyme-linked immunosorbent assay during monoclonal antibody production. For the application of monitoring bioactive and total monoclonal antibody concentrations in cell culture samples, a surface plasmon resonance assay using a target-monoclonal antibody model system was developed. In order to ensure the subsequent detection of bioactive monoclonal antibody concentrations, suitable immobilization strategies of the target were identified. A significant decrease of the limit of detection was achieved by using an adapted affinity method compared to the commonly used amine coupling. Furthermore, the system showed limit of detection in the low ng/mL range similar to control quantifications by enzyme-linked immunosorbent assay. Moreover, the comparison of total to bioactive monoclonal antibody concentrations allows analysis of antibody production efficiency. The development of an alternative quantification system to monitor monoclonal antibody production was accomplished using surface plasmon resonance with the advantage of low analyte volume, shorter assay time, and biosensor reusability by target-layer regeneration. The established method provides the basis for the technical development of a surface plasmon resonance-based system for continuous process monitoring.

KEYWORDS
antibody, bioactive and total antibody concentration, monitoring, surface plasmon resonance

Abbreviations: EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; GFP, green fluorescent protein; HBS, HEPES buffered saline; NHS, N-hydroxysuccinimide; NTA, nitrilotriacetic acid; PBST, phosphate buffered saline with polysorbate; R_{ligand}, immobilization level of the ligand; RU, resonance units; SPR, surface plasmon resonance.

1 | INTRODUCTION

Rapid advancements in mAb production technologies, including development of mAb producing cell-lines, new bioreactor

systems, and novel purification technologies [1–5] as well as improvements in the diagnostic and therapy of diseases [6,7] resulted in an explosive growth of the mAb market [8]. Worldwide sales will have an estimated volume of $125 billion by 2020 [4]. High mAb concentrations while maintaining activity during production are key issues for highly productive as well as cost and time efficient industrial manufacturing. Instead of solely enhancing antibody titers the focus shifts more and more towards controlling of process consistency and product quality to ensure efficiency [2].

The production of mAbs is mainly achieved by cultivation of mAb expressing mammalian cell lines with bioreactors and therefore subject to many manufacturing and processing regulations [9]. Next to general process parameters like temperature, CO_2, pH, or cell culture metabolites [2], other important parameters like specificity, purity but also total and active concentration of mAb need to be monitored during these processes. Due to the complexity of mAb expression using mammalian cell systems, changes in culture parameters can significantly affect process and product quality [10]. Therefore, direct measurements during mAb production with shorter assay times to regularly assess mAb concentration and activity are extremely important.

ELISA is recommended for the quantifications of mAb concentration in industrial production by the U.S. Food and Drug Administration (FDA). ELISA is broadly used for therapeutic mAb monitoring and represents a very well established and tested detection system [11,12]. Several other methods based on flow cytometry, reflectometric interference spectroscopy, or immuno flow injection analysis have been considered for fast monitoring [13–15]. Investigation of biomolecular interactions can also be achieved by SPR spectroscopy, a method based on refractive index changes at the sensor surface where one binding partner is immobilized and interacts with the analyte in solution delivered by continuous flow microfluidics. This process is also known as hydrodynamic isolation [16]. Technical enhancements over the last years in chip design, microfluidics, and automated sample preparation allow multi-spot measurements in parallel. Furthermore, an increase in the amount of analysis spots has been achieved by various manufacturers introducing new SPR devices, including SPR-32 from Bruker Daltonics SPR (formerly Sierra Sensors GmbH). Therefore, the SPR method is becoming a very suitable alternative to ELISA.

Developing independent bioactive sensor surfaces enables simultaneous, parallel monitoring of total and bioactive mAb concentration based on a partially mass transport limited interaction which is directly proportional to the antibody concentration. The obtained concentration can be used to perform affinity or kinetic characterization at the same surface allowing for increased data acquisition as well as simultaneous detection of various parameters. These additional information are not available using ELISA quantifications because it represents an end-point measurement. Furthermore, SPR spectroscopy is a label-free technique for real-time measurements. Bioactive sensor chips can be regenerated and reused and therefore allow the comparison of different batches of mAb productions with a single controllable sensor surface.

Both advantages eliminate time-consuming steps like repeated ligand immobilization or secondary detection methods which are necessary for ELISA quantifications [17].

Additionally, the reusability of SPR sensor chips decreases the cost per measurement when compared to ELISA where wells can only be used once and therefore require cost-intensive preparation of the bioactive surface with sufficient ligand for each analysis. Furthermore, the SPR technique operates independent of light scattering or adsorption [18] allowing analysis of crude culture solutions with turbid or colored samples. Moreover, studies have already shown that SPR is suitable to analyze complex mixtures [19]. Nevertheless, possible interactions of molecules from the media need to be evaluated and appropriate alterations for establishing the bioactive sensor surface or sample preparation should be implemented to avoid unspecific binding.

