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Review

Biopharmaceutical production: Applications of surface plasmon resonance biosensors☆

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ABSTRACT

Surface plasmon resonance (SPR) permits the quantitative analysis of therapeutic antibody concentrations and impurities including bacteria, Protein A, Protein G and small molecule ligands leached from chromatography media. The use of surface plasmon resonance has gained popularity within the biopharmaceutical industry due to the automated, label free, real time interaction that may be exploited when using this method. The application areas to assess protein interactions and develop analytical methods for biopharmaceutical downstream process development, quality control, and in-process monitoring are reviewed.

Contents

1. Introduction

The use of surface plasmon resonance (SPR) biosensor systems has rapidly gained popularity in the fields of quality control and biopharmaceutical production. Biacore® (GE Healthcare, Buckinghamshire, UK) is currently the major system provider for SPR biosensor systems and assays (based on this analytical platform) have been improved and optimised over the past 20 years [1–4]. A wide variety of methods to assess direct binding have been applied to examine protein–ligand interactions, investigate binding dynamics and rate kinetics (k_{on}, k_{off}), binding affinities (K_D) [5], antibody specificity and for the measurement of antibody and impurity concentrations to support in-process analysis or drug substance release. SPR data may provide an insight into the kinetics of binding and dissociation and can be used to quantify concentrations of analytes (antibodies, impurities) interacting with the bound molecules. SPR techniques coupled with miniaturized flow systems may be applied to identify target molecules that interact directly with a ligand immobilised onto a sensor surface [6] and

Abbreviations: EDC, *N*-ethyl-*N*-[(3-diethylamino)-propyl]carbodimide hydrochloride; ELISA, Enzyme Linked Immunosorbent Assay; Fab, antigen binding fragment; FIA, Fluoroimmunoassay; IgG, Immunoglobulin G; NGF, nerve growth factor; NHS, *N*-hydroxysuccinimide; QC, quality control; RIA, Radioimmunoassay; RU, response units; SPR, surface plasmon resonance.

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monitor the influences of other properties of the sample solution (pH, ionic strength) on binding and dissociation [7].

SPR assay systems have a number of advantages over alternative bioassay techniques such as ELISA, FIA or RIA which are traditionally used for biopharmaceutical process development and quality control (QC) [8–11]. As the SPR binding signal does not require reporter molecules such as enzymes/substrates, fluorochromes or radioisotopes, the interaction of a protein with the biosensor surface may be evaluated in real time, with the reactants typically retaining conformational integrity [12]. Following sample injection over the biosensor surface, continuous monitoring of information in the form of a binding sensorgram provides information on all steps in multi-step analysis (baseline signal, association, dissociation) and provides high throughput automated binding analysis of a large number of samples. SPR methods may also permit the analysis of low concentrations of product within a relatively crude sample [13–15]. There are however some potential disadvantages to the use of SPR systems. Although no labelling of molecules is required, the technique requires successful covalent immobilisation of sufficient amounts of ligand to the surface of the sensor chip. The chip surface with bound ligand must be robust enough to withstand several regeneration cycles to permit the injection and analysis of multiple samples. Unstable proteins bound to the chip surface may be denatured by subsequent regeneration steps, reducing the life time of the ligand surface. Also high sensitivities may be achieved using Biacore assays, but this may be at the sacrifice of assay robustness. Sufficient ligand density on the chip surface or a low molecular weight target protein binding to the immobilised antigen may also reduce the sensitivity of the SPR system (due to low chip surface densities) and impact the limit of detection, although many issues may be resolved using careful experimental design coupled with the use of instrumentation that shows improved sensitivity. The recent move of SPR instruments into the fragment screening arena has arisen as a direct result of these two factors [16–19]. The basic principles of SPR detection and its applications in the various stages of biopharmaceutical production and process development are described in more detail in the following sections.

