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REVIEW

Surface plasmon resonance in doping analysis

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Abstract Doping analysis relies on the determination of prohibited substances that should not be present in the body of an athlete or that should be below a threshold value. In the case of xenobiotics their mere presence is sufficient to establish a doping offence. However, in the case of human biotics the analytical method faces the difficulty of distinguishing between endogenous and exogenous origin. For this purpose ingenious strategies have been implemented, often aided by state-of-the-art technological advancements such as mass spectrometry in all its possible forms. For larger molecules, i.e. protein hormones, the innate structural complexity, the heterogeneous nature, and the extremely low levels in biological fluids have rendered the analytical procedures heavily dependent of immunological approaches. Although approaches these confer specificity and sensitivity to the applications, most rely on the use of two, or even three, antibody incubations with the consequent increment in assay variability. Moreover, the requirement for different antibodies that separately recognise different epitopes in screening and confirmation assays further contributes to differences encountered in either measurement. The development of analytical techniques to measure interactions directly, such as atomic force microscopy, quartz crystal microbalance or surface plasmon resonance, have greatly contributed to the accurate evaluation of molecular interactions in all fields of biology, and expectations are that this will only increase. Here, an overview is provided of surface plasmon resonance, and its particular value in application to the field of doping analysis.

Keywords Surface plasmon resonance · Doping analysis · Proteins and immunoassay

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Introduction

The definitions of doping are many, depending on the body or organisation issuing the document, but all are more or less as defined originally by the International Olympic Committee (IOC): "Doping is defined as the presence in the human body of substances which are prohibited according to the list published by the IOC and/or the international organisation of the member organization in question. The use of such substances, their presence in urine or blood samples, and the use of methods with the purpose of altering the result of an analysis of a urine or blood sample are prohibited." In 1999 the World Anti-Doping Agency

(WADA) was established as an international independent agency to battle the illicit use of a large number of substances that are included in the denominated prohibited list [1] and established doping as "the occurrence of one or more of the anti-doping rule violations set forth in article 2.1 through article 2.8 of the World Anti-Doping Code" [2]. In the aforementioned list a rapid classification can be made between prohibited methods and substances. Within the latter category the distinction is made between smaller molecules (here defined as less than 500 Da) and larger molecules (here defined as more than 500 Da); small molecules are the origin of the historically documented doping cases involving caffeine (1867), amphetamines (1920), amphetamines plus heroin (1967), and stanozolol (1988).

Two decades ago, the introduction of recombinant protein hormones enforced a different analytical approach for this new class of molecules that are sequentially identical to the endogenous counterpart and tremendously heterogeneous due to splicing and post-translational mechanisms. In this review we describe the development of analytical anti-doping strategies, principally addressed through antibody-based detection protocols, and how surface plasmon resonance (SPR) has contributed to established protocols.

Doping analysis

Small molecules, as defined above, possess well-defined structural characteristics, all the way from the basic physico-chemical properties to the unique three-dimensional (3D) disposition of the constituent atoms. Although in the early beginnings of anti-doping analysis these molecules were also targeted through radioimmunoassays [3–5], the use of chromatographic techniques soon became more popular. Eventually, the preference for gas chromatography (GC) was very much driven by the introduction of mass spectrometric (MS) analysers [6, 7]. In the beginning samples were purified by simple approaches such as thin-layer chromatography proceeded by gas chromatography with flame ionisation, electron capture or nitrogen–phosphorus detectors (NPD), and identification was accomplished through retention index by comparison with standard compounds [8, 9]. The transition from shorter packed columns with relatively low resolution to longer open tubular columns represented a major step forward in terms of efficiency, capacity, speed and reproducibility. One of the earliest mentions of the implementation of capillary columns that avoided the use of interfaces and the application of electron impact ionisation to obtain unambiguous structural information on anabolic steroids is from Cartoni et al. in 1983 [10]. They analysed 10 different steroids of commercial origin and reported a sensitivity as low as 1 ng/ml in urine. By 1984, for the Olympic Games in Los Angeles, the use of GC-MS was fully implemented for anabolic steroids, whereas other categories of substances were first screened by GC-NPD and only confirmed by GC-MS [11]. Surprisingly, liquid chromatography with UV detection was employed in those days only for the detection of 5-phenyl-2-imino-4-oxo-oxazolidine and quantification of caffeine. Cartoni et al.'s report appears to be the turning point as from that time on most studies reported in the literature included the mass spectrometric detection, thereby avoiding the identification on the basis of relative retention time and peak shape. The use of a mass fingerprint of any given substance, and later on the use of selected ion monitoring allowed for unambiguous identification and quantification. With the implementation of the mass-selective detector the instrumental development for GC had basically reached its endpoint and most of the improvements for GC-based analysis came from the optimisation of derivatisation chemistries during the 1990s [12]. A few years before the 1984 Olympic Games, in 1981, LC-MS made its first contribution to the equine anti-doping scene. Using a moving belt interface and negative ion chemical ionisation with ammonia the presence of synthetic corticosteroids in urine could be demonstrated [13]. The successor to the moving belt—the thermospray interface—was also applied in the field [14], but by that time the invention of 2002 Nobel awardee John B. Fenn, i.e. electrospray ionisation interface (ESI) [15], had already revolutionised the scene. This resulted in the first efficient interface that could be applied to a wide range of different molecules (small, large, polar, nonpolar, etc.), afforded information on the molecular species and also the molecular elements, and has ultimately been implemented in most anti-doping analyses. However, despite the obvious advantages of ESI, such as the lack of derivatisation requirements, the shorter analysis times and increased sensitivity, the first papers reporting the use of this technique did not appear till the turn of the century [16, 17]. Possibly, the serious consequences of erroneous analysis delayed the full acceptance of ESI until the technique had demonstrated its value. Recently, the introduction of fast chromatography [ultra-performance liquid chromatography (UPLC) and rapid resolution liquid chromatography (RRLC)] and ultrafast-scanning mass spectrometers have further consolidated this technique, shortening cycle times to only a few minutes, and thus increasing the analysis capacity. Around the same time that LC-MS was introduced onto the scene the scope of available pharmaceutical products also changed. In addition to the well-defined small molecules, protein pharmaceuticals were introduced as recombinant expression products [18–20] and also became accessible to elite athletes. With this development a new era started in the anti-doping field.