This study primarily focused on developing optimal immobilization procedures for bioactive SPR sensor surface fabrication using the antigen GFP (green fluorescent protein) and its respective GFP antibody as a model system for the analysis of total and bioactive mAb concentration. Different

PRACTICAL APPLICATION

The described technology where independent bioactive sensor surface is developed to measure multiple parameters like bioactive and total antibody concentration as well as established regeneration schemes for sequential measurements using a single bio-functionalized chip with multiple spots will promote the future use of SPR analysis during antibody production monitoring. Recent developments towards multi-channel measurements for new high-throughput SPR devices enable increased data acquisition as well as simultaneous detection of various parameters and binding kinetics. Advanced microfluidics and automation in sampling coupled with target layer regeneration and small analyte volume can provide shorter analysis time and significant cost savings when applied to SPR-dependent antibody monitoring in industry. Therefore, future SPR-based systems for continuous mAb production monitoring represent an excellent alternative to standard quantifications with ELISA.

immobilization techniques were evaluated and a combination of amine coupling and His-tag capturing has been established as most sensitive and reusable functionalized surface for bioactive mAb quantification. Total mAb detection using Protein A/G as ligand surface was established. For both surfaces, optimal regeneration regimes were identified and quantification of unknown mAb concentrations from cell culture broth was demonstrated. Sensitivity for the developed detection system was compared with ELISA as reference method. The experimental data highlights important parameters for SPR sensor surface development to further establish robust assay platforms for monitoring mAb production processes.

2 | MATERIALS AND METHODS

2.1 | Materials

GFP [2.1 mg/mL], anti-GFP antibody (purified, 2.69 mg/mL, mouse), cell culture samples with unknown anti-GFP concentrations, and the cell culture broth (DMEM-4.5 g/L Glucose; Gluta-max; Pyruvat, 10% IgG-stripped FBS, 100 µg/mL Gentamycin, 0.1% 2-mercaptothanol) were provided by Max Planck Institute of Molecular Cell Biology and Genetics (Dresden, Germany). GFP-His, Protein A/G, and anti-mouse-IgG-HRP were purchased from biomol GmbH (Hamburg, Germany). Anti-mouse-IgG (goat) was obtained from Sigma-Aldrich Chemie GmbH (Germany). The amine coupling kit (N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide [EDC], N-hydroxysuccinimide [NHS], ethanolamine) used to immobilize ligands was purchased from GE Healthcare Europe GmbH (Germany). Reference ligand BSA was obtained from VWR International GmbH (Germany). Glycine and EDTA were obtained from Carl Roth GmbH (Germany). HEPES buffered saline (HBS) (10 mM HEPES, 0.137 M NaCl at pH 7.5) and PBST, phosphate buffered saline with polysorbate 20 (10 mM PBS, 0.1% polysorbate-20, pH 7.4) were purchased from Sigma-Aldrich Chemie GmbH (Germany). SPR amine chip (carboxylic acid–COOH–surface, 2D chip) and high capacity amine chip (nitrilotriacetic acid [NTA] surface) were obtained from Bruker Daltonics SPR, formerly Sierra Sensors GmbH (Germany).

2.2 | SPR methods

SPR measurements were performed on a SPR 2/4 system (Bruker Daltonics SPR). The binding of analyte to immobilized ligand causes changes in refractive indices and thereby changes in the SPR angle. This change is reported as resonance units (RU). The evaluation of SPR data was performed using AnalyserR2 (Bruker Daltonics SPR).

2.2.1 | Immobilization of GFP to detect bioactive mAb concentrations

Immobilization of GFP by amine coupling

Immobilization procedure was based on the amine coupling kit protocol (GE Healthcare Europe GmbH, Germany) using 2D amine sensor chips with a carboxylic acid surface. For this purpose, the activation of the surface carboxyl groups was performed by injecting a 7 min pulse of EDC (400 mM)/NHS (50 mM). Subsequently, GFP (3 µg/ml) in 10 mM acetate buffer (pH 5) was immobilized at the activated chip surface for 11 min. The remaining active sites of the SPR sensor were blocked by 1 M ethanolamine (pH 8.5) for 7 min.

Immobilization of GFP-His by His-tag capturing

His-tag capture chips (NTA surface) have been applied for affinity immobilizations using His-dependent ligand orientation [20]. Hereby, NTA was used as a surface bound chelator which binds divalent transition metal ions (i.e. Cu^{2+} or Ni^{2+}). After ion immobilization (activation of the surface), proteins carrying a His-tag can bind the ions and therefore the carrier surface.

Activation of the NTA surface was achieved by injecting a 3 min pulse of $NiSO_4$ (200 µM). Subsequently, GFP-His (1.5 µg/mL) in HBS was applied to achieve non-covalent immobilization at the activated chip surface. Variation of GFP-His immobilization level was achieved by altering the injection time (60, 120, 240, 360, 600, 840, 1200 s).

