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Characterization and quantification of therapeutic ² monoclonal antibodies, anti-drug antibodies and their interactions for clinical applications

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Abstract: Therapeutic monoclonal antibodies (mAbs) are experiencing tremendous developments in terms of novel approved products and applications fields. Nonetheless, after administration, mAbs are submitted to a significant number of biological phenomena that may affect their quantity, structure and potency. Moreover, as mAbs are not exempt from adverse effects they can trigger immune responses leading to the expression of anti-drug antibodies (ADAs), potentially impacting their efficacy. This critical review details the different analytical techniques used for the characterization and quantification of mAbs and ADAs in different contexts from routine therapeutic drug monitoring to advanced clinical investigations. In addition, state-of-art approaches providing information regarding mAbs/antigen or mAbs/ADAs interaction such as binding affinities are described through impactful applications. Different analytical approaches are discussed including binding assays like ELISA and chemiluminescent immunoassay. Similarly, molecular interaction analyses are detailed like surface plasmon resonance and mass photometry. Finally, cutting-edge mass spectrometry-based analytical methods are presented with prior liquid chromatography and capillary electrophoresis separation, including the sample purification strategies required for the analysis of biological samples. Thus, the critical review discusses the technical requirements, advantages and limitations of the different analytical techniques in order to position them depending on the context and information required.

Abbreviations list:

ABT	Antigen Binding Test
ADA	Antibody drug-antibody
AE	Acridinium Ester
ALP	Alkaline Phosphatase
CDR	Complementary-Determining Region
CE	Capillary Electrophoresis
CID	Collision-Induced Dissociation
cIEF	Capillary Isoelectric Focusing
CL	Chemiluminescence
CLIA	Chemiluminescent Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
ESI	Electrospray
ETD	Electron-Transfer Dissociation
F _{ab}	Fragment antigen-binding
FC	Flow Cytometry
F _c	Fragment crystallisable
HRP	Horseradish Peroxidase
IFX	Infliximab
IP	Immunoaffinity purification
Ig	Immunoglobulins
K _D	Affinity Constant
K _{off} , K _d	Dissociation Constant
K _{on} , K _a	Association Constant
LBA	Ligand Binding Assay
LC	Liquid chromatography
LLOQ	Lowest Limit of Quantification
LOQ	Limit of Quantification
LOD	Lowest Limit of Detection
mAb	Monoclonal Antibody
mELISA	Microfluidic biosensor-based ELISA
MP	Mass Photometry
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
NADA	Neutralizing Antibody Drug-Antibody
nNADA	Non-neutralizing Antibody Drug-Antibody
PTM	Post-Translational Modification
RIA	Radioimmunoassay
SPR	Surface Plasmon Resonance
SRM	Selected Reaction Monitoring
SIL	Stable-Isotope Labeled
TDM	Therapy Drug Monitoring

1. Introduction

Monoclonal antibodies (mAbs) have demonstrated an undeniable success as therapeutic agents. In 2024, over 125 different mAbs are approved for therapeutic use, and more than 200 products are currently in clinical trials [1]. Thus, their constant development over the last few years has modified the paradigm in the pharmaceutical industry, enabling the emergence of the biopharmaceutical field as an important actor. The relevance of therapeutic mAbs can be explained by their high specificity for their corresponding antigen. Also, their pharmacological properties, like extended serum half-life, are particularly interesting for therapeutic applications [2]. The expansion of therapeutic mAbs was initially driven by oncology and the treatment of immune diseases [3]. However, their applications are constantly broadening with the treatment of more common conditions like asthma [4]. In addition, the introduction of other formats derived from mAbs such as bispecific antibodies, antibody-drug conjugates and fusion proteins, are demonstrating the interest of this type of therapeutic protein [5].

Due to their recent introduction as therapeutic agents and the increasing number of mAbs, insights regarding their evolution after administration remain relatively limited. Thus, after injection of an equivalent dose, some patients show a rapid elimination of the mAbs whereas in a minor fraction of the treated population, they may be detected in the serum several month after administration [6]. In addition, mAbs are not exempt from side effects which may take various forms. In particular, over time, a majority of treated patients are developing natural antibodies targeting the mAbs product, referred as anti-drug antibodies (ADA) [7]. As a consequence, mAb based therapies require monitoring during treatment in order to provide an improved understanding regarding of mAbs long-term effects and efficacy.

A significant number of analytical methods are focusing on the analysis of purified mAbs production, however, they do not allow the characterization of mAbs after administration [8]. The growing interest regarding the study of the evolution of therapeutic mAbs after their administration currently drives the development of innovative analytical methodologies able to tackle the complexity of the biological samples analysis containing mAbs.

This review aims to provide a critical overview of the different analytical techniques used for the quantification and characterization of therapeutic mAbs, their corresponding ADAs and measure their affinities in biological matrices. The first part is focusing on binding assays which represent the earlier approach for the analysis of mAbs in biological samples. In a second part, recent developments regarding molecular interaction analyses are described, especially concerning the possibility of using this type of techniques to obtain quantitative information and measure mAbs/ADAs interactions. Finally, the latest advances concerning mass spectrometry (MS) based analysis for the quantification and characterization of mAbs and ADAs are discussed. Thus, a particular emphasis on the potential and future developments expected concerning MS analysis is discussed for this type of clinical applications.

1.1 Monoclonal antibodies

Therapeutic mAbs are based on immunoglobulin G (IgG), which as such represent large biomolecules (molecular weight ~150 kDa). Because of their extended serum half-life, IgGs are the immunoglobulins type of choice for mAbs production [9]. Thus, mAbs are heterodimeric molecules composed of four polypeptides: two identical light chains (~25 kDa each) and two identical heavy chains (~50 kDa each) as emphasized in Figure 1. Each F_{ab} domain contains 3 complementary-determining regions (CDR) identified by a high degree of amino acid sequence variability and ensure mAb specificity for its antigen [10]. C_{H2} region incorporates a N-glycosylation site expressing a significant number of glycans whose structures can influence the effector functions of the antibody [11]. The different glycosylation forms of mAbs can affect their stability, immunogenicity and clearance rate [12,13]. The F_c part

interacts with F_c receptors found at the surface of cells such as immune cells involved in immune responses. For instance, it interacts with the neonatal F_c receptor which is responsible for the extended half-life of the mAb [14].

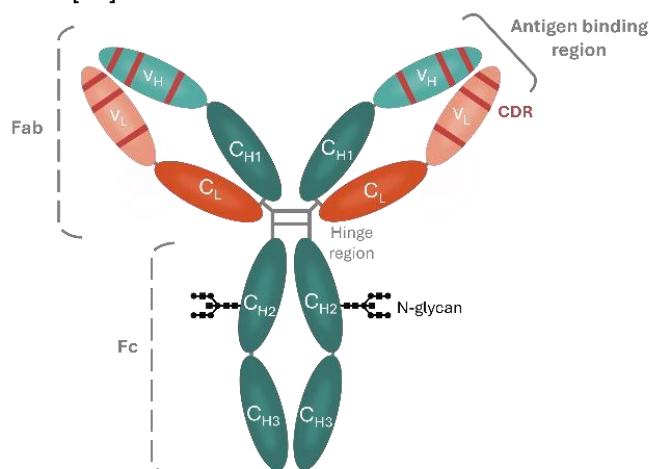


Figure 1. Structure of an immunoglobulin G (IgG). The different types of IgG differ in disulfide bond numbers and positions.

MABs are complex macromolecules susceptible to physical and chemical degradation such as aggregation, denaturation, or post-translational modifications (PTMs) [15,16]. Regarding PTMs, Asn deamidation, Asp isomerization, Met oxidation and C-terminal Lys clipping are some of the main types of modification. Their occurrence and modification level can have an impact on the stability and the potency of the mAb [17]. Also, the localization of PTMs is important as for example a change in the CDR region can have an influence on antigen binding affinity [18]. Therefore, PTMs including glycosylation represents critical quality attributes that are strongly regulated during the manufacturing and storage processes [19–21].

Therapeutic drug monitoring (TDM) defines the clinical follow-up of treated patients after administration of the drug. Currently, TDM relies essentially on the quantification of free mAbs circulating in the patient's serum, potentially to adjust the administration periodicity and dosage [22]. However, there is a crucial need to study chemical and structural changes of mAbs occurring after administration. Thus, it could provide information from a different perspective like the occurrence of major PTMs or compare the clearance of the different glycoforms.

