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REVIEW

Direct optical detection in bioanalysis: an update

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Abstract In biomolecular interaction analysis, direct optical detection is attracting increasing interest in academia and industry. Therefore, a previous review has been updated. Optical principles are given in brief, focussing especially on modern and frequently used techniques. Commercialized methods are listed with some specific applications. In addition, some of the many applications found in the literature are listed; others which have been reviewed elsewhere are cited. Overall, the growing interest in direct optical monitoring of biomolecular interaction is demonstrated and future trends are outlined. Because optical methods is a very wide field, the paper concentrates on the currently most common methods, microrefractometry and microreflectivity.

Keywords Optical biosensor · Refractometry · Reflectometry · Transduction · Optical monitoring

Introduction

A recent article in Science "Who needs labels?" [1] was the reason for considering the development of direct optical sensing during the past five years as an update to a previous review [2]. Especially, the growing interest in quantitative monitoring of protein–protein interactions, in miniaturization and parallelization, and in increasing applications make approaches of direct optical detection in bioanalysis competitive with fluorescence techniques which use labelled compounds. Thereby, besides innovations in optical techniques and improvements of transduction platforms, the potential application of such techniques to a huge variety of bioanalytical problems is the main innovation. Therefore, quite a few reviews have been published in recent years, either reviewing the different transduction methods [3] or reviewing applications and discussing trends [4–8].

In addition to analytical microarrays based on labelled compounds [9, 10], many direct optical techniques for microarray analysis have been reviewed. Loss of bioactivity and cost of labelling are increasingly regarded as disadvantages of fluorescence arrays, especially going from immunosensors to biosensors dealing with nucleic acids and whole-cell systems, besides protein–protein interactions. Therefore, for higher throughput, protein microarrays and, in proteomics [11, 12], direct optical techniques have had their first applications. Stimuli-responsive applications are certainly preferably performed by using direct optical techniques [13]. These will also help to obtain more information in the signalling chain or to discriminate between agonists and antagonists [14].

Quite a few systems using direct optical detection are now commercially available [7]. Commercialization started when Biacore entered the market [15, 16], using the surface plasmon resonance (SPR) technique [17, 18]. Accordingly, in recent years this technique has opened the market and, as will be discussed later, a large number of companies supply different instrumentation using various optical detection techniques.

Non-optical screening approaches [19] relying on other label-free techniques for example impedance spectroscopy, acoustic systems, or micro-electro mechanical sensors, will not be discussed here. Also, techniques such as FTIR [20],

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FTIR Microscopy [21], ATR-FTIR [22], Raman spectroscopy [23], surface-enhanced Raman spectroscopy [24], terahertz spectroscopy [25], circular dichroism [26], and the optical read-out of cantilevers [27] are not a subject of this paper. FTR chemical imaging is also a strongly emerging technology [28]. However, space restrictions force the author to concentrate on techniques known from direct optical sensing and based on refractometry and reflectometry in the UV–visible range.

Optical principles

Fluorescence-monitoring-based instrumentation relies on equipment well-established commercially, uses standard procedures and assays, and achieves a very low limit of detection, even down to single-molecule detection. For this reason, most routine assays use fluorescence read-out or plate readers. In pharmaceutical companies and in clinical diagnostics, this approach is commonly used. However, as mentioned before, expenditure and costs, and problems with bioactivity are a disadvantage in combination with the problem of photo-bleaching which normally does not allow time-resolved monitoring for evaluation of kinetics.

In contrast, this type of monitoring can easily be done with direct optical techniques which at present allow even the use of direct assays in which reagents are no longer necessary; this is an advantage in many routine applications [29–31]. The disadvantage is the problem with non-specific binding and the higher limit of detection compared with fluorescence techniques. However, photothermal refraction, known for many years [32, 33], is regarded as overcoming problems of high limits of detection and could even achieve detection of few molecules in very small volume elements.