Immobilization of GFP-His by His-tag capture coupling

For oriented and covalent GFP-His immobilization the previous His-tag capturing method has been combined with the amine coupling method (schematic representation, see Figure 4) [21,22]. First, activation of the NTA surface was achieved by injecting a 3 min pulse of $NiSO_4$ (200 µM) with subsequent EDC (400 mM)/NHS (50 mM) injection for 4 min. For oriented, covalent immobilization, GFP-His (2 µg/mL) in 10 mM HBS was immobilized at the activated chip surface for 5 min. Additional ligand crosslinking was achieved by subsequent incubation with EDC (400 mM)/NHS (50 mM) for 1 min and blocking of remaining active sites using 1 M ethanolamine (pH 8.5) for 3 min.

2.2.2 | Immobilization of Protein A/G by amine coupling to detect total mAb concentrations

Immobilization procedure follows the same protocol as described for GFP with the exception of ligand immobilization with 10 µg/mL Protein A/G in 10 mM NaOAc (pH 4.5) for 2 min.

2.2.3 | Anti-GFP antibody measurements and regeneration procedure

To obtain the SPR calibration curves, 2.69 mg/mL anti-GFP stock solution was diluted in PBST to achieve appropriate concentrations ranging from 19 to 9.6 µg/mL. Threefold analysis of samples was performed with a flow rate of 10 µL/min with 60 s contact and dissociation times using various functionalized sensor surfaces. Binding signals were obtained directly after anti-GFP (analyte) injection. The sensor surface was regenerated between experiments by dissociating any formed complex using a regeneration solution of 10 mM Glycine (pH 11.6) for GFP-His bioactive surface or 10 mM Glycine (pH 1.5) for Protein A/G bioactive surface for 1 min. Additionally, threefold blank injections of PBST and unknown samples directly from mAb production cell culture broth in various dilutions were measured.

2.3 | ELISA assay

Indirect ELISA was applied as a reference method to quantify bioactive and total mAb concentrations. After rinsing a 96 well MaxiSorp microtiter plate with coating buffer (10 mM PBS, pH 7.4), wells were coated with 100 µL of coating conjugates in PBS (10 mM, pH 7.4) and incubated at 4°C overnight (0.5 µg/mL GFP, 10 µg/mL IgG, 0.5/10 µg/mL BSA for control purpose). After washing the plate five times with 100 µL PBST, 100 µL of anti-GFP (mouse), or diluted cell culture sample (in 10 mM PBST) was added, and the assay was incubated at room temperature for 1 h. After washing steps with PBST, wells were incubated with 100 µL of diluted anti-mouse-HRP at room temperature for 1 h. Following the washing steps, the wells were incubated with 3,3′,5,5′-tetramethylbenzidin (TMB) substrate solution at room temperature in the dark for 15 min and the reaction was terminated by adding 100 µL H_2SO_4 (180 mM) after incubation. The absorbance was determined at 450 nm using a Tecan microplate reader (GENios, Tecan Group, Switzerland) and referenced with the absorbance at 580 nm.

3 | RESULTS AND DISCUSSION

3.1 | SPR assay development to detect bioactive mAb concentrations

In this study, GFP and anti-GFP as an antigen-antibody model system was used to establish an SPR assay to detect bioactive mAb concentrations from cell culture broth for mAb production processes. To achieve a reliable and functional SPR biosensor, the immobilization of the ligand on the SPR sensor surface is very crucial and the first step during assay development. Various methods exist using strategies involving biotin-avidin systems, antibody capturing, or direct amine coupling [23–25]. Some of those have been examined here to identify the most effective and suitable method to detect bioactive mAb concentrations.

3.1.1 | Immobilization of GFP by amine coupling

Amine coupling, a widely used immobilization technique, has been used in a first approach to couple the model antigen GFP on a suitable SPR chip with a planar carboxylated surface (alkanethiol monolayer coating). This method leads to a non-oriented immobilization due to the covalent coupling of any available amine group of the ligand to the carboxyl groups at the sensor surface. Therefore, the ligand is randomly oriented, rendering some of the epitopes unavailable for antibody binding. Additionally, to achieve high binding signals for the analyte (anti-GFP), sufficient amounts of immobilized ligand (R_{ligand}), measured in RU, are required for this method. To calculate the theoretical maximum binding capacity ($Rmax_t$), according to Eq. 1, R_{ligand} needs to be determined.

In our study, first immobilization tests showed a GFP immobilization level (R_{GFP}) of approximately 750 to 830 RU (data not shown). Subsequently, in-depth anti-GFP binding studies were carried out on a surface with R_{GFP} of 830 RU (GFP: 3 µg/mL; 660 s). BSA was immobilized at a different spot on the same sensor to obtain a reference surface with similar surface conditions [26], resulting in an immobilization level (R_{BSA}) of 803 RU. Additionally, blank measurements were performed to identify buffer specific effects and resulting data was subtracted from binding signals.

