Prospects of optical biosensors for emerging label-free RNA analysis

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Abstract

RNA is critical in countless cellular processes, and researchers are constantly discovering new types and attributing them different roles. Consequently, a growing interest in efficient RNA analysis has arisen. However, RNA detection is complicated and generally requires the use of labels. Major efforts are being devoted to conceive new approaches for RNA analysis with no need of markers. Optical biosensing is a highly sensitive approach that circumvents many of conventional methods' limitations. Lately, label-free applications with optical biosensors have been developed for short as well as for long RNAs. The low limits of detection at the pM level enabled by optical biosensors, together with a fast analysis, their reusability and the label-free scheme of operation, clearly highlight them among the most promising next-generation RNA screening platforms. This review covers the most relevant optical biosensor platforms and their potential for enabling sensitive and label-free RNA analysis.

Keywords: RNA, optical biosensor, label-free detection, microdevices

1. RNA, a key player in the biology of cells

Gene expression is finely regulated in cells for the proper management of protein production and their contents in the right place at the correct time, guarantying the perfect equilibrium necessary for ensuring cell survival. Great number of macromolecules and processes are involved, making it more and more difficult to elucidate which mechanism leads the cell to choose one pathway or another. Recently, researchers have focused on the multiple and variable function of RNAs[1], which play critical and diverse roles in countless cellular processes. Current methodological advances, including bioinformatics, microarray-based, biochemical and deep-sequencing studies, are revealing new insights into the roles that the regulation of RNA has in generating organism complexity from a relatively small number of genes.

Although they share the same nature, the RNA family is very diverse – in sequence and structure- having different and crucial roles in cell biology (see Table 1)[2-14]. Depending on whether they are involved in protein production or other regulatory functions, they have been classified into *protein coding* RNAs and *non-coding* RNAs (nc-RNAs)[15]. Protein *coding* RNAs are solely represented by the *messenger RNA (mRNA)*, though constituting a large family of RNA molecules that convey the genetic information from the DNA, directly serving as templates for the protein translation. On the contrary, the ncRNA family covers RNAs of many different lengths (from ~20 to ~several kb) and properties, most of whose functions are unknown. Although many classifications have been proposed the most widely accepted is that based on their structural or house-keeping roles and their regulatory role (Table 1). At the same time, ncRNAs playing regulatory roles are sub-classified according to their length in short (~20-200 nt) and long nc-RNAs (200 nt up to several Kb).

Until recently, ncRNAs were supposedly inert; but far from being of any usefulness, they have demonstrated to account for most of the cell's genomic output[9, 16] and display a very wide

repertoire of biological functions such as cell cycle regulation, pluripotency, retrotransposon silencing, meiotic entry, telomere length, imprinting or dosage compensation. To execute their function, ncRNAs can either control the transcription or translation of proteins via a number of mechanisms including provision of the machinery for translation (i.e., rRNAs and tRNAs), mediating in the splicing process (e.g., intronic RNAs) remodeling the epigenome (i.e., DNA methylation and histone modification) [17, 18] or by interacting with other RNAs and proteins. For example, miRNAs in the cytoplasm can interact with the mRNA product and inhibit its translation process. At the transcriptional level, nc-RNAs such as ln-ncRNAs can interact with the genomic DNA via formation of duplexes [13, 19-23] or triplexes [6, 9, 11]—an epigenetic pathway that has only recently been discovered [9, 11, 24], and recruit other protein regulator factors to alter gene expression. Importantly, these functions are not limited to the cell environment where the nc-RNA has been transcribed. Some non-coding RNAs can be exported out-of-cells via exosomes [25, 26] or freely circulate into the bloodstream [27]. This enables these ncRNAs to target other cells from distant organs. Exportation of RNA from cells is paramount in the onset of many diseases (*e.g.,* cancer); thus, researchers have endeavored to identify biomarkers in these pathways that can be exploited for early diagnosis and treatment [28].

Nonetheless, the remarkable plasticity of RNA can even surpass the coding/non-coding classification. Recent investigations [4] are showing that protein-coding and non-coding RNAs might not be mutually exclusive; some RNAs can function both as mRNAs and as functional ncRNAs, thereby RNA should be rather regarded as a multi-tasking molecule that can produce multiple products and that comprises a hidden stage of various levels of gene expression players and controllers.

Due to the increasingly recognized physiological importance of RNA in physiology and development, along with recent ground-breaking insights into its functions, RNA has been catapulted to the research forefront. RNA regulatory networks may determine most of our complex characteristics, which play a significant role in diseases and constitute an unexplored world of genetic variation both within and between species.

2. Challenging aspects of RNA detection

Regardless of the method employed for RNA analysis, there are intrinsic features on RNA molecules that deserve careful consideration for successfully designing a detection assay. These features can be summarized under the five "S's" criteria, i.e., *stability, sensitivity, specificity, size,* and *structure [29]*:

Stability: RNA is relatively unstable at room temperature, and it is extremely sensitive to the destructive action of ribonucleases and alterations in pH [30]. However, there are some exceptions, such as circulating RNAs in the bloodstream, which are stabilized and protected by other molecules and structures (e.g., exosomes).