2. Surface plasmon resonance (SPR) detection of protein interactions

A number of recent articles have detailed SPR methodology [20]. We will present an overview of the SPR technology which is deceptively simple: plasmons are the collective vibrations of an electron gas (or plasma) surrounding the atomic lattice sites of a metal; in the case of Biacore systems, this is a thin layer (50 nm) of gold coating each biosensor surface. Plane polarised light is shone onto the back of the biosensor chip creating an evanescent wave that excites electrons within the gold film. The resulting 'surface plasmons' (the fluctuation in the electron density at the interface of two materials of different refractive indices) result in a drop in the intensity of the reflected angle of light. When there is a change in mass at the surface of the biosensor, the angle of light shifts due to the increase in surface density. The SPR system exploits this technology to measure changes in mass on the sensor chip surface. These changes, due to bimolecular interactions are converted to optical signals. The resulting shift in surface plasmon angle is detected and measured and shown in real time as an SPR sensorgram (Fig. 1). When molecules bind to immobilised ligands attached to the surface of a biosensor, the refractive index at the chip surface alters the angle of polarised light; the change in angle caused by binding is directly proportional to the mass of bound material at the chip surface. The SPR sensorgram depicts the changes in SPR signal (directly proportional to changes in surface density) in real time, measured in response units (RUs). One response unit corresponds to a shift in the SPR angle by approximately 0.0001°.

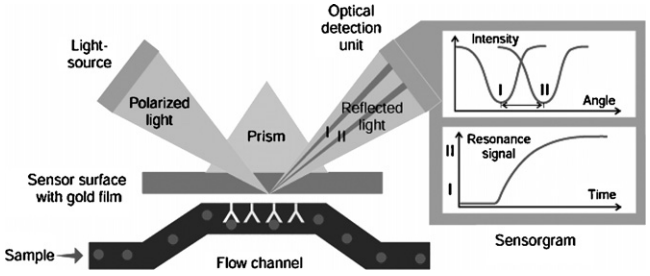

Fig. 1. The SPR detection system. Polarised light is focused and reflected at the interface of a gold coated glass slide. An increase in density at the chip surface (due to a binding event) results in a corresponding increase in refractive index, which alters the SPR angle and the change is displayed in real time as a resonance signal.

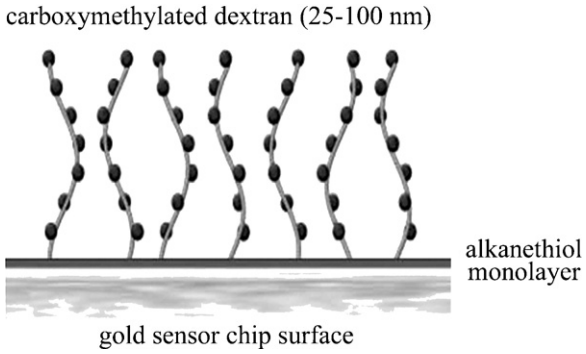

Fig. 2. A representative diagram of the surface of CM3, CM4 and CM5 (carboxymethyl dextran) biosensor chips (GE Healthcare). The dextran layer provides a hydrophilic environment favourable for the interaction with proteins and other biomolecules.

An SPR sensor chip is constructed by covering the gold surface with a layer of carboxymethylated dextran (Fig. 2) onto which a protein or ligand of interest is immobilised (Protein A, Protein G, IgG, polyclonal Fab) [21,22]. A microfluidic system injects the analyte solution containing the interacting molecule over the sensor surface. As the analyte binds to the immobilised ligand at the surface, the angle of incidence of light changes resulting in an increase in Resonance Units. As the binding of analyte to ligand reaches steady state, the SPR angle will cease to change and upon removal of analyte from the flowing buffer and under the appropriate elution conditions, the SPR angle will change as the analyte dissociates from the surface and return to its value prior to exposure of the surface to analyte (Fig. 3).

3. Ligand immobilisation chemistries

Biomolecular interaction using SPR begins with the immobilisation of a ligand to a chip surface. A variety of immobilisation

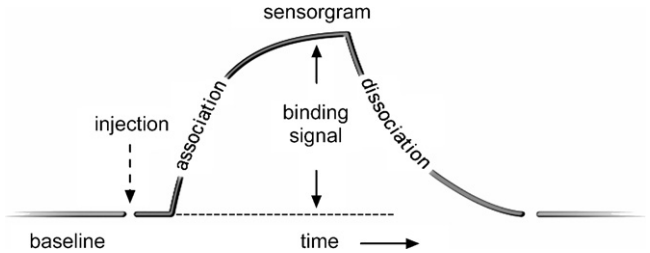

Fig. 3. The SPR sensorgram and sensorgram events. Initially buffer flows over the biosensor surface providing a baseline response. Following injection of analyte, an increase in SPR signal is observed proportional to complex formation and increasing density at the chip surface. After injection the bound analyte is dissociated from the surface using a regeneration solution.