Performing consecutive antibody binding experiments requires the regeneration of the ligand surface by completely removing the captured analyte while retaining the ligand activity. We utilized previous practical experience from a different SPR system conducting experiments with an aptasensor and identifying optimal regeneration regimes [27]. To find optimal conditions for the mAb specific sensor, a regeneration scouting was performed and results showed that regeneration with 10 mM Glycine (pH 11.6) solution was sufficient for reproducible results. The SPR signal after regeneration decreased to the baseline (−6 to −32 RU) and binding signals remained stable over several regeneration cycles (Supporting Information Figure S1). In our previous work, we demonstrated that a bioactive ligand surface using HSA-specific mAb [28] could be retained for at least 40 cycles over a period of one month [29].

Consecutive binding analysis with an anti-GFP antibody was performed with concentrations ranging from 9 to 9.6 µg/mL (Figure 1). Each concentration was analyzed in triplicate and all injections were conducted in random order. A calibration curve ranging from 19 to 2.4 µg/mL was obtained from the binding signals (Figure 2). A four-parametric logistic function (4PL) was fitted to the data points.

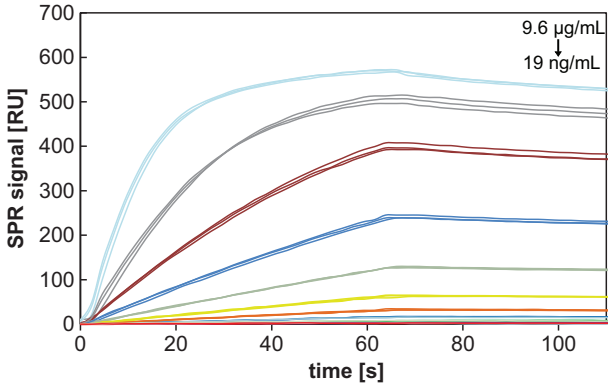

FIGURE 1 The SPR sensorgram for the detection of various anti-GFP antibody concentrations using an amine coupled GFP functionalized sensor surface. Increasing SPR signal for increasing concentrations of anti-GFP antibody (from bottom to top: 19 ng/mL, 37 ng/mL, 75 ng/mL, 150 ng/mL, 300 ng/mL, 600 ng/mL, 1.2 µg/mL, 2.4 µg/mL, 4.8 µg/mL, 9.6 µg/mL). Triplicates are depicted in the same colour. Signals of blank injections and signals over a BSA coupled reference spot were subtracted from the binding signals

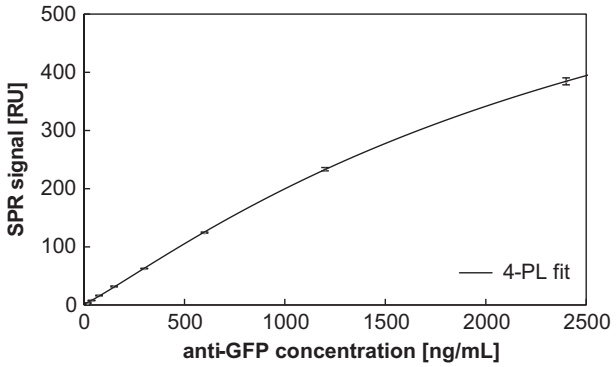

FIGURE 2 Calibration curve for detection of bioactive antibody concentration using an amine coupled GFP functionalized sensor surface. A calibration curve ranging from 19 to 2400 ng/mL anti-GFP was obtained from the binding signals after analyte injection. Each data point in the calibration curve represented the mean ± SD of three determinations. A four-parametric logistic function (4PL) was fitted to the data points

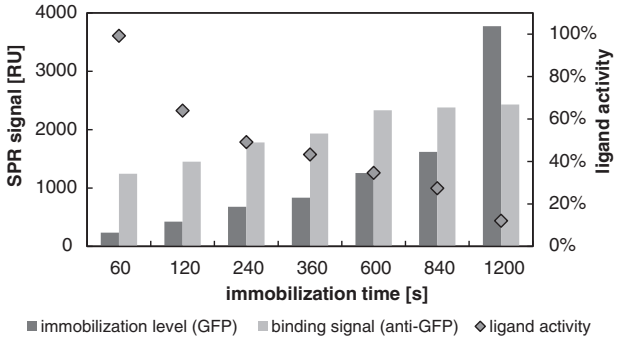

FIGURE 3 Comparison of GFP-His immobilization levels depending on incubation time and resulting SPR signals using anti-GFP. Depicted in dark grey bars is the GFP-His immobilization level ($R_{GFP\text{-}His}$ 1.5 µg/mL) and respective anti-GFP binding signals ($Rmax_{exp}$ anti-GFP: 20 µg/mL, light grey bars). Secondary axis shows calculated ligand activity in percent. $R_{GFP\text{-}His}$ = 234 RU (60s)/424 RU (120s)/677 RU (240s)/843 RU (360s)/1258 RU (600s)/1620 RU (840s)/3773 RU (1200s). $Rmax_{exp}$ = 1244 RU/1452 RU/1782 RU/1933 RU/2332 RU/2380 RU/2431 RU