Sensitivity: Cellular concentrations for many RNA types are typically very low. This, together with the low stability of RNA, leads to the need of very sensitive methods for RNA detection. However, many conventional techniques does not provide that sensitivity [31] requiring microgram quantities of total RNA —equivalent to over million cells—to achieve detection. This might become problematic when the sample comes from precious sources like biopsies or microdissections. The challenge of RNA detection goes forward when it is intended for capturing RNAs present in serum or plasma (i.e. circulating RNAs) which might exhibit extremely low concentration levels. In this regard, the method employed for extraction of

Specificity: RNA molecules of the same family (*e.g.,* mRNA) often share physicochemical properties [32] and even share portions of sequence. For example, the different isoforms generated by the alternative splicing of a gene share one or more exonic sequences that might complicate the specific detection of one of them (*i.e.,* detection without cross-talk from the other). The same problem arises with pre-microRNAs (pre-miRNAs) and its final miRNA product, since the miRNA sequence is already contained within the pre-miRNA one. Also close-related RNA sequences, such as those from members of the same miRNA family might only differ in one or few nucleotides, making their accurate detection challenging.

Size: RNA types vary greatly in length according to their function, they can go from 18 nucleotides (nt) to several thousands. For example, non-coding RNAs such as microRNAs can be as short as 18 to 20 nt, whereas other RNAs such as mRNAs, rRNAs and lnRNAs are very long (> 200 nt). Size affects detection in many ways: for instance, short RNAs are difficult to detect, as they have the same length as the primers used in most detection methods, whereas long probes hybridize well but have poor sequence specificity.

Structure: A hallmark of RNAs is that they tend to be very structured, especially as they increase in length. Unlike DNA, RNA has ribose within the nucleic acid backbone, which provides an extra oxygen atom that is free to promote further interactions between nucleotides. Pairing of local nucleotides generates secondary structures (*e.g.* hairpins and stem loops), while interactions among distantly located sequences generate tertiary structures [33]. The 3D-structure of an RNA molecule is essential to its biological function, but this inherent geometry can impair hybridization of RNA to a capturing probe [34].

3. Methods for RNA analysis

3.1 Conventional methods for RNA analysis

Current methods for RNA analysis are mostly based on processive PCR-based technologies whereby amplified products of reverse-transcribed RNAs are analyzed. This approach affords semi-quantitative data on a limited number of expressed genes. For the simultaneous test of thousands of expressed genes (*expression profiling*), micro- and nano-arrays are more appropriate tools [35, 36]. Other conventional methods, such as Northern Blot or Ribonuclease Protection Assay (RPA) are also common, but achieving a high level of multiplexing is more challenging.

Even the best of these RNA detection methods is limited by the 5 S's above mentioned, requiring especial strategies to deal with them. For example, to overcome sensitivity, most of them incorporate some kind of sample amplification and/or signal amplification [37]. While sample amplification strategies amplify RNA samples prior to detection, either exponentially or linearly (i.e., using PCR or RNA/DNA polymerase-based methodologies), signal amplification strategies amplify the signal from labeled targets (e.g. using 3DNA dendrimers, quantum dots or branched DNA). Labeling is indeed a major requirement for most RNA detection methods since they are not only critical for sensitivity, but for increasing specificity. They become even more essential when using multiplexed approaches such as microarrays. Conversely, the labeling process makes detection slower, costlier and less reliable, since it is often difficult to precisely control the number of labels on each molecule across different experiments. Moreover, due to some of the "5 S's" RNA intrinsic features, such as RNA size variation and 2D/3D structure; it is complex to equally label all species of a particular RNA type in the sample regardless of specific sequence or structure. For example small RNAs labeling can be particularly complicated to perform in a reliable way, as their short size impedes direct

incorporation of labels by ligation or chemical modification [38]. When any label signal bias arises, quantitative analysis becomes challenging.

In addition to the "5 S's" issues, there are also many others affecting the reliability of RNA analysis. Template preparation and the dispensing of reagents might be subjected to a certain degree of variability and contamination when different operators prepare multiple templates, which can only be reduced using expensive robotized systems [35]. For example, the reliability of RNA detection via micro/nanoarrays has been questioned, as they are influenced by array production, RNA extraction, probe labeling, hybridization conditions and image analysis [39-42].

As a consequence, standardization of RNA detection is non-trivial [30, 31, 37]. There is a real need for the development of novel detection methods tailored to deal with problematic intrinsic features of RNA, namely, easy-to-use, highly-sensitive diagnostic devices that provide quantitative data on RNAs from crude biological extracts, and that do not require reverse-transcription, amplification or labeling.

3.2 Biosensors as ideal platforms for label-free RNA analysis

The ideal screening platform for RNA would be, above all, label-free, enabling the access to the entire target RNA population in the sample; consequently, the results would be more reproducible and reliable, not depending on the number of labeled molecules.

Current label-free assay biosensor technologies provide a rapid and often real-time analysis, affording direct measurement of the affinity, kinetics and thermodynamics of biomolecular interactions *in vitro*—information that is difficult, if not impossible, to collect by other

methods. These technologies can provide the clearest—and most quantitative—biochemical characterization of molecular interactions available today [43].

