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# Monoclonal antibodies (mAbs) optical detection by coupling innovative imprinted biopolymers and magnetic beads: The case of therapeutic mAb anti-myostatin detection

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ABSTRACT

Immunotherapy has revolutionized modern medicine, becoming the largest part of the growing bio-drugs market. Along with the progress in this direction, the need for reliable monoclonal antibodies (mAbs) monitoring methods is a current foremost issue. In this scenario, the goal of this study was to design a straightforward beads-based plasmonic approach that combines magnetic beads (MBs) with a polynorepinephrine imprinted biopolymer (IBP) for real-time mAbs detection in biological matrices. Specifically, MBs-encoded by a specific antigen were exploited not only for the selective capture of the target mAb from human serum, but also MBs are directly involved in the molecular architecture of a sandwich assay. The mAb extraction from the real matrix occurred in a site-oriented manner by exploiting a paratope-epitope specific recognition, while leaving the constant mAb fragment (Fc) free to interact with an IBP specific for the mAb Fc portion. Anti-MYO-029, a recombinant monoclonal IgG1 antibody developed for myostatin inhibition and muscular-wasting disorders' treatment in clinical settings, was addressed as biological target to evaluate the analytical performances of the beads-based sensing assay. Myostatin-neutralizing antibodies may be misused as performance-enhancing drugs in sports competitions and their effective quantification has a central role also in the anti-doping control field. The optimization of the assay conditions led to establishing an assay able to achieve very good analytical performances in terms of repeatability and sensitivity, with negligible cross-reactivity with other Ig classes and IgG subclasses. In this frame, we proposed an extremely modulable assay, with potential large applicability, for multiplexing mAbs detection.

## 1. Introduction

Since the approval of the first murine monoclonal antibody (mAb, OKT3) used for the treatment of kidney transplant rejection, which occurred in 1985, approximately a hundred of monoclonal antibodies were authorized as new drugs by either FDA (Food and Drug Administration) and EMA (European Medicines Agency) [1,2]. Traditionally, therapeutic monoclonal antibodies (mAbs) are mainly produced in mammalian host cell culture systems, which are able to carry out a complex array of post-translational modifications (PTMs) necessary for the biological activity of many biopharmaceuticals. The therapeutic mAbs have revolutionized modern medicine, becoming the largest part of the growing bio-drugs market. Currently, the available mAbs have been applied to several therapeutic fields, including autoimmune diseases [3,4], asthma [5,6], cancer [3,7–8], bacterial [9–11] and viral infections [12]. In the last case, a few monoclonal antibodies were recently FDA-approved and/or -authorized for COVID-19 emergency treatment [13,14]. These mAbs belong to the IgG class, chiefly IgG1 isotype [15–17], and from a structural point of view they are constituted by variable fragment antigen-binding region (Fab) and constant fragment crystallizable region (Fc). The Fab maps the complementarity determining regions (CDR), responsible for the mAb selectivity, while the Fc mediates the cells response, including antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC), via binding interaction with Fc-effectors [18]. Concerning the latter item,

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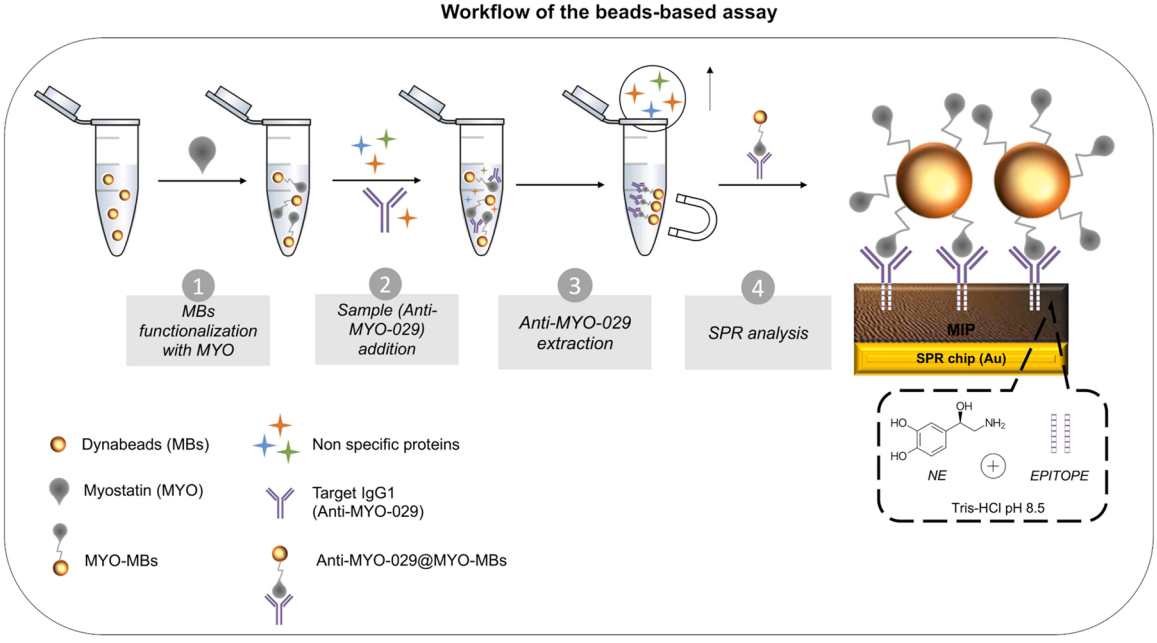
Fc-engineering strategies have been also developed to enhance the therapeutic value or to avoid interaction with immune system effectors, reducing possible adverse effects on patients [19–21]. Lately, nanobodies are under clinical investigation as new class of antigen-binding biodrugs, consisting of a single monomeric variable antibody domain, for a wide spectrum of human diseases (e.g., nanobodies targeting immune checkpoint molecules) [22,23], FDA-approved nanobody for thrombotic thrombocytopenic purpura treatment [22,24], by improving the target specificity and consequently reducing the unwanted immune response [25,26]. Thus, the detection of mAbs with increasingly sustainable, cheap, and easy-handling approaches, involving immune-free reagents, represents a real effective alternative to the commercially available immunoassays [27,28], in accordance also with the directive 2010/63/EU on the protection of animals used for scientific purposes [29]. mAbs quantitation is a key issue in different fields as therapeutics, anti-doping control, and large-scale industrial production. In this sense, antibody mimetics, chiefly aptamers [30–32] and imprinted polymers, represent a stimulating and promising solution for tracking the aforementioned mAbs [30–36]. Focusing on bioanalysis, here we aimed to design a modulable sensing plasmonic heterogenous bioassay (based on Surface Plasmon Resonance – SPR) for the real-time detection of IgG1-based drugs directly from human serum. Specifically, the continuous monitoring of the mAbs concentration in human serum is an essential part for both preclinical pharmacokinetic (PK) and toxicokinetic (TK) analysis, but also for the optimization of the therapeutic mAbs dosage regimens in patients. In this study, the planar gold surface of the Surface Plasmon Resonance (SPR) platform was modified with an Imprinted BioPolymer (IBP) constituted by a polynorepinephrine (PNE) film directed against the constant Fc portion of the mAb. PNE is a catecholamine-based biopolymer which is becoming increasingly appreciated in bioanalysis for diagnostic applications due to its outstanding features in terms of versatility, ease synthesis, and IBPs analytical performances [37–41]. Recently, we demonstrated the interesting behavior (in terms of affinity constants, stability, and reusability) of the PNE-based IBP in detecting very different analytes (e.g., gonadorelin, procalcitonin, Troponin I and human IgG) in biological matrices [37–41], which stimulate further exploration to emerging analytical problems with impact on pharmaceutical analysis as well as on novel therapeutic biological drugs used in immunotherapy for the treatment of important diseases. In detail, through the combination of beads-based magnetic solid phase extractors (dynabeads), "encoded" with specific antigens, the mAbs of interest can be selectively captured and pre-concentrated through the Fab region from the real matrix, and then directly assayed by the PNE-based SPR assay. To evaluate the feasibility of the beads-based plasmonic assay, a recombinant monoclonal IgG1 antibody, namely Stamulumab (Anti-MYO-029), was addressed as a case study. Anti-MYO-029 was initially developed for immunotherapeutic treatment of muscular-wasting disorders (e.g., cachexia, sarcopenia, and muscle dystrophy) [42]. Indeed, the specific antigen (MYO)-antibody (Anti-MYO-029) [43] reaction triggers the inhibition of myostatin activity/signaling by increasing the skeletal muscle mass. This biological effect could be improperly exploited by athletes in sport competitions thus, to prevent the suspected Anti-MYO-029 misuse, all myostatin-neutralizing antibodies (e.g., domogrozumab, landogrozumab and stamulumab) were already banned by World-Anti-doping Agency (WADA) and included among the prohibited substances in the S4 class of the relevant code [44]. To establish the analytical strategy, tosyl-activated magnetic bead (MBs) coated with myostatin has been combined with a PNE-based Fc imprinted optical sensor for the selectively one-step capturing and detection of the Anti-MYO-029 (Scheme 1) directly from human serum by optimizing several working parameters (e.g., imprinting technique, working temperature, and binding conditions). On this way, we proposed a versatile optical real-time beads-based assay, with potential large applicability, to assess also simultaneously (e.g., therapy based on co-administration of mAbs) the concentration of various mAbs, by using a unique imprinted Fc biopolymer-based system combined with a personalized 'on-beads' affinity tools able to suit to the specific molecular target investigated.