3.1.2 | Immobilization of GFP by His-tag capturing

To achieve oriented (site-directed) immobilization, and thereby increased availability of GFP epitopes for antibody-binding, a His-tag capturing method was utilized in order to gain maximum binding capacity. The advantage of the His-tag is its affinity towards metal ions like Ni^{2+}, which can be immobilized on nitrilotriacetic acid (NTA) sensor chips. His-tagged proteins can be made in-house or commercially obtained. In this study, we obtained commercially available His-tagged GFP (Biomol) and analyzed immobilization levels using NTA chips (Bruker Daltonics SPR). Increasing GFP-His immobilization levels ($R_{GFP\text{-}His}$) of 234–3773 RU were achieved with elongated incubation times, tested from 60 to 1200 s (Figure 3), using a constant GFP-His concentration (1.5 µg/mL). This resulted in anti-GFP binding signals of 1240 to 2440 RU (anti-GFP: 20 µg/mL). A saturation of the bioactive ligand surface was observed with immobilization levels above 1200 RU since the increase in binding signals (anti-GFP) reaches a plateau above this level (Figure 3).

At the end of the immobilization procedure, a reduction of the signal depending on the immobilization level was observed (Supporting Information Figure S2). High GFP-His immobilization levels showed a decrease in the immobilization level of 5–25% over a time period of 150 sec. This effect has been reported before [30] and is mainly due to relatively low affinity between His-tag and Ni^{2+}-NTA. Therefore, stable binding only occurs when enough ions are available for continuous rebinding between the two interaction partners, which is not the case for high amounts of ligand. In conclusion, incubation times during immobilization should be adjusted to result in GFP immobilization levels below 1300 RU to achieve a stable GFP sensor surface with minimal signal reductions of 1% (over 150 sec).

3.1.3 | Immobilization of GFP by His-tag capture coupling

The disadvantage of the His-tag capturing method described in the previous section is the non-covalent binding of the GFP onto the NTA surface. Therefore, the ligand surface is not susceptible for regeneration and re-usage of the bioactive surface.

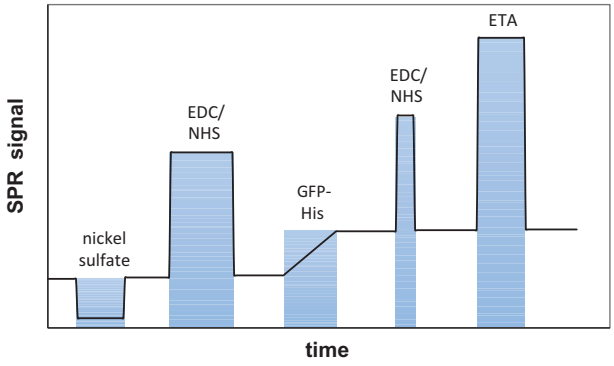

FIGURE 4 A schematic SPR sensorgram of the capture coupling method depicting the signal over time. Sensor surface is activated first by nickel sulphate following an EDC/NHS incubation. Subsequent injection of GFP-His leads to directed non-covalent binding to the Ni^{2+} ions as well as amine coupling between NH_2 groups and carboxylated sensor surface. Additional crosslinking between GFP-His molecules via a secondary EDC/NHS incubation are required for a stable bioactive sensor surface. Remaining active carboxyl groups are blocked using ethanolamine (ETA)

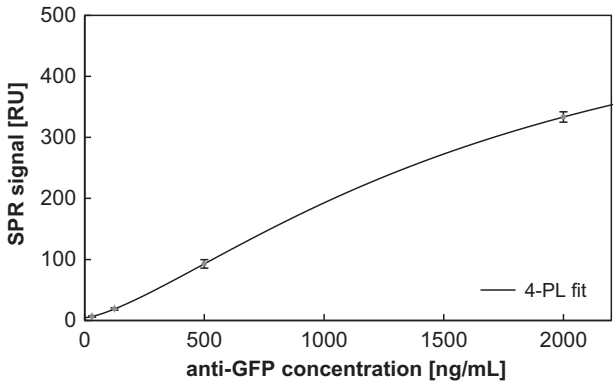

FIGURE 5 Calibration curve for detection of bioactive antibody concentration (anti-GFP) using a capture coupled GFP functionalized sensor surface. A calibration curve ranging from 31 to 2000 ng/mL anti-GFP was obtained from the binding signals after analyte injection. Each data point in the calibration curve represented the mean ± SD of three determinations. A four-parametric logistic function (4PL) was fitted to the data points

Each binding signal event requires a separate immobilization procedure rendering this process highly ineffective.