1.2 Anti-drug Antibodies

The toxicity of mAbs is fairly limited, nevertheless, their administration may still trigger undesired side effects such as infection susceptibility, autoimmune diseases development and immunogenicity [23]. Immunogenicity defines the immune response after administration of a foreign substance, identified by the immune system as an exogenous agent. In the case of mAbs, the immune system may express anti-drug antibodies (ADAs), sometimes early in the course of the treatment [24]. With regard to the first generation of mAbs, major adjustments have been implemented to their structure, notably by limiting the incorporation of parts from non-human species in order to reduce their immunogenicity [25]. ADAs can be divided into two categories: non-neutralizing and neutralizing anti-drug antibodies (NADAs) as shown in Figure 2. NADAs bind to the F_{ab} domain of the mAb which inhibits its interaction with the antigen [25]. Non-neutralizing ADAs (nNADA) bind to the F_c part of the mAb. This interaction induces the formation of immune-complexes with the mAb, which are rapidly cleared out of the system [26]. ADAs expression could be linked to abnormal decrease of the concentration of the mAb, a loss of response to the treatment or lower remission rates [28,29]. Also, the presence of residual ADAs after treatment can generate a negative response in case of further administration of another mAb, by increasing the sensibility to immune response [30,31]. Some mAbs have been identified as more prone to induce adverse immune response such as adalimumab and brolicizumab [27,32].

Concerning TDM, essentially the presence and/or concentrations of ADAs are monitored in order to provide individualized treatments for each patient [33]. In the vast majority of mAbs treatment, ADAs

production have been identified, however the impact of ADAs on the outcome of all treatments, for example in the case of cancer immunotherapy or new mAbs is still unclear [32,34]. In the case of infliximab, TDM in complement with adjusted dosing, has demonstrated to be more cost-effective than to increase the dose of the treatment in case of reduced response to the treatment [35]. However, ADAs analysis is particularly challenging because of their isotypes diversity and amino acid sequence variability. Nevertheless, detailed characterization of mAbs/ADAs complexes could be included to improve understanding of the interactions at the core of immune response in terms stoichiometry, affinity and localization.

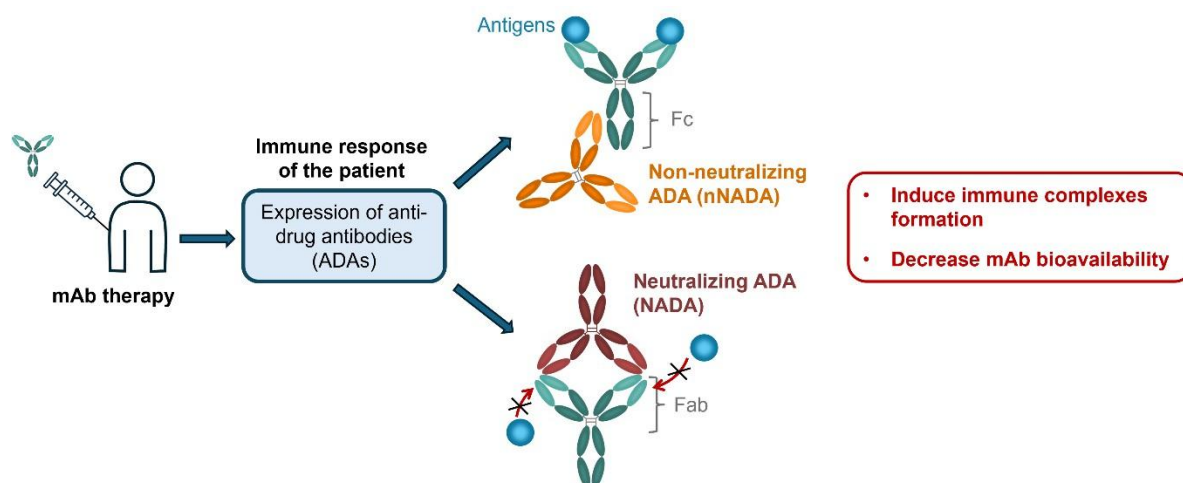


Figure 2. Schematic representation of the two types of ADA synthesized by the immune response from immunogenicity.

2. Binding Assay Analysis

2.1 Enzymatic-linked immunosorbent assay

Enzymatic-linked immunosorbent assay (ELISA) represents the standard technique for the quantification of proteins in biological samples due to its swiftness, sensitivity, throughput and requires relatively simple equipment. ELISA was developed for the quantification of infliximab demonstrating great precision and accuracy for concentration ranging from 0.10 to 8 $\mu\text{g}\cdot\text{mL}^{-1}$ corresponding to its therapeutic window [36]. Because of ELISA ease of use, relevant sensitivity and throughput for routine use, different kits were made commercially available for the quantification of the most common therapeutic mAbs, which include for example infliximab, adalimumab, rituximab, trastuzumab or ustekinumab [37]. However, commercial ELISA kits available represent only a small fraction compared to the extensive number of therapeutic mAbs approved.

ELISA can also be used for the quantification of ADAs, with bridging ELISA as the most common alternative [38,39]. The method was used for the detection of ADAs of infliximab, with the possibility to assign an immune response to a mAb concentration below 0.1 $\mu\text{g}\cdot\text{mL}^{-1}$ [36]. ELISA experiments often need to be developed in house for the detection of ADAs. Bridging ELISA experimental design allows to address analytical biases originating from the expression of various ADAs isotypes depending on treated individuals or species of origin [40]. Although, it is difficult to correctly quantify IgG4 isotypes as they are considered monovalent due to their F_{ab} arm exchange process [41,42]. Another challenge may be the presence of the mAbs drug in the sample as it can cause an underestimate of the ADA due to drug-ADA complex formation. The drug interferences can be minimized by acidic dissociation. For example, Du *et al* developed a two-steps acid dissociation/bridging ELISA method for the detection of ADAs directed against anti-LAG3 mAbs [43]. Also, acidic dissociation could be used in order to achieve the ELISA detection of ADAs targeting cabiralizumab in the presence of 200 $\mu\text{g}\cdot\text{mL}^{-1}$ of the

mAbs [44]. Those experiments showed the possibility to identify the presence of ADAs even for samples containing high concentration of the drug with improved sensitivity and robustness [45]. Recent instrumental developments concerning ELISA for mAbs and ADAs quantification have focused on improving analytical performances, especially for use as point-of-care analysis. Iria *et al* have developed a microfluidic biosensor-based on ELISA (mELISA) designed to quantify infliximab in the plasma of treated patients (Figure 3A). The mELISA required only 24 min to obtain the accurate and robust quantification of infliximab (Figure 3BC), representing a drastic reduction of analysis time compared to conventional ELISA [46].

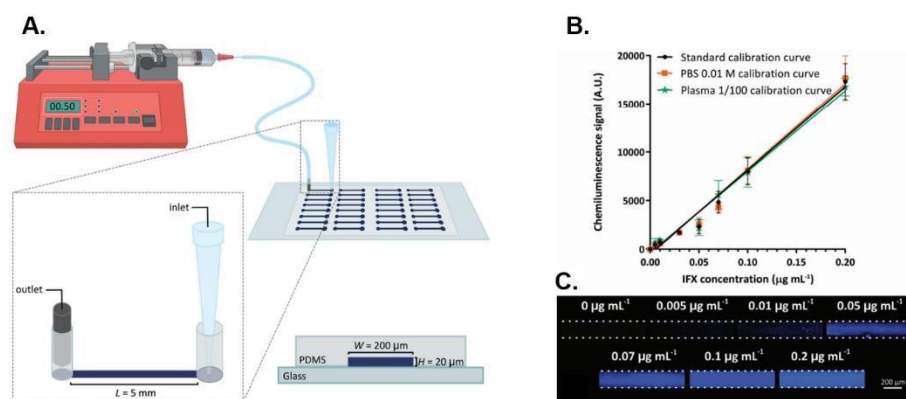


Figure 3. Schematic representation of A) instrumental setup used for the mELISA, where cross-section diagram of the microchannel are illustrated, B) standard calibration curves of IFX in mELISA and C) representative signal intensity for IFX calibration curves in phosphate buffered saline (PBS) in mELISA. Adapted from [46].

ELISA rapidly took the role of TDM reference technique for the quantification of mAbs and ADAs in biological samples, particularly due to the absence of sample preparation, the specificity of the incorporated ligand and the sensitivity of fluorescence detection. However, in some cases, its specificity may be compromised due to unspecific interactions between the ligand and the sample content, involving quantification biases to false positive in rare instances. Also, ELISA results showed significant variability indicating a need for standardization. Truffot *et al* compared two commercially available ELISA kits and liquid chromatography coupled to mass spectrometry (LC-MS) analysis for the quantification of rituximab and eculizumab. Results showed a negligible bias for ELISA quantification of rituximab, however the method demonstrated a significant bias of 69% in the case of eculizumab [47]. Similarly, several publications described a systematic underestimation of mAbs concentration using ELISA [48]. Due to the specific nature of the ligand implemented for the capture of the mAb, ELISA experimental settings cannot be directly transposed to the quantification of several types of mAbs. Complete method development is necessary when developing a new ELISA assay. As a consequence, currently ELISA cannot provide the quantification of mAbs and the corresponding ADAs using a single experiment. Instead, samples need to be submitted to two different ELISA in order to obtain information regarding the mAbs and the ADAs respectively. Finally, ELISA cannot provide any information regarding the structure of the mAbs and/or ADAs. This characteristic may be particularly limiting especially to investigate the evolution of mAbs post-administration.

