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Giuliano Zanchetta, Roberta Lanfranco, Fabio Giavazzi, Tommaso Bellini and Marco Buscaglia*

Emerging applications of label-free optical biosensors

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Abstract: Innovative technical solutions to realize optical biosensors with improved performance are continuously proposed. Progress in material fabrication enables developing novel substrates with enhanced optical responses. At the same time, the increased spectrum of available biomolecular tools, ranging from highly specific receptors to engineered bioconjugated polymers, facilitates the preparation of sensing surfaces with controlled functionality. What remains often unclear is to which extent this continuous innovation provides effective breakthroughs for specific applications. In this review, we address this challenging question for the class of label-free optical biosensors, which can provide a direct signal upon molecular binding without using secondary probes. Label-free biosensors have become a consolidated approach for the characterization and screening of molecular interactions in research laboratories. However, in the last decade, several examples of other applications with high potential impact have been proposed. We review the recent advances in label-free optical biosensing technology by focusing on the potential competitive advantage provided in selected emerging applications, grouped on the basis of the target type. In particular, direct and real-time detection allows the development of simpler, compact, and rapid analytical methods for different kinds of targets, from proteins to DNA and viruses. The lack of secondary interactions facilitates the binding of small-molecule targets and minimizes the perturbation in single-molecule detection. Moreover, the intrinsic versatility of label-free sensing makes it an ideal platform to be integrated with biomolecular machinery with innovative functionality, as in case of the molecular tools provided by DNA nanotechnology.

Keywords: optical materials; surface plasmon resonance; rapid analytical methods; imaging; DNA nanotechnology.

1 Introduction

Detecting and quantifying specific molecular compounds in solution is a ubiquitous need to analyze biological or environmental samples and industrial products. In general, a compound can be identified according to its physical or chemical properties or, alternatively, through the interactions with specific molecular partners. When the signal ascribed to the presence of a target molecule is directly or indirectly generated by the interaction with some biomolecule, the resulting system can be called a biosensor [1, 2]. A vast spectrum of biomolecular recognition elements is offered by nature and, if not already available, various biotechnology strategies can be exploited to produce novel biomolecules capable of binding virtually any possible compound [3, 4]. Besides the molecular recognition element, a fundamental part of a biosensor is the signal transduction mechanism, through which binding events are converted into detectable and quantifiable physical signals. In the last decades, there has been a large research effort aiming at the development of novel label-free detection methods. These are capable of generating a signal directly upon binding to the recognition element, without requiring additional interactions with other probes carrying a label that provides the signal [5, 6]. In this context, many different optical approaches have been proposed [7, 8]. A typical scheme is the measure of some optical signal sensitive to the change of refractive index in the proximity of the recognition element due to the binding of the target. In general, the label-free methods provide potential advantages in terms of simplicity and rapidity of the measurement procedure, which may not require washing steps and additions of reagents. Furthermore, these methods enable real-time monitoring

***Corresponding author: Marco Buscaglia,** Dipartimento di Biotecnologie Mediche e Medicina Traslazionale, Università degli Studi di Milano, 20090 Segrate, Italy,
e-mail: marco.buscaglia@unimi.it
Giuliano Zanchetta, Roberta Lanfranco, Fabio Giavazzi and Tommaso Bellini: Dipartimento di Biotecnologie Mediche e Medicina Traslazionale, Università degli Studi di Milano, 20090 Segrate, Italy

of the binding reaction, hence giving access to the kinetic parameters of the molecular recognition process [9].

Currently, the most common use of instruments performing label-free detection is for the characterization of specific molecular interactions or the screening of different compounds according to their interaction with molecular partners [10]. For these purposes, the use of instruments based on surface plasmon resonance (SPR) is widely consolidated, although several other techniques have been reported to enable high detection performance [11, 12]. Indeed, what makes a biosensing technology suitable for a particular application is not only the sensitivity of the detection method, but also other features that, in the end, must provide an overall practical benefit relative to other competing approaches. Since their introduction, the intrinsic versatility of optical label-free biosensors has facilitated their use in different kinds of applications. However, despite the continuous research on novel detection methods with improved performance, commercial products based on this class of biosensors are still proposed primarily to research laboratories for standard molecular characterization or screening. Therefore, the widely recognized potential impact of optical label-free biosensors on other application fields still seems largely unexpressed.

In this review, we propose a selection of particularly promising application fields, where the specific features of optical label-free biosensors are expected to provide significant competitive advantage relative to other approaches. We report a necessarily incomplete survey of either innovative technologies or novel uses of previously proposed methods that appeared in papers published in the last few years, as summarized

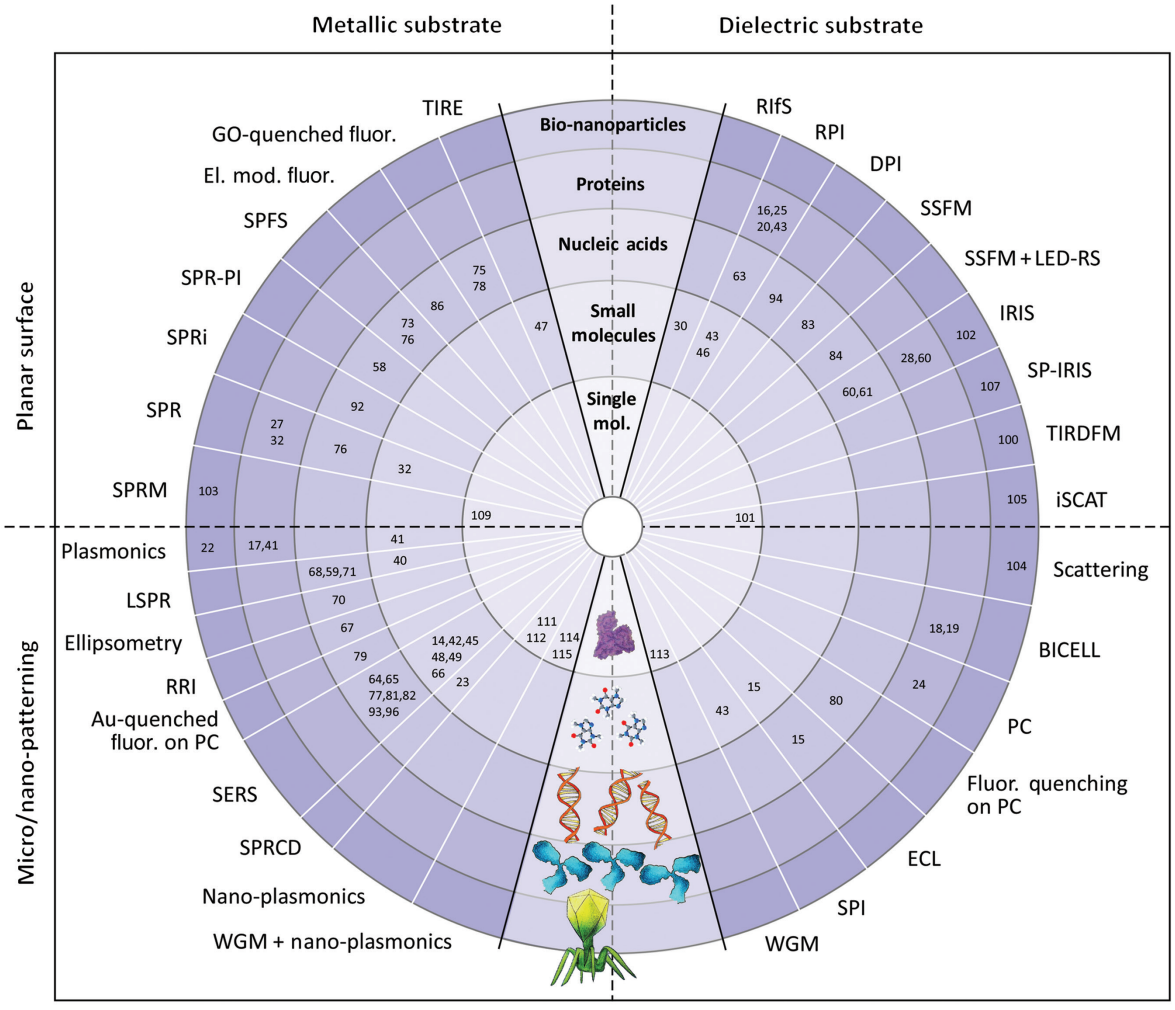

Figure 1: Graphical table reporting the biosensing methods and the corresponding references considered in this review.
The references are placed in the graphic according to the type of sensing substrate and the type of target. References to works where a metallic (dielectric) substrate is used are reported on the left (right) half of the table. References to works adopting a nano- or micro-patterned (planar) sensing surface are reported on the lower (upper) half of the table. The position along the radial direction indicates the target type among bio-NPs, proteins, NAs, small molecules, and single molecules.

in Figure 1. The following sections are organized according to five main classes of potential applications: biosensors for rapid, simple, and cost-effective detection of molecular markers – mainly proteins – in various kind of fluid samples (Section 2); detection of small molecules (Section 3); biosensor schemes targeting nucleic acids (NAs) or exploiting DNA structures as molecular tools for sensing (Section 4); digital detection of bionanoparticles (bio-NPs; e.g. viruses) (Section 5); and single-molecule detection (Section 6). We considered many different optical label-free technologies, which can be categorized according to the specific substrate that produces the sensing signal. Generally, the biorecognition elements are immobilized on a surface that can be made of different kind of materials with various structures at the nano- or micro-scale. Here, we propose to list these sensing substrates according to two main orthogonal features: the presence or absence of (i) a layer with optical properties typical of metals and of (ii) some kind of nano- or micro-patterning of the surface. Figure 1 shows the distribution of the techniques considered here in the quadrants defined by (i) and (ii) on a plane. The figure also presents a third axis in the radial direction that indicates the target type. Accordingly, Figure 1 enables to associate the properties of the substrate of a particular detection method with the reference reporting its recent validation for a particular type of target. Interestingly, the figure shows that the detection of each target type has been demonstrated for all combinations of (i) and (ii). Therefore, other features should be identified as the key determinants for the exploitation success of a given technique.