Albeit obviating the use of labels is crucial for improving current methods for RNA analysis, several other features are desired, such as greater specificity, sensitivity and reproducibility; amenability to the full range of RNA lengths; and the ability to detect structured targets. Label-free based biosensors are emerging as ideal tools for the next-generation of RNA detection methods since they can offer rapid, label-free detection of biomolecules of any type with excellent specificity, sensitivity and reproducibility [43-48]. In addition, recent advances in nanotechnology have triggered the development of novel nanobiosensors, which are gradually progressing from the lab to the clinic. Future lab-on-a-chip devices incorporating an array of nanobiosensors could be employed to rapidly and simultaneously screen various analytes at low cost and using minimum volumes of physiological sample [49-52].

The success achieved with biosensors derives from their basic composition: a biosensor contain a biological component, the *receptor*, which recognizes a target molecule in a specific way; and a signal-generating component, the *transducer*, which detects the resulting biomolecular interaction and converts this interaction into a measurable signal [53] (see **Fig. 1**). The presence and concentration of biological molecules in a given medium are measured by translating the biochemical interaction occurring at the sensing surface into a quantitative physical signal. This implies that the receptor(s) used must be previously immobilized onto the transducer surface and be highly specific for the target analyte(s). Biofunctionalization of the sensor surface is a critical step for ensuring high specificity and sensitivity [54]. Indeed, although sensitivity is highly dependent on the transducer method, astonishing results can be obtained when biofunctionalization is finely optimized [55, 56].

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The working principle of a biosensor depends on the type of transducer used, which is selected according to the analysis required. The most employed are electrochemical transducers and optical transducers [57-59]. Electrochemical biosensors offer excellent sensitivity and are highly amenable to miniaturization and to multiplexing detection; however they usually demand labeling and/or amplification strategies to generate a sensitive reaction, which in turn might complicate the detection scheme [60]. Optical sensors afford label-free detection with high sensitivity; they can be also miniaturized and multiplexed although the instrumentation can be complex [58]. As optical methods are the most feasible for label-free detection this review will focus only on recent advances in RNA detection achieved by optical biosensors.

3.2.2 Optical biosensor platforms

Optical biosensors based on the detection of changes in the refractive index (RI) [61] in the vicinity of the sensor surface are the most employed for label-free analysis. In these biosensors the binding of the analyte to its specific bioreceptor produces a local change in the refractive index at the sensing surface that is evaluated by the transducer. Refractive index changes are probed by the evanescent electromagnetic field that is generated in either guided or resonant optical modes traveling in the transducer. An evanescent wave is a near-field standing wave whose intensity exhibits exponential decay with distance from the boundary at which the wave is formed; it can range from a few tens to a few hundreds of nanometers, exhibiting the highest intensity at the sensor surface. As the optical detection of the local changes in refractive index is confined to the sensing surface—where the target is captured by the receptor—these methods are insensitive to the presence of unbound analyte or to other interfering molecules in the bulk solution. Moreover, detection is related to the sample concentration or surface density, instead of to the total sample mass, since the detection signal does not scale down with the sample volume. This characteristic is particularly attractive when a small detection volume is involved, and it is advantageous over other labeled-based

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methods, whose signal usually depends on the total number of analytes in the detection volume.

The main types of RI-based biosensors (extensively reviewed in refs [61-66]) are:

(i) Surface Plasmon Resonance (SPR) biosensors (see **Fig. 2A**): They are based on the generation of a surface plasmon wave, a charge density oscillation that occurs at the interface of a metal (the transducer surface, typically gold, onto which the bioreceptors are immobilized) and a dielectric [67]. There are several configurations for SPR sensors, the most widespread of which is the Krestschmann configuration. SPR is a well-known technology and is commercialized by several companies worldwide. SPR is a very sensitive biosensor technique, but is challenging for multiplexing. There are other plasmonic configurations with better multiplexing capabilities, such as Localized Surface Plasmon Resonance (LSPR) sensors (which use metal nanostructures) and Surface Plasmon Resonance imaging (SPRi) sensors (based on the spectroscopy analysis of the whole sensor surface where different receptors have been spotted in separated dots; Figure 2B). Other technique, Surface Enhanced Raman Scattering (SERS) (excited by localized surface plasmon resonances) can afford a high level of sensitivity and it is particularly interesting for multiplexing, but it is more complex to handle and integrate. In general, SPR-based biosensors can reach detection limits in the nM-pM range, and this limit can be even improved by using non-labeling target amplification strategies (*e.g.,* use of secondary antibodies, enzymatic reactions or nanoparticles).

(ii) Resonant waveguide based biosensors: In these sensors the incident light is generally coupled in a waveguide at a specific resonant angle, and the guided mode shows a strong evanescent field [62]. Consequently, the resonant angle is sensitive to the RI change near the waveguide surface. Examples include resonant mirrors [68, 69] (RM) and metal-clad waveguides (MCWG) [70].

(iii) Integrated interferometer-based biosensors: They are based on interferometric evaluation of the changes in the wave-vector of a guided mode as a consequence of the biomolecular recognition event. The most relevant examples are the Mach-Zehnder [71] and the Young interferometers [72]. They are extremely sensitive—orders of magnitude higher than SPR– but their integration and multiplexing are technologically complex. Recently, an innovative type of interferometer-based biosensor, the bimodal waveguide (BMW) sensor, has been introduced [52]. It offers the high sensitivity of interferometric biosensors, as well as greater amenability to integration, miniaturization and multiplexing (see **Fig. 2C**).