Most of the smaller molecules were of exogenous origin and their mere presence in a human specimen was sufficient to determine a doping offence. All protein pharmaceuticals, however, were made after the endogenous blueprint [except for human chorionic gonadotropin (hCG) that is also purified from the urine of gestating women [21]], enforcing a differentiation between endogenous and exogenous to call a positive doping case. Unfortunately, the straightforward use of mass spectrometry approaches for protein hormones has not been successful. In the deployment of the "omics" epoch, proteomics was initially addressed using two-dimensional gel electrophoresis (2DE) to separate the tremendously complex mixtures of proteins in biological specimens, followed by in-gel proteolytic digestions, and subsequent analysis by mass spectrometry [22]. With the development of multidimensional LC and fast-scanning mass spectrometric instruments the use of 2DE gels and peptide mass fingerprints was largely substituted by liquid sample handling and peptide sequencing information [23]. Nowadays, proteomics studies of complex samples scanning at 3 to 10 fragment spectra per second yield between 24,000 and 72,000 spectra for an average 2-h LC run [24]. Despite this tremendous wealth of information derived from such samples these studies rarely mention the identification of protein hormones of interest to the anti-doping community (Table 1). This is mainly because protein hormones are present only in minute amounts and at the lower end of the dynamic concentration range that spans 10 to 12 orders of magnitude [25, 26] and because proteomics data most frequently only cover the abundant proteins. Furthermore, direct analysis of complex samples is often impossible because of the intrinsic limitations of a complex matrix such as blood or urine. Thus, for the time being it seems unlikely that straightforward shotgun proteomics will find application in routine doping control. One dedicated study to precisely demonstrate the state-of-the-art instrumental development by one of the major manufacturers targeted human growth hormone. Although the efforts deserved merit, ultimately they could only detect the hormone when spiked into the sample at the nanomolar level [27]. Thus, only if directed front-end purification strategies are incorporated [28–30] might the LC-MS strategies be fruitful and WADA is already supporting research projects to explore this avenue (http://www.wada-ama.org/en/Science-Medicine/Research/Funded-Research-Projects/). Until that time immunoglobulin-based detection methods remain the standard to address protein doping offences.

Immunoassays

Today doping analysis of protein hormones is done through protocols in which the essential steps rely on the use of an antibody, preferably monoclonal and with well-defined specificity, to detect, identify and quantify the analyte of choice. Table 1 includes the analytical strategies that are employed to address the protein hormones relevant in the field. For a limited number of analytes the protocol relies on the use of assays that have been developed for clinical applications. As such, human chorionic gonadotropin [31] and luteinising hormone [32] are quantitated using either immunoradiometric assay (IRMA), immunochemiluminometric assay (ICMA) or enzyme immunoassay (EIA) assays and controversy exists because of the considerable variability of the measurements (vide infra). For example, Cole and colleagues evaluated up to nine commercial assays for hCG and found that the extreme variations encountered for the different purified hCG variants (intact, free β-subunit, β-core fragment, nicked hCG) could not be explained by the nature of the variant [33]. Similarly, Higgins et al. evaluated up to seven different methods with the fourth World Health Organization (WHO) standard for hCG and also human specimens finding an absolute bias of nearly 70% [34]. To this day much controversy exists as to which method is capable of providing the most accurate results for this particular hormone, with little coherence found between the intermethod variability encountered in different studies. Analysis of erythropoietin has been addressed in several ways [35], but the most widely employed method is based on isoelectric focussing (IEF) that results in a different IEF profile for endogenous and exogenous (rEPO, NESP and CERA) molecules [36–41]. Even though the differentiation is based on charge heterogeneity, principally due to the differences in degree of sialylation of these molecules, the detection of all relies on the use of an antibody. This immunoglobulin, AE7A5 from R&D, has demonstrated a hitherto unmatched sensitivity for which no second, confirmation, assay exists employing a different antibody. In order to present a second procedure to corroborate the IEF findings a molecular size separation by sodium dodecyl sulfate polyacrylamide–gel electrophoresis (SDS-PAGE) has been implemented [42], but employing the same antibody as in the IEF procedure. Even though this antibody has demonstrated its true value for anti-doping purposes, its biomolecular interaction characteristics required careful evaluation by SPR to establish the best analytical procedure for its employment (vide infra).

Finally, of all protein hormones that are of interest to the scientific community dedicated to doping analysis only insulin measurements are performed through mass spectrometric protocols [43], although immunoassays are also employed [44]. In this case a mass spectrometric approach is greatly facilitated because the protein is only 51 amino acids long (a B and A peptide held together by two disulfide bonds) without significant post-translational heterogeneity, and several pharmaceutical preparations contain unnatural modifications. Still, for all hormones addressed through

Table 1 Proteins of interest for anti-doping purposes, their concentration in plasma, and entry in the UniProt database

Protein	Protein mass (kDa)	Comment	PTM	Concentration	Detection	UniProt access no.
Ghrelin	3.37		Octanoyl	100–150 fmol/ml	Competition assay	Q9UBU3
Sermorelin	3.36				–	
ACTH	4.64		PO_3	5–46 pg/ml		P01189
Insulin	5.99	SS dimer			MS, IA	P01308
IGF-I	7.75			110–330 ng/ml[a]	IA	P05019
GHRH-1 GHRH-2	12.45 12.35		Amidated Amidated		–	P01286
GH-22	22.21		–	0.01–5 ng/ml	IA-SPR	P01241-1
GH-20	20.36	Splice variant	–	0.005–0.5 ng/ml	IA	P01241-2
EPO	18.48		3N+1O	0.5 ng/ml	IEF-WB	P01588
LH-β	13.29	Hetero dimer	1N	0.16–0.068 μg/ml	IA-SPR	P01229
LH-α	10.30		2N			P01215
hCG-β	15.52	Hetero dimer	2N+4O	33 ng–1.6 μg/ml	IA	P01233
hCG-α	10.30		2N			P01215
PIIINP	13.23			1.5–6 ng/ml	IA	P02461
HIF1–α	92.74		OH-Pro NO-Cys	–		Q16665
HIF3-α	72.79		OH-Pro	–	–	Q9Y2N7
MGF	2.89	Splice variant	–	–	–	
PDGF A	14.39		1N	250–370 pg/ml	IA	P04085
PDGF B	12.38		–			P01127
PDGF C	37.21	Latent form	2N			Q9NRA1
PDGF D	13.96		1N			Q9GZP0
PDGF AB	26.77		1N			
FGF 1	15.82		–	54–109 pg/ml	IA	P05230
FGF 2	16.40		–	0.34–2.4 pg/ml	IA	P09038
FGF 3	24.94		–	–	–	P11487
FGF 4	19.28		–	–	–	P08620
FGF 5	27.57		1N	–	–	P12034
FGF 6	18.97		1N	–	–	P10767
FGF 7	18.87		1N	<30 pg/ml	IA	P21781
FGF 8	24.19		1N	–	–	P55075
FGF 9	23.13		1N	–	–	P31371
FGF10	19.31		2N	–	–	O15520
FGF 11	24.99		–	–	–	Q92914
FGF 12	27.38		–	–	–	P61328
FGF 13	27.55		–	–	–	Q92913
FGF 14	27.81		–	–	–	Q92915
FGF 16	23.75		1N	–	–	O43320
FGF 17	22.50		1N	–	–	O60258
FGF 18	21.02		2N	–	–	O76093
FGF 19	21.42		–	31–554 pg/ml	IA	O95750
FGF 20	23.49		–	–	–	Q9NP95
FGF 21	19.40			n.d.–910 pg/ml	IA	Q9NSA1
FGF 22	17.16		–	–	–	Q9HCT0
FGF 23	25.32		1O	–	–	Q9GZV9
HGF-α HGF-β	53.65 25.98	α/β SS dimer	2N+1O 2N	670–2,000 pg/ml	IA	P14210