2 Reagentless distributed diagnostics

One of the main advantages of label-free detection methods is that they can avoid washing steps and addition of reagents to perform the measurement. This suggests a high potential for the development of simpler and more cost-effective diagnostic devices to be used outside the laboratory. The concept of decentralized diagnostics has been proposed long ago. The availability of sensitive and accurate analytical devices providing the results in a very short time without the need for specialized personnel or complex laboratory equipment is expected to enable a paradigm shift not only in human diagnostics but also in other fields, including veterinary, food safety, environmental monitoring, and biosurveillance [2]. In the last decades, many innovative technological solutions have been demonstrated to have the potentiality for a breakthrough in the quest for cost-effective, rapid, and simple-to-use analytical devices.

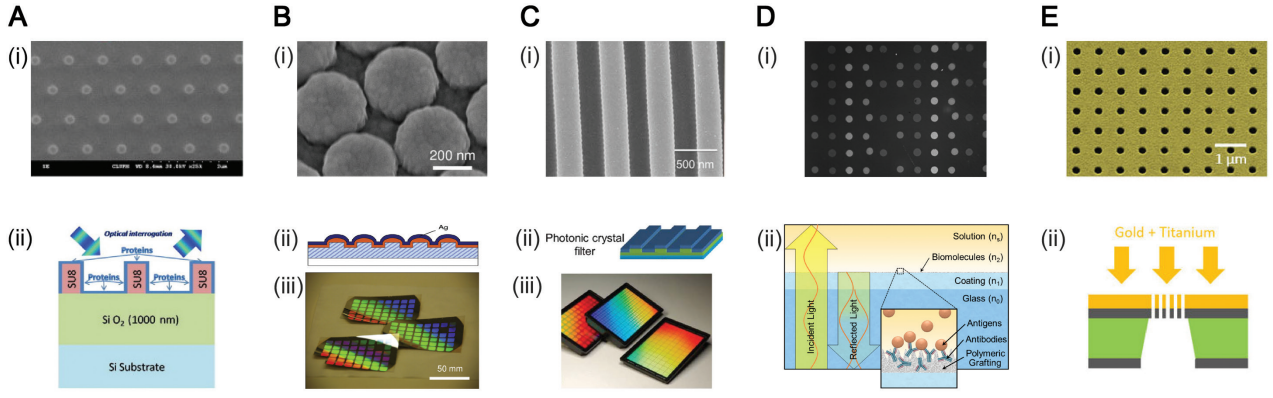

Figure 2: Material substrates for cost-effective and portable biosensors.
(A) Photonic structure made by nanopillars of SU-8 resist on SiO_2. (i) SEM image and (ii) schematic representation. Reprinted from Sensors Actuators B Chem, 176, Á. Lavín, R. Casquel, F.J. Sanza, M.F. Laguna, and M. Holgado, Efficient design and optimization of bio-photonic sensing cells (BICELLs) for label free biosensing, 753–760, Copyright (2013), with permission from Elsevier [13]. (B) Photonic structures formed by Ag-coated nano-domes on flexible polyethylene terephthalate (PET) sheets. (i) SEM image; (ii) cross-section diagram; and (iii) image of the complete array substrate. Adapted from Ref. [14]. Copyright IOP Publishing. Reproduced with permission. All rights reserved. (C) PC resonant reflector made by TiO_2-coated UV-curable polymer. (i) SEM image; (ii) schematic of the substrate; and (iii) image of the array in standard microplate format. Adapted from Ref. [15], with permission of The Royal Society of Chemistry. (D) RPI sensing surface made by SiO_2 anti-reflective layer on a glass slide. (i) RPI image of antibody spots with diameter of 150–200 µm; (ii) schematic of the sensing surface. Reprinted from Biosens Bioelectron, 74, M. Salina, F. Giavazzi, R. Lanfranco, E. Ceccarello, L. Sola, M. Chiari, B. Chini, R. Cerbino, T. Bellini, and M. Buscaglia, Multi-spot, label-free immunoassay on reflectionless glass, 539–545, Copyright (2015), with permission from Elsevier [16]. (E) Array of plasmonic nano-holes integrated in microfluidic. (i) SEM image; (ii) schematic of the final fabrication step. Adapted by permission from Macmillan Publishers Ltd.: Scientific Reports [17], copyright 2014.

Several detection methods have been proved to reach suitable sensitivities and specificities in the controlled laboratory environment. However, a few challenging requirements for a commercial product are rarely met. One of the main goals is the simplification of the measurement procedure, which should ideally consist only in the filling of a cartridge with the sample, without the need for further addition steps to perform the analysis. Other important technological challenges include the availability of cheap, disposable sensors; the realization of compact, semi-automatic devices; and the capability of working with real complex samples.

2.1 Toward disposable sensors

Key factors enabling novel bioanalytical platforms to become accessible to non-specialized users include the ease of use and a cost per sample comparable to current standard methods. Most of the tests based on antibody-antigen binding are generally performed in the laboratory by ELISA methods or in field by lateral flow systems [2]. Any novel approach should reach an average cost per sample and per analytical target at least comparable with those methods. An effective multiplex format, enabling the simultaneous measurement of multiple targets in a single sample, can largely reduce the cost per target. In contrast, in order to keep the cost per sample competitive, the sensor design should be based on cost-effective materials and compatible with a large-scale industrial production. Recently, an increasing number of research studies on innovative biosensing solutions have addressed these requirements. For example, sensing surfaces patterned with photonic structures have been realized with silica and SU-8 resist (Figure 2A) [18, 19]. An array of such structures, biophotonics sensing cells (BICELL), can be fabricated by standard deep ultraviolet (UV) lithography, a cost-effective method that enables a significant reduction of the production cost. The detection is provided by the shift of the spectrum of reflected light upon binding of biomolecules on receptors immobilized on the SU-8 surface. Similarly, processes based on UV-curable polymers on a silicon mold enable the fabrication of photonic structures on large plastic sheets. Arrays of Ag-coated nanodomes on flexible PET sheets have been demonstrated (Figure 2B) [14]. These nanostructures provide an enhancement of the Raman scattering (surface-enhanced Raman scattering, SERS) that enables the label-free quantification and identification of biomolecules adhering on the surface. A similar fabrication process has been employed to realize a photonic crystal (PC) resonant reflector made by UV-curable polymer coated with TiO_2 (Figure 2C). This reflector was used as a mirror of an external cavity laser, hence obtaining a narrow linewidth emission, which enabled to resolve small wavelength shifts due to the adhesion of biomolecules on the surface of the reflector [15]. A different class of optical label-free biosensors is based on simple dielectric coatings on planar surfaces, without nano-patterning. In particular, wedge-like glass slides coated with a thin layer of SiO_2 have been used to realize multispot biosensors based on the simple imaging of the intensity of reflected light (Figure 2D) [16]. High sensitivities are achieved by designing the sensing surface in order to have very low reflectivity in water. This principle is at the basis of the reflective phantom interface (RPI) method [20] that enables the fabrication of economic disposable cartridges for the direct detection of dozens of different biomolecular targets in a single sample, without requiring the addition of reagents nor washing steps.

2.2 Compact diagnostic devices

Another important requirement for a biosensor system to be used outside the laboratory is a reduced complexity of the instrument, which should be compact, while requiring an extremely simple procedure, suitable for untrained operators. In the field of medical diagnostics, these are common requirements for point-of-care (POC) systems [21]. Given the wide acceptance of SPR-based biosensors as characterization tools in the laboratory environment, several solutions aiming at the realization of POC systems are also based on plasmonic substrates with a variety of configurations [8]. However, to date, only a few truly compact devices have been demonstrated in the literature. Among these, a portable and simple biosensing device was exploited in combination with an array of plasmonic nano-holes integrated in a microfluidic cell (Figure 2E) [17]. Notably, a similar sensing substrate used in a different portable device enabled the detection and molecular profiling of exosomes [22]. As another example, in the field of food analysis, the quantification of different antibiotics in milk by a portable instrument was achieved exploiting the plasmonic response of a surface with gold diffraction grating embedded in a multichannel fluidic chip (surface plasmon coupler and disperser) [23].

An alternative and fascinating approach relies on the use of a smartphone to acquire and process the signal. One of the advantages of using a mobile device is the ease of developing an intuitive user interface, possibly connected with a remote support unit. Moreover, smartphones typically mounts a high-performance camera, suitable for the integration in sensitive label-free biodetection systems. In

Ref. [24], the smartphone camera is coupled to an external light source and used as a spectrometer allowing for measurements of shifts in the resonant wavelength of a PC fabricated on a plastic substrate and attached to a glass slide. In Ref. [25], both the camera and the LED flash of the phone are used to measure the image of the light reflected by a prism of perfluorinated plastics with refractive index very close to that of water. In this case, the multiplex, label-free quantification of biomarkers for HIV and hepatitis B infection in blood serum samples was demonstrated by RPI.