Direct optical detection methods have been reviewed quite frequently in recent years [2, 3, 8]. The optical principles have been presented in textbooks [34], handbooks [35], and monographs [36–39]. In general, these optical techniques can use optodes and measure colour changes, but they are more generally applicable using direct reflection techniques. These can be classified according to two principles—microrefractometry and microreflectometry. This means the spectroscopy of biomolecules at the surface is monitored by measuring the interaction between a thin biomolecular layer containing recognition sites and a ligand or an analyte in solution. The radiation reflectance in general measures changes in the so-called optical thickness which is the product of the refractive index n and the physical thickness d of this interaction layer. Fresnel

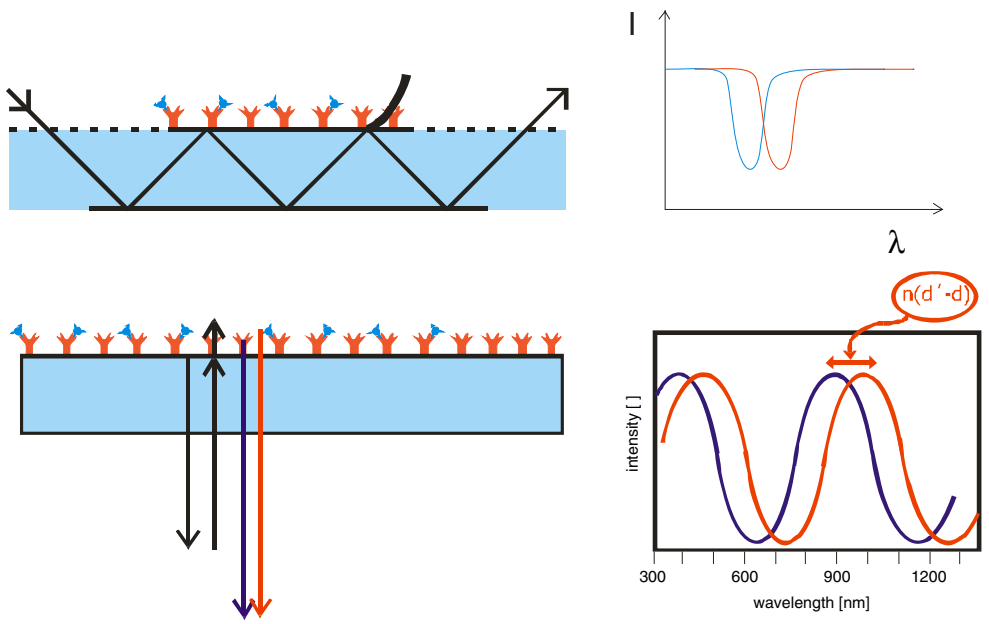

Fig. 1 Microrefractometry: radiation is guided in a waveguide; some methods couple the radiation into the waveguide. Many read-out techniques exist. The electric field vector of the guided radiation couples to an electric field outside the waveguide which exponentially decays into the layer next to the waveguide (in the interaction layer with the shielding biopolymer against nonspecific binding), the biomolecular receptor molecules and the solution with the analyte (ligand) molecules. Any change in this area causes changes in the refractive index influencing this decaying electric field and coupling back into the waveguide. The reflected beam reduces its intensity depending on angle or wavelength. Microreflectometry: radiation is partially reflected at interfaces given by the transducer (reference), the interface to the biomolecular interaction layer (including the shielding biopolymer), and the interface to the solution with the ligand. Interaction changes the physical thickness of the interaction layer and the superposition of the two superimposed partial beams of reflected radiation, shifting the interference spectrum (blue to red)

equations [34] can be used to explain these effects, which either result in changes of the evanescent field at the interface of the optical transducer or in white light interference at this layer.

Looking at microrefractometry, the refractive index of the optical thickness is especially interesting, because exponential decay of the evanescent field into the interaction layer causes inhomogeneous signal penetration of this interaction layer. This enables effects in restricted elements of the interaction layer, or close to it, and the bulk to be distinguished, but reduces the effects at a distance from the transducer surface. Furthermore, the refractive index is rather temperature-dependent which requires very strict temperature control (thermostatting) or very good referencing. Accordingly, surface plasmon techniques control the temperature down to 0.01 K. The principle of this waveguide-based principle is explained in Fig. 1a, where at the interface waveguide/interaction layer the electric field vector (coupled to the guided radiation within the waveguide) decays exponentially into this layer and analyte-containing solution. Among the various realizations of evanescent field effects readout, SPR [39, 40] (Fig. 2a), grating couplers [41, 42] (Fig. 2b), resonant mirror [43, 44] (Fig. 2c), Mach–Zehnder-Interferometer [45, 46] (Fig. 2d), Young interferometer [47] (Fig. 2e), and Bragg gratings [48] (Fig. 2f) are best known in research applications (to name just the most commonly used) and, as Table 2 demonstrates, in commercialization, too. All these optical principles have been frequently explained and reviewed [2–4, 36], therefore Table 1 only attempts to summarize the evanescent field techniques and to classify according to the read-out principle of effective refractive index caused in the waveguide by effects in proximity to the interface waveguide/sample bulk.