## 2. Material and methods

### 2.1. Chemical and instrumentation

L-norepinephrine hydrochloride (NE, ≧ 98.0%), tris(hydroxymethyl) aminomethane hydrochloride (Tris-HCl, ≧ 99.0%), 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), potassium chloride, sodium chloride, sodium phosphate monobasic monohydrate, sodium phosphate dibasic, boric acid, ammonium sulfate, hydrochloric acid, acetic




**Scheme 1.** Sketched outline of the Anti-MYO-029 extraction process with MYO-MBs. The resulting complex, Anti-MYO-029 @MYO-MBs, was directly injected onto the SPR platform to bind the PNE-based IBP modified sensor surface. The molecular architecture of the heterogeneous beads-based SPR assay is depicted.

acid (≧ 99.7%), sodium dodecyl sulfate (SDS), polyoxyethylene sorbitan monooleate (Tween-20), 11-mercaptoundecanoic acid (MUA), 6-mercapto-1-hexanol (MCH), and sterile-filtered human serum (from human male AB plasma) were all purchased from Merck (Darmstadt, Germany). Stamulumab Biosimilar (Anti-MYO-029) mAb of research grade (purity > 95%) was purchased from Proteogenix (Schiltigheim, France). The peptide used in the epitope imprinting approach, selected from the Fc region, with an HPLC purity above 95.0% (peptide sequence: $^{439}$KSLSLSPGK$^{447}$ (MW = 916.08 g mol$^{-1}$), was provided by GenScript (Leiden, Netherlands). GnRH1 monoclonal antibody (clone: LHRH13.327.8; Host/isotype: mouse/IgG1; Reactivity: human; acronym: Ab-1), monoclonal anti-Cardiac Troponin C antibody (clone: 7B9; Host/isotype: mouse/IgG1; Reactivity: human; acronym: Ab-2), Dynabeads® M-280 Tosyl-activated and human dimeric myostatin recombinant protein (Catalog # RP-8660) were purchased from Thermo Fisher Scientific (Milan, Italy). Monoclonal anti-Troponin C antibody (clone: 1F8-A9; Host/isotype: mouse/IgG1 kappa; Reactivity: human; acronym Ab-3) was acquired from Abnova (Taipei, Taiwan) while monoclonal Anti-BSA antibody (clone: 9E2-C2; Host/isotype: mouse/IgG1; Reactivity: human/bovine; acronym Ab-4) was obtained from GeneTex (California, USA). Anti-Troponin I Type monoclonal antibody (Clone: 4C2; Host/isotype: mouse/IgG2a; Reactivity: human) was obtained from Novus Biologicals (Italy). Human IgG2, IgG3, and IgG4 isotypes control (Host: mouse; Reactivity: human) were purchased from Bio-Techne (Milan, Italy). Ultrapure Milli-QTM water (R ≧ 18.2 MΩ • cm) was used to prepare all the buffer solutions. HBS-EP (composition: 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% Tween-20, pH 7.4) and PBS (composition: 140 mM NaCl, 2.68 mM KCl, 3.56 mM $NaH_2PO_4$, 6.44 mM $Na_2HPO_4$, pH 7.4), filtered through a microporous filter (0.22 μm Millipore filter), was used as dilution and running buffer for all SPR experiments. All chemicals used were of analytical grade. SPR measurements were all carried out by using Biacore X™ and Biacore X100 instrumentation, integrated with a temperature controller, working on gold sensor chips obtained from Cytiva Sweden AB (Uppsala). Data analysis was performed by using BIAEvaluation 3.1 Software and Origin 2022 Software (OriginLab).

### 2.2. Preparation of PNE-based receptors via bulk imprinting and SPR experimental setup

#### 2.2.1. IBPs synthesis

Polynorepinephrine (PNE) -based IBPs were directly synthesized on the gold SPR sensor chips, pre-cleaned with a piranha mixture (3:1 (v/v) $H_2SO_4/H_2O_2$), by dropping a polymerization solution (200 μL) containing the functional monomer norepinephrine (NE, 2 g L$^{-1}$ (9.72 mmol L$^{-1}$) in 10 mmol L$^{-1}$ Tris-HCl pH 8.5), in presence of the whole macromolecule mAb-1 (GnRH1 monoclonal antibody, IgG1 isotype), a model monoclonal antibody, used as template. Specifically, four IBPs were synthesized at 25.0 ± 0.5 °C in a thermostatic oven for 5 h [39–41], using different Ab-1 template concentrations (1.50, 8.00, 15.0, and 150 μg mL$^{-1}$). All the polymeric surfaces were modified with a water/ethanol solution (80:20, v/v) consisting of 1 mM MUA and MCH (Materials and Methods). The PNE-based films deposition was followed by a washing step with acetic acid (5% v/v), and deionized water to remove the template, leaving complementary binding cavities of polymeric matrices.

#### 2.2.2. SPR analysis

Real-time analysis of the analyte i.e., m-Ab-1 was achieved by Biacore X100 instrumentation using the IBP-based sensor chips. The selectivity of the PNE-based IBP towards the analyte was assessed by measuring the SPR responses against several IgG1 monoclonal antibodies (Ab-2, Ab-3 directed against TnC, while Ab-3 is an Anti-BSA, Materials and Methods) solutions at the same analyte concentration (20.0 μg mL$^{-1}$). The manual analysis was carried out by setting a contact time of 120 s (flow rate: 5 μL min$^{-1}$) and using HBS-EP pH 7.4 as ligands' dilution and running buffer. After each affinity binding interaction, the IBP surface was regenerated by injecting short pulses (24 s) of SDS 0.1% and 20 mmol L$^{-1}$ HCl.

### 2.3. mAbs (IgG1) recognition by epitope-based IBP

#### 2.3.1. Epitope-based IBPs synthesis

The PNE-based biomimetic receptors were synthesized according to the epitope imprinting approach [39–41] in which a small peptide sequence was involved as template/epitope in the whole procedure. Specifically, the epitope ($^{439}$KSLSLSPGK$^{447}$, acronym Fc $C_H$3) belongs to a well exposed portion located in the IgG constant domain of the heavy chain (UniProtKB Accession # P01857) and were selected according to structural considerations based on x-ray structure (well-exposed regions) and to antigen-based epitopes already available in the literature [41, 45–46]. The epitope imprinting approach was carried out according to the procedure described in the above paragraph, with the only difference that the macromolecule template (mAb-1) was replaced by a small peptide (400 μmol L$^{-1}$).