Table 1 (continued)

Protein	Protein mass (kDa)	Comment	PTM	Concentration	Detection	UniProt access no.
VEGF A	23.88		1N+3N6AcLys	46–666 pg/ml	IA	P15692
VEGF B	19.35		–			P49765
VEGF C	13.10		2N			P49767
VEGF D	13.10		2N			O43915
PIGF	22.77		2N			P49763

PTM post-translational modification, N,N- and O,O-linked glycosylation; *SPR* surface plasmon resonance; *IA* immunoassay; *IEF* isoelectric focussing; *PO_3* phosphorylation; *NO* nitroso; *OH* hydroxylation; *MS* mass spectrometry; *Ac* acetylation; *n.d.* not detected; *ACTH* adrenocorticotropic hormone; *IGF* insulin-like growth factor; *GNRH* growth-hormone-releasing hormone; *GH* human growth hormone; *EPO* erythropoietin; *LH* luteinising hormone; *hCG* human chorionic gonadotropin; *PIIINP* procollagen III N-terminal propeptide; *HIF* hypoxia-inducible factor; *MGF* mechano growth factor; *PDGF* platelet-derived growth factor; *FGF* fibroblast growth factor; *HGF* hepatocyte growth factor; *VEGF* vascular endothelial growth factor; *PIGF* placental growth factor

[a] Subject age between 20 and 40 years

biomolecular interactions using antibodies it is therefore important to know precisely how these immunoassays operate in order to fully understand the data produced.

Measuring biomolecular interactions

As stated above the measure of several protein hormones that are relevant in the context of anti-doping analysis is performed through immunoassays in which multiple incubations, washes and biomolecular interactions are involved. In essence this procedure resolves, in a stepwise manner, the successive hurdles posed to detecting the molecule of interest. The incubation with the secondary antibody (and subsequent steps) is especially necessary to enable detection of the analyte or to improve the sensitivity in the detection of the primary interaction. Thus, if interactions could be measured directly, without the need for labelling, or labelled molecules, the secondary antibody incubations would not be necessary and the interpretation of the results would be straightforward. This is one of the main reasons for the successful implementation of techniques such as atomic force microscopy (AFM), quartz crystal microbalance (QCM) or surface plasmon resonance (SPR). The technique of AFM was invented in 1986 and is considered one of the most sophisticated tools to scan nanoscaled surfaces [45]. A cantilever with a nanometer-sized tip is brought into proximity of a given surface and the forces, from either attraction or repulsion, result in a deflection of the cantilever according to Hooke's law [46]. If the cantilever is moved over the surface the deflection is translated into a surface image. One of the appealing applications of AFM consists in the immobilisation of a limited (all the way down to a single copy) number of molecules at the tip of the cantilever and bringing this in contact with a surface of complementary molecules. The forces derived from the breaking of the interaction can be translated into presence/absence of binding and the kinetic parameters of the interaction. However, the laborious cantilever–antigen preparation procedure, the fact that both entities are somehow surface bound, the limited scanning area (μm^2), and scan rate have impeded a broader application of this approach in the field of biomolecular interaction imaging.

A distinct principle is used in QCM in which changes in mass per unit area are detected through a change in frequency of a quartz crystal resonator. First described in the 1970s for gas-phase sensing, the technique has more recently been adapted to enable measurements in liquid too [47]. Given the piezoelectric properties of quartz, an alternating current induces oscillations that produce a standing shear wave with a quality factor in the range of 10^6. As a result of this narrow resonance the precision in the determination of the resonance frequency is very high which in combination with the high resolution (about 1 Hz on a 5-MHz scale) renders this technique suitable to measure minor changes at the surface. Although QCM is very promising it has only initiated its impact in the field of biomolecular interactions and applications are so far few [47–49] in comparison to other biosensing techniques. SPR has a more established history. The first cognizance of SPR dates back more than one century [50], but it was not until 1968 that two independent studies by Otto [51] and Kretschmann [52] addressed the phenomenon in a comprehensive manner. In the current SPR instruments the Kretschmann configuration is employed in which a metal film is evaporated onto the glass surface, rather than having two independent (glass and metal) entities. The technique relies on an optical phenomenon in which light photons interact with metal plasmons resulting in a diminished intensity of reflected light. The photon-to-plasmon interplay depends heavily on what is attached to the metal surface, and for an immobilised molecule the interaction with a complementary molecule (e.g. immobilised antibody with its antigen) will result in an alteration of

the conditions at which the photon-to-plasmon interplay occurs. The virtue of this technique, basically introduced in 1989 by a Swedish three-party consortium, is the ability to measure the interaction in real time, under dynamic and near-physiological conditions, without the need for labelling. In addition, the introduction of simultaneous multichannel measurement allowed for the inclusion of a reference surface measurement that granted tremendous credibility to the observed interactions.

Surface plasmon resonance

As indicated above SPR was one of the first techniques to be exploited for the direct measurement of interactions and this has greatly expanded specific knowledge on lipids [53], antibiotics [54], membrane proteins [55], antibodies [56] and carbohydrates [57], to name a few, and how they recognise complementary molecules. This technique has also found wide acceptance in the pharmaceutical industry, for example in the characterisation of therapeutic antibodies [58], or in fragment screening assisted by NMR spectroscopy [59]. This is principally because one of the major manufacturers of SPR instruments has rendered the complex technology in a user-friendly ready-to-execute medium-throughput format that swiftly produces data.

As indicated earlier SPR is based on an optical phenomenon that employs monochromatic, plane-polarised light. When this light hits a boundary through a prism in which the refractive index at the other side of the boundary is lower than that of the material from which the beam proceeds, light will be partially refracted and partially reflected (indicated in Fig. 1 with the solid line and angle θ_1). At a particular critical angle of incidence, light will no longer be refracted and we speak of total internal reflection (TIR). This is indicated in Fig. 1a by the dashed line and θ_2. The detection in SPR is based on the reflected light. Thus, light source and detector are at the same side of the prism (Fig. 1b).

Under conditions of TIR incident light photons from a monochromatic and plane-polarised beam still penetrate about a quarter of a wavelength into the next medium and may excite in a resonant manner the electron charge oscillations in the metal layer that covers the glass surface. This surface plasmon resonance occurs, under TIR, only at a particular angle (the SPR angle θ), when the impulse of the plane-parallel vector matches that of the plasmon. Hence, the plane-perpendicular part dissipates as evanescent wave into the medium opposite of the light source as indicated in Fig. 1b. The evanescent wave interacts with the medium into which it penetrates and the extent to which this happens depends largely on the nature of the medium. As a consequence the SPR angle will also be determined by the nature of this medium. A logical effect of the fact that photon energy is transformed into plasmons and an evanescent wave is that the reflected light intensity at θ will diminish.