To circumvent this disadvantage, a combination of the two previously described methods has been established. In this study, this combinational method was termed His-tag capture coupling. Various parameters were tested and resulted in an assay procedure for the immobilization described in Figure 4. Thereby, the NTA sensor surface is activated by nickel(II) sulfate and EDC/NHS which leads to a partial oriented immobilization of GFP-His compared to a solely amine coupled sensor surface and simultaneously a covalent binding of free amine groups of GFP-His with carboxyl groups of the sensor surface.

Similar to the amine coupling, the established bioactive surface was tested for appropriate regeneration conditions to release anti-GFP from the bioactive surface with remaining ligand binding activity. A regeneration scouting was performed and results showed that regeneration with a 10 mM Glycine (pH 11.6) solution was sufficient for reproducible results. The SPR signal after regeneration decreased to the baseline (−5 to −33 RU) and binding signals remained stable over several regeneration cycles (Supporting Information Figure S3).

Consecutive binding analysis with an anti-GFP antibody was performed with concentrations ranging from 31 to 2 µg/mL (Supporting Information Figure S4). Each concentration was analyzed in triplicate and all injections were conducted in a random order. A calibration curve ranging from 31 to 2 µg/mL was obtained from the binding signals (Figure 5). A four-parametric logistic function (4PL) was fitted to the data points.

3.1.4 | Comparison of different immobilization methods, sensitivity, and LOD

Using three different immobilization methods we were able to detect SPR binding signals of anti-GFP with a bioactive ligand surface using either GFP or GFP-His. Comparison of these methods using similar ligand immobilization levels and saturated levels of anti-GFP (20–25 µg/mL) to achieve binding signals have shown that oriented immobilization increases the binding capacity of the bioactive surface. The theoretical maximum binding capacity ($Rmax_t$), based on the immobilization signal, needs to be calculated to assess ligand activity for the different methods. $Rmax_t$ represents the theoretical binding signal where each immobilized ligand molecule is fully accessible and functional for analyte (anti-GFP) binding. $Rmax_t$ was calculated by Eq. (1).

$$Rmax_t = \frac{M_{analyte}}{M_{ligand}} \cdot R_{ligand} \cdot valency_{ligand} \quad (1)$$

$Rmax_t$: theoretical Rmax
R_{ligand}: immobilization level of the ligand
$M_{analyte}$: molecular mass of the analyte
M_{ligand}: molecular mass of the ligand
$valency_{ligand}$: binding stoichiometry

Subsequently, the ratio of experimental obtained $Rmax_{exp}$ to $Rmax_t$ represents the ligand activity (overview in Supporting Information Table S1). Low $Rmax_{exp}$ compared to $Rmax_t$ implies that considerably fewer immobilized ligand molecules are able to bind the analyte.

Higher amounts of ligand at the sensor surface (high immobilization level) often result in decreasing ligand activity because too densely distributed ligands lead to a lower

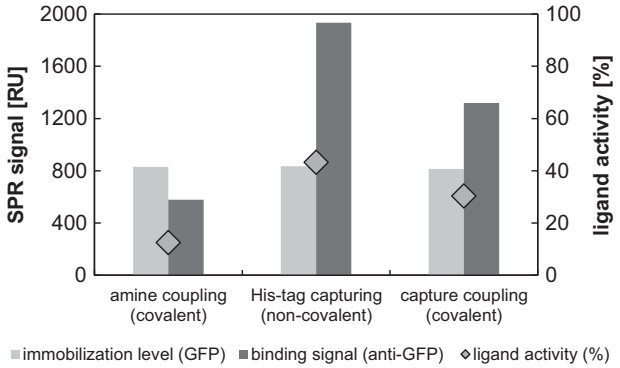

FIGURE 6 Comparison of the immobilization methods. At similar GFP immobilization levels (appr. 800 RU), the anti-GFP (20 µg/mL) binding signal using amine coupling is 578 RU (ligand activity 12%), using His-tag capturing is 1933 RU (ligand activity 43%) and using capture coupling is 1318 RU (ligand activity 30%)

probability of each ligand to bind the analyte, mainly due to increasing sterical hindrance [31]. Indeed, with our results we could show that higher amounts of ligand at a planar surface increased the probability of interferences between ligand molecules resulting in decreased ligand activity. Due to random orientation of the ligand and therefore rendering a percentage of ligand unavailable for analyte binding, $Rmax_{exp}$ for amine coupling was only 12% (Figure 6, Supporting Information Table S1) in this study.