#### 2.3.2. SPR analysis

In this case, the study of the binding interactions between IBP and Ab-1 was performed at three different temperatures of 10, 25, 35 °C. The system calibration was carried with mAb-1 in a concentration range spanning from 0.63 to 10 μg mL$^{-1}$ (contact time: 120 s and flow rate: 5 μL min$^{-1}$). The IBP vs. Ab-1 binding was followed by a regeneration step performed as reported above. Additionally, all the SPR measurements were performed by setting the multi-cycle analysis (MCK) method available on the Biacore X-100 instrumentation, where all the SPR responses are acquired for a series of analyte concentrations and considered as a single experimental data set. This data set was subtracted of the mean blank value and processed with Origin 2022 software (OriginLab) using a linear regression fitting model (y = mx + b) to estimate all the analytical parameters. Once the working temperature has been optimized, the selectivity of the imprinted surface was studied in terms of ability to discriminate a set of targets monoclonal IgG1 antibodies (Ab-1, Ab-2, Ab-3, Ab-4) from the other subclasses IgG2/IgG3 and IgG4 (isotypes control). In this case, all the mAbs were tested at a fixed concentration of 2.5 μg mL$^{-1}$ in HBS-EP pH 7.4 (as dilution/running buffer). A control non-Imprinted biopolymer (NIBP) surface was produced by polymerizing PNE in absence of the template, following exactly the same experimental procedure of the IBPs, to evaluate potential non-specific biomolecular adsorption. The regeneration of the NIBP surface was achieved as for imprinted IBP, i.e., short injections (24 s) of SDS 0.1% and 20 mmol L$^{-1}$ HCl.

### 2.4. Epitope-IBP based sensing for Anti-MYO-029 detection: system calibration

Anti-MYO-029 standard solutions were prepared in PBS pH 7.4 within a concentration range of 1–8 μg mL$^{-1}$ and analyzed by setting an MCK method (120 s, flow rate: 5.00 μL min$^{-1}$, T = 25.0 ± 0.5 °C). After each measuring cycle, the IBP surface was regenerated with a single-shot injection (contact time: 24 s) of 0.5% SDS solution. Beads-based plasmonic assay on an imprinted surface for Anti-MYO-029 analysis in human serum. Human serum samples, spiked with Anti-MYO-029, were analyzed using a beads (MBs)-based extraction. MBs functionalization with myostatin: tosyl-activated magnetic beads were functionalized with a myostatin (MYO) coating to selectively extract the specific analyte (Anti-MYO-029) from human serum samples spiked with different concentrations (4 – 20 μg mL$^{-1}$). All the experimental procedures were performed according to a protocol adapted from manufacturer's instructions. Briefly, the MBs were properly washed in 0.1 M borate buffer pH 9.5 and collected using a magnetic rack, discarding the supernatant. Then, 100 μL of myostatin diluted in 20 mmol L$^{-1}$ HCl was added to the MBs (5 mg) suspension within 100 μL of 3 mol L$^{-1}$ ammonium sulfate

pH 7.4 and the tube containing the solution was incubated under shaking on a roller at 37 °C for 20 h. The MYO-MBs were sequentially washed and resuspended several times with PBS pH 7.4 containing 0.1% (w/v) and 0.5% (w/v) BSA, to prevent non-specific adsorption on the MBs' surface, before being used to isolate the target protein from five serum samples.

#### 2.4.1. Analyte extraction from human serum using MYO-MBs

The MYO-MBs were finally used with human serum samples spiked with different amounts of the Anti-MYO-029 mAb (4 – 20 μg $mL^{-1}$). The serum samples were thus added to the MYO-MBs suspension and incubated under shaking on a roller at room temperature for 30 min. Then, the Anti-MYO-029 @MYO-MBs complex was washed two more times (PBS pH 7.4 with 0.1% BSA) and resuspended in PBS pH 7.4.

#### 2.4.2. SPR analysis

The resulting Anti-MYO-029 @MYO-MBs complex was directly injected into the SPR system to study its binding interaction with the Fc $C_H3$ imprinted PNE-based film on the sensor chip surface. All the negative controls were performed by injecting the naked magnetic beads and the beads modified with the myostatin in a single step, both 1:10 diluted in PBS pH 7.4, to assess the presence of non-specific adsorption in assembling the molecular architecture of the final complex. After each injection, the biochip surface was regenerated with a single-shot injection (contact time: 24 s) of 0.5% SDS solution.

## 3. Results and discussion

The final goal of this study is to design a general 'two-steps' strategy to detect monoclonal antibodies, i.e., immunotherapeutics, in complex matrices such as human serum, using a polynorepinephrine (PNE) biomimetic receptor integrated into a high throughput plasmonic sensing platform. Recently, we studied the promising PNE analytical features of imprinted PNE as biomimetic receptors for the polyclonal IgG detection [41]. Here, the same IBP was exploited for the development of a novel beads-based assay directed to immunotherapy drugs' recognition (i.e., Stamulumab - Anti-MYO-029). Specifically, two PNE-based imprinting techniques were investigated. Firstly, the whole IgG1 mAb-1 was finger-(im)printed in the biopolymer matrix with the final idea of generating specific imprinted binding sites for a particular mAb, used as template, eventually driven by complementarity determining regions (CDR), the key portion to achieve the required mAb fingerprinting selectivity. Secondly, an epitope-directed approach, in which specific peptides belonging to the IgG1 macromolecule are selected as imprinting templates, was used to synthesize an IBP receptor. In this case, the effectiveness of the aforementioned technique to be applied to different catecholamines-based polymers (such as polydopamine, PDA, and polynorepinephrine, PNE) has been successfully demonstrated for a range of biomolecules (TnT, TnI, IgG and PD-L1) [37, 40–41,47].

### 3.1. Finger-(im)printing of mAb-1 into a PNE-based matrix

Firstly, the bulk imprinting technique was involved to synthesize the mimetic receptors for IgG1 (mAb-1), since therapeutic mAbs belong to the IgG class (mainly IgG1 isotype). Bulk imprinting, at first glance, appears as the simplest approach in which the whole target molecule (mAb-1) is imprinted in the polymeric network to generate a unique conformational fingerprint of the target monoclonal antibody, here the mAb-1 (IgG1). In this frame, four different PNE-based IBPs were synthesized keeping constant the concentration of the monomer, norepinephrine (NE), and tuning the template (mAb-1) concentration, therefore varying the density of the imprinted binding cavities distributed on the PNE polymeric surface. The optimization of the latter parameter is extremely important to avoid potential mAbs' crowding effects on the biopolymeric surface, leading to inaccessible binding sites. Specifically, four mAb-1 concentrations were used during the imprinting process, i.e., 1.50, 8.00, 15.0 and 150 μg $mL^{-1}$, theoretically increasing the polymer imprinted surface area. The resulting imprinted surfaces were thus tested in their ability to selectively bind the target/template analyte (Ab-1). The cross-reactivity of the fingerprint-imprinted polymers were evaluated by testing other three mAbs (Ab-2, Ab-3, Ab-4, details in Materials and Methods), all belonging to the same isotype of the target analyte mAb-1, at a fixed concentration, namely 20 μg $mL^{-1}$. According to the selectivity coefficients (k), expressed as $k = (\Delta RU)_{competitor}/(\Delta RU)_{target}$, (where the competitors are Ab-2, Ab-3 and Ab-4) reported in Fig. 1, the biomimetic receptors produced for mAb-1 detection via bulk imprinting seem very selective (Fig. 1a-c) for the analyte mAb-1 over Ab-2 and Ab-3, since low k values are associated to less interference by competitor molecules. Conversely, all the IBPs showed a significant cross-reactivity with Ab-4 (as shown by k values above 1, Fig. 1), probably due to a high similarity between Ab-1 and Ab-4 in terms of amino acid sequence (unknown), and/or conformational structure. Considering this issue, the PNE-based finger-(im)printing technique will be deeper explored by testing nanobodies, single-domain antibodies, which due to their smallest functional fragments could provide a potential high IBPs' selectivity. Thus, in this specific case, we left behind the idea of whole mAb finger-(im)printing for moving towards an epitope-mediated imprinting approach, according to a recent study [41].