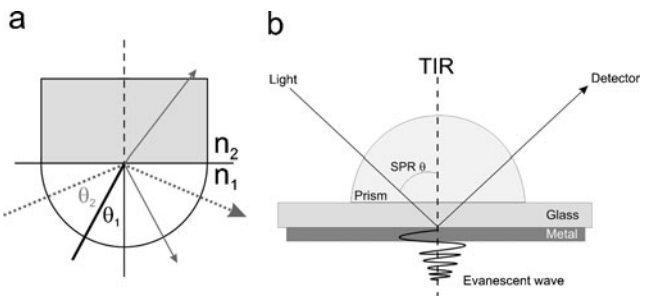

Fig. 1 **a** Schematic of light penetrating through a medium of refractive index n_1 to refractive index n_2. **b** Kretschmann configuration that is still in use for SPR measurements

Most SPR instruments do measure θ of the reflection minimum in time. Figure 1b shows the schematic set-up of an SPR instrument with the light source, prism and detector on one side of the gold surface (gold is used in most cases, but silver, copper, titanium, chromium and aluminium also support surface plasmons), and the evanescent wave on the other. Immobilising any molecule onto the metal surface will change the properties of this medium and as such the θ of the reflection minimum. In this respect a change of 0.0001° in θ corresponds to 1 pg of material deposited on the surface (standardised for globular proteins and expressed as 1 resonance unit or RU) and the current instruments can measure down to 10–15 RU of substances with fairly low molecular weights (~500 Da). Unfortunately, this does not necessarily mean that with as little as 15 pg of material an interaction can be observed, but it indicates the resolving power and capability of SPR.

In addition, the physical separation between the reflected light measurement and the phenomenon that affects the angle of minimum reflection has permitted an instrumental design with a continuous solvent delivery in which sample injection and regeneration can be alternated. This configuration is tremendously practical as a single surface can be employed to produce several measurements, provided the generation is optimised to release non-covalently bound analyte from the surface without affecting the integrity of the covalently bound ligand. Figure 2a–d shows how a typical sensorgram is obtained on a single flow cell, demonstrating that repetitive measurements using the same immobilised ligand are possible. In every panel the arrow indicates the incident light under TIR, the centre part the sensor chip with a metal–glass construction and a fluidic circuit through which sample is delivered. At the bottom the sensorgram is drawn as a function of time. In Fig. 2a the situation is depicted in which only buffer flows across; in panel b the injected analyte reaches the sensor surface and the SPR θ changes; in panel c the analyte injections are finished

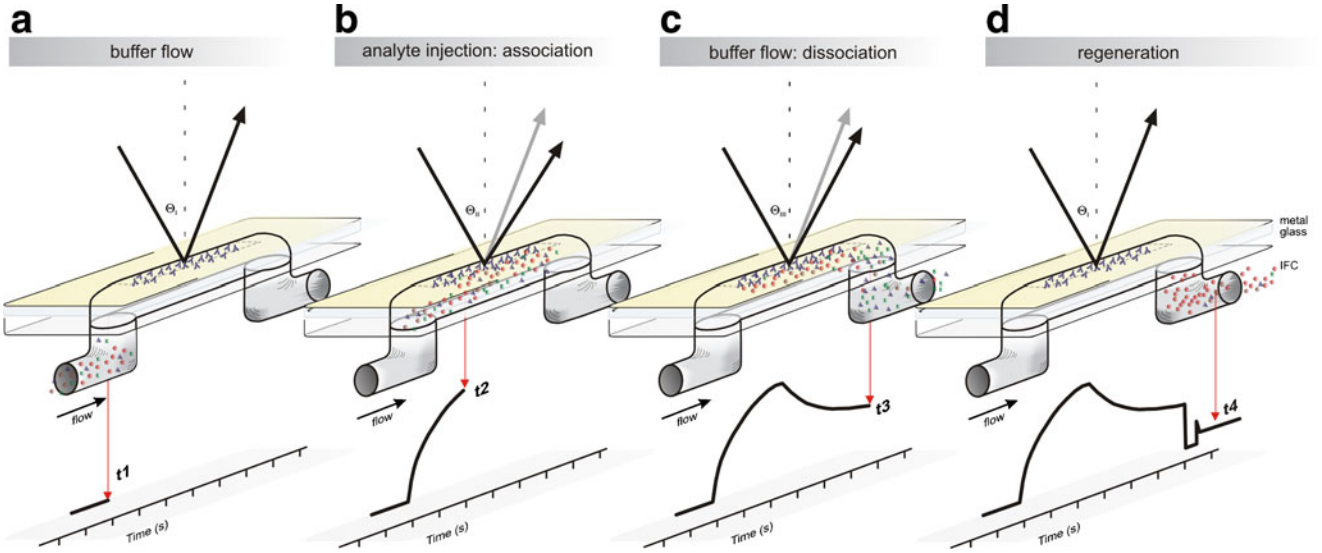

Fig. 2 **a**–**d** Schematic representation of how a sensorgram is generated

and all non-specifically bound material is eluted away with again a change in SPR θ; Finally, in panel d the surface is regenerated returning to the initial state and SPR θ.

Moreover, depending on the manufacturer and instrument model several flow cells are operated in series or in parallel, enabling simultaneous measurements of interactions between a single analyte and distinct ligands. A maximum of 400 simultaneous measurements can be achieved (flexchip from Biacore) but in this matter the total number of flow cells is not nearly as relevant as the fact of having two as it means that the non-specific binding can be addressed. In this setup, a representative ligand that does not display specific binding is immobilised in the first flow cell and the specific ligand is attached in the second flow cell. Both surfaces can display non-specific binding of the analyte, especially if this consists in a complex mixture of proteins, whereas the specific ligand can also display specific binding to the analyte. Subtracting both interactions as they occur provides a direct measure of the specific interaction in real time. Figure 3 displays how this differential measurement results in a specific sensorgram. Panel a displays the individual flow cells and the corresponding sensorgram for the non-related ligand (reference flow cell) and the specific ligand (ligand flow cell). In panel b both sensorgrams are displayed in overlay and the subtracted trace in which non-specific binding and buffer-induced bulk refractive index changes are eliminated, displaying only the response corresponding to the true binding event in which the height of the signal corresponds to the absolute amount of material bound during the experiment and the shape of the sensorgram is dictated by the kinetics of the interaction. Either feature is of utmost value when it comes to the characterisation of a given antibody that is used in a single immunoassay, or when the comparison of different immunoassays is required.

SPR-assisted immunoassays

One of the first glycoproteins targeted at a worldwide level for anti-doping purposes was erythropoietin (EPO). Until 2000 the tools used to assess the suspected illicit use of this substance were based on blood parameters such as the concentration of haemoglobin (a colorimetric assay) or the haematocrit (proportion of blood volume that is occupied by red blood cells). In that year the French team headed by Prof. de Ceaurriz published an application of the isoelectric focussing principle for EPO [36]. Using an ultra-concentration and an ingenious double blotting system they demonstrated that the recombinant and endogenous (i.e. urine-secreted) EPO have different isoelectric focussing profiles that may largely be explained by the sialic acid content present in either molecule [38, 39].