In contrast, using microreflectometry, the signal is nearly independent of temperature because a volume increase of the interaction layer with temperature is compensated by a decrease of the refractive index with temperature. In addition, reflectometry concentrates on measuring changes in the physical thickness of this interaction layer, using an approach that is independent of the layer thickness because exponential decay of the evanescent field is not essential for the signal. In principle, white light is reflected at the interfaces of the interaction layer, the reflected radiations superimpose to form an interference spectrum (Fig. 1b) which shifts with wavelength upon changes in the optical thickness (in linear dependence on the physical thickness between a few hundred nanometres and micrometres). Therefore this method is called reflectometric interference spectroscopy (RIfS) [2]. Thus, microreflectometric methods are also predominant in measuring cell-based assays, interaction at thick layers shielding the transducer against non-specific binding, and in arrays without cross-talk from spot to spot. Accordingly, an increasing number of applications are being published (see later).

A family of reflectometric sensors has been developed, starting with white light interference, using the total interference spectrum [49], or measuring just a few wavelengths in the case of parallelized detection in microtiter plates [50], using four wavelengths for chemical detectors [51], and ending with the measurement at a single wavelength together with a reference which also enables modern quantitative imaging techniques [52]. Reflectometric interference turns out to be an optically very robust and simple method which enables direct determination of the interaction of small molecules and gives good results for parallelised measurements on chips, as will be demonstrated below.

A rather special device is the picoscope, which is, in principle, based on correlation of two different interferometer positions [53]. It can be combined with Fabri–Perot,

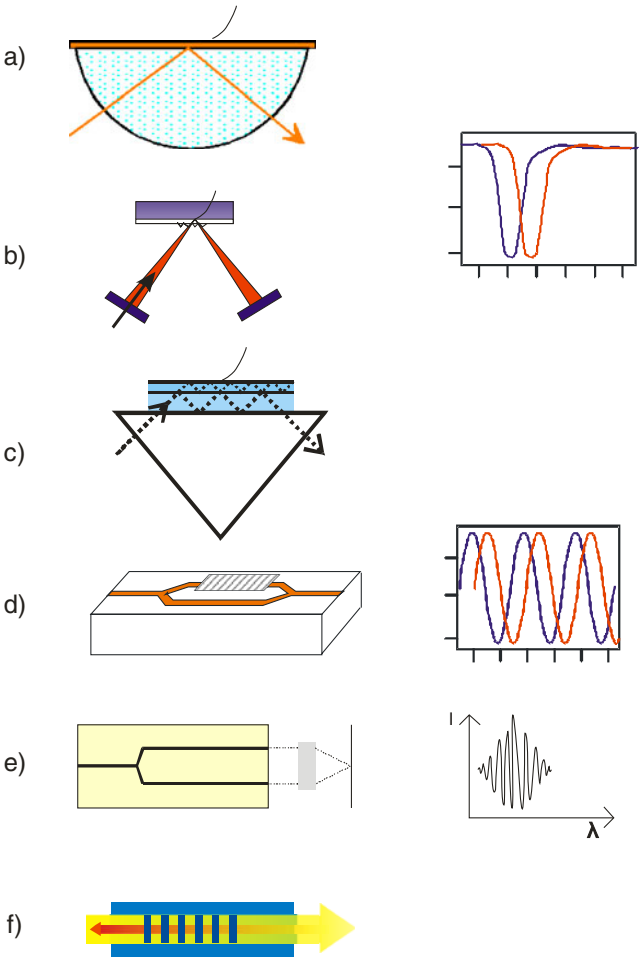

Fig. 2 Schematic diagrams of the different mentioned evanescent field techniques (microrefractometry): **(a)** surface plasmon resonance (SPR); **(b)** grating coupler; **(c)** resonant mirror; **(d)** Mach–Zehnder interferometer; **(e)** Young interferometer; **(f)** Bragg grating