(iv) Micro-ring resonators based biosensors (see **Fig. 2D**): they constitute an emerging sensing technology where ring-shaped waveguide structures (micro-rings) are optically coupled to one or more linear waveguides, patterned on a planar surface. The light propagates inside the micro-ring in the form of whispering gallery modes (WGMs) which generate an evanescent field at the resonator surface. The effective light-analyte interaction length of a ring resonator sensor is determined by the number of revolutions of the propagating light supported by the resonator [73].

(v) Optical fiber-based biosensors: Like planar waveguide biosensors, optical fibers exhibit an evanescent field along their structure that penetrates into the external medium, where the biosensor reaction occurs. Interesting types include nanofibers, Bragg fibers and Fabry-Perot based fibers [74].

(vi) Photonic crystal (PC)-based biosensors (see **Fig. 2E**): photonic crystals have a periodic dielectric structure with a periodicity on the order of the light wavelength that forms a photonic bandgap. When a photonic "defect" is introduced within the bandgap, the light resonant with the defect mode can propagate in the PC. Since the spectral position of the defect mode is highly sensitive to the change in the local environment around the defect, it can be used as the sensing transduction of the binding of the analyte molecules to the

defect. Photonic crystal-based biosensors have the advantage of strong light confinement, provided by the photonic cavity, and high flexibility for adjusting the defect mode wavelength across the photonic bandgap [75].

4. Current applications for detection of RNA using optical biosensors

To date, most of the optical biosensor applications for RNA analysis have chiefly focused on miRNAs, m-RNAs, r-RNA and recently, on RNAs potentially interacting with the genomic DNA via formation of triplexes. Despite the extensive literature on many different biosensor schemes employed to detect nucleic acids, most are mainly devoted to DNA oligonucleotides [76]. In principle, given the similarities between DNA and RNA, the results obtained for DNA oligonucleotides could be considered valid for the homologous RNA sequence. However, extraction is really a challenging aspect of RNA determination, which can strongly influence the analytical performances of biosensing tools when addressing detection directly from real biological samples. Therefore, since the RNA-detection capability of these schemes has not been demonstrated with real RNA samples, applications using DNA oligos have not been considered in this review.

There are also examples of indirect RNA detection, whereby the RNA sample is first converted into cDNA by methods such as RT-PCR. Given that the conversion step complicates the detection and limits the biosensing technology from offering any unique advantage over reverse-transcribed based conventional methods, these applications have not been considered either. This review has therefore strictly focused on biosensor applications for directly detecting different types of RNA (including synthetic RNA) in biological samples without any reverse-transcribed amplification. A summary of the main outcomes and analytical performances of each method in the detection of the different RNA types is depicted in Table 2.

4.1 Detection of small RNAs

Several types of optical biosensors have been employed to detect small RNAs (mostly in the range of 20 nt) such as miRNAs. Surface Plasmon Resonance biosensor and its microarray format homolog, SPRi, have shown to be incredibly versatile and effective for direct sensing of miRNA. However, the short length of small RNAs translates to a small mass to be detected, which reduces their capability to achieve high sensitive detection (sensitivity is typically in the nM range). To enhance sensitivity researchers have employed a variety of non-target labeling amplification strategies, which enable detection limits at the pM or even fM levels. The disadvantage of these strategies is that the addition of one or more post-detection steps requires longer measuring times. Typically, standard SPR detection takes less than few minutes; however, addition of an amplification strategy can extend the read-out under 1 h, which is still reasonable. For example, Sipova *et al.* [77] achieved rapid, label-free detection of miRNA-122 from mouse liver tissue at 0.2 nM, using a novel, high-performance portable SPR sensor. But they managed to lower their previous detection limit by two orders of magnitude—down to a reported value of 2 pM—by performing an amplification step in which the captured miRNA is subsequently recognized by an antibody that exhibits affinity to the RNA*DNA hybrids. Nonetheless, the linear range of evaluation only extends to miRNA concentrations up to 10 nM and 0.1 nM for the hybridization and antibody-recognition steps, respectively (**Fig. 3A**). The sensitivity for evaluating RNA concentrations under these values is limited and, probably, unreliable. Despite the additional antibody step, the total time for miRNA detection was only 35 min. A similar strategy was developed by Nasheri *et al.* [78]. They directly detected synthetic miR-122 using a SPRi biosensor achieving a detection limit of 40 nM. After hybridization, the signal was amplified using the protein p19, which has affinity towards the hybrid RNA/RNA duplex. Although this strategy is interesting for demonstrating the utility of p19 for the recognition of miRNA-bound probes, this approach does not appear to be as effective as the antibody-recognition one: Nasheri *et al.* were only able to lower their

detection limit by one order of magnitude, whereas Sipova *et al.* achieved two orders of magnitude. However, the worse detection limit of the former could also be due to the technique itself, as SPRi is less sensitive than standard SPR.