2.3 The challenge of complex matrices

Real samples can contain large amounts of interfering substances and, typically, the type and quantity of these contaminants can be highly variable from sample to sample. For this reason, many analytical procedures require multiple steps for sample preparation. However, if the ease of operation is an important requirement, biosensing systems capable of performing direct measurements even in complex matrices are needed. Ideally, a suitable detection method should be almost insensitive to the composition of the solution and the sensing surface should be effectively passivated to limit the background signal provided by non-specific binding. The latter feature is typically achieved by coating the sensing surface with a suitable anti-fouling layer [26]. For example, in the field of diagnostics, the complex composition of blood poses a tremendous challenge on label-free detection systems. Despite this, multispot detection of protein biomarkers has been demonstrated in 10% plasma by SPR on surfaces passivated by covalently immobilized bovine serum albumin (BSA) [27] and up to 60% serum by RPI on surfaces coated with a multifunctional copolymer of dimethylacrylamide [16, 20, 25]. Examples of other kinds of measurements performed in very complex matrices include the detection of cytokines in cell culture medium by interferometric reflectance imaging sensor (IRIS) [28], the chemical analysis of food samples by SERS [29], the quantification of testosterone in milk by reflectometric interference spectroscopy [30], and the early detection of virus biomarkers in vegetables extracts by RPI [31].

From a different point of view, label-free methods are also extremely powerful tools to characterize the interactions of complex matrix components with the surface. An important example is provided by the study of complement activation in controlled experimental conditions. SPR has been used to monitor the activation of the complement system of proteins, either for biomedical studies [32] or to guide the development of biomaterials [33].

3 Small-molecule detection made simpler

Designing a standard label-based assay for the detection of small molecules (typically <1 kDa) can be complex because their chemical conjugation is typically challenging and because the common sandwich immuno-assay format may not be feasible. For these reasons, information gained from label-free methods with adequate sensitivity is regarded as potentially more accurate and straightforward.

The category of small molecules typically includes compounds of either natural or pharmaceutical origin, biologically active because of their interaction with biomolecules. In particular, the small structure can favor their penetration through the cell membrane. Accordingly, they can be extremely dangerous when present in the environment. Despite the increasing attention to the control of molecular pollutants, only a few technologies nowadays are able to identify small amounts of this kind of compounds in real samples. The classical and most robust techniques for small-molecule detection include colorimetric methods [34, 35] and liquid chromatography, possibly coupled to mass spectrometry [36]. In contrast, label-free methods can be exploited to propose new paradigms for small-molecule detection. In particular, optical label-free methods represent a good candidate for the realization of novel classes of environmental sensors, possibly embedded into autonomous devices providing real-time monitoring with relatively low detection limits. Here, we present the most recent methods to detect directly the presence of small molecules in real samples with particular attention to the final goal of realizing reliable portable sensors. Different works have exploited localized SPR (LSPR) and SERS for this purpose. Recently, two general strategies are used to increase the detection capability of these optical label-free methods: engineering the optical response of the sensing surface [37] and coupling different techniques sensitive to complementary molecular properties [38]. Depending on the method, the selectivity of detection is provided either by biomolecular recognition or by specific spectral features of the optical signal.

3.1 Engineered substrates for enhanced sensitivity

The nano-pattering of the substrate, on which the detection takes place, is one of the most successful approaches to enhance the sensitivity of both SPR and SERS devices.

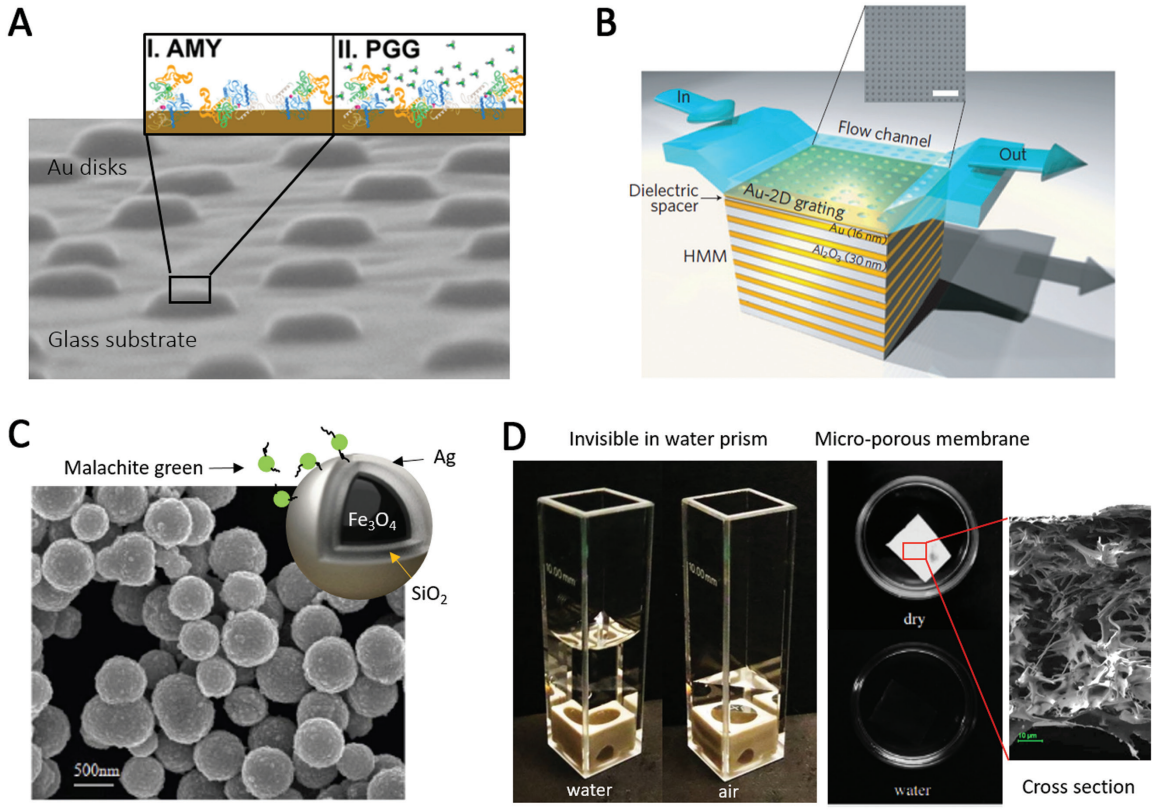

Figure 3: Substrates for small-molecule detection.
(A) Detection of small molecules with LSPR. SEM images of the Au nano-disks fabricated on glass substrate and a scheme of the binding process. Adapted with permission from ACS Nano, 2014, 8 (8), pp. 7958–7967. Copyright 2014 American Chemical Society [40]. (B) Schematic representation of the HMM sensor device and SEM image of the two-dimensional sub-wavelength gold diffraction grating on top of the HMM. The grating has an average period of 500 nm and whole size of 160 nm (scale bar, 2 μm). Reprinted by permission from Macmillan Publishers Ltd.: Nat Mater 15, 621–627 (2016), Copyright 2016 [41]. (C) SEM images of Fe_3O_4@SiO_2@Ag magnetic particles and schematic representation of their composition. Adapted from Sci Rep 6, 22870 (2016) [42]. (D) Perfluorinated prism and micro-porous membrane immersed in water and air, as indicated, and a SEM image of the cross section of the micro-porous membrane. Adapted with permission from R. Lanfranco, F. Giavazzi, M. Salina, G. Tagliabue, E. Di Nicolò, T. Bellini, and M. Buscaglia, APS, Phys Rev Appl 5, 054012, 1–15, Copyright 2016 by the American Physical Society [43].

In particular, if the surface plasmon of SPR is confined in nanostructures with size comparable to or smaller than the wavelength of light, a higher sensitivity to small molecules can be achieved [37, 39]. In this context, LSPR, based on tracking the local refractive index changes due to biomolecular reaction, represents a promising technique. As an example, gold nano-disks with a proper surface functionalization allow the creation of specific spots of probe molecules on a glass [40] (Figure 3A). The immobilization of R-amylase on the nano-disks enables the LSPR detection of pentagalloylglucose also in wine samples with a detection limit of 400 ng/ml.

Recent progress in nanofabrication has encouraged the development of novel label-free plasmonic biosensors. In this framework, meta-materials are earning increasing attention. They are typically made from the assembly of multiple elements of metals or plastics. Their peculiar response to electromagnetic fields derives from their specifically designed shape, geometry, size, orientation, and arrangement [44]. Reference [41] proposes a plasmonic biosensor platform based on hyperbolic meta-materials (HMMs), substrates with hyperbolic dispersion that provide extreme sensitivity from visible to near-infrared wavelengths (Figure 3B). Thanks to the different response of bulk plasmon polariton modes, target molecules with different mass can be discriminated. An extremely high sensitivity was achieved with streptavidin-biotin interaction with a limit of detection (LOD) of 0.2 pg/ml, in a rather short detection time of <1 h. These performances, in combination with the multiplexing capability, make this substrate suitable for integration in sensitive analytical devices for various applications.