Table 1 Classification of the read-out principles of evanescent field effects for waveguide sensors

Interference effects			Plasmons
Grating	Modes of polarized radiation	Waveguide arms	
Grating coupler (PC)	Resonant mirror	Mach-Zehnder	SPR
Bragg		Young	
Photonic crystals (PC)			
Phase dependency of reflected in and out-coupled radiation on effective refractive index gives at certain angle or wavelength optimum constructive interference (peak). In case of GC, the grating is at the waveguide surface, for Bragg within the waveguide, for PC a 3D variation of effective refractive index exists	Propagation in waveguide different for TE and TM depending on effective refractive index, incidence of 45° polarised radiation, read-out shift in state of polarisation	Measurement and reference arm experience different effective refractive index, either recombination in waveguide or in free space, resulting in an interference pattern, shifting with change in effective refractive index	Thin gold film at interface between prism and sample, incident radiation excites plasmons (electron density fluctuations), resonance reduces intensity of reflected radiation, (dip), shift with change in effective refractive index

Mach–Zehnder or Michelson set-ups, and has also been used as a biosensor.

Commercial instrumentation

Screening of recent literature and of company presentations on the internet reveals a large variety of commercially available instrumentation, as given in Table 2. As demonstrated, especially surface plasmon resonance and grating couplers have been commercialized. Formerly, a resonant mirror system was available commercially; however, the company has recently stopped supporting this system [54]. A recent development of grating couplers is the use of photonic crystals which are used in a commercialized system from SRU Biosystems in their BIND approach [55, 56]. Embedded gratings in fibres, for example Bragg gratings are also a possibility not yet really used in bioapplications, but more in remote sensing and security aspects [48]. Surface plasmon resonance is the method which many companies have commercialized as can be seen in Table 2.

Later than SPR and commercialized by fewer companies, reflectometry started a few years ago. Reflectometric interference is a stripped-down ellipsometry method [101] which uses polarised radiation and is especially useful to characterise biolayers for biosensing [102]. However, ellipsometry is rather complex and not suitable for simple biosensing. Nevertheless, based on this principle Maven Biotechnologies has developed LFIRE (label-free internal reflection ellipsometry) [103], which enables precise, real-time measurement of specific interactions between molecular entities in a microarray or well-plate format and combines the principles of ellipsometry and evanescent wave detection. However, the instrumentation thereby loses the advantages of simple RIfS of negligible temperature dependence. A typical application is given for reversed-phase protein arrays measuring antigen–antibody interaction or glycobiology [104]. ForteBio [105] avoids evanescence problems and utilizes what they call proprietary bio-layer interferometry (BLI). Coated fibres dip into the wells of a microtiter plate in this OCTET system, read-out shifts in interference spectra for 16 wells are simultaneously measured to calculate binding curves [106]. Biametrics [107] intends to bring a family of reflectometric interference instrumentation to the market, covering simple, robust single-wavelength devices, spectral measurements and quantitative imaging systems.

A rather interesting modification uses two parallel waveguides in a chip in which radiation is coupled in by a grating and coupled out by another grating forming a free-space Young interferometer. This principle can be identified in instrumentation commercialized by Farfield in Crewe (UK) [108]. The *Ana*Light instrument series supplies a variety of instruments for biophysics and surface analysis [109]. It is used to interrogate DNA immobilization and DNA−small-molecule interactions in real time [110].

New applications

In the area of evanescent field techniques, surface plasmon resonance applications dominate the literature. Some publications give good reviews [18, 39, 40, 69] and outline future trends [70]. A number of other applications, using specific commercial instruments, can be seen in Table 2. An interesting application using multiwavelength SPR is the study of conformational and electronic changes induced by the electron-transfer reaction in cytochrome c