Detection can also be largely improved through the use of more effective strategies for amplification and biofunctionalization. For example, Fang *et al.* analyzed three types of synthetic miRNAs (miR-16, miR-23b, and miR-122b) by SPRi achieving an incredible detection limit of only 5 attomoles (10 fM) [56]. They optimized the biofunctionalization by (i) using locked nucleic acid (LNA) probes, which have demonstrated to increase both the sensitivity and the specificity of hybridization [79] and (ii) subsequently recirculating the miRNA target solution over the LNA-derivatized sensing surface for 4 h. Although this is a long assay period, it enabled better saturation of the receptors for a given miRNA concentration, thus obviating the use of higher concentrations. Regardless, the cornerstone of their success was probably the impressive degree of amplification that they obtained using a two-step approach involving enzymatic polyadenylation extension of captured miRNAs followed by recognition of the generated polyadenylated tails by nanoparticles coupled to polythymidine oligonucleotides (see **Fig. 3B**). The real-time capability of this sensor enabled monitoring the entire procedure in real-time, including the polyadenylation reaction, in which the incorporation of each adenine was observed. The use of total RNA sample extracted from mouse liver tissue as a source for miRNA determination instead of synthetic miRNAs did not affect significantly the analytical performance of this method, which still could demonstrate detection of these miRNAs at femtomolar concentrations. Moreover, they tested the same amplification strategy on a novel optical biosensor technique based on Nanoparticle-Enhanced Diffraction Gratings (NEDG). In this sensor the optical properties of surface plasmons generated on gold gratings are coupled to the optical properties of adsorbed gold nanoparticles [80]. Using this sensor, synthetic miR-122b targets were detected at a concentration of 50 pM, which was higher than when using

the SPRi sensor. However, the NEDG sensor could be improved by the ability to tune the plasmon excitation wavelength in the gratings using different types of nanoparticles.

Zhou *et al.* [81] devised another interesting amplification strategy for SPRi, for the analysis of three synthetic miRNA mimics. They employed DNA-modified silica nanoparticles complementary to the miRNA target for capturing. The target was then fixed to the nanoparticle-DNA sequence by using a T4-ligase enzymatic reaction. Finally, the read-out of the nanoparticle-target pair was done by SPRi using receptor probes arrayed on the sensor surface that were also complementary to the target RNA sequence. The silica-nanoparticle step plays a double role towards enhancing target detection: firstly, it enables purification and concentration of the target, as they can be centrifuged, separated, washed and reconstituted in a smaller microliter volume format; and secondly, it enhances the target recognition signal, as it amplifies the mass loading at the sensor surface. The final result is a biosensor method able to detect multiple ssRNA sequences at concentrations as low as 100 fM. Recently, Joshi *et al.* [82] developed a technology based on solid-state localized surface plasmon resonance (LSPR) consisting of highly sensitive nanostructures (gold nanoprisms). They achieved the direct detection of miRNAs 21 and 10b at a LoD 23-35 fM in plasma extracted total RNA. Detection of the same miRNAs from raw plasma samples without performing the RNA extraction step showed that miRNA concentration in extracted samples was at least 2-fold lower than in the pure plasma samples. This outcome puts in evidence the large impact of the methodology employed for RNA extraction in the accuracy of detection since it can cause significant loss of miRNAs during the RNA extraction process. The large sensitivity of LSPR, which enables performing detection without the need for sample purification and pre- or post-amplification steps place this type of biosensing technology at the forefront of miRNAs detection technologies.

Microring resonators based biosensors demonstrated to be as sensitive as SPR for detecting the changes in refractive index that accompany the binding of a miRNA target. Qavi

et al. [83] employed arrays of silicon microring resonators to analyze four miRNAs from a clinically-relevant sample of a cell-line model of glioblastoma, with minimal sample preparation (see **Fig. 3C**). They reached a detection limit of 150 fmol (~2 nM) without any amplification, a similar value to that obtained by SPR; however, they achieved a wider linear dynamic range of more than two orders of magnitude. To achieve this wide range, rather than utilize the absolute wavelength shift, which saturates as miRNAs hybridize to all of the available ssDNA capture probes, they determined the rate at which the resonance peak changes immediately after target introduction, and they used the initial slope response for quantification. The advantages of this approach were the generation of a linear calibration and a decrease in the total assay time (~10 min).

Driskell *et al.* [84] employed Surface-Enhanced Raman Spectroscopy (SERS) to detect five different synthetic miRNAs at μM level (but it only requires 1μL of RNA sample) in very short acquisition time (only 10 s). Interestingly, they identified single nucleotide polymorphisms that enable discrimination among eight members of the let-7 miRNA family. SERS is a promising method for miRNA detection that enables label-free detection of multiplexed miRNA. However, due to the subtle differences in peak intensity as a function of distinct—but related—sequence composition, the identification of specific sequences requires extensive multivariate analysis. A novel integrated optical biosensor based on a slot waveguide Mach-Zehnder interferometer (MZI) configuration was also developed for multiplex miRNA detection by Liu *et al.*[85]. It allowed a rapid and accurate detection of two types of miRNAs (miR-21 and let-7a) in human urine samples in a single reaction down to 1fM level of sensitivity. Moreover, this biosensing platform also demonstrated the ability to identify the single nucleotide polymorphism of the let-7 family of miRNAs. This large sensitivity and specificity alongside its capability for integration put this biosensor a step forward for the development of a point-of-care (POC) test for disease-associated miRNAs.