2.2 Radioimmunoassay

Radioimmunoassay (RIA) is a binding assay with the characteristic of using labeled biomolecules with gamma-ray emitting isotopes for the detection of the analyte [49]. For instance, the determination of ADAs in a biological sample can be done by adding radiolabeled F(ab)₂ fragments of the mAb they are targeting. The non-bound F(ab)₂ fragments are then washed and the radioactive signal of the complexes formed is measured as shown in Figure 4 [50]. In other cases, the radiolabeled molecule that did not bound can also be measured and give a quantitative information on the complexes formed [51]. During the development of RIA assays, some characteristics of the radioligand (stability, lifetime) are important to study due to their influence on the experiment. Barta *et al* did an *in vitro* study on concentration, labelling efficiency and stability for ¹³¹I-cetuximab using time-resolved RIA. They

demonstrated the fast and efficient radioiodination of cetuximab using an incubation of 1 min, and the labelling showed to be stable up to 96h providing sufficient time to perform experiments [52]. They encourage the characterization of stability and the binding affinities of the labeled mAbs with real-time method before further use in RIA. ^{125}I and ^{131}I isotopes are relevant as radiolabeling agents because they combine high natural isotopic abundances and provide relevant sensitivity [53].

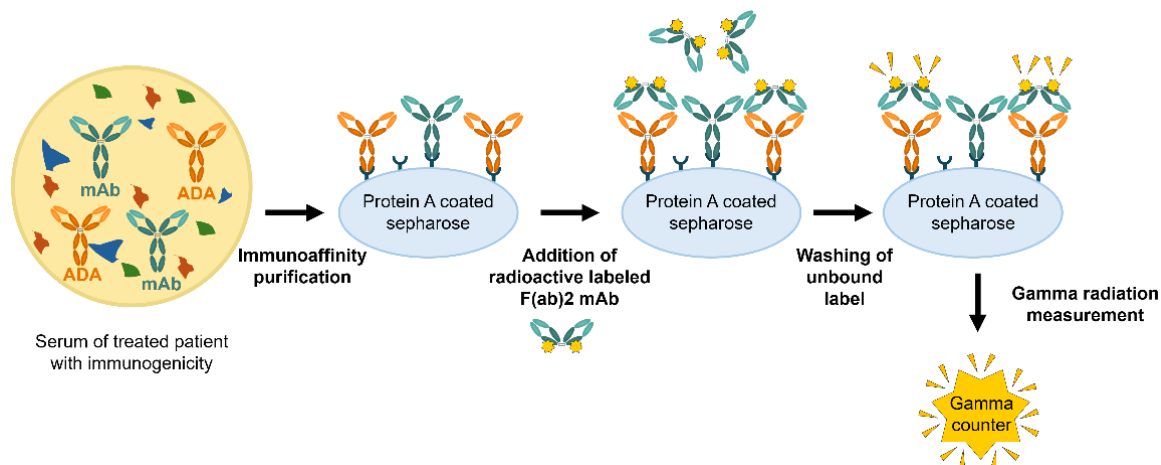


Figure 4. Schematic representation of the radioimmunoassay strategy commonly used for the quantification of ADA in human serum.

RIA could be successfully used for the quantification of edrecolomab from serum samples originating from patients treated for pancreatic cancer [54]. More recently, the implementation of RIA was described for the quantification of infliximab in serum [51]. Also, RIA could be successfully used to detect ADAs corresponding to adalimumab for rheumatoid arthritis samples from patients. Note, that the measurement of ADA and adalimumab levels proved to be an early predictor of the therapeutic response to the treatment [50].

Due to its experimental design, RIA allowed to reduce quantification biases compared to ELISA by limiting the impact of residual non-specific interaction. In addition, radioactive detection enables to achieve lower LOD whereas maintaining excellent specificity which is relevant for the analysis of mAbs and ADAs in biological samples [38]. Thus, for the detection of ADA, RIA sensitivity was also due an improved drug tolerance of the assay, associated with the co-presence of adalimumab [55]. However, safe handling of radioactive materials and the need for expensive equipment [56] represents important constraints that complexify its implementation compared to ELISA [52,55]. Still, the development of RIA allowed from a conceptual point of view, to pave the way in order to further improve the specificity and sensitivity of binding assays for the detection and quantification of mAbs and ADA.

2.3 Chemiluminescent immunoassay

Chemiluminescence (CL) is based on the detection of photons produced from a chemical reaction. This effect is used in chemiluminescent enzyme immunoassay (CLIA), where the species used for detection can generate light emission. For the analysis of mAbs and ADA, CLIA often relies on an antibody labeled with an enzyme. Typically, horseradish peroxidase (HRP) or alkaline phosphatase (ALP) is used with luminescent substrate like luminol for HRP or 1,2-dioxo-cyclohexane derivatives for APL [57]. The CL generated from HRP or APL reaction are usually not intense, thus various developments were realized to intensify the signal [58,59]. Darwish *et al* have developed an ultrasensitive CLIA for the quantification of durvalumab using HRP-luminol and hydrogen peroxide reaction in presence of 4-(1,2,4-triazol-1yl)phenol [60]. Using a similar approach, they used 4-(1-imidazolyl)phenol as a CL enhancer for the CLIA quantification of atezolizumab achieving high sensitivity and compatible with high throughput analysis [61].

Enzymes used to perform CLIA can also create variabilities in term of reproducibility, significant background signal and potential inhibition of the interaction between antigen and antibody [62]. Another type of chemiluminescent reaction for mAbs quantification uses acridinium ester (AE) labeled antibodies, to remove the use of enzymes [63]. These characteristics have led to the commercialization of AE labeling kits, that were used for the analysis of infliximab, adalimumab and the corresponding ADAs [64]. Consequently, the AE-label CL method could be further integrated in an easy automated system for high-throughput analysis [65]. Compared to conventional ELISA and RIA methods, CLIA has recently attracted much interest due to its better sensitivity, wider dynamic range and low background [61,63]. In clinical practice, CLIA has gained attention due to its rapid analysis and the increase of standardization practice enabled by the automated process. Another major advantage has also been the possibility to analyse in biological samples multiple drugs or a mAb and its ADA in a single analysis [65].

3. Molecular Interaction Analysis

3.1 Flow cytometry

Flow cytometry (FC) is a real-time analysis technique based on the measurement of light scattering events and fluorescence emission of cells or particles in solution passing in front of a focused laser. FC provides quantitative and qualitative analysis of mAbs. FC quantification of mAbs could be achieved for rituximab, providing accurate and sensitive quantification for the concentration range 5-250 $\mu\text{g}\cdot\text{mL}^{-1}$ [66]. Furthermore, FC could also be used to determine the affinity between adalimumab and its corresponding antigen TNF- α [67]. Affinity measurements using FC could be applied to compare different mAbs biosimilars, emphasizing potential differences in potency and functional differences [68]. Similarly, FC can be used to characterize the interaction of mAbs with effector function receptors, which can contribute to the optimization of mAbs design [69]. Also, recent advances further improved the ability of FC to evaluate nonspecific binding interactions between mAbs and non-antigen biomolecules in order to assess mAb polyspecificity [70].

FC could also be used to investigate mAbs immunogenicity in the form of ADAs expression. Thus, a FC method was developed for the simultaneous quantification of adalimumab and the corresponding ADAs in plasma samples. Using polystyrene beads functionalized with either TNF- α or adalimumab F(ab')₂, robust quantification could be demonstrated between 79-600 and 1-150 $\text{ng}\cdot\text{mL}^{-1}$ for adalimumab and ADAs respectively [71]. Thereby, FC could potentially provide improved sensitivity for the quantification of mAbs or ADAs compared to conventional bridging ELISA, with reduced interferences from the matrix. Nevertheless, FC applications remain limited due to the complexity of the method development, which in addition requires detailed assessment in order to ensure absence of biases and cannot be transposed to different mAbs and/or ADAs.

3.2 Surface plasmon resonance

Surface Plasmon Resonance (SPR) is an analytical technique used to monitor molecular interactions in real time [72]. Thus, the monitoring of binding provided by SPR enables the experimental measurement of kinetic constants, especially the association rate (k_{on} , k_{a}) and the dissociation rate (k_{off} , k_{d}) corresponding to the interaction. Also, SPR sensorgrams allow to determine equilibrium dissociation constants (K_{D}) that define affinity. As a consequence, SPR has gradually become an essential tool for the characterization of protein-protein interactions.