In the first blotting procedure a specific monoclonal anti-EPO antibody is employed that was produced nearly 30 years ago when the amino acid sequence of the purified endogenous material was first sequenced [60]. This antibody has shown an unmatched sensitivity to this day and is still in use. Even though the antibody is directed against the N-terminal 26 amino acids (Fig. 4c) the optimal use of this immunoglobulin required prior denaturation which was extremely well exemplified through SPR monitoring of the interaction between the ligand and the analyte directly. In Fig. 4a the interaction between native recombinant EPO and this antibody is depicted in green. For comparative purposes the interaction between the same molecule and a polyclonal anti-

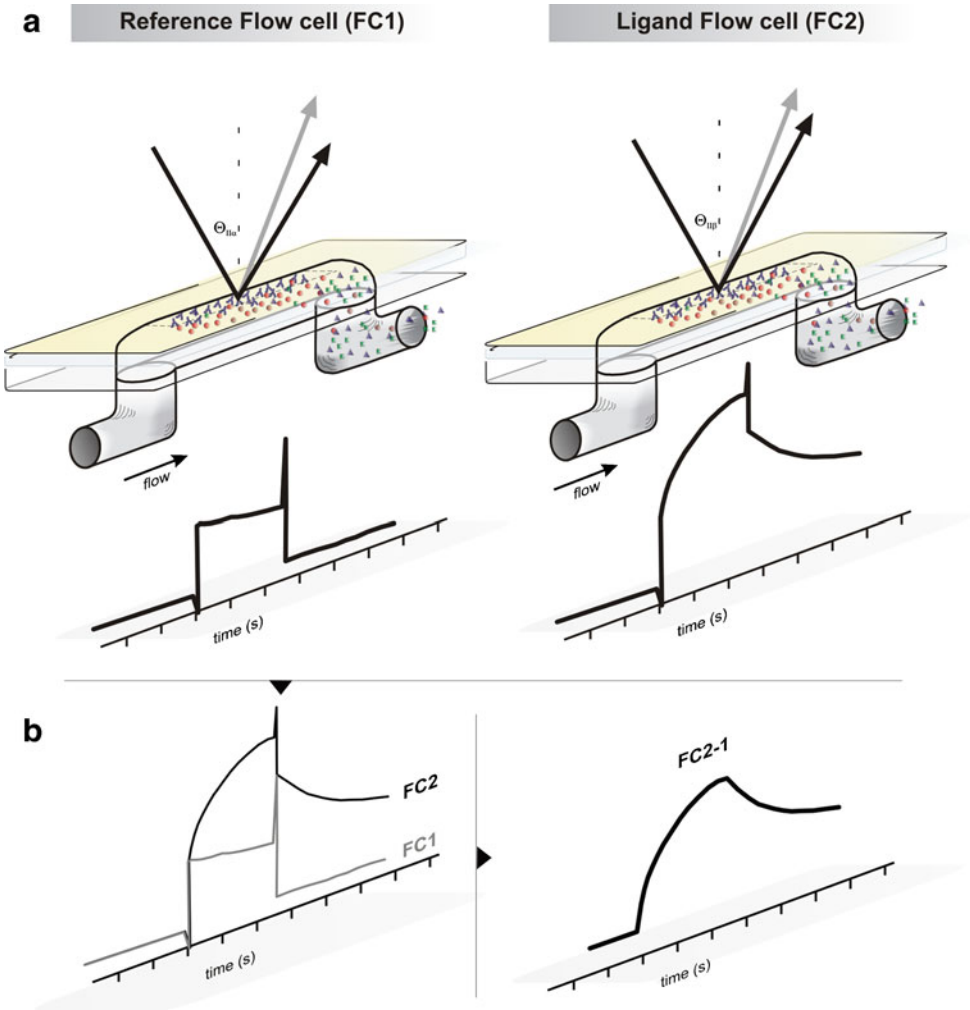

Fig. 3 Schematic representation of the simultaneous and differential binding. Panel **a** depicts the two flow cells and panel **b** the overlaid (*left*) and subtracted (*right*) sensorgrams

EPO antibody, used for standard EPO measurements (in blue), is included, yielding approximately 50 RU. Clearly no signal is observed for the green trace, indicating no binding whatsoever, whereas the blue trace shows a weak but distinctive response. Even though the response for the blue trace was very clear-cut, this corresponded to approximately 2% of the maximum response at saturation, providing some insight into the orientation of this polyclonal antibody at the surface and the difficulty of recognising EPO in the native conformation. Figure 4b displays the interaction of the same rEPO after reduction, alkylation and filtration (beginning from the same initial concentration) where clearly the relative responses are inverted. Whereas the blue trace still represents the same 2% of maximum response (approximately 60 RU), that for the monoclonal antibody has drastically improved yielding not only a very strong signal of 510 RU (~15% of the maximum response) but also an extremely stable interaction as judged from the near-zero dissociation in time. A careful evaluation of the peptide sequence used for the generation of the antibody revealed that the original sequence contained two mistakes; position 7 and 24 were erroneously identified as Asn7 and Lys24 which should have been Cys7 and Asn24 (see Fig. 4c). These two amino acids are apparently not crucial for the recognition by the antibody (judged from the SPR response) but represent one partner in a disulfide bond (Cys7) and an occupied N-glycosylation site (Asn24), rendering these two residues crucial in the tertiary structure of the native molecule and as such very important, at least from the point of view of approaching the antibodies' epitope.

A second field in which SPR has and still plays an important role is the development of anti-doping analytical methods for human growth hormone (GH). The first steps in the direction of what is today the operational protocol [61] were published more than one decade ago when Dr. Strasburger and co-workers described the use of two parallel immunoassays for a single sample to detect GH [62]. According to the readings of each assay a ratio was computed to detect use of recombinant