4.2 Detection of long RNAs

4.2.1. Messenger RNA (mRNA). Messenger RNA (mRNA) analyses are required for understanding certain biological mechanisms, for example, to determine whether changes in mRNA levels correlate to the translation of a given protein and to pinpoint the cell types that exhibit said changes, which in turn might be related to the onset of many diseases. Albeit the number of copies of mRNA in a cell type varies drastically depending on the mRNA type, the number of copies of any given mRNA in a cell extract is usually very low. Thus, mRNA detection requires high sensitivity. Although challenging, the advantage of mRNA biosensing relative to short ones, such as miRNAs, is that they have larger mass and generate larger refractive index changes. As a result, detection of cellular levels of mRNAs does not always require post-capture amplification strategies. Conversely, detection of mRNA suffers from certain drawbacks: for instance, since they are longer they exhibit extensive secondary structures that, together with their large size, might increase the probability of inadequate orientation and poor accessibility. Also, diffusion of mRNA is considerably slower than that of smaller RNA molecules, which translates into longer assay times for comparable sensor responses [86, 87]. Another problem is specificity: discrimination among the huge variety of mRNAs within a given cell is not trivial; especially among gene-related isoforms (these are generated by transcription of the same gene during alternative splicing and can share one or more exons).

Taken together, the aforementioned difficulties might explain why the literature contains only a few examples of mRNA detection by optical biosensors. One such example is the work reported by Kindt *et al.* [87] on the detection of three full length mRNA transcripts (c-myc, β-actin, and IL-8, in HL-60 cells) using microring resonators. The authors circumvented the problem of accessibility to target regions caused by mRNA structuring by employing chaperone DNA probes (small DNA helper strands complementary to the adjacent matching region) creating a localized duplex region with reduced secondary structures. To ensure specificity during detection, authors selected various capturing sequences for simultaneous use as receptors designed by BLAST searches (to target unique identifier regions of the primary sequence and successfully eliminate cross-hybridization among other mRNA species) and by Mfold prediction (to reduce the likelihood of secondary structures at the matching region). They also employed a two-step, bead-based, signal enhancement strategy to increase the sensitivity and to widen the linear range up to four orders of magnitude, reaching a detection limit of 512 amol (~2.5 pM). Finally, to fully demonstrate the capabilities of the biosensor array for mRNA analysis, Kindt *et al.* monitored the change in mRNA expression of the three transcripts upon differentiation of HL-60 cells. They detected changes in both myc and ꞵ-actin mRNAs, but were unable to detect IL-8 mRNA transcripts due to their lower abundance. This result highlights the need for novel approaches offering greater sensitivity.

A second example of mRNA detection by optical biosensors is a direct-detection strategy recently developed by Huertas et. al [88] using a portable SPR biosensor. The authors explored the analysis and quantification of the two main mRNA isoforms generated by alternative splicing of the pro-apoptotic gene *Fas* with no need of cDNA conversion and amplification steps, obtaining comparable levels of sensitivity than conventional qRT-PCR-based methodology. In this study, to overcome accessibility problems due to the long length of the mRNA isoforms they performed a simple fragmentation step based on RNA alkaline hydrolysis prior detection. Cross-hybridization was reduced to background levels by

Optical biosensors have also been employed for the detection of viruses, by the direct determination of their typically long single-stranded RNA (ssRNA). Griffin *et al.* [89] demonstrated for the first time that the non-linear optical (NLO) properties of gold nanoparticles (hyper-Rayleigh scattering) could be used for screening and quantifying HCV RNA without any modification, offering an excellent detection limit (80 pM) and selectivity (at the single base-pair mismatch level).

4.2.2. Triplex-forming RNAs and highly structured RNAs. Triplex-forming RNAs (TF-RNAs) and highly structured RNAs are typically long; and can be included in the category of long RNAs. Triplex-forming RNAs sequences contain a poly-purine or poly-pyrimidine motifs that can form a triple helix with a DNA duplex via Hoogsteen base-pairing rules [90]. These motifs are common in many coding and non-coding RNAs sequences [91] and, therefore, are interesting for RNA analysis. They enable RNA sequences to interact with genomic DNA or with proteins that bind triple-helices [90, 91].

Long, highly structured RNAs were explored by Carrascosa *et al.* [92] using a standard SPR sensor. They reported the label-free detection of a highly structured purified 96-mer RNA by exploiting that many ln-RNAs contain a triplex motif within their sequence (in this case a 12 nt triplex motif) and could be therefore captured by duplex-receptor, with the subsequent formation of a triplex helix (see **Fig. 5B**). Their triplex-based strategy, which can be extended to detect TF-RNAs of any type, entailed the use of *aminoadenine-modified tail-clamps*, as

capturing duplex-probes. The main advantage incorporated by this new type of tail-clamps is that the duplex receptor largely enhances the stability of a given triplex, maximizing the chances for RNA target capture. With this method, they reported a detection limit of 200 pM (50 fmol) twice lower than with a conventional duplex approach. The most outstanding feature of this method is that it works better for long (*e.g.* mRNAs or lnc-RNAs) and structured RNA sequences than for short and less structured ones.

The advantages of SPR sensors to monitor biomolecular interactions in real-time and label-free have also proven useful in the discovery of the mode of binding of potential TF-lnc-RNAs, such as promoter-associated lnc-RNAs. These RNAs have demonstrated to bind DNA by triplex interaction and coordinate regulation of adjacent genes via epigenetics [9, 11]. Recently, SPR has been strategic for uncovering the binding mechanism of Particle, a lnc-RNA involved in regulation of the methionine adenosyltransferase (MAT2A) gene in response to low-dose radiation [9]. Experiments carried-out using conventional SPR demonstrated that Particle creates a triplex structure to regulate the expression of MAT2A via epigenetics.