### 3.2. Epitope-mediated PNE imprinting: temperature effect on the IgG1-IBP binding interaction

Since a cross-reactivity effect was observed by using the bulk imprinting approach (i.e., finger-(im)printing technique) here we

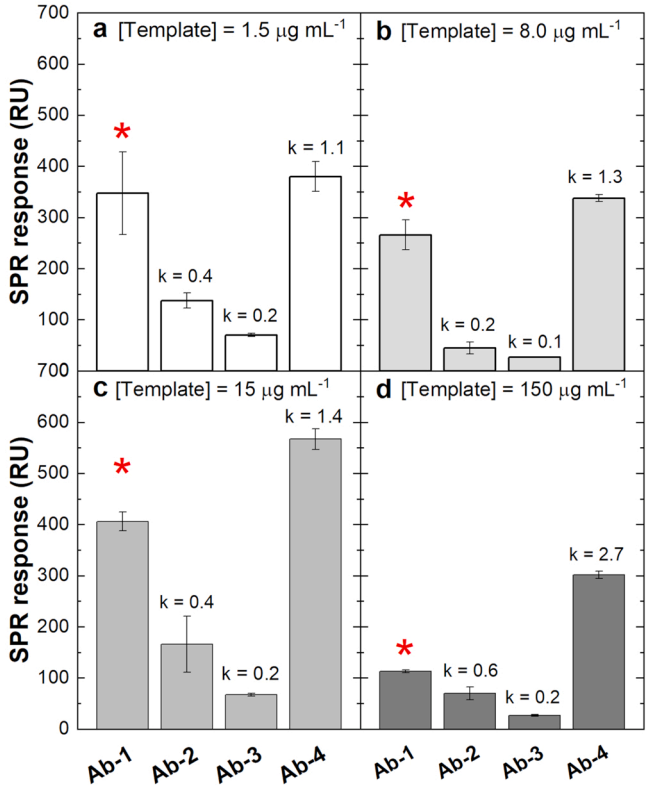



**Fig. 1.** Evaluation of the mAbs (Ab-1, Ab-2, Ab-3, Ab-4 - isotypes: IgG1, 20 μg $mL^{-1}$) cross-reactivity on PNE-based receptors imprinted with different increasing template (m-Ab1) concentrations, in a clockwise direction:1.50 μg $mL^{-1}$ (a), 8.00 μg $mL^{-1}$ (b), 15.0 μg $mL^{-1}$ (c) and 150 μg $mL^{-1}$ (d). The analyte Ab-1 mean response (n = 3) is marked with a red asterisk. The bar chart indicates $RU_{mean}$ ± SD recorded in triplicate for each IgG1 binding interaction.

moved towards an epitope-mediated strategy to prepare a selective mimetic receptor for the model mAb-1 (IgG1). The epitope approach is based on addressing the Fc region of the mAb according to our recent study [41]. Detailing, a nonapeptide ($^{439}$KSLSLSPGK$^{447}$, acronym Fc $C_H3$) belonging to the IgG heavy constant domain, whose sequence is shared by all the immunoglobulins (Ig) G isotypes, was selected as imprinting epitope for mAb-1 (IgG1) detection. In this case, the rational choice to imprint this short peptide, inspired by the good analytical IBP's performance observed in our previous study [41], is dictated by the impossibility of finding the variable primary sequence of all mAbs idiotypes. In this frame, we have firstly investigated the influence of the temperature on the binding interaction between mAb-1 vs. the IBP. Three different working temperatures (10, 25, and 35 °C), commonly exploited in bioassays, were set-up (using a temperature controller integrated into instrumentation) to calibrate mAb-1 in a concentration range spanning from 0.63 μg mL$^{-1}$ to 10 μg mL$^{-1}$. The imprinted surfaces were able to interact with the analyte at any working temperature. However, the temperature affects the specific recognition of the biopolymeric mimetic receptor, as expected (Table 1). In detail, at 25 °C significantly higher absolute mean SPR response was observed than the ones recorded at 10 °C and 35 °C, resulting vastly superior, also in terms of analytical performances, as highlighted by the larger slope (sensitivity), the smaller limit of detection (LOD), and repeatability (coefficient of variation $_{av}$CV%) (Table 1). This behavior can be tentatively ascribable to an increased conformational stability of both proteins (mAb-1) and the binding sites, occurring at 25 °C, which jointly benefit the affinity interaction. Considering these findings, 25 °C was selected as working temperature for further investigation.(Fig. 2).

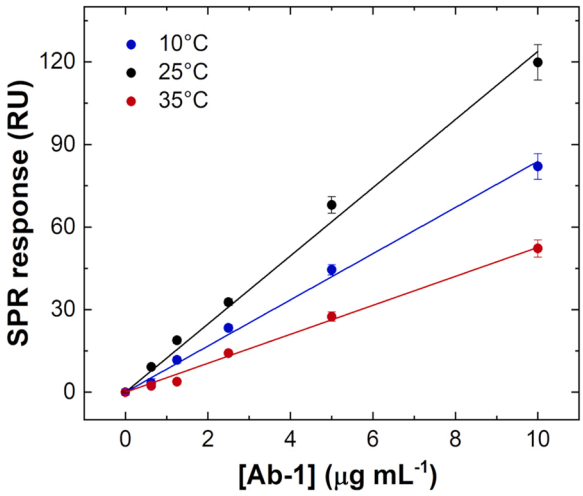



**Fig. 2.** Influence of temperature on the affinity binding between mAb-1 and the IBP imprinted with Fc $C_H3$ epitope integrated into the SPR sensing platform. mAb-1 calibration curves in the concentration range of 0.63 – 10 μg mL$^{-1}$) were carried out using three working temperatures (10 °C blue line, 25 °C black line, and 35 °C red line).

### 3.3. Fc-imprinted sensor selectivity over G subclasses of different mAbs

The IBP receptor, directed towards the Fc $C_H3$ portion, was tested both for its ability in binding other mAb belonging to the same IgG1 class (Ab-1, Ab-2, Ab-3, and Ab-4), thus sharing the imprinted epitope, and versus the multiple IgG antibody subclasses (IgG2, IgG3 and IgG4). Fig. 3b shows that all the IBP recognizes all the IgG1 mAbs, tested at the same concentration, as shown by similar SPR responses (Fig. 3b), expressed as normalized mean values respect to the highest (Ab-4). Additionally, the effectiveness of the affinity interactions between IgG1 Abs and the specific binding sites distributed on the IBP surface was corroborated by the large mean imprinting factor (IF =13.5) calculated as the IBP/NIBP SPR response ratio. The level of repeatability of the measurements resulted excellent as demonstrated by the average coefficient of variation, $_{av}$CV% = 0.1% (Ab-1), 1.4% (Ab-2), very good for Ab-3 $_{av}$CV% = 4.8%, and good in the case of Ab-4 $_{av}$CV% = 8.3%. Contrarily, the binding interactions between the biopolymeric imprinted surface and the other IgG subclasses (IgG2, IgG3, and IgG4) is markedly reduced, albeit the template sequence (KSLSLSPGK) is conserved among the heavy chains of the IgG subclasses (residues 439–447 IgG1 # P01857; residues 318–326 IgG2 # P01859; residues 369–377 IgG3 # P01860; residues 319–327 IgG4 # P01861). These unexpected results could be related to the difference in the hinge/$C_H2$ portions (Fig. 3a), influencing the overall conformation and plasticity, and allowing the recognition of IgG1 mAbs only probably because their $C_H3$ portion turns out to be better-exposed. Finally, we moved on exploring the ability of the system to detect a therapeutic mAb acting as myostatin inhibitor (Anti-MYO-029) as a case study.