SPR could be successfully used for the characterization of a mAb-antigen interaction. Wang *et al* developed a SPR method to determine the kinetic and affinity characteristics for the interaction between CD20 receptor and respectively rituximab or obinutuzumab F_{ab}. Thus, they demonstrated

different CD-20 affinities of the two proteins with K_D measurements of 0.2 and 62 nM respectively [73]. Similarly, SPR could be implemented to measure the affinity between pembrolizumab and the targeted PD-1 receptor [74]. SPR provides crucial information regarding the affinity for the targeted antigen which showed to be suitable for the biosimilarity assessment in the case of biosimilar mAbs having distinct glycosylations profiles [75].

SPR could also be implemented for the detection and analysis of mAbs-ADAs interactions. For example, Real-Fernández *et al* developed a strategy to determine the affinity of anti-adalimumab antibodies present in the sera of treated patients using immobilized adalimumab [76]. Also, SPR could be used to measure the affinity of ADAs expressed in rats exposed to rituximab. The study also highlighted that ADAs expressed from different individuals exhibited variable affinities illustrating differences of immune response [77]. Similarly to binding assays, the presence of important quantities of mAbs in the sample may generate drug interferences when performing SPR analysis. Weeraratne *et al* showed the benefits of using a secondary confirmatory detector antibody to improve drug tolerance and signal sensitivity of the method. Results obtained for the fusion protein trebananib demonstrated a more accurate assessment of ADA pharmacokinetic with trebanabib than CLIA [78].

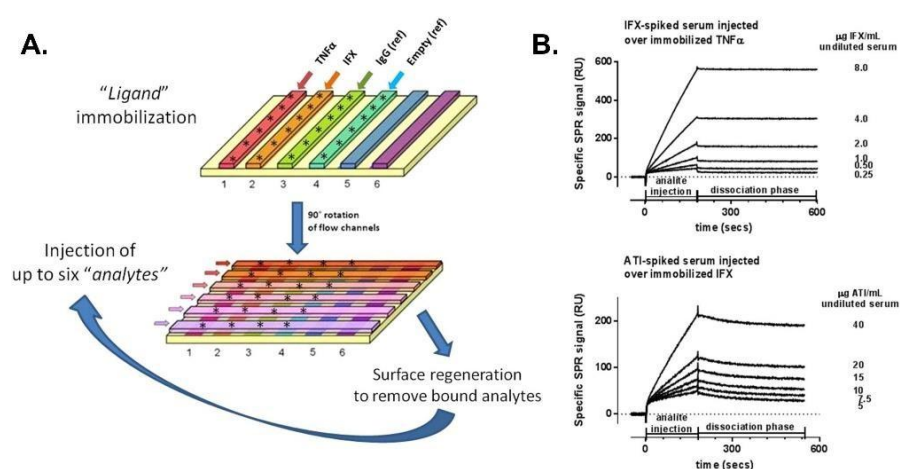


Figure 5. A) General scheme of the SPR-based assay for simultaneous determination of infliximab (IFX) and antibodies directed against IFX (ATI) concentrations in serum. The SPR apparatus has six flow channels which can immobilise up to six ligands on parallel strips of the same sensor surface. B) Representative sensorgrams obtained injecting simultaneously six solutions of IFX-spiked sera over immobilised TNF α , or six solutions of ATI-spiked sera over immobilised IFX. Adapted from [79].

Various developments were recently reported to enrich the applicability of SPR. Beeg *et al* developed a novel SPR instrumental technology to enable the simultaneous quantification of a mAb and ADAs in a single analysis. On a single sensor surface, several flow channels were used to immobilize different types of ligands on parallel stripes, and samples were introduced in parallel over all the immobilised ligands (Figure 5A). Therefore, multiple ligands and solutions could be measured in a single analysis (Figure 5B) [79]. Consequently, they identified significantly higher concentration of ADAs and less false negative when performing quantification using SPR compared to commercial ELISA assay. These differences were attributed to long incubation times during ELISA experiment and the presence of low affinity ADAs in sample originating from patients as opposed to high affinity IgGs used for ELISA calibration [80]. To prevent similar phenomenon in SPR, a calibration-free SPR method was also developed for the quantification of ADAs in serum samples [81].

SPR has revealed to be a particularly powerful technique for the characterization of protein-protein interaction, capable to provide detailed characterization of such subtle phenomenon. Also, the instrumental versatility of SPR progressed significantly with the possibility to immobilize different types of partners. Such characteristics are interesting for mAbs and ADAs that interact with multiple antigens and receptors. SPR analysis is not impacted by eventual matrix effects and the method does not involve sample labeling or pretreatment. Moreover, due to the low sample preparation required and real time monitoring of interactions, this method has proven to be capable of detecting ADAs with lower affinities compared to ELISA for serum samples originating from patients treated using panitumumab [82]. The possibility of using SPR to measure precisely the affinity between a mAb and

the corresponding ADA could be successfully correlated to the therapy outcome of patients [83]. As a consequence, SPR can provide information critically important for TDM, enabling an earlier discovery of ADA in order to provide more patient-specific therapeutic decisions.

3.4 Mass photometry

Mass photometry (MP) is an analytical technique recently introduced, capable to measure the mass of single biomolecules and complexes in solution. MP is based on an optical microscope system modified to measure the interference between light scattered by the solubilized analyte and light reflected from the surface. The analyte binds non-specifically to the cover glass surface of the microscope, resulting in a change in the refractive index at the interface between glass and water (Figure 6A). The visual signal undergoes image processing (Figure 6B) to obtain a measure of the interferometric contrast (Figure 6C), which can be correlated to the molecular weight of the analytes (Figure 6D). MP enables to estimate with a 2% accuracy the molecular mass of compounds presents at nanomolar concentrations using only 10 μ L of sample. Also, it does not require sample preparation and therefore represents a high-throughput technique as analysis time is commonly about 5 to 30 sec per sample [84]. MP could be used to analyse a variety of biomolecules such as viral vectors [85], nucleic acid [86] and membrane proteins [87].

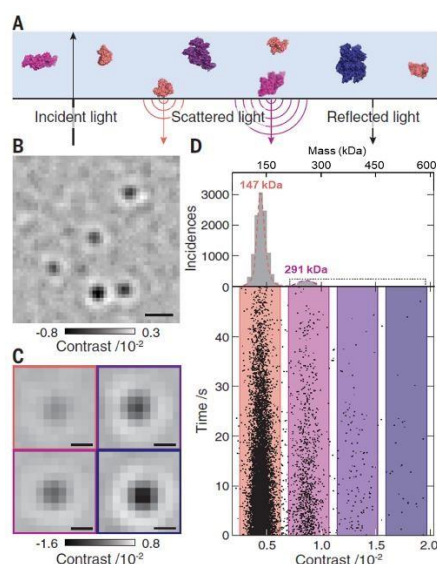


Figure 6. Principle of single-molecule counting by mass photometry. A) Label-free single-molecule detection by imaging the interference of scattered and reflected light arising from individual landing events at a glass-water interface over time. B) Differential interferometric scattering image of the binding events. C) Representative images of molecules with different masses (larger molecules have more intense contrasts). D) Scatter plot of single-molecule contrasts and resulting mass distribution (indicative mass values). Adapted from [84].

MP characteristics are particularly interesting for the study of antibodies because it allows direct observation of antibody binding events. Thus, MP could be successfully used to characterize the formation of complexes associating an anti-thrombin mAb to its target antigen or FCGR3A receptor respectively. Data allowed to determine unambiguously their different stoichiometries. Moreover, K_D dissociation constants could be calculated from measurement of free and bound analytes [88]. Another study highlighted differences of the k_{off} and k_{on} kinetic constants for the formation of complexes between glycosylated and deglycosylated trastuzumab with the Fc γ R1a receptor [89]. When compared to size exclusion chromatography-native MS and charge detection-MS for the analysis of protein-protein interactions, MP demonstrated the ability to overcome some limitations of native MS regarding the measurements of highly heterogeneous antibodyantigen co-occurring complexes [90]. Thus, MP could be implemented concomitantly with charge detectionMS for the characterization of the complex formed between SARS-CoV-2 proteins and IgGs. The study gave further information on the binding

behaviour of IgGs towards the SARS-CoV-2 and the kinetics of the different stoichiometry complexes that are formed [91].