Structured RNAs have also been analyzed by Boucard *et al.* [93] using a SPR sensor. They employed a novel binding ligand, a bifunctional-binding aptamer, to simultaneously bind to the two stem–loop structures of the RNA target and reported affinity analyses of these interactions. Nair *et al.* [94] explored structured RNAs in a completely different way. They were interested on developing a model system for understanding RNA loop–loop interactions using SPR sensors. To address it they used the HIV TAR–TAR* ("kissing hairpin") RNA complex and determined its formation kinetics. By immobilizing one of the hairpin loops (TAR*16) onto a streptavidin chip (by first biotinylating it at the 5´-end), they accurately determined the relative stabilities of two very tight RNA complexes (TAR–TAR* and TARs2U–TAR*) and they also investigated how divalent metal ions affect the RNA complexes.

Finally, Mandir *et al.* analyzed the RNA-accessible sites of three pre-miRNAs (miRNA precursors of ~75 nt in length) by SPR (**Fig. 6**). They used an oligonucleotide array fabricated

with a thin layer of amorphous carbon deposited onto a SPR-active gold substrate [95]. With this approach, the group developed a single "universal array" that contains all possible 6-mer sequences that can bind to any accessible site of RNA molecules of any sequence in a rapid and label-free way. Identification of accessible sites for hybridization within the RNA sequence is an interesting approach for identifying antisense-mediated gene suppression sites. The rich secondary and tertiary structure adopted by RNA molecules leaves only a small portion of its sequence available for hybridization; therefore, those sites are considered to be strongly implicated in binding and regulation during RNA interference (RNAi).

4.2.3. Ribosomal RNA. Ribosomal ribonucleic acid (rRNA) is the RNA component of the ribosome, the cell's essential protein factory. Ribosomal RNAs comprise two subunits: a large subunit (LSU) and a small subunit (SSU). The molecular weight and genetic composition of the subunits differ among organisms, a feature that can be exploited to identify microorganisms. Since rRNA is present in a large amount within the cell, rapid ribosomal RNA analysis of the type of bacterial pathogens in clinical specimens is easy [96].

Two interesting manuscripts report on rRNA analysis using optical biosensors. In the first one, Joung *et al.* [97] used an SPR sensor and a peptide nucleic acid (PNA) as a capture probe for the detection of *E. coli* 16s rRNA. They employed a signal-enhancing method in which the 16s rRNA, once hybridized onto the PNA receptor layer, interacts with a cationic gold nanoparticle achieving a detection limit of ~58 pg/mL (10 fM).

Nelson *et al.* [98] used an SPR imaging array containing DNA probes (15 to 21 bases) designed to be complementary to 16S rRNA gene sequences of *E. coli* and *B. subtilis* as well as to a highly conserved sequence found in the rRNA of most bacteria. Using a standard commercial kit for RNA extraction, they were able to detect the RNA in less than 2 hours (including 1 hour for RNA hybridization). They achieved a detection limit of 2 µg/mL (3.8 nM) for fragmented *E. coli in* total cellular RNA.

5. Conclusions

Optical biosensors have been garnering increasing attention due to their potential for rapid, direct and label-free analysis of RNA. Although not many examples of RNA detection have been reported to date, the methodology is applicable to a wide range of RNA types. Very interesting approaches have been developed for long RNAs in the monitoring of relevant biological situations, such as alternative splicing or in deciphering their biological mechanism of interaction with target DNA sequences. Promising approaches for the detection of structured RNA targets and small RNAs have also been devised. However, in most of the proposed strategies, detection of small RNAs at an adequate level of sensitivity required non-target labeling amplification approaches. The principal objectives now facing researchers are the development of more accurate, sensitive and specific biofunctionalization methods, and the creation of more integrated and multiplexed platforms to allow simultaneous monitoring of numerous RNA targets with minimum sample pre-treatment, which should make the devices accessible to non-skilled users.

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Figure Captions

Box 1. Schematic of RNA types and functions

Figure 1. Scheme of the working principle of a biosensor device

Figure 2. Different types of optical biosensors. **A)** Scheme of a standard SPR biosensor and **B)** of an SPR imaging biosensor. **C)** Scheme of an integrated interferometer biosensor based on bimodal waveguides (BiMW): (a) Working principle showing the distribution of the electromagnetic field in the single mode and the bimodal sections; (b) envisioned lab-on-a-chip platform based on BiMW sensors; and (c) photograph of a chip (30 mm×10 mm) containing 16 BiMW interferometers. *Reprinted with permission from The Royal Society of Chemistry (ref [52])* **D)** Photograph of a ring resonator biosensor. *Reprinted with permission from ref* [99] **E)** Photograph of a photonic crystal-based biosensor. *Reprinted with permission from ref [75].*

Figure 3. Detection of small RNAs with optical biosensors. A) Scheme of the two-step assay developed by Sipova *et al.* for the detection of miRNA-122 with SPR. Examples of the temporal sensor response to the miRNA-122 and its selective antibody, and the corresponding calibration curves, are included below. *Reprinted with permission from ref [77]* **B)** Schematic representation of the three-step assay developed by Fang *et al.* for the detection of miRNA 16, miRNA-122b and miRNA-23b with an SPR imaging platform. The monitoring of polyadenylation after capture of the miRNAs targets along with the calibration curves is also shown. *Reprinted with permission from ref [56]* **C)** Multiplexed detection of several miRNAs with the ring resonator based biosensor by Qavi *et al.* An example of the absolute wavelength shift after miRNA recognition and real-time evaluation of the selected miRNAs are shown. *Reprinted with permission from ref [83](Copyright Wiley-VCH Verlag GmbH & Co. KGaA)*