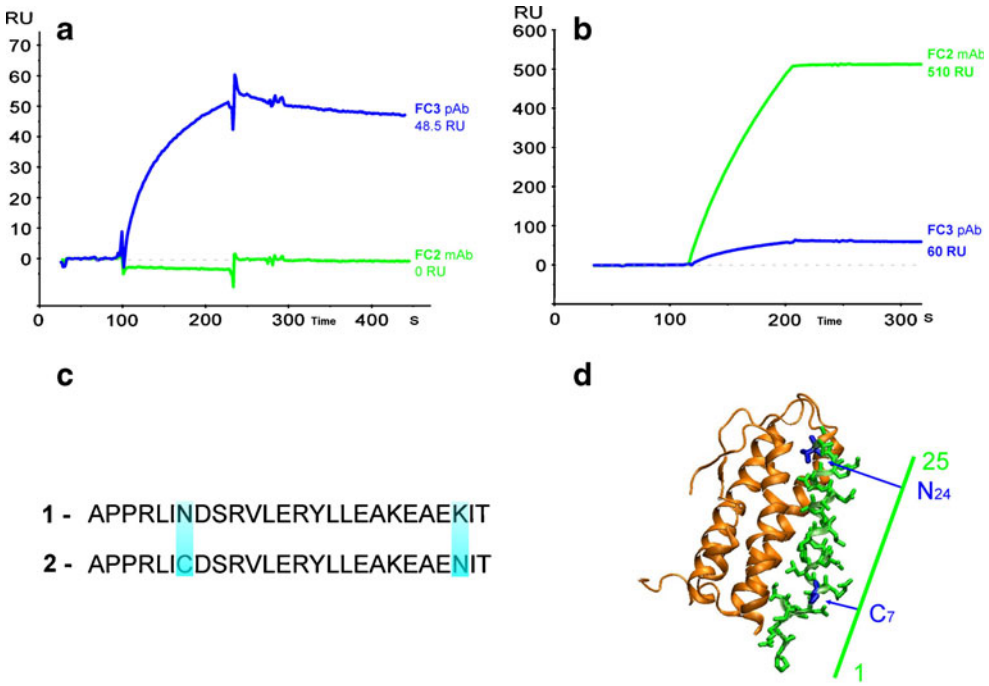

Fig. 4 SPR sensorgrams of the interaction between recombinant EPO and two different antibodies. **a** Interaction for native rEPO. **b** Interaction after reduction and alkylation. **c** N-terminal 26 amino acids of EPO (*1*) and the sequence used for immunisation (*2*). **d** Tertiary structure of EPO

human GH. Also the family of pituitary GH comprises several members [63]: the most abundant isoform consists of a single chain, 191 amino acid, 22-kDa protein that contains two disulfide bonds and no glycosylation. The second most abundant, but only representing approximately 5–10%, is a splice variant in which the amino acids 32–46 (in the 22-kDa sequence) are not included. Other variants include point mutations, N-terminal acetylation or glycosylation; the last of these modifications only contained a single core-2-type sialylated O-glycan [64]. Finally, a considerable portion (ca. 25%) consists of dimers, oligomers and so-called non-dissociable entities that could be established through disulfide bond crosslinking [65]. In circulation the relative proportions of the different pituitary variants are different and also the presence of GH binding proteins plays an important role. Ultimately, the main differences are a significant decrease in the proportion of 22 kDa and the appearance of fragments, allegedly of proteolytic origin and produced in circulation [66, 67]. The relative proportion in circulation is different between male and female but appears to be stable over time, under conditions of food intake, fasting and exercise, and does not display significant variations in different populations. Given the total number of distinct isoforms, aggregates and complexes it is not surprising that conventional immunoassays for GH display a high level of disparity in the comparative results [68]. Precisely this heterogeneity is the basis for anti-doping assays as the recombinant pharmaceutical expressed principally in *Escherichia coli*, but also in CHO cells, is a single 22-kDa variant. As such, the administration of this product alters the relative ratio of the different GH variants in circulation both through the introduction of a single variant and also the negative feedback effect on the release of variants from the pituitary gland. Thus, measurement of this recombinant variant through a specific assay and all variants through a second assay should allow for such ratio determination. However, generating an antibody that is specific for one of the variants is not straightforward as individual variants have few unique features. Perhaps the easiest immunogen in that regard is the 20-kDa variant in which the junction between amino acids 31 and 47 (of the 22-kDa sequence) produces a single structural trait. A limited number of monoclonal anti-20-kDa GH antibodies are known and available to the scientific community and comparison of their binding properties exemplifies what is also applicable to other antibodies that address a single analyte within a family of related molecules. Figure 5 shows five overlaid sensorgrams corresponding to the analysis of a single 20-kDa GH injection over five monoclonal anti-20-kDa IgGs produced in four different laboratories. All antibodies were deposited at similar densities on the SPR sensor surface for precise comparison. All antibodies appear to bind the analyte with similar strength, judged from the similar slopes in the dissociation phase. However, the large differences in the association phase indicate striking differences in avidity and possibly binding capacity. Whereas the former is a strict binding property of the antibody that will dictate to a large extent what minimal incubation times are required, the latter may reflect the result of an inappropriate immobilisation chemistry that renders a significant portion in a non-optimal orientation. In the standard immobilisation procedure used for SPR, the primary amine functionalities in a given molecule are targeted. Figure 5 highlights the residues with a primary amine in an IgG molecule (1IGT) demonstrating that such an immobilisation

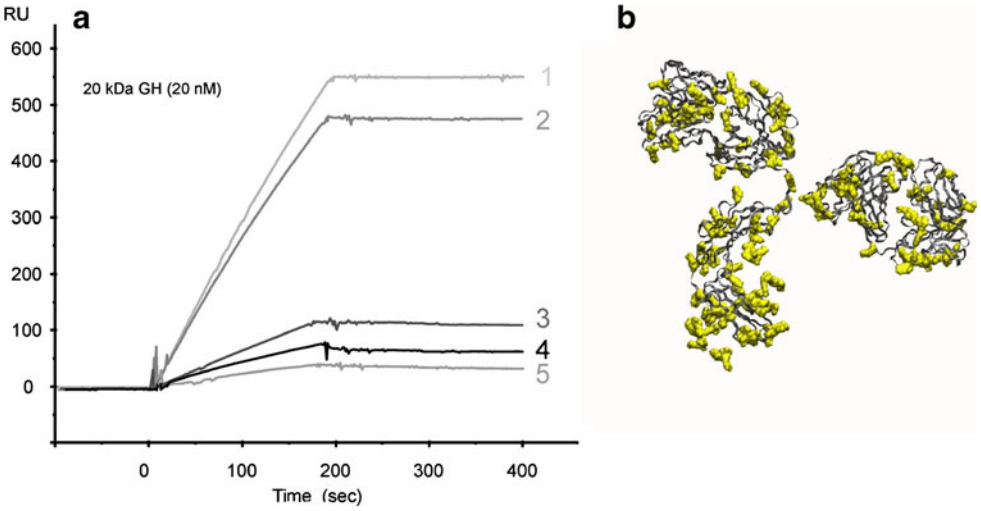

Fig. 5 **a** SPR sensorgrams of the interaction between recombinant 20-kDa GH and five different anti-20-kDa GH antibodies. **b** X-ray structure of an IgG (1IGT) is depicted with all lysine and arginine residues highlighted in *yellow*

procedure most probably renders a random distribution of differentially oriented molecules at the surface which is allegedly also the case in the preparation of immunoassays where mostly the passive adsorption procedure is applied.

As such, the results obtained for this type of SPR experiment can be extrapolated directly to what will occur in a plate- or tube-based immunoassay. With the clear example of antibodies to the 20-kDa GH variant, it is not difficult to imagine that some antibodies to the other, more akin, variants will display more licentious binding characteristics and that not every variant shall attach and detach similarly. As such, in a "black box" experiment, as is an immunoassay, only the final outcome of the delicate interplay between immobilised antibody and the different variants, present at different concentrations, with different affinities and avidities to both the antibody and the growth hormone binding proteins will be observed, whereas the alteration of one of these components may lead to a different result. If all variants are available as well-defined structural entities one may embark on assaying all separately, preferably using a technique that is capable of monitoring the interaction directly. Figure 6 displays the kinetics experiments performed on two different monoclonal anti-GH antibodies using a number of GH variants from either pharmaceutical origin, produced through recombinant technologies (20 kDa) [69], or produced through limited proteolysis [67].