Detection methods based on Raman scattering offer an intrinsic selectivity due to the specific spectral response

of different target molecules. Recent developments of sensors based on SERS detection also focused on the micro-structuring of the sensing surfaces. The availability of a portable Raman spectrometer favors the realization of compact SERS devices for on-site direct detection of small molecules in real samples. In Ref. [42], a widely used fungicide and pesticide, malachite green (MG), was detected using nanosilver-coated magnetic particles (Figure 3C), reaching an extremely low LOD of 2 fg/ml. Regeneration and stability of the sensing surface are also evaluated considering the potential matrix effect in tap water, bottled water, and secondary sedimentation water. In Ref. [45], the fabrication of disposable screen-printed SERS arrays was demonstrated. This kind of array is an ideal candidate for analytical application of SERS given their high detection performance, cost-effective fabrication, and portability. Various small molecules (tryptophan, phenylalanine, adenosine, and flavin mononucleotide) are specifically detected with LOD ranging from 0.3 to 100 ng/ml.

More recently, it has been demonstrated that the reflective phantom interface (RPI) or scattering phantom interface signals originating from perfluorinated materials with a refractive index similar to that of water also provide adequate sensitivity to detect small molecules [43]. The simple adsorption on the bare surface of a prism or a microporous membrane enables to discriminate among molecules with different extensions of hydrophobic moiety and different charges (Figure 3D). In this case, the substrate preparation is extremely simple because surface functionalization is not required and the sensor can be easily regenerated by washing with suitable solvents. This method was also applied to real river water samples [46], where 0.4–0.7 μM of surfactants was detected.

3.2 Combined methods for improved detection

The combination of different methods is a standard way to detect, quantify, and discriminate small molecule in the laboratories, as in case of liquid chromatography-mass spectrometry [36]. Optical label-free techniques can be coupled with other methods sensitive to different molecular properties, as the molecular mass or the polarizability, in order to enhance the device performance. In Ref. [47], a standard SPR prism was modified in order to gain sensitivity to the amplitude and phase shift of the p- and s-polarized light reflected by the sensing surface, as in a classical ellipsometry measurement. This method, called total internal reflective ellipsometry, combines the selectivity of spectroscopic ellipsometry and the sensitivity of Kretschmann SPR. Toxins with low molecular weight, such as T-2 and zearalenone mycotoxins, are specifically captured by surface immobilized antibodies, enabling the detection of concentrations down to 0.1 ng/ml. In Ref. [48], classical SERS measurements were performed on an engineered substrate formed by a suitably designed micro-resonator (Figure 4A), demonstrating the detection of MG and p-ATP. The proposed multimodal sensing system incorporates two detection methods: the capture of the target molecules yields both a shift in the resonant frequency of the cantilever due to the change of mass and a specific SERS spectrum. Both signals contribute to the selectivity of detection. Another method that can be combined to label-free SERS to provide on-site detection of small molecules is the one proposed by Li et al. [49]. A convenient thin layer chromatography (TLC) protocol enables the separation of aromatic compounds that are detected using a portable SERS spectrometer (Figure 4B). Although the LOD of 8–200 ng/ml achieved with this approach is not yet suitable for most environmental monitoring applications, the robustness of detection and the simplicity of sample preparation make it an interesting candidate for the realization of portable devices for on-site detection of water pollutants.

4 Nucleic acids: prey and predator

NAs are the molecules of life, with countless roles in storage, transmission, and expression of information and in process regulation. Numerous diseases can be traced back to defects in NA sequence, pairing, and replication. For this reason, there is an intense effort to develop biosensing techniques for detection, quantitation, and discrimination of NA sequences in biological fluids. NAs of interest feature a wide variety of molecular masses, from short oligonucleotides such as microRNA (miRNA) of 20–22 nucleotides (nt) (i.e. around 6 kDa in molecular weight and few nm in size), through polymerase chain reaction (PCR) products, to large molecules as cDNA and ribosomal RNA of thousands of nt (i.e. up to MDa and hundreds of nm), with very different requirements in terms of sensitivity and specificity. Differently from other molecular recognition processes, NA detection typically relies on hybridization, and therefore the target and the probe are complementary NA sequences. Both the affinity and specificity of the hybridization can be controlled and enhanced through a suitable design of the probe sequence. However, NA detection can be challenging relative to other molecular targets because of different

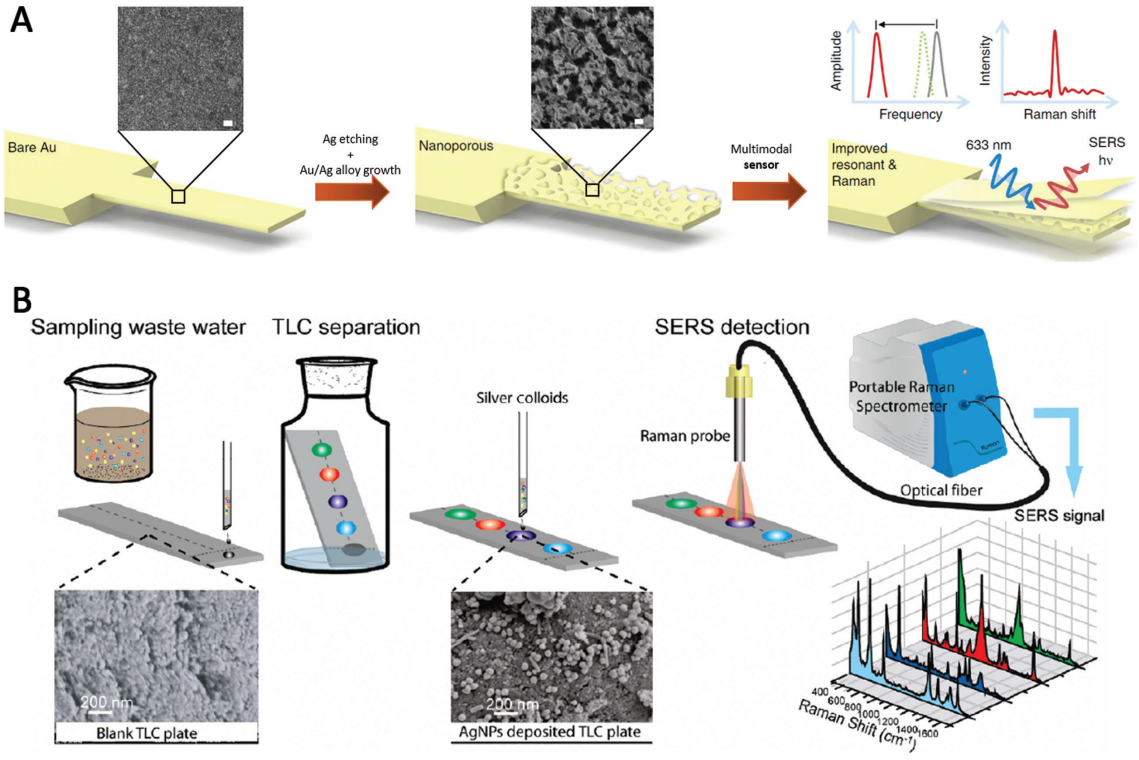

Figure 4: Combined techniques for small-molecule detection.
(A) Combination of SERS detection on a micro-resonator. The two images represent the resonator surface before and after the chemical treatments performed to produce a layer of porous gold. Adapted by permission from Macmillan Publishers Ltd.: Nat Commun 5: 3456, copyright 2014 [48]. (B) Schematic illustration of TLC-SERS for on-site detection of aromatic pollutants in wastewater. The two images show the SEM characterization of the blank TLC plate and the silver NPs deposited on the TLC plate, respectively. Reprinted with permission from Environ Sci Technol, 2011, 45 (9), pp 4046–4052. Copyright 2011 American Chemical Society [49].

reasons: the extremely low concentration of some NA strands like miRNA, which also display expression levels differing by several orders of magnitude; the distributed binding area and the sequence-based recognition, which can be weakened by competing local interactions with low affinity; and, at least for RNA, the fast degradation and the distinctive variety of three-dimensional structures [50]. Recent reviews are available on specific label-free optical techniques applied to NA detection, like SPR [51, 52] or SERS [53], and on detecting specific classes of NA like miRNA [50, 54, 55].

On the other hand, the peculiar properties of NA polymers, with unique versatility in terms of flexibility, shape tuning, and specific binding, make them an ideal toolbox to control molecular interactions, sensing environmental changes through conformational switches, or even for the assembly of nanostructures and triggers for signal amplification [56]. Therefore, depending on the application, NA can just take the role of the final target of the detection or they can also provide the molecular tool to perform the detection itself. In the following, we provide a necessarily partial path through the most recent, ever-growing literature on label-free optical biosensors either targeting NA or exploiting NA structures as a detection tool.

4.1 NA in the crosshairs: label-free detection of specific sequences

Since the 1990s, NA microarrays relying on fluorescent labeling of target strands have been used for genomic-scale analysis and detection of NA sequences with extremely low LOD. However, despite tremendous advancements, NA microarrays can only be semiquantitative and are typically used for self-consistent comparison of expression levels. Therefore, various classes of label-free optical techniques are being explored for the detection of NA hybridization. These techniques can offer the advantages of ease of use, access to kinetics, and absolute quantitation of bound targets.

An example of a simple approach for one-step, real-time detection of DNA-binding events is proposed in Ref. [57]. A colloidal PC structure was embedded in hydrogel beads functionalized with probe strands, so that binding of

complementary target strands caused the distance between beads to change and their Bragg diffraction peak to shift accordingly (Figure 5A). Binding of methylated or unmethylated 18mer strands was discriminated with 3 μg/ml sensitivity. Another simple method providing direct access to real-time events is offered by reflectance-based techniques. In this case, the signal is due to the amplitude or the spectrum of the light reflected by the sensing surface upon hybridization of immobilized probe strands. In the spectral reflectance imaging biosensor – later renamed IRIS [60] – hybridization of DNA 20mers was detected through the spectral analysis of light reflected by a silicon substrate coated by a silicon oxide layer, with good discrimination of base mismatches [61, 62]. More recently, using the RPI method, the reflectance from an index-matched interface provided multiplexed access to kinetics of DNA hybridization process and enabled addressing the overall stability of fully complementary and mismatched 12mer strands on the sensing surface [63].