MP was also used to characterize the interaction between mAbs and ADAs. Reinert *et al* used MP to characterize the interaction between infliximab and a corresponding ADA. MP data allowed to precisely determine the stoichiometries of the resulting complexes and demonstrated the specificity of infliximabADAs interaction (Figure 7A). Finally, study of the infliximab-ADAs complexes formation according to the molar equivalent of the two species could be achieved (Figure 7B) [92]. Regardless of its recent introduction, MP represents a quite promising technique for the characterization of mAbs and ADAs.

The study of biomolecular interactions between a mAb and its target is important, for example to determine their stoichiometries and their affinities. By following the formation of complexes in solution, MP provides highly accurate information on the binding affinity, heterogeneity and dynamics of protein-protein interactions involving therapeutic mAbs. The light-scattering method is a reliable technique for the insolution analysis of protein complexes and can fill the gaps of others characterization techniques. In particular, the possibility to characterize interactions in-solution maintains *in vivo* dynamics compared to other techniques like X-ray crystallography and cryogenic electron microscopy, implying static analytes for the analysis. Still, some limitations of MP have also been highlighted such as the low mass resolving power. Moreover, MP has not been yet used for the analysis of protein complexation in biological matrices. Nevertheless, further technological and methodological improvements concerning MP should be expected in the near future in order to place it as an essential tool to understand mAbs complex interactions.

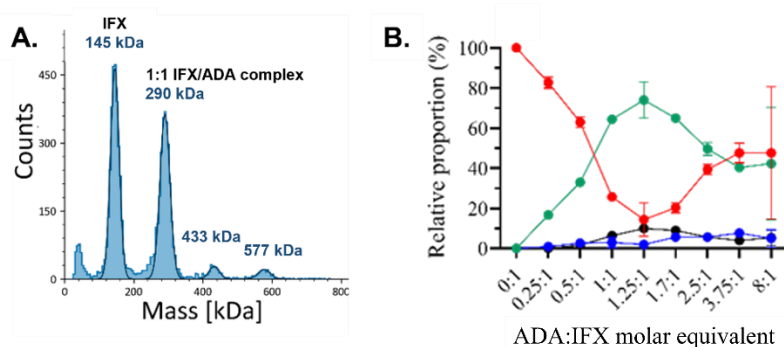


Figure 7. A) MP analysis of the complex formation of infliximab (IFX) and ADA in PBS. B) Evolution of species relative proportion for different IFX:ADA molar equivalents using MP in PBS. Red dots : Free mAbs (signal at 145kDa), green dots: 1:1 stoichiometry (signal at 290kDa), blue dots: 1:2 or 2:1 stoichiometry (signal at 433kDa) and black dots: 2:2 stoichiometry (signal at 577kDa). Adapted from [92].

4. Mass Spectrometry Based Analysis

4.1 Sample preparation

The analysis of therapeutic mAbs in biological samples also integrates an additional complexity. Indeed, it represents the analysis of a therapeutic IgG, present at nanomolar range concentration, in an extremely complex matrix containing up to 120 μ M of natural IgGs in the case of serum, and several thousands of different molecules and biomolecules [93]. Therefore, sample preparation becomes a pivotal element in order to achieve a robust detection and accurate quantification of mAbs and ADA. Sample preparation should take into account the complexity of the biological sample, and the characteristics of the subsequent analysis regarding injection volume and mass analyzer instrumental sensitivity for instance. Usually, an initial step is incorporated to reduce the sample complexity using depletion or isolate therapeutic mAbs from the matrix (Figure 8).

Sample depletion represents the removal of highly abundant proteins composing the sample. Different protocols are available to perform albumin removal of biological samples, they often rely on albumin immune-affinity using short column formats [94,95]. Protein precipitation can also be used to reduce

the complexity of the sample. Indeed, it represents a cost-effective way to remove a large variety of small organic compounds and other contaminants potentially present in serum or plasma. Addition of organic solvent causes a brutal reduction of the dielectric constant of the sample, disrupting the hydrophobic interactions of proteins leading to their precipitation [96]. Nguyen *et al* used only organic solvent protein precipitation for sample preparation in order to perform the absolute quantification of remdomab-B1 in serum and plasma samples compatible with pharmacokinetic studies [97]. Protein precipitation is particularly simple to perform but often does not provide specificity. Therefore, various developments were made in order to induce partial specificity toward the precipitation of mAbs, in particular using different types of anionic polyelectrolytes [98]. The implementation of polyanetholesulfonic acid as a precipitation agent allowed to achieve highly effective extraction in model biological matrices [99]. More recently, Schäfer *et al* described the use of ammonium sulphate for the precipitation of different types of mAbs. They could be consequently quantified using a bottom-up approach by analytical format LCMS/MS using a triple quadrupole instrumentation [100].

Sample preparation can also be designed to perform the extraction of therapeutic mAbs from biological matrices, usually with minimized steps while providing medium to high specificity. Immunoaffinity purification (IP) is particularly adapted for the isolation of therapeutic mAbs from biological samples. To perform IP, ligands interacting with the protein of interest are immobilized on a static solid media. Different reactions can be implemented to allow the immobilization of ligands on the surface of adequately prepared media like porous resins, agarose or ferromagnetic beads [101]. Different proteins, interacting with the F_c region are commonly used to perform the extraction of IgGs from biological samples. Protein A immobilized on sepharose resin was used to perform IP of trastuzumab and bevacizumab from human plasma followed by nanoflow LC-MS/MS for their bottom-up quantitative analysis [102]. Similarly, protein G immobilized on ferromagnetic beads was used for the IP extraction of different therapeutic mAbs from serum samples followed by LC-MS/MS analysis using a quantitative bottom-up strategy [103,104]. Protein A and protein G represent versatile partners for the IP of different types of therapeutic mAbs [105]. They provide efficient purification from abundant proteins. However, after purification, samples will still contain a large number of natural IgGs co-extracted from the matrix. Their presence at high concentrations could potentially result in the saturation of the IP media, preventing the optimal extraction of the mAbs of interest, and generate interferences during MS analysis.

To address limitations of protein A/G IP-based extraction, ligands specifically interacting with the therapeutic mAb of interest can be incorporated to the surface of the IP media. Purified antigens are used which allows to drastically improve extraction specificity and prevents unforeseen saturation of the extraction media. Moreover, it reduces the probability of MS signal interferences originating from other proteins. Jourdil *et al* reported the immobilization of ligands in pipet tips for IP extraction step to immobilize TNF- α in order to obtain the purification of infliximab from plasma [106]. Similarly, streptavidin-coated 96 wells plates was used to immobilize biotinylated TNF- α for the IP extraction of infliximab followed by LC-MS/MS absolute quantification [107]. Also, Reinert *et al* described the immobilization of TNF- α on the surface of streptavidin-coated magnetic beads to perform the extraction of infliximab and adalimumab in human serum. They demonstrated infliximab extraction yield systematically superior to 95% using indirect ELISA quantification [108]. The usage of immobilized antigen IP delivered outstanding recovery yields even for complex biological samples. The implementation of antigen-based IP has permitted the quantification of mAbs in biological samples using analytical format instrumentation readily available and MS instrumentation incorporating different types of mass analyzers [100]. Other ligands were recently explored for the specific IP extraction of mAbs in biological samples. Thus, Sun *et al* developed the implementation of aptamers immobilized on streptavidin agarose beads for the purification of trastuzumab from serum samples [109].

The characterization and quantification of mAbs using MS is conventionally performed by the intermediate of peptide-centric analysis. As a consequence, proteolytic digestion is realised consequently to sample purification detailed previously. Similarly to quantitative proteomics analysis,

trypsin remains the preferred enzyme to perform the digestion [110]. Indeed, trypsin exhibits a good activity capable to achieve high digestion yield after a few hours of incubation. In addition, generating peptides systematically having C-terminal basic residues is favourable to protonation during the ESI ionization process which guarantees optimal MS signal sensitivity [111].

4.2 Liquid chromatography

Liquid chromatography hyphenated to mass spectrometry (LC-MS) demonstrated to be particularly adapted for sensitive, highly specific and robust analysis of biomolecules in complex matrices. Concerning mAbs analysis in biological samples, peptide centric quantification derived from bottom-up proteomic strategy is commonly used. Peptides obtained from proteolytic digestion can be conveniently separated using standard reverse phase columns to achieve their sequential elution to MS instrumentation (Figure 8). To ensure optimal specificity, quantification peptides are selected based on their amino acid sequence singularity screened on databases, and absence of potential PTMs [112]. Therefore, selected peptides are commonly located in the F_{ab} domain of mAbs [113]. LC-MS/MS analysis of peptide mixtures can be performed using multiple reaction monitoring (MRM) or selected reaction monitoring (SRM). In part due to the extensive sample preparation required, a stable-isotope labeled (SIL) mAb is used as an internal standard.