Table 2 Systems on the market (past and present) with some applications

Company	Optical realization/description of company	Newer published applications of devices
Grating coupler		
Artificial Sensing Instruments (ASI)	Instrumentation uses a precision-sensitive CCD array which avoids mechanical angle scanning for optimum coupling [57, 58]; bi-diffractive coupler [41]; polycarbonate gratings as disposable chips varying either the grating constants across the chip or the waveguide height [59, 60]	
Microvacuum OWLS 210	Optomechanical grating coupler [61]; details of the technology can be found in Ref. [62]	Biomolecular interactions [42]
Corning Epic	Broadband source grating coupler with a high-throughput platform [63]	Examines regulations of cell functions [64] or label-free cell-based screening of GPCR agonists and antagonists [65]
SRU Biosystems	Broadband source coupler [55]; instead of gratings a BIND system (biomolecular interaction detection) [66] is used	Biochemical and cell-based applications [67]; photonic crystals: a platform for label-free biomolecular and cellular assays [56] or DNA detection [68]
Surface plasmon resonance		
BiaCore GE Healthcare	Dual-channel instruments with varying specifications [72]; Biacore Flexchip (array-based parallel kinetic profiling instrument with 400 spots) [72]	Huge database of applications [73]; label-free protein-interaction analysis [16]
IBIS technologies IBIS-iSPR	Developed in the Netherlands at the Twente Technical University [74]; vibrating mirror system [75]	An imaging application is given in Ref. [76]
Texas Instruments, Sensdata Technologies Spreeta	Low-cost miniaturised SPR [77].	Refractive index sensor for a direct assay for human creatine kinase MB (CK-MB, a marker for heart attacks) [78]; monitor endocrine-disrupting chemicals (EDC) in aqueous samples [79]
Reichert Lifescience SR7000DC	Simultaneous 2-channel surface plasmon resonance measurements [80]	Small molecule assay: 4-carboxybenzenesulfonamide (201 Da) binding to carbonic anhydrase II [81].
Bi Biosensing Instruments BI-2000, BI 3000	Biosensing Instrument Incorporated is devoted to providing high-performance SPR instruments for creative research and efficient analysis. Flow-injection SPR compatible with electrochemistry SPR [82]	By controlling the potential of the "working electrode" (SPR metal sensor) with respect to a reference electrode using a potentiostat, electrochemical processes can be studied by monitoring both the current and the SPR signal at the electrode [83]
Mivitec GmbH (Analytical μ-Systems)	Binocular 6 system [84] combining SPR and electrochemistry	Offers to measure interaction of biomolecules (antigen–antibody and others) and binding constants [85]. probing antigen–antibody binding processes by impedance measurements on ion-sensitive field-effect transistor devices and complementary surface plasmon resonance analyses: development of cholera toxin sensors [86]
GenOptics, HORIBA Jobin Yvon	GenOptics [87], a technology innovator in surface plasmon resonance, now distributed by Horiba Yvon scientific	Automated SPR imaging system for assay measurements [88]
Ecochemie	A company in the Netherlands [89], a daughter of Metrohm Autolab	Has brought, with Esprit, Springle and Twingle, a product family to the market dedicated to label-free measurement of biomolecular interaction processes, even in combination with electrochemical analysis [90]
XanTec SR7000DC	A company highly specialized in biosensor and bio-chip development [91]	Provide for their SPR biosensor applications and selling a variety of SPR chips, specialized to tailor sensing surface as the central part of evanescent field biosensors [92]
GWC instruments Madison	Offers Fourier transform and imaging SPR with applications to life science and material research [93]	The SPRimagerII, SPR100, and SSD systems support a broad range of applications in life science and materials science research and development [94]
Sierra Sensors	Sells, besides quartz micro balance sensors, also SPR equipment [95]	Application and technical notes not accessible without user login

Table 2 (continued)

Company	Optical realization/description of company	Newer published applications of devices
DKK-TOA Corporation	In Japan has entered the market with model SPR-20 [96]	
Biolin Scientific KSV instruments	KSV is part of Biolin Scientific [97], the leading manufacturer of instruments for characterization of nanoscale films and study of surface molecular interactions	Real-time characterization of polyelectrolyte; multilayers using surface plasmon resonance [98]
Nanofilm Accurion	In Göttingen; has developed an ellipsometric platform EP^{3}SPR using a goniometer of their ellipsometer equipment with a gold SPR platform [99]	Protein binding was monitored in real time by imaging SPR in the ellipsometric mode [100]

[111], the detection of pathogenic microorganisms [112], and the analysis of food samples [113]. Other applications are the combination of SPR microscopy and imaging to analyse kinetics quantitatively [114], fragment-based screening [115], the discrimination of mutants [116], and the quantitative analysis of small molecule interactions with nucleic acids [117].