A different strategy toward direct detection of DNA strands without labeling steps relies on deciphering their intrinsic signatures in the Raman spectrum, which show distinctive dependence on sequence and chemical modifications. Indeed, SERS spectra were obtained from DNA 23/25-nt single strands non-specifically adsorbed onto the enhancing surface of silver colloids in a flattened configuration, and single base polymorphisms were resolved [64]. To adsorb double-stranded DNA onto silver NP without causing denaturation, these were coated with positively charged spermine molecules [65]. In this way, negatively charged DNA promoted NP aggregation into stable clusters, which produced field hotspots while preserving the double helix structure. In such a configuration, it was possible to detect sequence mismatches and discriminate their degree of methylation. Although these steps are largely insufficient to reach the sensitivities required for diagnostic purposes, the results are encouraging. Notably, DNA origami structures were recently used to precisely tune metal NP structures for SERS detection of small molecules, also demonstrating sensitivity to DNA strand sequence composition [66].

For many applications, an extremely high sensitivity to NA hybridization is clearly the most stringent requirement. Different methods based on plasmonic substrates

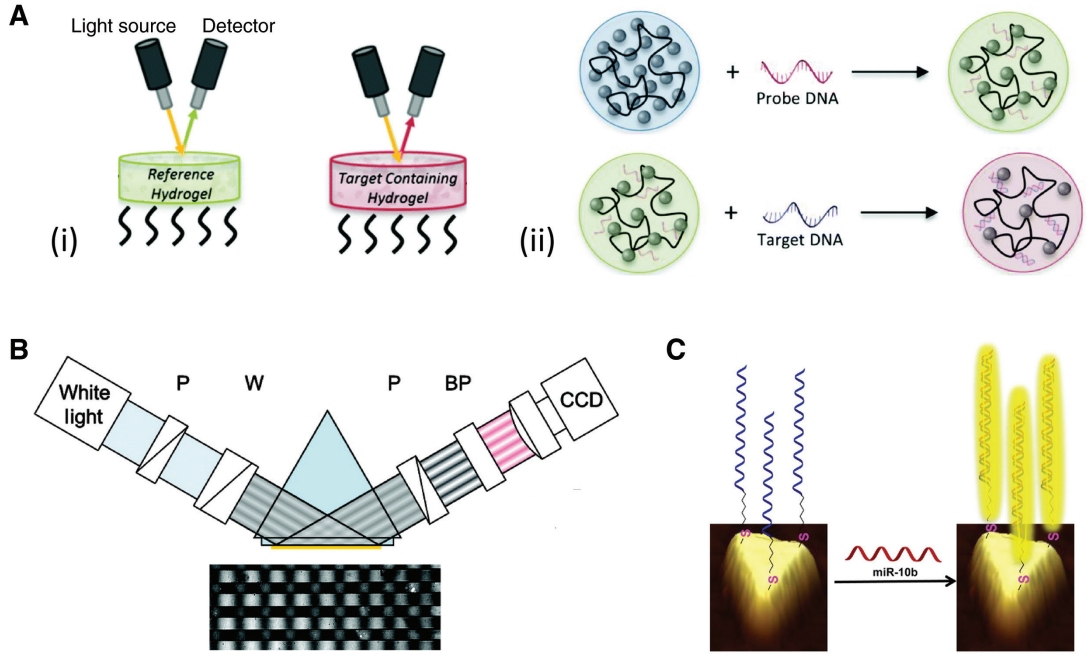

Figure 5: Label-free techniques for detection of NAs.
(A) Hydrogel-embedded PC allows simple detection of DNA hybridization based on reflected light (i). Functionalization with probe strands and binding of target strands affect relative distance of PC beads and cause color shift (ii). Adapted from Ref. [57] with permission of The Royal Society of Chemistry. (B) Optical configuration of surface plasmon resonance phase imaging (SPR-PI), with polarizer P, wedge retardance plate W, and bandpass filter BP. An example of phase shift differences is also shown: horizontal stripes correspond to patterning with different biomolecular interactions, while vertical stripes are the imposed sinusoidal phase fringes. Adapted with permission from Ref. [58]. Copyright 2011 American Chemical Society. (C) Schematic representation of miRNA detection based on LSPR, superimposed to AFM image of nanometric gold nanoprisms functionalized with single-stranded DNA complementary to target RNA. Adapted from Ref. [59].

have been recently demonstrated to reach very low LODs. Relative reflected intensity from a gold nanostructured surface with immobilized primer strands allowed real-time optical monitoring of PCR process, with much higher sensitivity than the typical fluorescence-based methods [67]. As regards SPR, two main strategies are adopted to increase the sensitivity without adding labels [51]: the use of alternative optical configurations and the design of nanostructured surfaces to produce localized plasmons. An interesting example of the first strategy is reported in Ref. [58], where the phase shift of p-polarized light interacting with surface plasmon was measured by creating an interference fringe image on the interface, which changed upon hybridization of DNA 30mers, yielding a LOD of 50 pg/ml (Figure 5B). As for the second, localized SPR can instead be obtained with various types of patterning, like gold nanoprisms with 30–50 nm edges [68, 59], which allowed miRNA quantitation with an impressive LOD as low as 0.25 fg/ml (Figure 5C).

Plasmon modes were also exploited in a system of gold nanoparticle (NP) dimers linked by a stem-loop DNA strand and anchored to a glass substrate. The loop sequence was able to bind a miRNA target sequence in solution, which changed the distance between the NP and produced a spectral shift in the scattered light [69], allowing a detection limit of 70 fg/ml. Other materials were also explored as substrates for localized plasmons, like gallium NP whose resonance can be tuned from the UV to the infrared range by varying their shape and size. By exploiting a condition of reversal of polarization handedness as measured through ellipsometry, it was possible to detect DNA strands of 10–100 nt with 20 pg/ml LOD, also discriminating single nucleotide mismatches [70].

4.2 Switch on the light: detection through fluorescence modulation on smart DNA surfaces

Typical strategies to enhance optical signals imply fluorescent labeling, addition of NP, or target recycling. However, any secondary binding would easily impair access to kinetics and absolute quantitation. A different kind of system relies on the change of signals obtained by labeled NA probes immobilized on a surface upon binding with label-free complementary strands. Although the classification of these approaches as label-free is arguable, because the signal is generated indirectly as a consequence of the binding, in practice they share the main features of truly label-free biosensors. The target in solution is detected and quantified without the addition of secondary probes or reagents. Therefore, these methods combine the merits of a simple one-step procedure with the intrinsic sensitivity of fluorescence signals. This elegant approach, made possible by the very nature of the NA structure and interactions, is typically implemented through the so-called DNA molecular beacon [71]. In the classic configuration, a DNA hairpin is double labeled with a fluorophore and a quencher at the paired terminals, so that fluorescence is quenched when the target is absent, while fluorescence emission is restored upon binding and opening of the hairpin. This simple idea, widely explored in solution also for DNA-protein interactions [72], has recently been extended to surface-based techniques. Gold [73] and graphene oxide (GO) [74, 75] surfaces can also act as quenchers, providing sensitivities as low as 1 ng/ml for 20mers. Furthermore, in surface plasmon fluorescence spectroscopy (SPFS), excitation of the fluorophore through an evanescent wave allows to directly combine and compare this approach with classical SPR measurements [76] (Figure 6A). Various combinations of molecular beacons with PC were reported to further enhance fluorescence emission [79, 80], yielding DNA and miRNA detection with a 0.2 ng/ml limit.

An interesting, complementary application of beacon concept to Raman measurements are the so-called SERS sentinels [77]: a DNA hairpin is immobilized onto a nanostructured SERS substrate and a Raman reporter attached to it is held close to the substrate, so that SERS emission is on. Upon target binding, the reporter separates from the surface and the SERS signal decreases (Figure 6B). As compared to fluorescent dyes, Raman reporters have sharper peaks and are not subject to bleaching. This strategy was scaled down to a microfluidic channel with SERS sentinels on a nanoporous gold disk [81], achieving the resolution of single hybridization events. Furthermore, a chip with a "switch on" version of SERS sentinel – with a placeholder strand – was developed for one-step detection of specific tracts of the dengue virus sequence [82].