**Table 1**
The analytical figures of merit for the IBP-based sensing system working at different temperatures.

| T (°C) | [1]m (μg mL$^{-1}$) | [2]LOD (μg mL$^{-1}$) | [3]LOQ (μg mL$^{-1}$) | $R^2$ | $_{av}$CV% |
|---|---|---|---|---|---|
| 10 | 8.39 ± 0.18 | 0.390 ± 0.008 | 1.31 ± 0.60 | 0.997 | 14 |
| 25 | 12.38 ± 0.32 | 0.070 ± 0.002 | 0.25 ± 0.01 | 0.996 | 6 |
| 35 | 5.26 ± 0.13 | 0.250 ± 0.006 | 0.40 ± 0.01 | 1.34 | 10 |

[1]SPR (RU) = m× [IgG1]; [2]LOD = 3 ×$SD_{blank}$/m; [3]LOQ = 10 ×$SD_{blank}$/m

### 3.4. Anti-MYO-029 therapeutic mAb detection using a PNE-based plasmonic assay

At this stage, the monoclonal antibody-directed myostatin inhibitor (Anti-MYO-029) was tested as a case of interest on the epitope-based mimetic bioreceptor. A direct measurement of five Anti-MYO-029 standard solutions in the concentration range spanning from 1 μg mL$^{-1}$ to 8 μg mL$^{-1}$ were prepared in PBS pH 7.4. The dose-response obtained (Fig. 4) was fitted with a one-site binding model ($R^2$ = 0.998) using OriginLab software, RU = $R_{max}$ x [Anti-MYO-029]/($K_D$ + [Anti-MYO-029]), where $R_{max}$ is the maximum response ($R_{max}$ = 291 ± 28 μg mL$^{-1}$), and the $K_D$ is the equilibrium dissociation constant Anti-MYO-029 vs. IBP ($K_D$ = 50.9 ± 6.5 nmol L$^{-1}$). The PNE-based sensor exhibited excellent repeatability, namely $_{av}$CV% = 5.5%, achieving a LOD and a LOQ value of 0.037 ± 0.004 μg mL$^{-1}$ and 0.13 ± 0.02 μg mL$^{-1}$, respectively, established using the following equations LOD = 3 $SD_{blank}$ x $K_D/R_{max}$ and LOQ = 10 $SD_{blank}$ x $K_D/R_{max}$, respectively.

### 3.5. Anti-MYO-029 therapeutic mAb detection in human serum: a beads-based plasmonic assay

The final aim of this study was the detection of the therapeutic Anti-MYO-029 in human serum. It is worth noting that the developed Fc-imprinted surface is able to recognize polyclonal IgG antibodies, massively present in human blood (i.e., mg mL$^{-1}$ physiological range), that share a common Fc $C_H3$ epitope sequence along the proper sequence [41]. To selectively address the Anti-MYO-029 detection in such a complex matrix as human serum, we combined the Fc capturing IBP of the mAb with a simple extraction step by using a biomagnetic separation approach. In detail, magnetic beads (MBs) conjugated to myostatin molecules (MYO-MBs) were employed to extract the myostatin-specific antibody (Anti-MYO-029) from reinforced human serum specimens. The resultant Anti-MYO-029 @MYO-MBs complex was directly injected into the microfluidic system of the SPR instrumentation, to bind the PNE-based IBP, generating a real-time interaction signal. An effective interaction between the bioreceptor, directed against the mAb Fc, and the Anti-MYO-029 mAb, selectively bound to MYO-MBs, was recorded in real-time. It is important to note that the

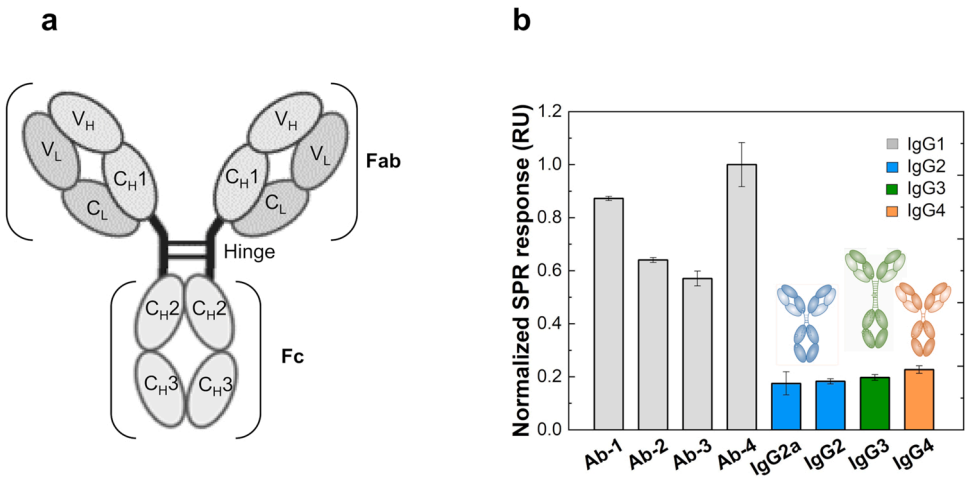



**Fig. 3.** (a) Schematic structure of an antibody. (b) Cross-reactivity among monoclonal antibodies (2.5 μg $mL^{-1}$) on a PNE-based mimetic receptor. The bar chart indicates normalized mean values ($RU_{mean}$ ± SD) recorded in triplicate for each mAb-IBP binding interaction analysis. The gray-, orange-, red-, and black-colored bars respectively indicate the following mAbs' isotypes: IgG1, IgG2, IgG3 and IgG4.

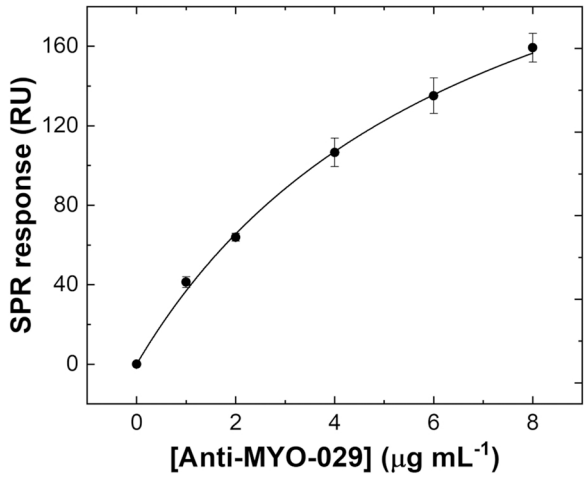



**Fig. 4.** Anti-MYO-029 detection (T = 25 °C) via a PNE-based sensor and the calibration curve (concentration range: 1–8 μg $mL^{-1}$) was fitted with a one-site binding model. Each point is representative of three measurements ($RU_{mean}$ ± SD). The SPR signal of the blank sample was subtracted from each measurement.