Bragg grating-assisted plasmon–polariton fibres are new approaches, and their quality is demonstrated for bio-medical applications [118]. Newer approaches of resonant mirrors use porous silicon for measurement of very low DNA concentrations [119]. Further applications of Mach–Zehnder chips have been implemented in bioanalysis [120]. Integration of micro fluidics and the use of standard CMOS compatible processed lab-on-chip systems have been realised to measure DNA [121]. To reduce the limit of detection, magneto–optic modifications have been patented [122] and parallelised. Because zero compensation is more

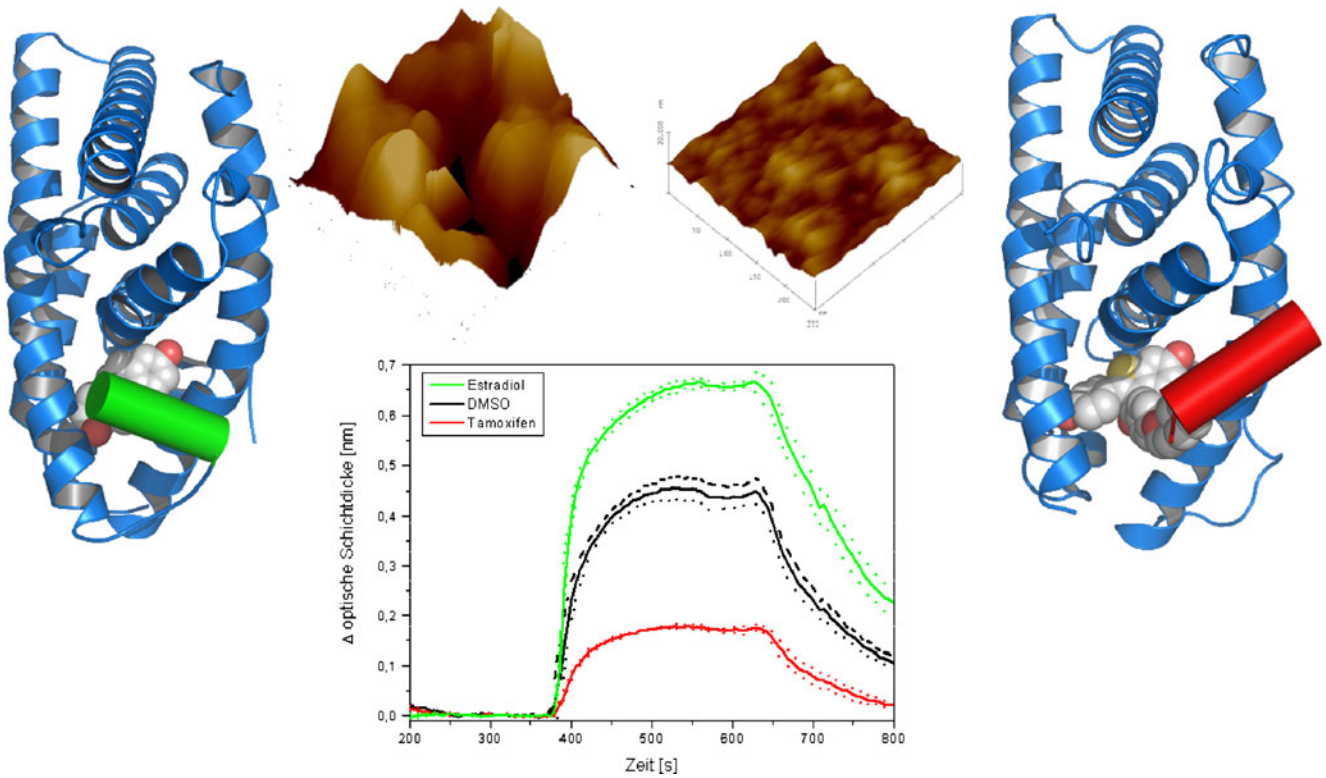

Fig. 3 Biotinylated peptide α/β I binds to the streptavidin-coated surface, ERα-LBD (ligand binding domain of estrogen receptor) was incubated with (*green, red*) or without (*black*) different ligands, and rinsed over the surface. No presence of ligands results in medium binding effect. β-Estradiol causes the ERα to adopt a conformation which enables increased interaction between the receptor in solution and the peptide immobilized on the surface (*green curve*, left structure, agonist). Tamoxifen causes the receptor to adopt a conformation less recognized by the peptide (*red*, right structure, antagonist). The AFM picture (*top*) reveals increased and reduced binding capability