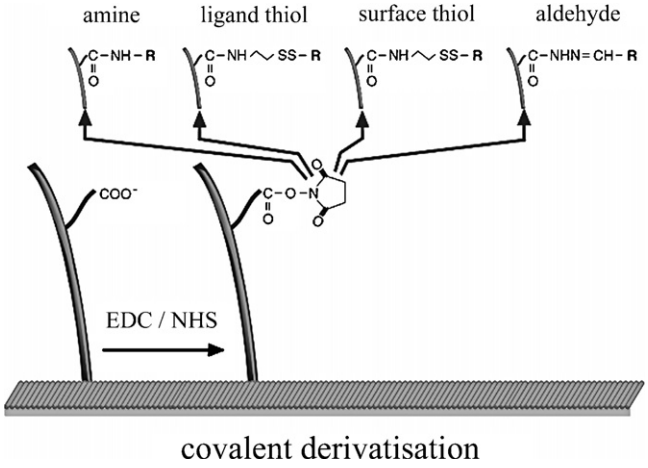

Fig. 4. Ligand immobilisation to a biosensor surface. Ligands may be coupled directly to the chip surface (covalent immobilisation), or via capture approaches that couple the ligand to the surface. Cross-linking has the advantage of stabilising the chip surface to prevent elution of the ligand with the bound analyte during regeneration steps.

chemistries are available to covalently couple ligands to a biosensor surface (Fig. 4). The most frequently reported and widespread technique used to bind proteins is amine coupling in which the protein is immobilised to a chip surface via free primary amine groups present in lysine residues or at the N terminus of the protein. Other coupling chemistries include thiol coupling, based on the exchange reaction between thiol and active disulphide groups, and less frequently used maleimide and aldehyde coupling. As reactive groups are often abundant within a protein sequence, the orientation of protein ligands directly coupled to a biosensor surface may affect subsequent ligand binding and represents a potential disadvantage of direct coupling methods. To overcome this limitation, indirect (capture) immobilisation techniques utilising ligands such as monoclonal antibodies may be used to create a homogenously oriented surface of bound ligand (Fig. 5). A limitation of this method however is that regeneration often removes the ligand from the captured surface, in which case fresh ligand needs to be re-captured prior to each binding cycle. Loss of surface ligand during regeneration may be overcome by adding a cross-linking step into the immobilisation procedure to create a permanently oriented surface where ligand stability becomes less of an issue [23,24]. The wide variety of coupling chemistries may be applied to immobilise non-protein ligands. Interactions involving small organic molecules, such as drug candidates or large molecular assemblies including whole viruses can also be evaluated using SPR techniques [25,26]. Methods for covalent ligand coupling are not covered in detail as part of this article but have been discussed previously [1].

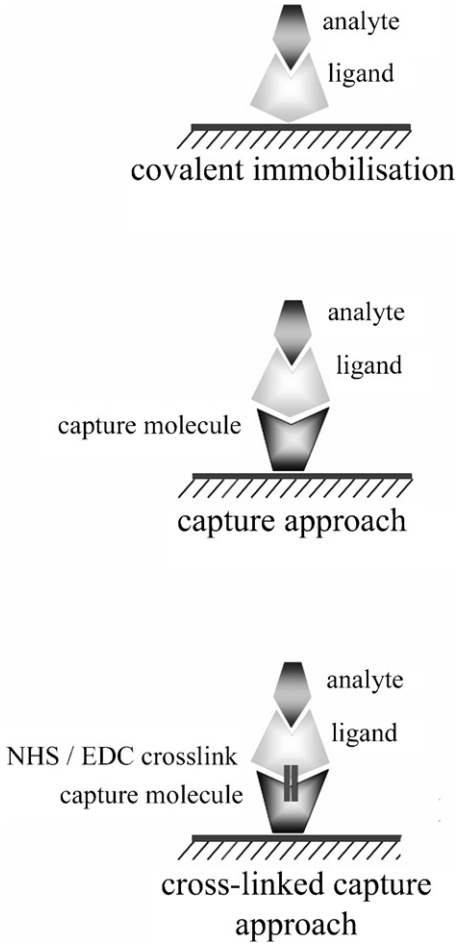

Fig. 5. Direct immobilisation techniques used to couple proteins to a biosensor surface. Chip surfaces may be activated using EDC/NHS to produce reactive succinimide esters. Proteins may be coupled using a variety of chemistries.