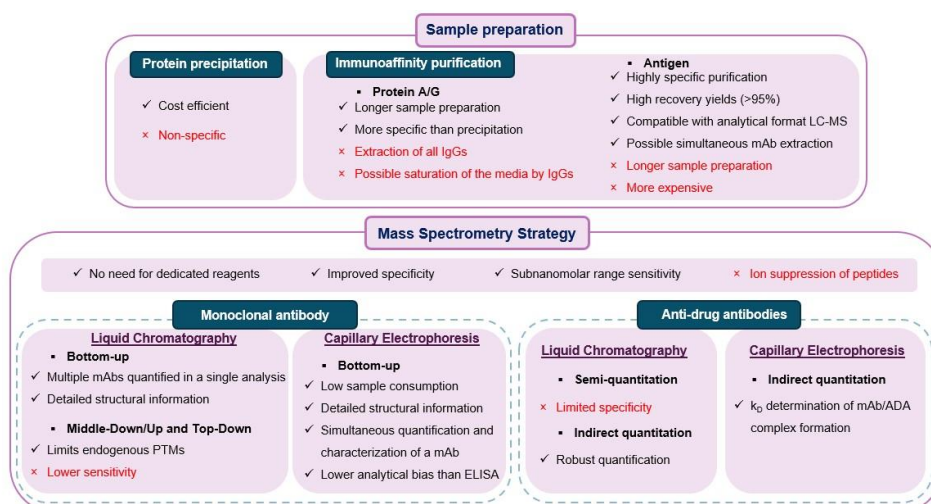


Figure 8. Overview diagram of the benefits and drawbacks of the mass spectrometry strategy for mAb and ADA analysis.

Quantification using LC-MS/MS analysis was developed for different types of mAbs present in various biological matrices including plasma [103], serum [114], tissue [115], cerebrospinal [116] and nasal lining fluid [117]. Ligand binding assays (LBA) like ELISA are typically dependent of specific anti-idiotype reagents that may not be always available for newer mAbs. Therefore, another advantage offered by LC-MS/MS analysis is the possibility to develop the quantification for a variety of mAbs using minimal method adaptation. For instance, El Amrani *et al* described a conventional methodology for the development of LC-MS/MS quantification of mAbs [118]. Thus, LC-MS/MS quantification could be achieved for eleven different mAbs using the same sample preparation [115]. The versatility provided by MS analysis is also a major benefit for the quantification of mAbs using concomitantly in a treatment. For example, trastuzumab and pertuzumab used in bi-therapies could be quantified in serum sample in a single analysis using LC-MS/MS [119]. Also, infliximab and adalimumab are both targeting TNF- α , therefore a common LC-MS/MS method could be developed for the quantification of the two mAbs in plasma samples. The method could be used without prior information regarding the type of mAb product present in the sample [120]. Quantification of infliximab and adalimumab in serum sample from Crohn's disease patients allowed to identify a cut-off concentration potentially associated with remission [121,122]. Using a similar approach, absolute quantification of mAbs REGN10933 and REGN10987 used for the treatment of SARS-CoV-2 could be demonstrated in serum samples using

LC-MS/MS [114]. LC-MS/MS analysis could be implemented for the simultaneous quantification of seven different mAbs used in the treatment of cancer in plasma sample. Results showed similar performance for the different mAbs quantification compared to ELISA reference methods [123]. Thereby, LC-MS/MS methodologies were recently reported to be able to achieve performances a minimum on par with ELISA concerning accuracy and sensitivity [124]. However, MS/MS data have provided additional specificity. Almost thirty different mAbs were successfully quantified using LC-MS/MS in biological matrices (Table 1), sometimes preceding the availability of the anti-idiotypic of the mAb necessary for LBA development [117]. LBA like ELISA present some limitations due to occasional interferences originating from the biological matrices or with ADA interfering with the quantification causing epitope binding [125–127].

Table 1: Mass spectrometry quantification strategy depending on the mAb or ADA quantified and the matrix.

Mab or ADA quantified	Sample volume of matrix	Purification method	Internal standard	Quantification method	LOQ/LLOQ	Reference
IFX	125 µL (human serum)	antibody precipitation (ammonium sulphate)	Horse IgG / SIL-peptides	2 peptides (SRM)	1.0 µg.mL ⁻¹	[128]
	5 µL (human serum or plasma)	filter plate	SIL-peptides	2 peptides (MRM)	0.72 µg.mL ⁻¹	[124]
Natalizumab	2 – 100 µL (human serum)	immunoaffinity (TNF-α coated plate or beads)	SIL-IFX	1 peptide (SRM or MS signal)	0.5 µg.mL ⁻¹ 0.22 µg.mL	[107] [129]
ADM	Human serum and CSF	-	Murine Ig	3 peptides (MRM)	1 µg.mL ⁻¹	[116]
Toripalimab	10 µL (human plasma)	non-antibody serum proteins binding support	SIL-ADM	Light chain (MS signal)	1 µg.mL ⁻¹	[130]
RTX	35 µL (human plasma)	protein precipitation (centrifugation)	SIL-peptide	1 peptide (MRM)	5.03 µg.mL ⁻¹	[131]
CTX	20 µL (human serum)	non-antibody serum protein binding support	VDZ	Light chain (MS signal)	9.7 µg.mL ⁻¹	[132]
TZM	50 µL (human plasma)	protein precipitation (methanol)	SIL-peptides	2 peptides (SRM)	1 µg.mL ⁻¹	[133]
TZM	50 µL (human serum)	protein precipitation (methanol)	-	1 peptide (MRM)	0.05 fmol.µL ⁻¹	[113]
ECZ	100 µL (human serum)	immunoaffinity (aptamer coated beads)	SIL-peptide	1 peptide (MRM)	0.5 µg.mL ⁻¹	[109]
USM	50 µL (human serum)	immunoaffinity (spin Column IgG4 affinity)	PBZ	Light chain (MS signal)	5 µg.mL ⁻¹	[134]
BVZ	35 µL (human serum)	cation exchange plate	Murine IgG	1 peptide (MRM)	0.4 µg.mL ⁻¹	[135]
DNX	2,25 µL (human plasma)	size Exclusion Spin Column	SIL-peptide	1 peptide (MRM)	1.8 µg.mL ⁻¹	[112]
Canakimumab	10 µL (human plasma)	antibody precipitation (ammonium sulphate)	SIL-peptide	1 peptide (MRM)	1 µg.mL ⁻¹	[136]
HDIT101 antibody	20 µL (human plasma)	immunoaffinity (protein G coated plate)	CTX or RTX	1 peptide (MRM)	0.1 µg.mL ⁻¹	[111]
TZM or BVZ	25 µL (human plasma)	immunoaffinity (protein A resin)	SIL-peptide	1 peptide (MRM)	20 µg.mL ⁻¹	[110]
TXG and CGV	10 µL TZM 5 µL BVZ	immunoaffinity (protein G coated beads)	TCZ	1 peptide (MRM)	2,5 µg.mL ⁻¹ TZM 10 µg.mL ⁻¹ BVZ	[104,137]
REGN10933 and REGN10987	20 µL (human serum or swab for NLF)	immunoaffinity (protein A/G coated beads)	SIL-peptides	1 peptide/mAb (MRM)	0.3 µg.mL ⁻¹ (serum) 5 ng.mL ⁻¹ (NLF)	[117]
ADM and IFX	5 µL (human serum)	-	SIL-peptides	1 peptide/mAb (SRM)	20 µg.mL ⁻¹ REGN10933 10 µg.mL ⁻¹ REGN10987	[114]
TZM and PTZ	<50 µL (human plasma)	immunoaffinity (TNF-α coated tips or plate)	SIL-IFX and SIL-ADM	1 peptide/mAb (MRM or SRM)	1 µg.mL ⁻¹	[120] [138]
RTX and ECZ	5-10 µL (human serum)	immunoaffinity (protein A coated beads or resin)	SIL-mAb or SIL-peptide	1 peptide/mAb (MRM)	0.25 µg.mL ⁻¹ TZM 0.3 µg.mL ⁻¹ PTZ	[119] [139]
BVZ, TZM, TCZ and NVM	20 µL (human plasma)	immunoaffinity (protein G coated plate)	SIL-ADM	1 peptide/mAb (MRM)	1 µg.mL ⁻¹ RTX 5 µg.mL ⁻¹ ECZ	[47]
IFX, RTX, CTX, DPL, DNX, VDZ and EMZ	DBS (human blood)	immunoaffinity (protein G coated beads)	PBZ	1 peptide/mAb (MRM)	5 µg.mL ⁻¹ BVZ - 10 µg.mL ⁻¹ TZM - 13 µg.mL ⁻¹ TCZ - 8 µg.mL ⁻¹ NVM (LOQ)	[103]
BVZ, CTX, PBZ, RTX, TZM, IPLC and NVM	10 µL (human serum or plasma)	antibody precipitation (ammonium sulphate)	SIL-peptides	1 peptide/mAb (SRM)	1 µg.mL ⁻¹ IFX DNX VDZ 2 µg.mL ⁻¹ CTX DPL 4 µg.mL ⁻¹ RTX EMZ	[140]
IFX, CTX, RTX, PTZ, ECZ, BVZ, DVL, NVM, VDZ, OCZ, USM	20 µL (human plasma)	immunoaffinity (affinity resin to IgG)	SIL of each mAb	1 peptide/mAb (MRM)	2 µg.mL ⁻¹	[123]
NADA against IFX	20 µL (human plasma)	immunoaffinity (protein G resin)	SIL-ADM	1 peptide/mAb (MRM)	1 µg.mL ⁻¹ RTX 5 µg.mL ⁻¹ ADM CTX IFX TZM TCZ SCK	[122]
ADA (IgG1-4, IgM, IgE, IgA1, IgA2)	50 µL (human serum)	protein precipitation (caprylic acid)	SIL-peptides	1 peptide/mAb (MRM)	12.8 µg.mL ⁻¹ PBZ - 20 µg.mL ⁻¹ ECZ - 4 µg.mL ⁻¹ IFX CTX RTX BVZ NVM VDZ OCZ USM DVL(LLOQs)	[115]
ADA against IFX	5 µL (human plasma)	immunoaffinity (TNF-α coated plate)	SIL-ADM	1 peptide (SRM)	3-96 µg.mL ⁻¹	[141]
ADA against IFX	95 µL (human plasma)	immunoaffinity (protein Z beads / mouse mAb directed against human IgG coated beads)	SIL-peptides	1 peptide/isotype (MRM)	0.5 µg.mL ⁻¹ IgG1 - 0.25 µg.mL ⁻¹ IgM - 0.1 µg.mL ⁻¹ IgE IgG4 IgA1 IgA2	[142]
ADA against IFX	5 µL (human plasma)	immunoaffinity (protein A coated beads)	SIL-IFX F(ab') ₂	1 peptide (SRM)	0.1 µg.mL ⁻¹	[143]