sensitive in principle, better results are achieved [123]. However, experimental expenditure increases. The resulting interference fringes of the superimposed partial beams leaving the two arms of a Young interferometer are used to monitor an anti-human serum albumin–human albumin immunoreaction in a micro fluidic sensing system [124].

Starting in reflectometry with antigen–antibody interaction, RIfS enables quantification of DNA–ligand interactions [125]. The ligand-induced assembly of the type I interferon receptor on supported lipid bilayers has been examined [126] and the adsorption of proteins on biomaterial surfaces has been quantitatively evaluated [127]. A modification of RIfS has been used to detect nucleic acid targets on optically coated silicon [128] and label-free oligonucleotides [129]. Biochips have been examined by use of a Fabri–Perot type interferometer [130]. Some additional applications have recently been reviewed in Ref. [8]. SPR and RIfS have been compared, giving practical tips for data evaluation and obtaining kinetic constants [131]. Another interesting approach is a Fourier transform version used as LC detector [132] or for characterizing antibodies on the basis of their affinity constants in affinity chromatography [133]. As a result of surface modification, affinity constants of LNA and DNA duplex formation have been determined [134], endocrine receptors have been examined [135], and cell morphology has also been examined by RIfS [136]. Binding of ubiquitin to short peptide segments of hydrolase has been studied in comparison with fluorescence correlation spectroscopy, isothermal calorimetry, and NMR [137].

Hyphenated techniques are frequently used in analysis. It is easy to couple SPR to MALDI-TOF using the metal transducer. Coating the glass type surface with ITO (indium tin oxide) to achieve a matrix for laser desorption; mixtures of the emergency antibiotic vancomycin have been examined [138]. The spectroscopic detector can also be substituted with RIfS. Electrophoretic flow conditions can be monitored directly [139], because no metallic film, which would cause a breakdown of any applied electrical potential, is involved. Combining electrokinetics and reflectometric interference furnishes insight into hydrophobic and electrostatic interactions of fibronectins on biofilms and electrostatics of biopolymers [140, 141].The combination of interferometry with AFM has been reported recently [142]. Interesting is a combination of infrared spectroscopy with RIfS which besides interaction kinetics also provides sample identification [143].

Label-free detection in high-content screening [144] and the trends in fragment-based screening [145] have been reported recently. In a very recent publication reflectometric interference spectroscopy cannot only be used to monitor and quantify bio-interaction processes but can also be used to discriminate agonists and antagonists (Fig. 3) [14].

Conclusion

Direct optical detection has been developed as an interesting tool, not only to determine binding constants, i.e. study of thermodynamics and their equilibrium, but also to enable measurement of kinetics, i.e. the rate constants of processes even in competitive reaction schemes. As mentioned, these techniques enable the use of simpler assays (direct detection without reagents), are less expensive because labelling is avoided, and can overcome problems with loss of bioactivity (by labelling). Thus, these methods open an approach to signalling and even monitoring of system biology. Accordingly, besides antibody–antigen detection, hybridisation studies and measurements for drug design, protein–protein interaction, the measurement of nuclear receptors is now possible. This means the recent interest in bioanalysis is aimed at effect-directed analysis, i.e. at dose-related effects, and also considers membranes and cell-based assays as essential. Direct optical transduction has achieved a high standard; many instruments are available commercially and the principles have proved their feasibility in many applications. The trend in interaction monitoring of large molecules, complex structures, and proteins is directed to direct optical detection. Although fluorescence assays achieve far lower limits of detection and quantification, modern instrumentation provides possibilities of examining even interactions of small molecules and reaching LOD and LOQ down to 1 pg mm^{-2} in the case of appropriate binding constants and molecule size. Thereby, reflectometry is far less dependent on temperature effects and a high degree of parallelisation has been proved.

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