Other techniques exploit modulation of the fluorescence of labeled probes to assess binding of label-free targets. In spectral self-interference fluorescence microscopy (SSFM) [83], the average height and conformation of surface-bound DNA probes can be estimated with sub-nanometer resolution from the fluorescent spectrum as reflected by the Si-SiO_2 substrate. The technique has also been applied to detection of protein-induced conformational changes of double-stranded DNA in conjunction with reflectance spectroscopy (SSFM + LED-RS) [84]. Oligonucleotides tethered at surfaces can also be controlled in their conformations and proximity to the surface via application of a weak external voltage. Switching

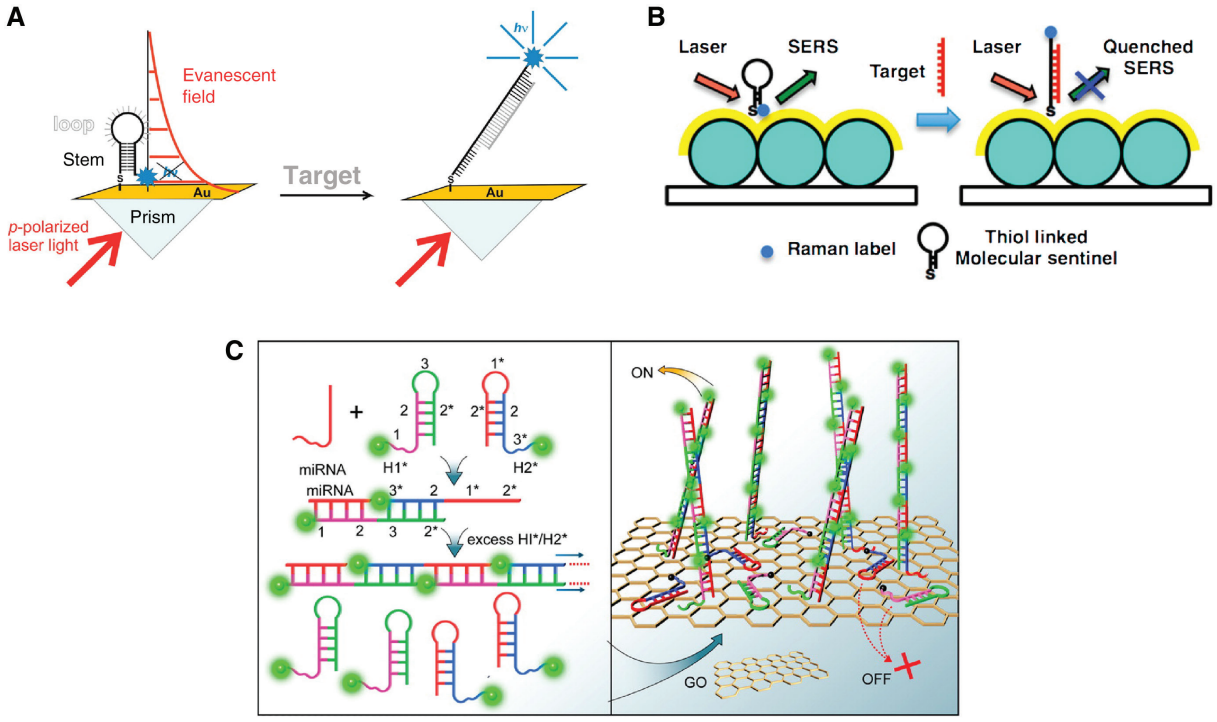

Figure 6: Detection of NAs based on DNA molecular beacons and/or DNA self-assembly.
(A) Working principle of SPFS: an evanescent field from surface plasmons impinges on a molecular beacon adsorbed at a gold-coated glass prism. In the closed state, the fluorophore is close to the gold surface and fluorescence is quenched, while target hybridization opens the hairpin and switches fluorescence signal on. Reproduced with permission from Ref. [76]. Copyright 2014 American Chemical Society. (B) Schematic behavior of a so called SERS "molecular sentinel": a DNA hairpin linked to a thin gold film – deposited on packed nanospheres – keeps a Raman label close to the surface when folded. Upon target binding, the label is separated from the surface and the SERS signal is quenched. Reproduced with permission from Ref. [77]. Copyright 2013 American Chemical Society. (C) Combination of HCR and molecular beacon concepts: two hairpins carrying emitting fluorescent tags assemble into chains only in the presence of a target miRNA, while fluorescence is quenched in proximity of the GO surface. Reproduced with permission from Ref. [78]. Copyright 2012 American Chemical Society.

amplitude and dynamics of probe strands is significantly affected by binding events, so that analyzing such behavior at different frequencies, a wealth of information can be retrieved about affinity, kinetics, and bending flexibility [85, 86].

4.3 DNA-based amplification strategies

By nature, DNA is an excellent building block for self-assembly of predictable shapes with controlled and reversible interactions. Indeed, DNA is increasingly used to create nano-scaffolds on surfaces for controlling probe density, position, and accessibility with nanometric resolution [87], hence enabling to minimize the effect of competing interaction with the surface. Furthermore, selected sequences can act as aptamers capable of binding specific targets, ranging from metal ions [88] to proteins [72, 89]. One more notable application of DNA as a molecular toolbox is "one-pot" signal amplification. Although also in this context, the boundary between label-based and label-free techniques is somewhat fuzzy, these studies represent the premise for the future fabrication of smart, reconfigurable surfaces with enhanced sensing performance and dynamic range.

Two main examples of such DNA-based amplification strategies are hybridization chain reaction (HCR) [90] and rolling circle amplification (RCA) [91]. In HCR, self-assembly of two different single strands forming separate hairpins is triggered by a third filament, yielding a long double-stranded structure. This strategy has been used to increase the bound mass in SPR imaging detection of DNA binding [92] and in SERS detection based on gold NPs [93]. Direct access to kinetics is, however, hindered by the cascade of secondary binding events or by the need of multiple steps, and real-time detection is thus a current challenge. Interestingly, dual-polarization interferometry has been recently used for direct monitoring of the kinetics of HCR evolution [94]. Various attempts were also made to combine one or two different molecular beacons with self-assembly. Interestingly, this strategy was used to improve the signal contrast on a GO surface (Figure 6C) [78].

A different amplification strategy can be obtained by RCA. This is based on DNA isothermal polymerization performed by certain enzymes whose activity can be triggered by binding events. Currently, this approach is typically exploited in sandwich assays in conjunction with NPs for colorimetric tests in solution [95] or with nano-rods for SERS detection [96] on a surface. Although, in principle, this kind of amplification can tremendously improve sensitivity (down to fg/ml), no real label-free detection has yet been demonstrated.

5 Digital detection of bio-nanoparticles

The class of NPs of biological relevance, with size roughly between 10 and 100 nm, is very broad, including, for example, synthetic NPs, designed for drug delivery or as probe for biosensing, atmospheric particulates with impact on human health, and natural bio-NPs [97, 98]. In this section, we focus on label-free optical detection with single particle resolution (i.e. digital detection) of naturally occurring biological NPs, in particular viruses and exosomes (extracellular vesicles). From a technical point of view, a first rough distinction that can be introduced is between imaging-based and resonant cavity-based methods [97]. In this section, the focus will be mainly on the first class, while a more in-depth discussion of resonant cavity-based methods will be provided in the next section, where single-molecule detection is discussed.

In general terms, the optical sensing of single NPs typically involves the illumination of the particle with a suitable light beam, the collection of the light scattered by the NPs with a suitable optical system, and its detection. The amplitude of the electric field E_s elastically scattered by a small spherical particle can be written as [99] $E_s = cE_0|\Delta\epsilon|R^3$, where E_0 is the amplitude of the incident field, $|\Delta\epsilon|$ is the difference in dielectric permittivity between the particle and the medium, R is the radius of the particle, and c is a constant depending on the illumination and collection geometry. In the presence of a (wanted or unwanted) "reference" field E_r, independent of the particle and superimposing to the scattered field, the total field amplitude E_d on the detector is given by $E_d = E_r + E_s$, while the detected intensity reads

$$I_d = |E_r|^2 + c^2|E_0|^2\,\Delta\epsilon^2 R^6 + 2c|E_r||E_0||\Delta\epsilon|R^3\cos(\theta). \qquad (1)$$

In Eq. (1), the first term is a constant optical background, while the last two terms depend on the presence of the particle. Specifically, the second term corresponds to the intensity scattered by the particle, while the last one accounts for the interference between the reference and the scattered light, θ representing the relative phase of the two fields. Many of the microscopy methods discussed in this section can be categorized according to the strategy adopted to maximize the contribution of the particle-dependent terms to the detected intensity [98].

A first strategy consists in "killing" the first and the third term in Eq. (1), formally by letting $E_r \cong 0$. This corresponds to the so-called dark-field condition, where, in principle, only the scattered light is collected by the imaging optics. In this case, high sensitivity is obtained by minimizing any straight light contribution to the image (low E_r) and optimizing the sample illumination (maximizing E_0). Recently, this approach has been exploited for the detection of single viral particles (H1N1) by Enoki et al., in the so-called total internal reflection dark field microscopy (TIRDFM) [100]. In this work, the authors obtained an effective rejection of the straight light, high power, and spatially selective illumination, by adopting a through-the-objective total internal reflection illumination scheme (Figure 7A). A similar principle is used in Ref. [104], although in a completely different experimental geometry. In this work, the solution containing the particles to be detected fills the core of a single-mode fiber. Particles are illuminated by the guided mode of the fiber, which provides an intense and spatially localized illumination. The light scattered by the particles at 90° with respect to the fiber axis is then detected with a conventional optical microscope. The study of the trajectory of each particle allows calculating its diffusion coefficient and the particle's size via a suitable Stokes-Einstein relation. Although affected by the typical limitations related to the use of micro- or nano-channels in real-life applications (e.g. clogging), this approach offers a very good sensitivity in the detection of and accuracy in the determination of the size of NP. In general, the major limitations of dark-field methods rely on the extreme sensitivity to stray light, in the difficulty of detecting small intensities, and in their narrow dynamic range in terms of particle size, due to the strong R^6 dependence of the scattered intensity that enormously amplifies the typical dispersity and heterogeneity of natural NPs.