Abbreviations: ADM : Adalimumab ; BVZ : Bevacizumab ; CGV : Cilgavimab ; CSF : Cerebrospinal fluid ; CTX : Cetuximab ; DBS : Dried blood spot ; DNX : Dinutuximab ; DVL : Durvalumab ; DPL : Dupilumab ; ECZ : Eculizumab ; EMZ : Emericizumab ; IFX : Infliximab ; IPL : Ipilimumab ; NLF : Nasal Lining Fluid ; NVM : Nivolumab ; OCZ : Ocrelizumab ; PBZ : Pembrolizumab ; PTZ :

Pertuzumab ; RTX : Rituximab ; SCK : secukinumab ; TCZ : Tocilizumab ; TXG : Tixagevimab ; TZM : Trastuzumab ; USM : Ustekinumab ; VDZ : Vedolizumab.

On contrary, LC-MS/MS quantification was not subjected to significant matrix effects or the presence of ADAs, however the incorporation of an internal standard is mandatory to obtain a suitable quantification [144]. LC-MS/MS analysis also showed reliable quantification of IgG4, that can be problematic due to the occurrence of F_{ab} arm exchanges. Indeed, because the quantification is strictly based on specific peptides, their quantity remains equivalent even in case of arm exchanges [116]. In contrast, LBA can be significantly impacted by IgG4 F_{ab} arm exchange leading to important quantification biases [145]. MS based quantification of mAbs is linked to the availability of a protein specific peptide consequently to proteolytic digestion. However, ionization efficiency is dependent on the chemical nature of the analytes which significantly impacts signal intensity and sensitivity. Therefore, the performances of the LCMS/MS may be affected depending on the type of mAbs, even though the inherent sensitivity of MS instrumentation provides relevant quantification for treated patients.

One of the trends regarding recent analytical developments for the quantification of mAbs using LCMS(/MS) analysis is driven by using other methodologies as alternative to peptide centric experiments. Thus, a middle-up analysis could be developed for the quantification of rituximab using LC-MS analysis [132]. Using a middle-up approach, the μ LC-MS quantification of eculizumab could also be developed demonstrating similar performances [134]. The quantification of adalimumab was also developed using 2D-LC-MS analysis. Heavy chains and light chains separation was achieved by cation exchange followed by reverse phase chromatography [130]. The second dimension of separation was needed only to place the heavy and light chains in solvent conditions compatible with ESI ionization. In most cases only the signal corresponding to the light chain could be considered due to the inherent heterogeneity of heavy chains from N-glycosylations. The identification was usually based on MS measurements, therefore it required high resolution MS analysis in order to prevent signal misinterpretation and false positive. To further improve the confidence of the identification, middle-down analysis can be performed. Collision induced dissociation (CID) routinely used for peptide fragmentation cannot deliver sufficient energies to enable free chains fragmentation. Therefore electron-transfer dissociation (ETD) providing higher energies must be incorporated. For instance, adalimumab extracted using affinity purification was submitted to limited proteolysis to generate large fragments consequently analysed by nanoflow LCMS/MS using CID/ETD fragmentation on a FT-ICR instrument. Results showed confident identifications by means of systematic sub-1ppm mass accuracies and MS/MS sequence coverage up to 81%. Still, sensitivity remained limited to important concentration ranging from 29 to 2436 $\mu\text{g}\cdot\text{mL}^{-1}$ [146]. Limited sensitivities observed for the analysis of large fragments is explained by a lower ionization efficiency in the case of larger macromolecules, and the occurrence of ion suppression effect originating from the sample content.

The quantification of mAbs was also explored by the intermediate of intact proteins analysis using topdown strategy, in order to remove time-consuming proteolysis and limit endogenous PTMs [147]. However, at the moment lower sensitivities observed for intact proteins are still preventing the application of top-down MS analysis for the quantification of mAbs in biological samples. Nevertheless, significant improvements concerning sample preparation and MS instrumentation should be expected in the future to envisage top-down MS quantification in clinical applications. Especially, methodology compatible with native MS should be developed in order to further characterize mAbs in conditions close to its *in vivo* state. Because the problematic regarding biological sample complexity will still be present, it appears essential to develop sample purification enabling to maintain purified mAbs in their native conditions. Addressing this analytical challenge will provide the possibility to explore further the evolution of therapeutic mAbs after their administration for example.

Concerning ADAs which represents a trending aspect of LC-MS(/MS) development for clinical analysis, dedicated methodologies were designed in order to enable ADAs quantification using MS based analysis. Semi-quantitation of ADA was recently developed using affinity-based purification of ADAs followed by proteolytic digestion and LC-MS/MS analysis. ADAs quantification was achieved by the intermediate of peptides from the IgG constant domain as they are naturally produced IgGs. This

methodology was used for the quantification of ADAs in plasma, showing a minimal concentration of $0.5 \mu\text{g}\cdot\text{mL}^{-1}$ [142]. The specificity of this type of analytical strategy may be limited. As a consequence, recent developments described indirect quantitation of ADA based on the immunocapture of IgGs in sera by adding mAb F_{ab} fragment. The F_{ab} fragments involved in interactions are quantified in order to deduce the concentration of ADAs in plasma samples [143]. Another study described the indirect quantification of ADAs based on the proportion of mAbs neutralized after addition of a fixed concentration of infliximab. After equilibrium, the fraction of infliximab not forming complexes with ADAs, was extracted using immobilized TNF- α . Results showed the possibility to achieve a robust quantification of ADAs for concentration as low as $1 \mu\text{g}\cdot\text{mL}^{-1}$ and ADA quantification in agreement with ELISA [141].

Contrary to LBA and molecular interaction analysis, MS analysis is able to provide structural information regarding mAbs. Thus, LC-MS/MS was extensively used for the characterization of major PTMs occurring on mAbs, either using bottom-up [148] or middle-up approaches [149] as part of *in vitro* stability studies. Furthermore, such feature was used to determine the level of a single Asn deamidation after administration of Anti-CRTh2 mAbs. LC-MS/MS analysis of the peptide mixture allowed to perform the relative quantification of deamidation considering the affected peptide over a period of 12 weeks [150]. Using a similar methodology, precise *in vivo* monitoring of three modifications affecting an asparagine located in the CDR domain in the case of trastuzumab could be performed. The specific modification of this residue characterized by LC-MS/MS could be correlated to a loss of trastuzumab recognition in ELISA assays, attributed to an altered affinity for the HER-2 antigen [151]. Lately, the LC-MS/MS analysis of trastuzumab *in vivo* led to the identification of amino acids prone to PTMs in regions crucial for mAbs binding, that could potentially impact its potency and affinity [152]. It is important to note that sample treatment commonly used for mAbs bottom-up analysis, may induce endogenous PTMs especially Asn deamidation. Hopefully, careful optimization of sample preparation can be realised in order to limit drastically undesired modifications of the protein. Moreover, the *in vivo* and *in vitro* comparison study clearly identified the occurrence of PTMs after administration of therapeutic mAbs [152]. Thus, the implementation of LC-MS/MS analysis is particularly interesting due to the possibility to perform highly specific and sensitive quantification. In addition, this technique can provide detailed characterization which could be used in the future to understand their impact on mAbs activity.