Ligand immobilization levels have been studied here in detail for His-tag capturing using NTA chips as previously described and showed that in terms of ligand activity low $R_{GFP\text{-}His}$ (234 RU) resulted in almost 100% ligand activity whereas an immobilization level of 3773 RU achieved only 12% ligand activity (compare Figures 3 and 6). Hence, low immobilization levels reduced hindering steric effects of the analyte and negative interactions between ligands and therefore yielded high ligand activity. The reactivity of proteins, especially antibodies, towards metal ions of the NTA surface is also relevant in this context due to intrinsic metal chelating residues like His or Cys [20]. This can lead to unspecific binding of the analyte to the sensor surface. Higher immobilization levels, meaning less free metal ions, and addition of EDTA to the buffer can partially counteract this effect. Ligand activity is therefore not the only aspect for the development of a sensitive detection system but rather the identification of a good balance of directed immobilization, adequate ligand coverage and optimized buffer conditions.

In our study, optimization of these parameters resulted in increased ligand activity (Figure 6, Supporting Information Table S1) for His-tag capturing (43%) and the capture coupling method (30%). Simultaneous His-tag dependent orientation and amine coupling in the last method showed slightly decreased ligand activity due to additional ligand crosslinking rendering more epitopes unavailable for analyte binding.

In contrast to His-tag capturing (non-reusable bioactive surface), functionalized sensor chips established with amine coupling or capture coupling allow fast, online determination of bioactive antibody concentration during production processes due to the ability to regenerate the functionalized chip surface. Subsequently, the sensitivity to detect the GFP antibody with reusable surfaces has been determined to compare these two methods. We achieved a significant decrease of the LOD by using the adapted His-tag method (LOD: 8 ng/mL) compared to the commonly used amine coupling (21 ng/mL). This demonstrates that the improvement of ligand orientation increases the sensitivity of the ligand surface.

3.2 | SPR assay development to detect total mAb concentration

To detect total amounts of antibodies in solution Protein A/G, a recombinant fusion protein that combines Protein A and Protein G antibody binding domains was used. Due to its properties to bind human and mouse antibodies (IgG) at the Fc domain (constant region), Protein A/G is used for purification and capturing of antibodies [32]. In contrast to GFP with one epitope for GFP antibody binding, Protein A/G contains eight binding sites. Hence, oriented immobilization for Protein A/G is not necessary for high biosensor sensitivity and therefore the standard amine coupling procedure has been used for detection of total antibody concentrations.

Successful covalent immobilization of Protein A/G with an immobilization level of 1142 RU was achieved. Similar to previous experiments, a regeneration scouting to identify appropriate conditions for the Protein A/G functionalized surface was performed. Results showed that regeneration with a 10 mM Glycine (pH 1.5) solution was sufficient for reproducible results. The SPR signal after regeneration decreased to the baseline and binding signals remained stable over several regeneration cycles (Supporting Information Figure S5). Consecutive binding analysis with an anti-GFP antibody was performed with concentrations ranging from 31 to 2 µg/mL (Supporting Information Figure S6) based on the results of the binding analysis using amine coupled GFP. Each concentration was analysed in triplicate and all injections were conducted in random order. A calibration curve ranging from 31 to 2 µg/mL was obtained from the binding signals (Figure 7). A four-parametric logistic function (4PL) was fitted to the data points.

3.3 | Detection of bioactive and total mAb concentrations directly from cell culture broth

Towards the goal of monitoring mAb quality directly during production, the established SPR methods to detect bioactive and total mAb concentrations have been tested using anti-GFP producing cell culture samples. In a first step, possible cross

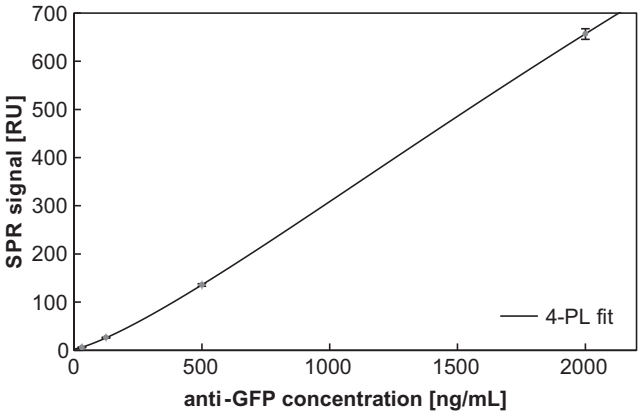

FIGURE 7 Calibration curve for detection of total antibody concentration (anti-GFP) using an amine coupled Protein A/G bioactive sensor surface. A calibration curve ranging from 31 to 2000 ng/mL anti-GFP was obtained from the binding signals after analyte injection. Each data point in the calibration curve represented the mean ± SD of three determinations. A four-parametric logistic function (4PL) was fitted to the data points

reactions with components of the cell culture media at the bioactive sensor surface were analyzed. Injections of blank cell culture media using Protein A/G and GFP coupled sensor surface showed that the binding signal after incubation with media remained at similar levels to buffer injections (Supporting Information Figure S7). Therefore, components from the production media do not show unspecific interactions with the sensor surfaces allowing the direct measurement of untreated samples.