These problems are less relevant in interferometric approaches, where the intensity of signal associated to the particle is generally larger and the size dependence is less pronounced. In this case, a large reference field ($E_r >> E_s$) is present and the relevant term becomes the last one in Eq. (1). One example of a conceptually simple, and, at the same time, effective method in this class is provided by

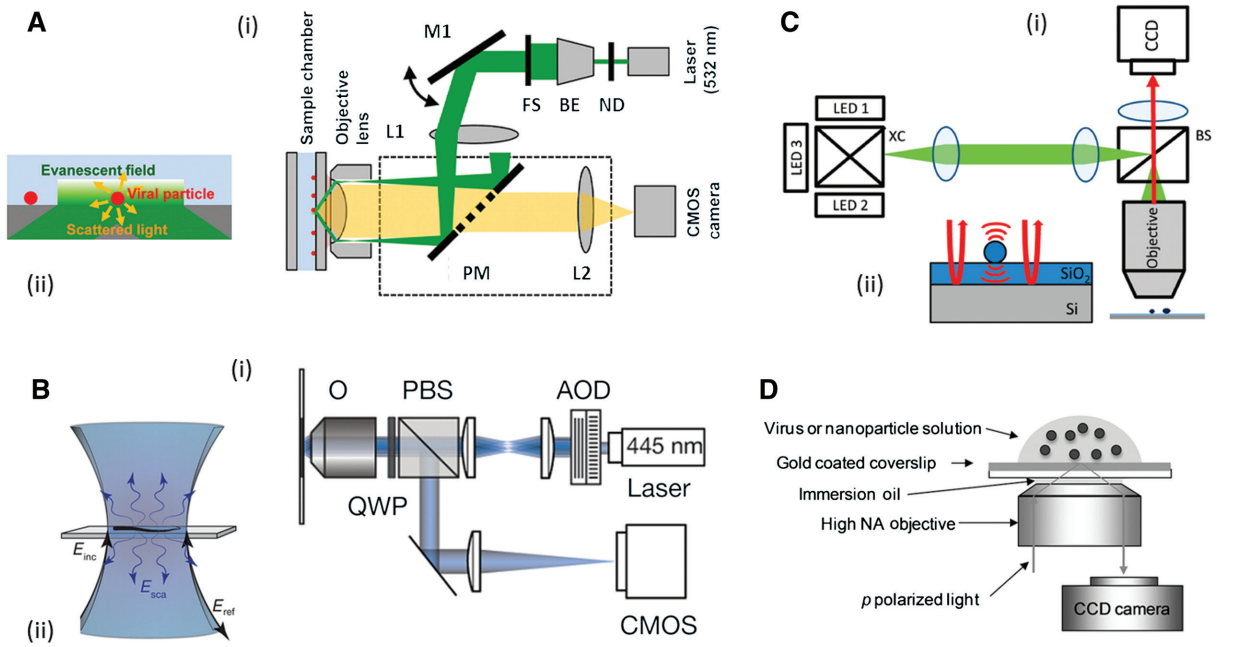

Figure 7: Label-free imaging methods for single bio-NP detection.
(A) (i) Schematic representation of the experimental setup for TIRDFM: ND, neutral density filter; BE, beam expander; FS, field stop; M1, mirror; L1 lens; PM, perforated mirror; L2, objective lens. (ii) Pictorial representation of the detection event. Adapted from Ref. [100]. (B) (i) Schematic representation of the iSCAT optical setup: O, objective; QWP, quarter wave plate; PBS, polarizing beam splitter; AOD, acousto-optic deflector. (ii) Pictorial representation of the sample region, where the incident E_{inc}, reflected by the substrate E_{ref}, and scattered by the biomolecule E_{sca}, are also shown. Adapted from Ref. [101]. (C) (i) Schematic representation of the optical setup for IRIS: XC, x-cube used to combine the beam of three different LED sources; BS, cube beam splitter. (ii) Pictorial representation of the layered substrate and of the optical paths leading to the interference signal. Adapted with permission from Ref. [102]. Copyright (2010) American Chemical Society. (D) Schematic representation of the SPRM experimental setup. Adapted from Ref. [103].

interference scattering microscopy (iSCAT). The optical setup of the iSCAT imaging system (Figure 7B) consists of a confocal microscope with a laser illumination where, differently from a standard fluorescence confocal microscope, the collected light is given by the coherent superposition of light elastically backscattered by the sample and light reflected by the glass slide, the latter playing the role of the reference field. This method has been demonstrated by Kukura et al. [105] for the high spatial and temporal resolution detection and tracking of single viral particles. More recently, it has been successfully used in conjunction with suitable time average schemes and differential algorithms to image single actin filaments and single myosin 5a HMM molecules [101]. The uses of a coherent light source and of a sophisticated microscopy setup are probably the major limitations of the application of this technique outside of a laboratory. A very interesting alternative is provided by the so-called IRIS method. Initially proposed by Ozkumur et al. in 2008 [106], this method relies on the presence of a thin-film coating on a reflecting Si substrate. The thickness of the layer, as well as the illumination wavelength, can be tuned to modulate the interference term, optimizing the response of the sensor to specific targets. Recently, this method has been successfully applied to the high-throughput parallel detection of single viruses by Daaboul et al. [102]. A schematic overview of the IRIS experimental setup with multicolor incoherent (LED) illumination is shown in Figure 7C. In this work, the target particles are selectively immobilized on the surface and the observation is performed in air, after washing and drying the surface. A major step forward toward the real-life application of this method is represented by the so-called single-particle IRIS, presented in a recent work by Scherr et al. [107]. In this paper, by coupling the IRIS sensing surface with a suitable microfluidic chip, the authors demonstrate the real-time detection and discrimination of single bioparticles, a recombinant vesicular stomatitis virus that expresses the surface glycoproteins of the Zaire strain Ebola (rVSV-ZEBOV), in a complex medium (serum). In the context of interferometry-based detection schemes, optical heterodyne detection is also worth mentioning. In this method, a prescribed frequency shift, corresponding to the so-called detuning frequency, is introduced in the reference beam, the so-called local oscillator. This allows to separately determine the phase and amplitude

of the interferometric term. Exploiting this approach, Mitra et al. [108] demonstrated real-time, single particle detection of synthetic dielectric NPs as well as several different viruses flowing through a microfluidic channel.

As already mentioned in previous sections, SPR is probably one of the most mature and widely used label-free sensing mechanisms, used both commercially and in research. In a typical configuration, SPR relies on the excitation of plasmonic waves on a thin planar metallic layer sandwiched between a dielectric substrate and the solution containing the sample to be probed. The light impinges on the surface with an angle close to that of minimal reflection, after being refracted by a prism coupled with the sensing surface and the reflected light is collected. A space-resolved map of the refractive index distribution over the sensing surface can be obtained by forming an image of the plane on an imaging sensor. The strongly tilted illumination and the aberrations introduced by the prism strongly limit the resolution of the image that can be obtained. An elegant solution to overcome this limitation is the one exploited by Wang et al. [103], by adopting an illumination/collection scheme similar to the one used in total internal reflection imaging (see Figure 7D) and compatible with a high-magnification, high-numerical-aperture objective. With this method, named SPR microscopy (SPRM), the authors demonstrated the real-time detection of the binding of single viral particles (influenza virus) on the sensing surface. Moreover, the measurement of the intensity associated with the image of a particle allows estimating its mass, providing an important tool for particle sizing and discrimination. This method, complemented with a differential processing of the images aimed at removing the optical background, has been recently pushed to the single-molecule level and applied to the detection of single λ-DNA molecules stretched on the gold surface [109].

A last important class of microscopy methods that are widely used for NP characterization is represented by absorption microscopy [110]. These techniques typically rely on the existence of large imaginary dielectric permittivity in correspondence of specific wavelengths, leading to large absorption cross-section. However, we will not discuss these methods here, as naturally occurring bio-NPs have negligible absorptions at optical frequency.

6 Label-free single-molecule detection

Since the possibility of detecting single fluorophores was demonstrated in the 1990s, sensitivity to single non-labeled molecules became the ultimate goal for ultra-sensitive biosensing. Although it originally appeared as a virtually impossible achievement, label-free single-molecule detection was demonstrated in the last 5 years with molecules of progressively smaller weight. This impressive accomplishment has been made possible by the development of two distinct technologies: nano-plasmonics and high-Q resonators. While both techniques were shown to enable recognizing surface binding of single molecules, the latest developments indicate that the future of label-free single-molecule detection will probably be in their combination.

When the longitudinal plasmonic resonance of gold nanorods is excited, the local field in proximity of the caps is enhanced by many orders of magnitude, and the effect of adsorbed molecules on the resonance peak is proportionally increased. In 2012, by using gold nanorods of diameter 9–20 nm and axial ratio approximately equal to 3, two distinct groups could demonstrate detectable shifts (0.2–1 nm) as single massive proteins (streptavidin conjugate of 300 kDa and fibronectin of 450 kDa) bind to the functionalized surface of the nanorods [111, 112]. As shown in Figure 8A, the individual events of binding lead to discontinuous redshifts of the plasmonic resonance. Such a shift is measured after total internal reflection (TIR) illumination with a white laser light [112], or through a photothermal effect [111].

Alternatively to local field enhancement, various groups have been considering the amplification provided by high-Q resonators, typically lithographically shaped dielectric material or dielectric spherical beads, in which photons circuit the structure with a long residence lifetime. This condition leads to the formation of a resonant wave dubbed "whispering-gallery mode" (WGM) from the notorious acoustic effect experienced in the whispering gallery of circular domes. In these systems, the linewidth of the resonance is extremely narrow, a condition that brings about a strongly increased sensitivity to variations in the dielectric properties of the surrounding medium. The exploitation of high-Q resonators to single-molecule label-free detection was demonstrated by a pioneering work carried out in CalTech [113] using a micro-ring resonator with $Q > 10^8$. In that work, the binding of single-protein interleukin-2 to antibodies previously attached to the resonator is detected as tiny resonance shifts of the order of 0.1 pm (Figure 8B). However, although small, such shifts are still much larger than expected, an observation that led the authors to investigate further and interpret the observed effects as thermal. The single-molecule detection was, in this case, mediated by light-adsorption-induced heating of the micro-ring.