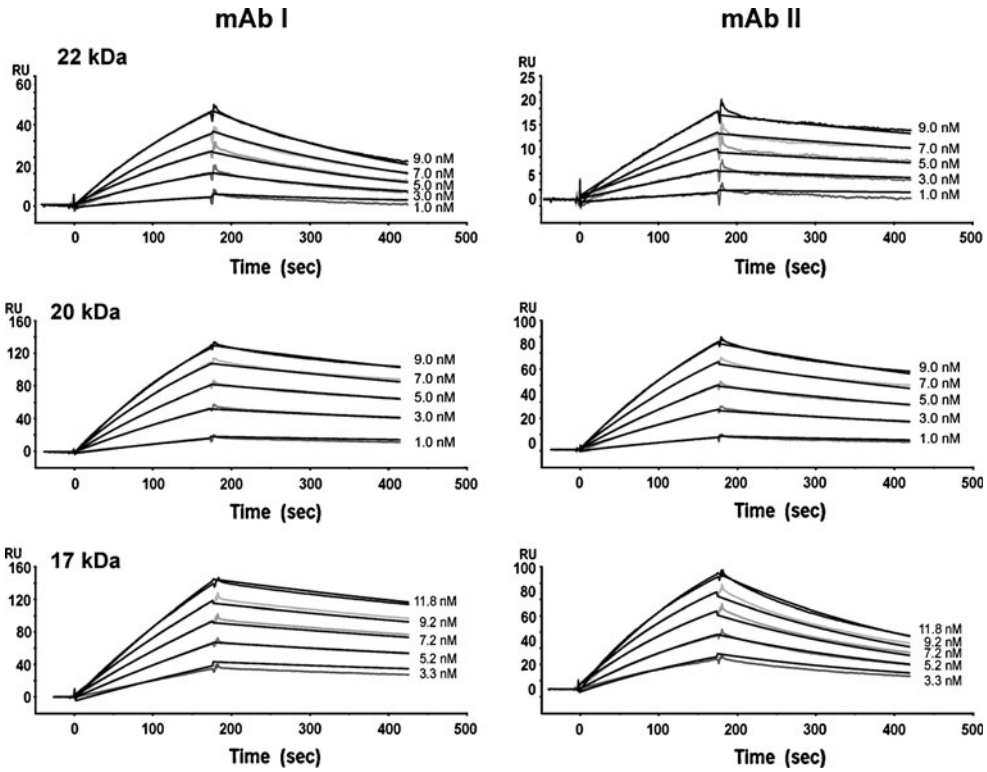

Fig. 6 SPR sensorgrams corresponding to kinetic experiments of two different antibodies (mAb I and mAb II) with 22-, 20- and 17-kDa GH

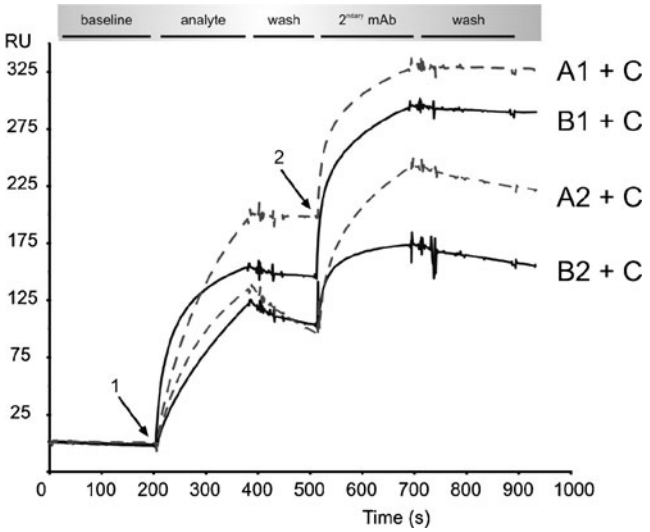

Fig. 7 SPR sensorgrams corresponding to a simulation of a differential immunoassay employing a single secondary antibody and up to four different primary antibodies

From a straight comparison of both antibodies it can be clearly seen that antibody I binds faster to the 17- than the 20-kDa variant albeit both to approximately the same extent. In contrast the 22-kDa variant is less well recognised and also with lower affinity. In comparison, antibody II binds less well to the 22-kDa variant but with the highest affinity given the shallow slope of the dissociation phase. Both 20- and 17-kDa variants are better bound, but the latter dissociates readily and both faster than the 22-kDa variant. With this example it may be clear that the direct monitoring of interactions facilitates to a great extent the understanding of how the antibodies work with respect to the different analytes. Moreover, multiple binding events may be programmed in an SPR setting providing a simulation of the final immunoassay. Figure 7 displays such a setting and in each sensorgram two association phases and two dissociation phases are present. The injection time point of the analyte is indicated with a "1" and the injection time point of the secondary antibody with a "2" . A well-defined mixture of analytes flows across four different antibodies (at similar immobilisation levels) yielding the response for the capture antibody. The shape of the sensorgrams, indicative of the affinity, implies that antibody B1 is reaching saturation while the others are not (the slope of the signal in the initial phase of the first injection is the steepest and starts levelling before the others do, indicating approximation to equilibrium binding or saturation under the given conditions). This feature needs to be taken into account when setting up the assay to ensure that the binding capacity will not be the limiting factor. The different dissociation rates dictate the speed of processing in the intermediate steps of the immunoassay. Clearly, the dissociation rate constant for A2 is detrimental in the case of prolonged incubations. Finally, the incubation with the secondary antibody provides similar information but on a more dynamic surface as it builds on a dissociation phase. In this application it becomes evident that the binding of the analyte by B2 does not provide the best exposure for binding to C given the lowest increment in response units.

This will evidently also condition the final sensitivity of the assay. As such, the total evaluation of the immunoassay, dissecting the components, provides extremely valuable information, not only on the assay performance but also on which steps are amendable for improvement.