4.3 Capillary electrophoresis

Capillary electrophoresis (CE) enables a high-resolution separation of charged species in solution, particularly relevant for the analysis of proteins and peptides. Thus, various CE methods could be developed for the characterization of mAbs over the different levels defining their structure [153]. Due to a relevant combination of their characteristics, CE coupled to MS has emerged as a powerful technique for therapeutic mAbs products characterization [154]. Direct CE-MS hyphenation could also be used for the study of glycoforms and charge variants composing several therapeutic mAbs products [155]. CE-MS instrumentation is experiencing major technical improvements as well, like the development of 2D-CE-MS for the detailed identification of mAbs charge variants in their intact form [156].

Simultaneous quantification and structural characterization of infliximab in serum samples using CEMS/MS analysis could be successfully developed (Figure 8). The analytical strategy, based on a bottomup strategy, was composed of antigen-affinity purification and proteolytic digestion, followed by CZEESI-MS/MS analysis [129]. Compared to the corresponding ELISA assay, CE-MS/MS showed consistent quantification and significantly lower analytical biases (Figure 9A). CE-MS/MS data allowed to simultaneously identify different types of PTMs including N-glycosylations, Asp deamidation and Asp isomerization. Also, a novel MS signal normalization was incorporated to data treatment in order to address any eventual endogenous PTMs generated during sample preparation. Results allowed to monitor the evolution of the glycan distribution after administration, enabling to identify fast clearance

variants independently. Interestingly, the comparison of the concentration of active infliximab depending on the anteriority of the administration did not demonstrate any conclusive correlation (Figure 9B). Whereas the residue Asn⁵⁷, located in the CDR regions, exhibited gradual modification depending on time since administration potentially affecting its interaction with TNF- α (Figure 9C). Thus, they

demonstrated that clinical follow-up considering exclusively free mAbs concentration may not be sufficient to fully understand the phenomenon undergone by mAbs during their residence time. The performance of the CE-MS/MS analytical method clearly demonstrated the added value advanced MS analysis can bring to study complex biological phenomenon [129]. For CE-MS/MS analytical workflow, comprehensive assessment and optimization of the sample preparation was realised in order to provide

an optimal sample compatibility with both CE separation and ESI-MS/MS analysis [108]. Using a similar

analytical strategy, CE-MS/MS analysis could be used to perform the biosimilarity assessment between 722 different infliximab products concerning *in vivo* stability. In addition, infliximab PTMs characterization 723 was performed for serum samples from treated patients [157].

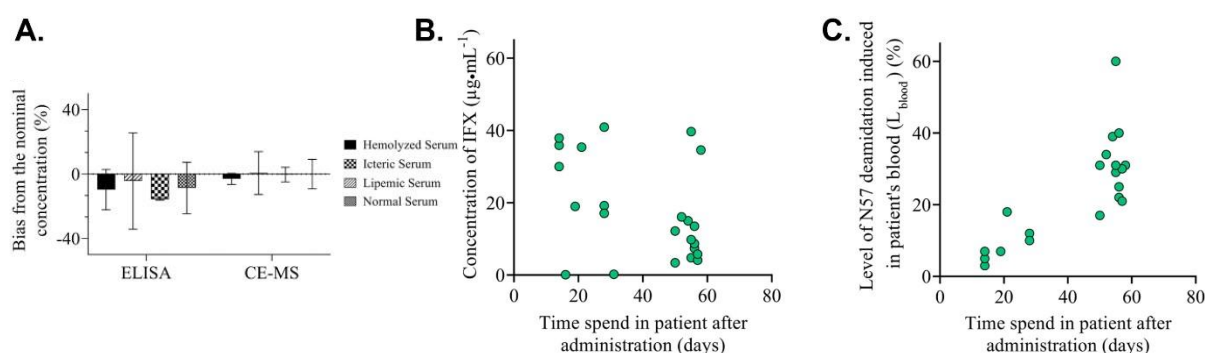


Figure 9. A) Infliximab quantification accuracy and repeatability for different types of spiked serums measured using ELISA assay and CE-MS/MS analysis, evolution of B) infliximab concentration in patient serums and C) deamidated level of N57 with anteriority of the administration. Adapted from [129].

CE-MS/MS was also recently described to investigate the interaction between infliximab and a corresponding NADA. In order to determine the quantity of NADA in a serum sample, fixed quantities of internal standards SIL-infliximab and SIL-adalimumab were introduced to the sample. Afterward, affinity purification was performed followed by subsequent proteolytic digestion. CE-MS/MS signal corresponding to the two mAbs was used in order to determine the proportion of neutralized SILinfluximab compared to unaffected SIL-adalimumab, and deduce the initial concentration of NADAs in the serum. Experiments allowed to determine the dissociation constant K_D of 14.4 nM between infliximab and NADAs [92]. In particular, this methodology designed to determine K_D value from CE-MS/MS data took into account the bivalence of both infliximab and NADAs due to their IgG nature. Infliximab-NADAs interaction was generated in serum samples to provide an environment similar to *in vivo* conditions. Another advantage of this methodology was the ability to investigate infliximab-NADAs interaction without any information regarding the amino acid sequence of neutralizing ADAs [92]. Considering the excellent characteristics of CE-MS/MS analysis, further developments could be envisaged in the near future to achieve the simultaneous quantification of therapeutic mAbs and corresponding NADAs in biological samples.

5. Challenges summary and future prospects

Considering the complexes phenomena undergone by mAbs, it appears crucial to investigate their outcome after administration in the context of treatment follow-up and/or clinical applications. Similarly, the so-far unpredictable expression of ADA and their implication urges the implementation of relevant approaches for their detection and characterization. Different analytical methodologies can be used

depending on the nature of the information required (e.g. quantification, structural modifications, interactions characterization) and the clinical context.

LBA, with ELISA as the most common alternative, provide mainly quantitative information of mAbs and ADA in biological samples with a cost-effective and rapid analysis with commonly available instrumentation. However, CLIA sensitivity, compared to ELISA, appeared particularly relevant for the early identification of adverse immune response in the form of ADA expression. Thus, automated instrumentations were recently developed, opening the wide scale use of CLIA for routine quantification of mAbs and ADAs. Even though LBA remains mainly for quantitative analysis, ELISA can be used to measure molecular interactions with complex experiments and dedicated assays. Therefore, SPR represents the method of choice to study mAbs interactions with other proteins like the targeted antigen, receptors and even ADAs. SPR is capable to measure in a precise manner kinetic and equilibrium binding constants to study the affinity of mAbs with different types of proteins in a versatile manner. However, various parameters need to be considered for the implementation of SPR which may be relatively complex especially for neophytes. The recent introduction of MP analysis represents an excellent opportunity for the characterization of mAbs molecular interactions. Compared to other techniques like SPR or FC, it represents an easy-to-handle technique that does not require protein immobilization for the analysis. Thus, MP has been used to study complex formation between mAbs and antigens or ADAs, while providing precise information regarding the stoichiometries in comparison to SPR. Due to the recent introduction of MP, further developments taking full profit of the potential of this technique are expected in the future to improve its applicability to study protein-protein interactions.

MS emerged over the last years as a pivotal tool to study the evolution of mAbs after administration. Indeed, in the context of quantification in biological samples, MS showed its ability to prevent signal interferences from other proteins that was observed in ELISA and to limit drastically the occurrence of matrix effects. In complement, MS analysis prior hyphenated to a chromatographic or electrophoretic separation demonstrated the possibility to obtain primary structure characterization like the identification of PTMs. Therefore, MS based methodologies may provide a novel perspective regarding the structural evolution of mAbs after administration. Nonetheless, the implementation of MS for the analysis of mAbs and ADAs in biological samples may be restricted at the moment to fundamental and investigational clinical applications due to the extensive sample treatment required. To further envisage MS analysis for the routine follow-up of patients, methodological developments should be performed in the future to reduce the complexity and time necessary for the analysis. Moreover, native MS represents one of the most advanced types of MS experiments, with demanding experimental conditions. Native MS in biological samples could help tremendously to improve the knowledge concerning protein-protein interactions involving therapeutic mAbs following their administration.

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