Determination of unknown product concentrations (anti-GFP) using the established calibration curves showed a bioactive anti-GFP concentration of 6.77 ± 0.33 µg/mL and a total antibody concentration of 7.71 ± 0.22 µg/mL (Supporting Information Table S3). This means that 12% of the mAb which was produced during cell culture is not active. Future integration of the SPR analysis in the monitoring of mAb production processes with appropriate automated sampling will benefit industrial mAb manufacturing. Processes can be managed by setting specific thresholds for bioactive mAb concentrations allowing feedback control. Thereby optimal conditions will be achieved leading to higher process efficiencies.

For comparative analysis a method for detecting bioactive and total mAb concentrations using ELISA was established (Supporting Information Figure S8). Due to cross reactivity, an antibody against mouse IgG was used for total antibody detection instead of Protein A/G. Quantification of unknown mAb concentration of cell culture samples with ELISA showed bioactive anti-GFP concentration of 6.82 ± 0.27 µg/mL and a total antibody concentration of 6.92 ± 0.12 µg/mL and are comparable to SPR results.

4 | CONCLUDING REMARKS

Industrial mAb production requires monitoring of antibody quality and quantity. The established method using the SPR technology from Bruker Daltonics SPR (formerly Sierra Sensors GmbH) provides an alternative quantification system to simultaneously monitor bioactive and total mAb concentration in cell culture processes. With the reusability of the functionalized sensor surface using the identified regeneration procedure, measurements can be performed at various process steps. The technical properties of the SPR system like low analyte volume, shorter assay time, and label-free analysis in combination with an autosampler, which has been optimized during assay development, provides the basis for the technical development of a SPR-based system for continuous monitoring of mAb production. Future device development could incorporate automated interfacing of the analysis into production processes including automated sampling. Possible technical solutions include a method described by Chavane et al., 2008 [10], which uses a decantation column, filters, and pumps for sterile sample delivery from a bioreactor into a SPR device rack.

Although the presented methodology is not dependent on sample purification due to analyte specificity, samples need to be diluted due to relatively narrow dynamic analysis ranges and high mAb production titres. Nevertheless, the same drawbacks also apply for standard ELISA mAb quantifications. In general, reducing manual operations is key to achieve faster and more reproducible results in research, diagnostics, and industrial manufacturing. Therefore, automated processes are already integrated in modern devices and platforms for a variety of methods, e.g. liquid handling systems [33,34]. Appropriate automated technologies for sample dilutions have been developed for ELISA already [35] and can be adapted to SPR devices.

Due to the fact that there is no final technical solution as well as no routine application with a standard SPR device, an exact time and cost saving calculation is difficult. A definite advantage is the mass dependent detection of the analyte using SPR without the need of secondary detection methods. Therefore, compared to ELISA, the binding of the analyte can be directly measured within seconds to minutes depending on injection time and flow rate. Previous studies compared time of complete SPR and ELISA analysis and showed that SPR measurements can determine 100 samples in 16 h, with hands-on time of approximately 1 h and automated analysis overnight. In contrast to this, ELISA was performed in this study for IgA and IgGsc with a total time of analysis of 10 samples in 6 h each, with hands-on time of approximately 4 h. [36]. Although, automated microfluidic ELISA systems have been shown to decrease assay time to 30 min [37].

Concerning time, the process of establishing the bioactive surface is similar for both methods but the SPR technique

has the advantage of bioactive surface regeneration. Depending on the temperature within the SPR reaction chamber and the properties of the immobilized ligand, the functionalized sensor surface can be utilized for prolonged times. While applying the system, it is required that the stability of the bioactive surface is monitored. Therefore, SPR analysis of a few standard concentrations is conducted and compared with the calibration curve within a defined time frame or after a defined amount of binding cycles. If the error exceeds a specific threshold then there is the need to recalibrate by repeating the complete calibration series. In general, it is possible to use a functionalized sensor surface over a period of one month and over 1000 binding cycles can be conducted, respectively (unpublished data, Bruker Daltonics SPR). Other systems can compensate reasonable changes in surface activity using calibration trend functionality (Biacore T200) and have shown reusability of the same immobilized surface for approximately 250 binding cycles during a time period of 12 days [36].

In conclusion, it was shown here that the SPR results are similar to ELISA quantifications and with the aforementioned advantages SPR-based biosensing can be a competitive alternative to the conventional immunoassays for antibody production monitoring for industrial applications.

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CONFLICT Of INTEREST

The authors have declared no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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