4. Probing binding kinetics and mechanisms

A number of biosensor platforms (including SPR techniques) have been used to evaluate the binding mechanisms and affinities of therapeutic monoclonal antibodies. Optical biosensors have been developed to measure binding affinities of antigen–antibody interactions in real time. The binding kinetics of human nerve growth factor (NGF) to a humanised NGF-neutralising monoclonal antibody (Tanezumab, Pfizer Inc., New York, USA), an antibody developed as a therapeutic for chronic pain has recently been reported [27]. Using a combination of multiple biosensor platforms, the *in vitro* biophysical characterisation of the interaction was evaluated. Although the analytical requirements for drug substance and drug product release are driven by current regulatory guidelines [28,29], SPR biosensors have the potential to provide a rapid and automated alternative to cell based assays that often demonstrate a high degree of variability and may be relatively complicated and difficult to validate.

The use of an immobilised antigen covalently coupled to an SPR biosensor surface to determine binding affinities of therapeutic antibodies does however create a number of technical challenges. The binding affinities of biotherapeutics are often matured over several orders of magnitude during product selection and screening, with binding affinities (K_D) after the initial stages of development in the single picomolar range [30]. As a result of the high affinity interactions, relatively harsh regeneration conditions (acid, base, salts) are typically required to remove the bound protein (from the immobilised ligand on the chip surface) and re-generate the biosensor for subsequent injections. As a result, degradation of the chip surface may occur. To overcome this limitation, software is available for a number of SPR systems (Biacore, GE Healthcare) that permits single cycle kinetic measurements. As an increasing concentration of analyte is injected over the chip surface (without chip surface regeneration), binding affinities are calculated by the level of equilibrium binding and the step increase in binding response [31,32]. Another potential issue may be the orientation of the chip surface chemistry may impact the apparent binding kinetics of a therapeutic protein, particularly when the multimeric antigens are bound to the chip surface. Although cross-linking cycles may be used to stabilise antigen subunits bound onto the biosensor surface, binding affinities may be reduced and additional immobilisation strategies or additional analytical methods may need to be con-

sidered to ensure the subunit cross-linking has not affected the interaction between the target protein and the bound ligand.

5. Quantification of biotherapeutics and minor impurities

A wide variety of qualified or validated analytical methods are utilised to evaluate concentrations and in-process product characteristics within process development and QC laboratories. SPR techniques can distinguish between conformationally active and inactive proteins and generally do not require highly purified samples, so the concentration analysis may be used for intermediate process steps during the manufacture of a biotherapeutic. Instrument automation also reduces operator involvement and as no labels or probes are required to measure the binding interaction, samples may be injected directly over the chip surface. As a result, the need for reagent preparation, stability and characterisation is reduced during assay qualification and validation. The use of SPR for the quantification of total IgG in hyper-immunised ovine serum for use within the biopharmaceutical industry for production of polyclonal Fab fragments has recently been described. Serum IgG concentrations were evaluated using recombinant *Streptococcus* protein G immobilised to a CM5 sensor chip [33]. SPR techniques have also been reported by co-authors to develop two assays, one to with the potential to measure the polyclonal anti-digoxin Fab fragments and the other assessed for the potential to evaluate eluted or leached ligand from the affinity column used to purify digoxin-specific antibody fragments.

A number of factors should be considered when using SPR techniques for in-process analysis of samples during the manufacture of a biotherapeutic. The sample matrix (pH, conductivity, buffer type) may impact the interaction of the ligand with the target protein and dilution or sample preparation may be required. The limit of quantification may also be dependent on the mass of the impurities of interest and this may also limit the amount of dilution or type of sample preparation used for the assay. Such limitations may often be easily resolved with careful assay design (increasing ligand density on the chip, reducing flow rates to extend injection times and adding enhancement steps to boost signal) but should be considered when using SPR techniques for biopharmaceutical analysis.