Anti-MYO-029 extraction procedure is overall effective with significant concentration-dependent changes of the SPR responses (Fig. 5). Several analyses may be performed on the same IBP surface (around 10 cycles, data not shown) by dissociating the binding interactions between PNE-based IBP receptor and Anti-MYO-029 @MYO-MBs complex with a single shot (24 s) of 0.5% SDS. This represents an interesting and time saving approach where analyte extraction and detection consist in a fast single step, thanks to the modified MBs' binding ability to recognize the mAb of interest from the Fab region, responsible for its binding selectivity. In the latter situation, the MYO-MBs bind the analyte in a site-specific oriented manner leaving the constant fragment (Fc $C_H3$) free to interact with epitope (Fc $C_H3$) imprinted biopolymer. Specifically, here the modified magnetic particles were exploited not only to pre-concentrate mAbs from biological samples, but also to design the first, to our knowledge, beads-based plasmonic assay targeting mAbs, which involves an effective PNE-based mimetic receptor. The assay principle is displayed in Scheme 1. In the molecular architecture of the assay, the secondary receptor carrying the analyte in a site-specific oriented manner helps to improve the capturing of the mAb constant fragment ($C_H3$) used as an imprinting template into the PNE matrix. A calibration curve of Anti-MYO-029 mAb was carried out by directly extracting the analyte from spiked human serum samples over a final concentration range spanning from 4 to 20 μg $mL^{-1}$. Additionally, a blank sample (unspiked serum) underwent the same procedure with MYO-MBs and it was tested for its SPR signal on the same IBP-based sensor. A low mean SPR response was recorded and subtracted from the entire data set. The assay achieved a LOD = 0.211 ± 0.002 μg $mL^{-1}$ and a LOQ = 0.710 ± 0.007 μg $mL^{-1}$, extrapolated from the linear regression curve (Fig. 5a, $R^2$ = 0.97), with a repeatability ($_{av}$CV%) of 11.4%. Accordingly, we have designed an extremely modulable assay involving an effective single biopolymeric receptor that has shown a good sensitivity, as demonstrated by the LOD value, theoretically, appropriate to dose therapeutic mAbs, which are usually administered at high concentration (1–10 mg $Kg^{-1}$), reaching the mg $mL^{-1}$ range in serum [27,48]. Conversely, to the best of our knowledge, for anti-doping purposes, a threshold values or MRLP (minimum required performance level) for myostatin-neutralizing antibodies (e.g., domogrozumab, landogrozumab and stamulumab) misuse is not reported by WADA; eventually, this approach could be also applied to other mAbs with myostatin inhibition activity, potentially available on the market. Remarkably, the approach developed here displays a general validity and could be used to detect several mAbs, also allowing their simultaneous analysis. Specifically, the mAbs multiplexing detection may be accomplished by a first synchronized samples' treatment using MBs modified with the suitable antigen, followed by SPR analysis on a single surface modified with an epitope (Fc $C_H3$) imprinted polymer.

## 4. Conclusions

The rising interest and use of biological drugs for immunotherapies has strongly pushed towards the necessity of developing simple and rapid diagnostic assays for their detection in biological complex matrices. In this study, we designed a beads-based plasmonic assay on an SPR platform, which embedded a polynorepinephrine-based mimetic receptor (the polymerization process is not completely known yet [49–52]) combined with myostatin-encoded beads, to address the case study of Anti-MYO-029 therapeutic mAb quantification in human serum, both for clinical and anti-doping purposes. The beads-based affinity tool is not only used to selectively capture the specific mAb-idiotype (Anti-MYO-029) from the biological matrix, but it is also directly involved in the molecular architecture of the sensing detection platform. Firstly, the applicability of the PNE-based IBP in IgG1 recognition was studied, from the analytical point of view, on a model monoclonal antibody by optimizing the main experimental conditions.

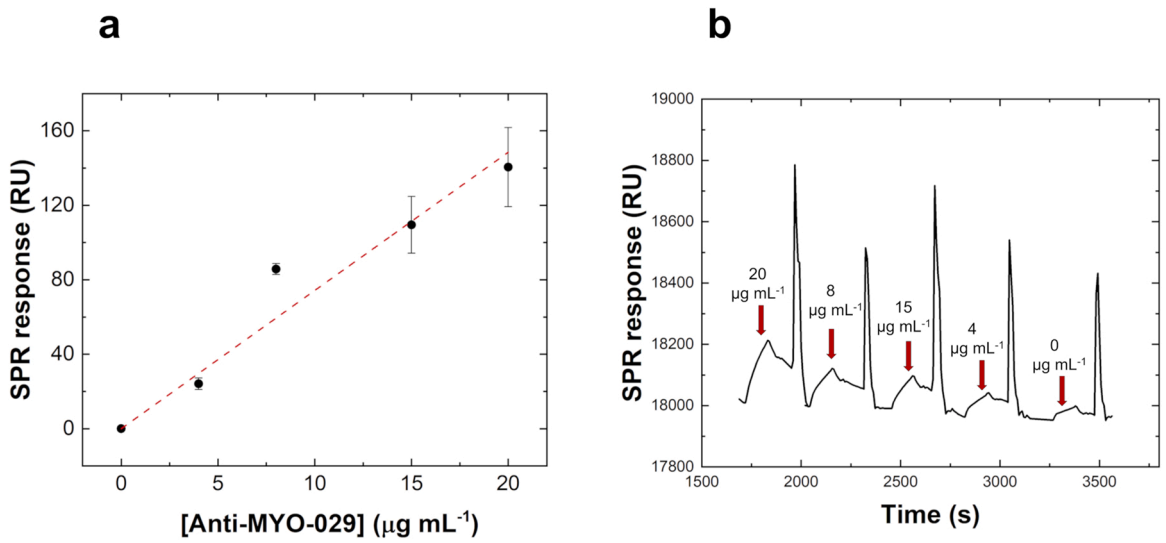



**Fig. 5.** (a) SPR data resulting from the analysis of different Anti-MYO-029 mAb concentrations (4 – 20 μg $mL^{-1}$) extracted from human serum samples. Each point represents the mean of three measurements ($RU_{mean}$ ± SD). (b) Representative sensorgram of a measurements' cycle in which different Anti-MYO-029 mAb concentrations were injected onto the biosensor, followed each time by a single regeneration step.

The biopolymeric mimetic receptor showed a marked selectivity for mAbs belonging to IgG1 isotype, as expected, while negligible binding for the other mAb subclasses (e.g., IgG2, IgG3 and IgG4). This interesting evidence will be further investigated by combining structural conformation and aminoacidic sequence analysis. The biosensor calibration was performed on the therapeutic bio-drug of interest, Anti-MYO-029 mAb. Comprehensively, the analytical strategy enables a high-throughput real-time analysis of a myostatin-neutralizing mAb, in line with the indicative range reported in clinical trials for the immunotherapeutic' administration, providing new insights into the actual approach for mAbs monitoring. The beads-based assay was tested directly in human serum achieving a satisfactory repeatability ($_{av}$CV% =11.4%), a LOD of 0.211 ± 0.002 μg $mL^{-1}$ and a LOQ of 0.710 ± 0.007 μg $mL^{-1}$. The designed approach is extremely modulable, showing a general validity for mAbs analysis. In particular, the assay is independent from the complementary determining regions (CDR), and for this reason could potentially be applied to different mAbs, simply by introducing a fast pre-analytical sample processing with magnetic beads (MBs)-encoded with the suitable antigen. This affinity tool confers the proper selectivity to the system, being flexible at the same time, made of fully interchangeable parts, which can be modified according to the mAb of interest. In this scenario, further development will be in the direction of mAb multiplexing magnetic beads-and IBP-based assay.

## CRediT authorship contribution statement

**Francesca Torrini** Conceptualization, Methodology, Investigation, Data curation, Writing – original draft preparation, Writing – review & editing. **Federica Battaglia** Conceptualization, Investigation, Data curation, Writing – original draft preparation. **Davide Sestaioni** Conceptualization, Investigation, Data curation. **Pasquale Palladino** Conceptualization, Data curation, Writing – review & editing. **Simona Scarano** Conceptualization, Methodology, Investigation, Data curation, Writing – review & editing, Supervision. **Maria Minunni** Conceptualization, Methodology, Investigation, Data curation, Writing – original draft preparation, Writing – review & editing, Supervision, Project administration.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data Availability

Data will be made available on request.