A significant step toward an even further sensitivity to the binding of unlabeled molecules was recently achieved

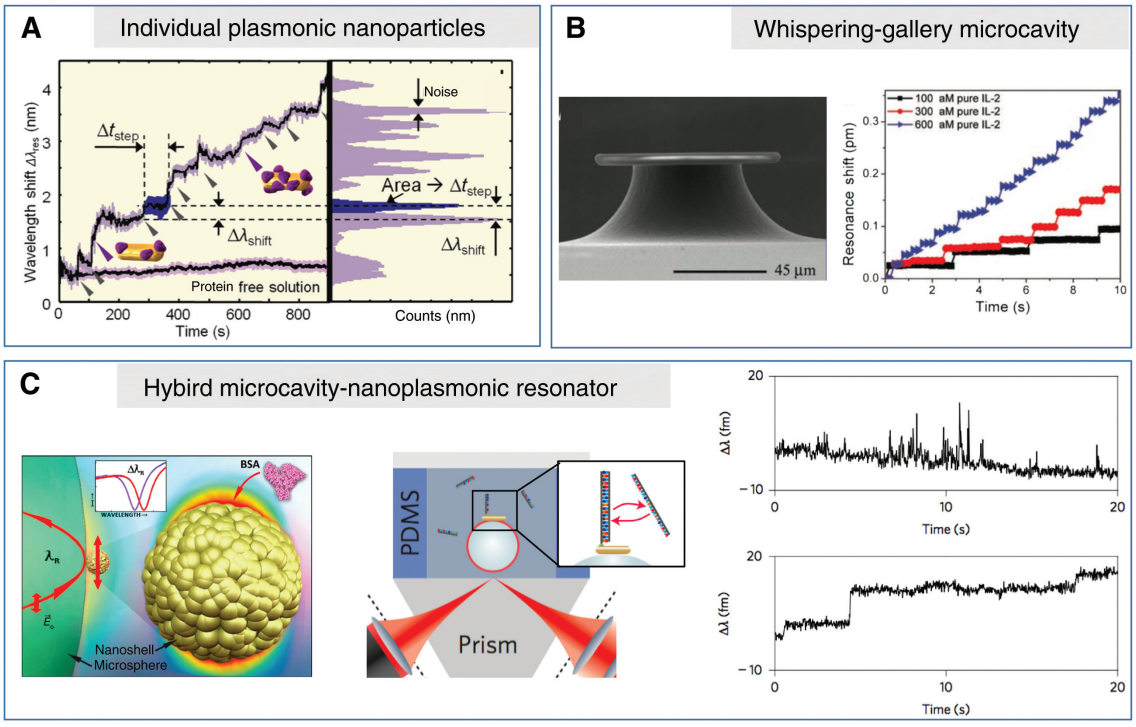

Figure 8: Detection of non-labeled single molecules.
(A) The adhesion of individual fibronectin proteins shifts the plasmonic resonance peak of the gold nanorods (left). The discontinuous behavior clearly appears on the histogram of the same time traces (right). Reproduced with permission from Ref. [112]. Copyright 2012 American Chemical Society. (B) Silica micro-toroid whispering-gallery resonators (left). As individual interleukin-2 proteins bind to the surface, the resonant wavelength position discontinuously shifts. The rate of adhesion steps depends on protein concentration (right). Reproduced with permission from Ref. [113]. Copyright 2007 The American Association for the Advancement of Science. (C) Pictorial illustrations of a protein adhering to a gold nanosphere with irregular surface, which, in turn, is in contact with a microsphere supporting WGM (left panel, reproduced with permission from Ref. [114]. Copyright 2013 American Chemical Society) and of the coupling scheme to a microsphere supporting WGM decorated by a gold nanorod with DNA 16-base long oligomers grafted on it (central panel, reproduced with permission from Ref. [115]. Copyright 2014 Nature Publishing Group). As complementary DNA strands are placed in solution, they bind to the grafted oligomers either transiently or permanently depending on the conditions of the solution, producing either spikes (right upper panel) or steps (right lower panel) in the WGM frequency.

by the combination of the WGM of dielectric resonators with nanoplasmonics. Indeed, when the narrow mode of the resonator matches the plasmonic resonance of the NP, and when the NP is located in the equatorial plane of the resonator where the mode is localized, a particularly favorable condition of strong local fields and hence strong sensitivity to the presence of molecules is attained. With this strategy, Arnold and coworkers detected the binding events of single BSA proteins [114]. Their sensitivity was increased by having chosen gold NPs with an irregular surface, providing an even stronger localized field enhancement. In 2014, Vollmer and coworkers [115] detected the hybridization of single oligonucleotides to complementary strands previously bound to NPs (Figure 8C).

Along the same line, but with a distinct strategy, there are approaches that exploit nanostructured metal-dielectric materials ("meta-materials"), in which the high Q is provided by the periodic metal structures instead of the WGM in a dielectric resonator [41]. Although these materials have not yet achieved single-molecule sensitivity, various attempts are under way.

7 Conclusions and outlook

Over the past decades, many technological solutions have been proposed to realize label-free optical biosensors with various properties. At the same time, given their flexibility, this kind of biosensors has been used to detect different kinds of targets, in different matrices and conditions. This review provides an overview on recent works on label-free optical biosensors on the basis of their potential

exploitability in a few specific application fields. These applications were selected because they take advantages of the peculiar features of label-free biosensors, and therefore a significant impact on these application fields is expected by further developments of biosensor technology. The selected application fields are: (i) rapid, simple, and compact analytical devices; (ii) small-molecule detection; (iii) sensing schemes targeting or mediated by NAs; (iv) digital detection of viruses; and (v) single-molecule detection.

In the case of rapid and compact analytical devices, label-free methods enable minimal measurement procedure, because they may not need the addition of secondary probes or other reagents to perform the analysis. The ideal system is formed by cheap sensors, either disposable or reusable, and by a compact, semi-automatic device. Many studies on rapid analytical solutions focused on protein biomarkers. However, NAs, small molecules, and bio-NPs can also be important targets. Therefore, this promising application field basically includes all the possible targets. A common challenge for this class of devices is to reach the capability of performing the measurement in real complex samples, avoiding or limiting the sample preparation phase.

For small-molecule detection, the sensitivity of the sensor is one of the most important features to reach suitable performance. In general, methods based on fluorescence or enzymatic amplification can provide better sensitivity relative to label-free sensors. However, the use of secondary probes often is not feasible in the case of small-molecule targets. Therefore, label-free sensors with suitable sensitivity and selectivity can represent competitive solutions, especially in the case of environmental monitoring or food safety analysis. To reach lower LOD, novel solutions typically rely either on suitably designed sensing surfaces or on the combination of different detection techniques. Nevertheless, in order to become exploitable technologies competitive with the current approaches, the novel sensors must be cost-effective and robust enough for uses outside the laboratory. These conditions represent the current challenges for the effective exploitation of optical label-free methods to small-molecule detection.

A specific section is dedicated to biosensors involving NA hybridization. As for other target types, also in the case of DNA or RNA detection, label-free methods can offer advantages like ease of use, access to kinetics, and absolute quantitation of bound target. What remains often challenging is to reach a suitable sensitivity to very low molecular concentrations, especially for diagnostic applications. In this respect, extremely high sensitivities have been currently demonstrated for different label-free methods based on nano-structured plasmonic surfaces. A particularly interesting class of systems is also provided by smart detecting surfaces realized with responsive DNA probes conjugated to fluorescent labels. The fluorescence signal is modulated by the conformational change of the probe strand upon target binding, without the addition of secondary probes or reagents. The research on this class of biosensors is expected to grow in parallel to the development of novel DNA-based responsive materials, in the frame of the so-called functional DNA nanotechnology. A new format of label-free optical biosensors can be envisioned, in which the biorecognition element not only provides the binding but also is somewhat responsive and generates an amplification of the signal transduced by the optical sensor.

Another promising application field is the identification and enumeration of biological NPs, such as viruses or exosomes, in various fluid samples. The feasibility of a "digital" detection of bio-NPs has been demonstrated for different methods based on label-free imaging. At the present stage, in the perspective of exploitation of these methods also outside the research laboratories, the main challenges include selectivity, namely the ability to discriminate the target NPs in a "sea" of other objects similar in size and optical properties; flexibility and high dynamic range, to account for the large natural variability of the targets, even within a single sample; ease of use; and automation of the particle recognition and size/mass quantification steps. Indeed, devices enabling the digital detection and screening of bio-NPs are expected to have a high impact in various contexts, including diagnostics and environmental monitoring.

Finally, a recent application of label-free optical biosensors is the detection of single molecules. This represents an unexpected breakthrough with very high potential of further development. In this case, extremely high sensitivities are the main requirement. To date, only a couple of techniques have reached suitable performance. These are based on plasmonic nanostructures and high-Q resonators. In particular, the best results have been achieved by a combination of the two approaches. In general, the access to optical signals originated by a single molecule, without the perturbation induced by fluorescence labels, is expected to bring about an important contribution to the fundamental researches aiming at unraveling the behavior of biomolecules.

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