6. Purification development and ligand leakage from chromatography columns

At first glance, the application of micro-scaled-down SPR technologies for downstream bioprocess development may appear to be limited, as the physical characteristics of a ligand coupled biosensor surface are unlikely to be analogous to the ligand coupled surface of chromatography beads (such as Protein A Sepharose) used for the primary capture of a therapeutic antibody. Clearance of impurities such as host cell protein (HCP) and DNA [34] is unlikely be comparable to residual levels present within an antibody solution following the elution from an affinity column and the flow rates and ligand densities used for the SPR experiments are unlikely to be representative of those used for the purification of an antibody using a chromatography resin. Finally, chromatography bead specific effects (such as pore diffusion) may be difficult to mimic and the orientation of the ligand on the chip surface (due to the method of coupling used) may not be representative of the chromatography media used for the process scale separation.

However, ligand coupled biosensor surfaces (such as Protein A) may still be useful tools for bioprocess development. The binding of an antibody to a chip surface may be monitored in real time and the properties of buffer solutions used for product elution may be evaluated and optimised. Using this approach it is possible to rapidly screen solution characteristics (pH, conductivity, salt concentration) that permit complete dissociation of the antibody from the bound ligand. This technique may also be applied when attempting to identify the mildest elution conditions permitted or tolerated to minimise issues with antibody or ligand stability or solubility or when a pre-elution wash step is required to remove low affinity or non-specifically bound antibodies or impurities. Eluted antibody may be collected and product quality attributes (aggregate, acidic species) evaluated. Biosensor re-use studies may also be useful to evaluate the robustness of a potential affinity ligand. Preliminary SPR re-use studies prior to a resin re-use study may offer a number of advantages as the method may be automated and small amounts of material (antibody, ligand, buffers) are required and may provide an early indication of potential re-use and column lifetime prior to undertaking a resin re-use study at laboratory scale. As a range of coupling chemistries are available to permit capture and covalent coupling of ligands, SPR techniques may also be applied to optimise the coupling of affinity ligands to activated chromatography beads. Immobilisation chemistries, buffer properties and washing procedures may be rapidly evaluated using small sample volumes and evaluate potential conditions for ligand coupling to activated chromatography adsorbents.

7. At-line monitoring of biopharmaceutical manufacture using SPR

An emerging application for SPR technology is the at-line (real time) monitoring of protein concentrations and bioactivity levels during upstream processing. Real time monitoring of a bioreactor using at-line measurements is a feasible option with SPR technologies as lengthy preparation and analysis times associated with alternative techniques (ELISA, Western blot, bioassays) are not required. An automated at-line SPR detection system has recently been described to monitor secreted protein in a bioreactor culture of transiently infected embryonic kidney cells [35] and automated SPR methods have also been used to quantify levels of bioactive monoclonal antibody produced using a hybridoma cell line. The at-line SPR method described applied a pump that slowly and continuously delivered filtered culture medium to a biosensor tube rack; at defined intervals an automated SPR system injected samples over the biosensor surface [36]. With the development of process analytical technologies (PAT) within the biopharmaceutical industry, bioprocess monitoring using SPR methods may permit the development of new methods for the automated, real time monitoring of critical quality and performance attributes of a biotherapeutic throughout a bioreactor process. The analysis could be used to accelerate cell line screening and optimise cell culture parameters and could be extended to monitor multiple bioreactors run in parallel by utilising the four biosensor surfaces on a typical biosensor chip [35].

8. Conclusion

Surface plasmon resonance is a rapid, label free detection system used to monitor the interaction of a protein of interest with a target ligand. Although SPR systems are typically used within analytical groups for the quantitative measurement of antibody and impurity concentrations, SPR techniques may be valuable tools to aid biopharmaceutical development. SPR systems may be applied to monitor binding mechanisms and affinities of therapeutic monoclonal antibodies, assess levels of ligand leakage from chromatography columns, quantify product and minor impurity levels and permit real time monitoring during upstream processing within a bioreactor. Within this review only a small number of application areas have been discussed. The development of new SPR platforms and technologies will no doubt permit new application

of automated, label free interaction analysis for biopharmaceutical development and manufacture.

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