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## References

[1] P.A. Todd, R.N. Brogden, Muromonab CD3. A review of its pharmacology and therapeutic potential, Drugs 37 (1989) 871–899, https://doi.org/10.2165/00003495-198937060-00004.
[2] The Antibody Society. Therapeutic Monoclonal Antibodies Approved or in Review in the EU or US. www.antibodysociety.org/resources/approved-antibodies/ (Accessed 20 September 2022).
[3] U. Hafeez, H.K. Gan, A.M. Scott, Monoclonal antibodies as immunomodulatory therapy against cancer and autoimmune diseases, Curr. Opin. Pharmacol. 41 (2018) 114–121, https://doi.org/10.1016/j.coph.2018.05.010.
[4] A.C. Chan, P.J. Carter, Therapeutic antibodies for autoimmunity and inflammation, Nat. Rev. Immunol. 10 (2019) 301–316, https://doi.org/10.1038/nri2761.
[5] M.G. Matera, L. Calzetta, P. Rogliano, M. Cazzola, Monoclonal antibodies for severe asthma: pharmacokinetic profiles, Respir. Med. 153 (2019) 3–13, https://doi.org/10.1016/j.rmed.2019.05.005.
[6] S. Chaplin, COVID-19: a brief history and treatments in development, Prescriber 31 (2020) 23–28, https://doi.org/10.1002/psb.1843.
[7] M.X. Sliwkowski, I. Mellman, Antibody therapeutics in cancer, Science 341 (2013) 1192–1198, https://doi.org/10.1126/science.1241145.
[8] D. Zahavi, L. Weiner, Monoclonal antibodies in cancer therapy, Antibodies 9 (2020) 34, https://doi.org/10.3390/antib9030034.
[9] M.P. Motley, K. Banerjee, B.C. Fries, Monoclonal antibody-based therapies for bacterial infections, Curr. Opin. Infect. Dis. 32 (2019) 210–216, https://doi.org/10.1097/QCO.0000000000000539.
[10] Y. Lee, W.I. Lim, C.I. Bloom, S. Moore, E. Chung, N. Marzella, Bezlotoxumab (Zinplava) for Clostridium Difficile Infection P & T. 42 (2017) 735–738. PMID: 29234211.