A last example of the use of immunoassays for anti-doping purposes is human chorionic gonadotropin (hCG); the analysis of this hormone can be performed by a variety of different assays, provided by distinct manufacturers, which have been put in place to analyse this molecule within a clinical context that ranges from the simple confirmation of a pregnancy to the detection of certain fetal chromosomal abnormalities, gestational trophoblastic disease, or choriocarcinoma. As with most proteins hCG also comprises a family of molecules. Functional hCG is a non-covalently bound αβ-heterodimer in which both subunits are glycosylated, generating a number of different glycoforms [70]. The β-subunit can be found free in circulation, but hyperglycosylated (an additional~5 kDa in triantennary complex-type N-glycans and larger O-glycans [71]), nicked hCG, nicked β-subunit and β-core hCG can also be found [72]. It is therefore not surprising that numerous studies have appeared in the literature in which the different methodologies have been compared, either pairwise [73] or comparing more methods. In all reports the variation encountered consistently ranges from approximately −40% to +40% [34, 74, 75]. Attempts have been undertaken to assess these differences by employing proficiency testing material [76], calibration material from WHO or isolated, well-purified isoforms [33] without a clear answer to what is measured by which assay. Surprisingly, fairly little has been done in this field using surface plasmon resonance. In 2005, the group of Prof. Homola described the development of a novel SPR imaging system for high-throughput screening of biological samples including hCG [77]. This prototype, based on the combination of polarisation contrast imaging and a spatially patterned multilayer SPR arrangement, consisted of 108 sensor channels capable of monitoring all these simultaneously. In that paper conventional immobilisation chemistry for antibodies was employed and hCG was detected at concentrations of 500 ng/ml. In 2010, the same group published an update in which the total number of sensor channels was increased to 120 (0.2×0.15 mm, each) and the immobilisation chemistry for the antibodies varied (sensor surfaces were microspotted with short oligonucleotide sequences and antibodies were conjugated to the complementary sequence for clean immobilisation procedures). Using diluted blood samples (10%) they established detection limits for hCG of 100 ng/ml, nearly reaching the lower limits of endogenous concentration in circulation (see Table 1) [78].

This latter example indicates that the direct application of SPR for either clinical or anti-doping purposes appears to be merely a matter of time.

Direct application of SPR for anti-doping purposes

In the previous section the introduction of an SPR imaging system was illustrated by the application to hCG. In the past few years other applications have emerged. A Spanish manufacturer of SPR instruments (Sensia S.I.) has recently focussed on the analysis of pituitary hormones. One of the hormones addressed is GH and the method is both different and elegant. Their approach consists in the covalent immobilisation of recombinant GH on the sensor surface and subsequent binding of a non-specific anti-GH antibody to the immobilised GH. The biological specimen then flows across this prepared surface and the signal reduction is indicative of the presence of GH [79]. Trevino et al. employed spiked serum samples in the concentration range between 4 ng/ml to 40 μg/ml and established the LOD as 6 ng/ml. Overall, this assay displayed good inter- and intra-assay variability (<5%) and the chip to chip reproducibility was claimed to be excellent. The approach, distinct in the way that the displacement of the antibody is measured rather then the interaction with the antibody itself, adds robustness as the regeneration of the surface affects the immobilised 22-kDa GH, which has proven to be compatible with repetitive pulses of a wide array of harsh chemical conditions such as HCl at pH 1 or 2 M NaCl at pH 11. Even though the approach may be employed in a clinical setting it has a few drawbacks. One is evidently the continuous consumption of antibody which is higher than in a standard well-per-sample antibody consumption for immunoassays. The main limitation for doping purposes, however, resides in the sensitivity. Although 6 ng/ml may well be within the diurnal fluctuations of circulating GH levels it remains too high to pick up the lower basal values as established for a larger cohort [61]. Further work by Trevino et al. addressed, in addition to GH, thyroid stimulating hormone (TSH), follicle stimulating hormone (FSH) and luteinising hormone (LH) in both serum and urine [80, 81]. In a similar approach the recombinant molecules were covalently immobilised on individual sensor chips and samples were spiked with the hormone of interest. Evidently, the detection limit for GH remained the same (vide supra) but that for LH was established as less than 3 ng/ml, a value that is well below the normal concentration range found in healthy individuals (Table 1). Given that this method was applied to both serum and urine its implementation for anti-doping purposes should be facile if a medium to high throughput can be guaranteed. With a cycle time for each sample of 3,000 s (5 min) and a demonstrated robustness of 100 cycles per sensor surface the first, promising steps have been made.

A different class of molecules has recently been addressed through SPR by the group of Profs. Botrè and Mazzei [82]. They optimised the sensor surface preparation to minimise non-specific adsorption that could foul the desired readout through the immobilisation of a hydroxyl/thiol-functionalised polyamidoamine dendrimer encapsulated gold nanoparticle that, in turn, was submitted to a thiol–hydrazide conversion for the conjugation to a desired protein. In their description they focussed on insulin that was coupled using the conventional amine coupling reaction. Following the same principle as described for the pituitary hormones the insulin surface was subsequently swaddled with an anti-insulin antibody and subjected to a competition with diluted serum samples from healthy control subjects and diabetic insulin-treated patients. Experimental findings were matched against a radioimmunoassay evaluation demonstrating a superior sensitivity (0.5 pM) for the SPR-based approach.

A further indication that SPR is slowly finding its way into the most stringent analytical environments, represented by anti-doping laboratories, is that the first approximation for the detection of non-protein material was published recently [83]. Using the most conventional approach, i.e. immobilising an antibody and flowing across an untreated urine or saliva sample, Frasconi et al. analysed cortisol and cortisone with detection limits of 2 and 9 μg/l, respectively, demonstrating that smaller molecules can also be addressed. Moreover, the SPR analysis results correlated well with those of a conventional LC-MS approach, rendering the immunosensing approach, with a 15-min total cycle time (no sample preparation required) and at least 100 cycles per antibody, an attractive alternative for the future.

The versatility of SPR-based biosensors for anti-doping purposes is evident from the fact that for a relatively new topic such as gene-doping the potential application of the technique has already been described [84]. In an applied study the same authors set up a hybridisation-based SPR sensor for two markers of transgenosis, i.e. the enhanced green fluorescence protein gene and the promotor of cytomegalovirus, and applied this approach to transfected cultured cells and a QCM instrument [85]. This approach still requires several hurdles to be overcome to allow its potential application, but already it opens an avenue to tackle the problem of traceability of transgenosis.

Concluding remarks

Surface plasmon resonance as a direct sensing technique has come a long way since it was introduced nearly three decades ago. Initially engineered to monitor the interaction between two proteins, ideally an antibody and its antigen, SPR has been employed in many other settings covering possibly the entire spectrum of entities that may engage in some kind of

interaction. For the anti-doping community the lack of sensitivity, due to the instrumental design employed by most manufactures, has impeded an earlier recognition of its potential. One commercial SPR instrument allows a single 3D sensing surface to be functionalised with as much as 20 ng of immunoglobulin, which is equivalent to a binding capacity of approximately 300 fmol if all IgG molecules are fully functional after immobilisation. Although this would be sufficient to capture all endogenous GH from 3 ml of serum even the best binding antibodies require solutions at K_D concentrations (~100 pM) to effectively monitor the interactions. It seems that the sensitivity improvement required for direct monitoring may be achieved in the near future. Meanwhile, the initial contributions of SPR have taken place mostly at a secondary level to better understand and develop new immunoassays through the determination of the binding profiles (specificity, surface properties, robustness, kinetics, effect of antibody labelling, etc.) of the IgGs. The increasing number of recent scientific reports indicate that several research groups have realised that this technique also harbours promise for the direct fight against doping not only for protein hormones but also for the steroids and gene doping.

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