[11] D.V. Zurawski, M.K. McLendon, Monoclonal antibodies as an antibacterial approach against bacterial pathogens, Antibiotics 9 (2020) 155, https://doi.org/10.3390/antibiotics9040155.
[12] G. Salazar, N. Zhang, T.-M. Fu, Z. An, Antibody therapies for the prevention and treatment of viral infections, NPJ Vaccin 2 (2017) 19, https://doi.org/10.1038/s41541-017-0019-3.
[13] V.P. Chavda, R. Prajapati, D. Lathigara, B. Nagar, J. Kukadiya, E.M. Redwan, V. N. Uversky, M.N. Kher, R. Patel, Therapeutic monoclonal antibodies for COVID-19 management: an update, Expert Opin. Biol. Ther. 22 (2022) 763–780., https://doi.org/10.1080/14712598.2022.2078160.
[14] U.S. Food , Drug Administration (F.D.A.) website. https://www.dfa.gov/drugs/emergency-preparedness-drugs/coronavirus-covis-19. (Accessed 21 September 2022).
[15] J.T. Ryman, B. Meibohm, Pharmacokinetics of monoclonal antibodies, CPT Pharmacomet. Syst. Pharmacol. 6 (2017) 576–588, https://doi.org/10.1002/psp4.12224.
[16] Y. Tang, P. Cain, V. Anguiano, J.J. Shih, Q. Chai, Y. Feng, Impact of IgG subclass on molecular properties of monoclonal antibodies, Mabs 13 (2021) 1993768., https://doi.org/10.1080/19420862.2021.1993768.
[17] R. Yamin, A.T. Jones, H.H. Hoffmann, A. Schäfer, K.S. Kao, R.L. Francis, T. P. Sheahan, R.S. Baric, C.M. Rice, J.V. Ravetch, S. Bournazos, Fc-engineered antibody therapeutics with improved anti-SARS-CoV-2 efficacy, Nature 599 (2021) 465–470., https://doi.org/10.1038/s41586-021-04017-w.
[18] C.D. Dellisanti, Putting antibodies into shape, Nat. Immunol. 17 (Suppl 1) (2016) S11, https://doi.org/10.1038/ni.3606.
[19] H.J. Van der Horst, I.S. Nijhof, T. Mutis, M.E.D. Chamuleau, Fc-engineered antibodies with enhanced fc-effector function for the treatment of B-cell malignancies, Cancers 12 (2020) 3041, https://doi.org/10.3390/cancers12103041.
[20] T. Schlothauer, S. Herter, C.F. Koller, S. Grau-Richards, V. Steinhart, C. Spick, M. Kubbies, C. Klein, P. Umaña, E. Mössner, Novel human IgG1 and IgG4 Fc-engineered antibodies with completely abolished immune effector functions, Protein Eng. Des. Sel. 29 (2016) 457–466, https://doi.org/10.1093/protein/gzw040.
[21] I. Wilkinson, S. Anderson, J. Fry, L.A. Julien, D. Neville, O. Qureshi, G. Watts, G. Hale, Fc-engineered antibodies with immune effector functions completely abolished, PloS ONE 16 (2021), e0260954, https://doi.org/10.1371/journal.pone.0260954.
[22] I. Jovčevska, S. Muyldermans, The therapeutic potential of nanobodies, BioDrugs 34 (2020) 11–26., https://doi.org/10.1007/s40259-019-00392-z.
[23] P.S. Kozani, A. Naseri, S.M.J. Mirarefin, F. Salem, M. Nikbakht, S.E. Bakhshi, P. S. Kozani, Nanobody-based CAR-T cells for cancer immunotherapy, Biomark. Res. 10 (2022) 24, https://doi.org/10.1186/s40364-022-00371-7.
[24] C. Morrison, Nanobody approval gives domain antibodies a boost, Nat. Rev. Drug Discov. 18 (2019) 485–487., https://doi.org/10.1038/d41573-019-00104-w.
[25] P.D. Skottrup, H. Døssing, N.W. Andersen, L. Buch-Rasmussen, Nanobody-based microfluidic human Fc assay for preclinical plasma quantification of IgG1/1.1 and IgG1-Fc-conjugates, J. Immunol. Methods 502 (2022), https://doi.org/10.1016/j.jim.2022.113214.
[26] M.S. Valdés-Tresanco, A. Molina-Zapata, A.G. Pose, E. Moreno, Structural insights into the design of synthetic nanobody libraries, Molecules 27 (2022) 2198, https://doi.org/10.3390/molecules27072198.
[27] K. Todoroki, H. Mizuno, E. Sugiyama, T. Toyo'oka, Bioanalytical methods for therapeutic monoclonal antibodies and antibody-drug conjugates: a review of recent advances and future perspectives, J. Pharm. Biomed. Anal. 179 (2020), https://doi.org/10.1016/j.jpba.2019.112991.
[28] C.W. Damen, E.R. de Groot, M. Heij, D.S. Boss, J.H. Schellens, H. Rosing, J. H. Beijnen, L.A. Aarden, Development and validation of an enzyme-linked immunosorbent assay for the quantification of trastuzumab in human serum and plasma, Anal. Biochem. 391 (2009) 114–120., https://doi.org/10.1016/j.ab.2009.05.030.
[29] B. Gorovits, A. Hays, D. Jani, C. Jones, C. King, A. Lundequist, J. Mora, M. Partridge, D. Pathania, S.S. Ramaswamy, D. Rutwij, H. Shen, G. Starling, Perspective on the EURL Recommendation on the use of Non-Animal-Derived Antibodies, AAPS J. 23 (2021) 34, https://doi.org/10.1208/s12248-021-00567-z.
[30] S. Miyakawa, Y. Nomura, T. Sakamoto, Y. Yamaguchi, K. Kato, S. Yamazaki, Y. Nakamura, Structural and molecular basis for hyperspecificity of RNA aptamer to human immunoglobulin G, RNA 14 (2008) 1154–1163., https://doi.org/10.1261/rna.1005808.
[31] T. Saito, Y. Shimizu, K. Tsukakoshi, K. Abe, J. Lee, K. Ueno, R. Asano, B.V. Jones, T. Yamada, T. Nakano, J. Tong, A. Hishiki, K. Hara, H. Hashimoto, K. Sode, T. Toyo'oka, K. Todoroki, K. Ikebukuro, Development of a DNA aptamer that binds to the complementarity-determining region of therapeutic monoclonal antibody and affinity improvement induced by pH-change for sensitive detection, Biosens. Bioelectron. 203 (2022) 114027, https://doi.org/10.1016/j.bios.2022.114027.
[32] T. Yamada, T. Saito, Y. Hill, Y. Shimizu, K. Tsukakoshi, H. Mizuno, H. Hayashi, K. Ikebukuro, T. Toyo'oka, K. Todoroki, High-throughput bioanalysis of bevacizumab in human plasma based on enzyme-linked aptamer assay using anti-idiotype DNA aptamer, Anal. Chem. 91 (2019) 3125–3130, https://doi.org/10.1021/acs.analchem.8b05725.
[33] O.S. Ahmad, T.S. Bedwell, C. Esen, A. Garcia-Cruz, S.A. Piletsky, Molecularly imprinted polymers in electrochemical and optical sensors, Trends Biotechnol. 37 (2019) 294–309., https://doi.org/10.1016/j.tibtech.2018.08.009.
[34] Z. Bognár, R.E. Gyurcsányi, Aptamers against immunoglobulins: design, selection and bioanalytical applications, Int. J. Mol. Sci. 21 (2020) 5748, https://doi.org/10.3390/ijms21165748.
[35] O.I. Parisi, F. Francomano, M. Dattilo, F. Patitucci, S. Prete, F. Amone, F. Puoci, The evolution of molecular recognition: from antibodies to molecularly imprinted polymers (MIPs) as artificial counterpart, J. Funct. Biomater. 13 (2022) 12, https://doi.org/10.3390/jfb13010012.
[36] H. Yoshida, K. Sato, T. Ishikawa, T. Sakamoto, K. Yamagishi, Binding interaction analysis of RNA aptamer-Fc region of human immunoglobulin G using fragment molecular orbital calculation, Chem. Phys. Lett. 738 (2022) 136854, https://doi.org/10.1016/j.cplett.2019.136854.
[37] V. Baldoneschi, P. Palladino, M. Banchini, M. Minunni, S. Scarano, Norepinephrine as new functional monomer for molecular imprinting: An applicative study for the optical sensing of cardiac biomarkers, Biosens. Bioelectron. 157 (2020) 112161, https://doi.org/10.1016/j.bios.2020.112161.
[38] F. Battaglia, V. Baldoneschi, V. Meucci, L. Intorre, M. Minunni, S. Scarano, Detection of canine and equine procalcitonin for sepsis diagnosis in veterinary clinic by the development of novel MIP-based SPR biosensors, Talanta 230 (2021) 122347, https://doi.org/10.1016/j.talanta.2021.122347.
[39] F. Torrini, P. Palladino, V. Baldoneschi, S. Scarano, M. Minunni, Sensitive 'two-steps' competitive assay for gonadotropin-releasing hormone detection via SPR biosensing and polynorepinephrine-based molecularly imprinted polymer, Anal. Chim. Acta 1161 (2021) 338481, https://doi.org/10.1016/j.aca.2021.338481.
[40] F. Torrini, G. Goletta, P. Palladino, S. Scarano, M. Minunni, A LysLysLys-tag as trigger in polynorepinephrine epitope imprinting: The case study of soluble PD-L1 detection in serum by optical-based sensing, Biosens. Bioelectron. 220 (2023) 114806, https://doi.org/10.1016/j.bios.2022.114806.
[41] F. Torrini, F. Battaglia, P. Palladino, S. Scarano, M. Minunni, Imprinted biopolymers as green abiotic route in immunoglobulin affinity plasmonic sensing, Biosens. Bioelectron. 217 (2022) 114706, https://doi.org/10.1016/j.bios.2022.114706.
[42] M. Saitoh, J. Ishida, N. Ebner, S.D. Anker, J. Springer, S. von Haehling, Myostatin inhibitors as pharmacological treatment for muscle wasting and muscular dystrophy, JCSM Clin. Rep. 2 (2017) 1–10, https://doi.org/10.17987/jcsm-cr.v2i1.37.
[43] J. Suh, Y.-S. Lee, Myostatin inhibitors: panacea or predicament for musculoskeletal disorders? J. Bone Metab. 27 (2020) 151–165, https://doi.org/10.11005/jbm.2020.27.3.151.
[44] List of prohibited substances. Wada-ama.org/2021. (Accessed 20 September 2022).
[45] E. Moczko, A. Guerreiro, C. Cáceres, E. Piletska, B. Sellergren, S.A. Piletsky, Epitope approach in molecular imprinting of antibodies, J. Chromatogr. B 1124 (2019) 1–6, https://doi.org/10.1016/j.jchromb.2019.05.024.
[46] S. Schwark, W. Sun, J. Stute, D. Lütkemeyer, M. Ulbricht, B. Sellergren, Monoclonal antibody capture from cell culture supernatants using epitope imprinted macroporous membranes, RSC Adv 6 (2016) 53162–53169, https://doi.org/10.1039/C6RA06632A.
[47] P. Palladino, M. Minunni, S. Scarano, Cardiac Troponin T capture and detection in real-time via epitope-imprinted polymer and optical biosensing, Biosens. Bioelectron. 106 (2018) 93–98, https://doi.org/10.1016/j.bios.2018.01.068.
[48] T. Willeman, J.F. Jourdil, E. Gautier-Veyret, B. Bonaz, F. Stanke-Labesque, A multiplex liquid chromatography tandem mass spectrometry method for the quantification of seven therapeutic monoclonal antibodies: Application for adalimumab therapeutic drug monitoring in patients with Crohn's disease, Anal. Chim. Acta 1067 (2019) 63–70, https://doi.org/10.1016/j.aca.2019.03.033.
[49] S. Hong, J. Kim, Y.S. Na, J. Park, S. Kim, K. Singha, G.I. Im, D.K. Han, W.J. Kim, H. Lee, Poly(norepinephrine): ultrasmooth material-independent surface chemistry and nanodepot for nitric oxide, Angew. Chem. Int. Ed. Engl. 52 (2013) 9187–9191, https://doi.org/10.1002/anie.201201646.
[50] J.-Y. Hong, X. Yu, B.M. Bak, C. Pang, H.S. Park, Bio-inspired functionalization and redox charge transfer of graphene oxide sponges for pseudocapacitive electrodes, Carbon 83 (2015) 71–78, https://doi.org/10.1016/j.carbon.2014.11.020.
[51] J. Chen, R.P. Liang, L.L. Wu, J.D. Qiu, One-step preparation and application of mussel-inspired poly(norepinephrine)-coated polydimethylsiloxane microchip for separation of chiral compounds, Electrophoresis 37 (2016) 1676–1684., https://doi.org/10.1002/elps.201600054.
[52] T. Iwasaki, Y. Tamai, M. Yamamoto, T. Taniguchi, K. Kishikawa, M. Kohri, Melanin precursor influence on structural colors from artificial melanin particles: PolyDOPA, polydopamine, and polynorepinephrine, Langmuir 39 (2018) 11814–11821, https://doi.org/10.1021/acs.langmuir.8b02444.

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