Figure 4. SPR methodology for quantitative evaluation of Fas gene alternatively spliced mRNA isoforms. (A) Schematic representation of the different RNA and protein isoforms generated by alternative splicing of *Fas* gene/CD95. (B) Scheme for the label-free and direct detection of alternative spliced variants with an SPR biosensor. (C) SPR calibration plots for *Fas*57 (up) (R2= 0.99) and *Fas*56 (down) (R2=0.99) DNA-probes using the optimized hybridization and detection conditions. (D) SPR analysis of different HeLa

Figure 5. Detection of TF-RNAs developed by Carrascosa *et al.* using an SPR biosensor. **A)** Triplex-detection strategy using amino-modified tail-clamp receptors; and **B)** folding structure of the RNA target. **C)** Calibration curve of the detection of a purified RNA, and the two-folded enhanced signal obtained with the triplex strategy as compared to the duplex one. *Reprinted with permission from Ref [92].*

Figure 6. Universal SPR imaging array developed by Mandir *et al.* comprising all possible 6-mer combinations for high throughput screening of RNA accessible sites. **(A)** SPRi difference image showing spots of hybridization for a solution 1 µM pre-let-7a3. **(B)** Histogram of the hybridization data obtained from the difference image. The y-axis corresponds to the average change in % reflectivity (Δ%R), and the x-axis corresponds to the location of the surface probe along the RNA sequence (*Location 1 corresponds to nucleotides 1 through 6*). The dotted horizontal line represents the cutoff line. Features with intensity changes greater than the cutoff line are considered accessible sites. **(C)** The lowest-energy structure of pre-let-7a3 predicted by Mfold. The red line indicates the 6-mer accessible sites determined from the hybridization experiment. *Reprinted with permission from Ref [95].*
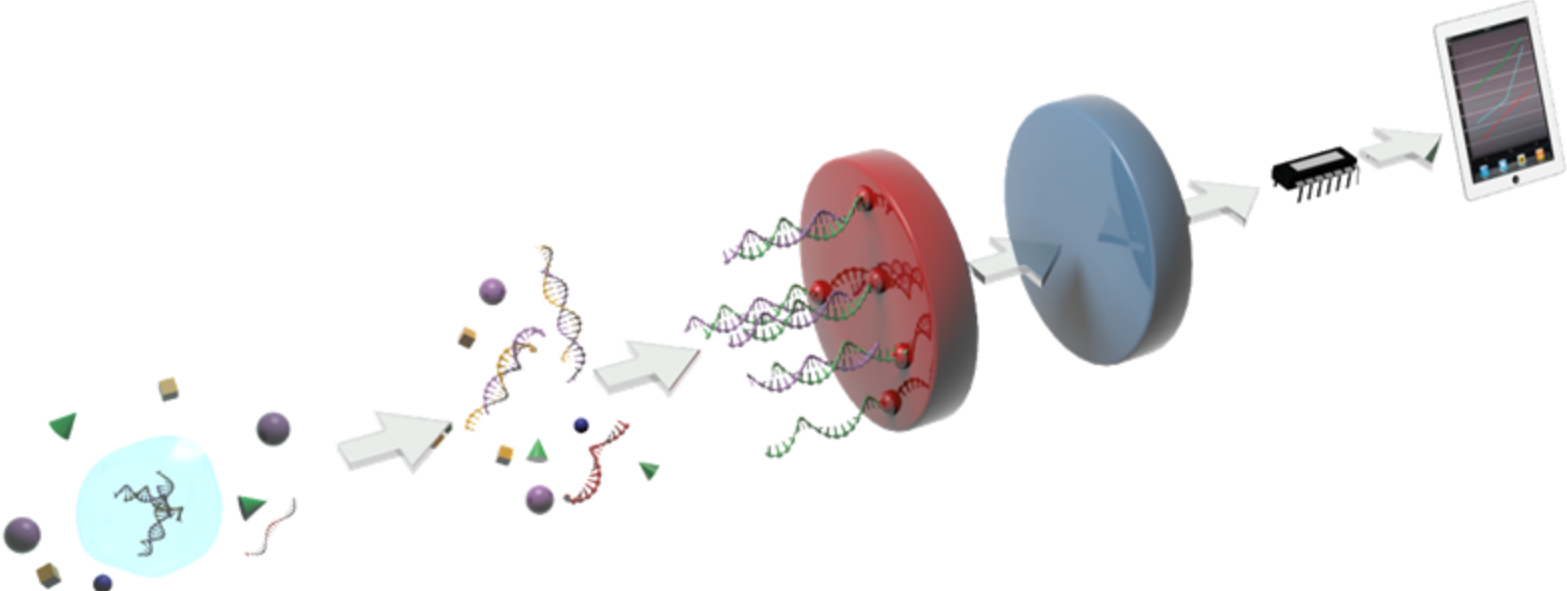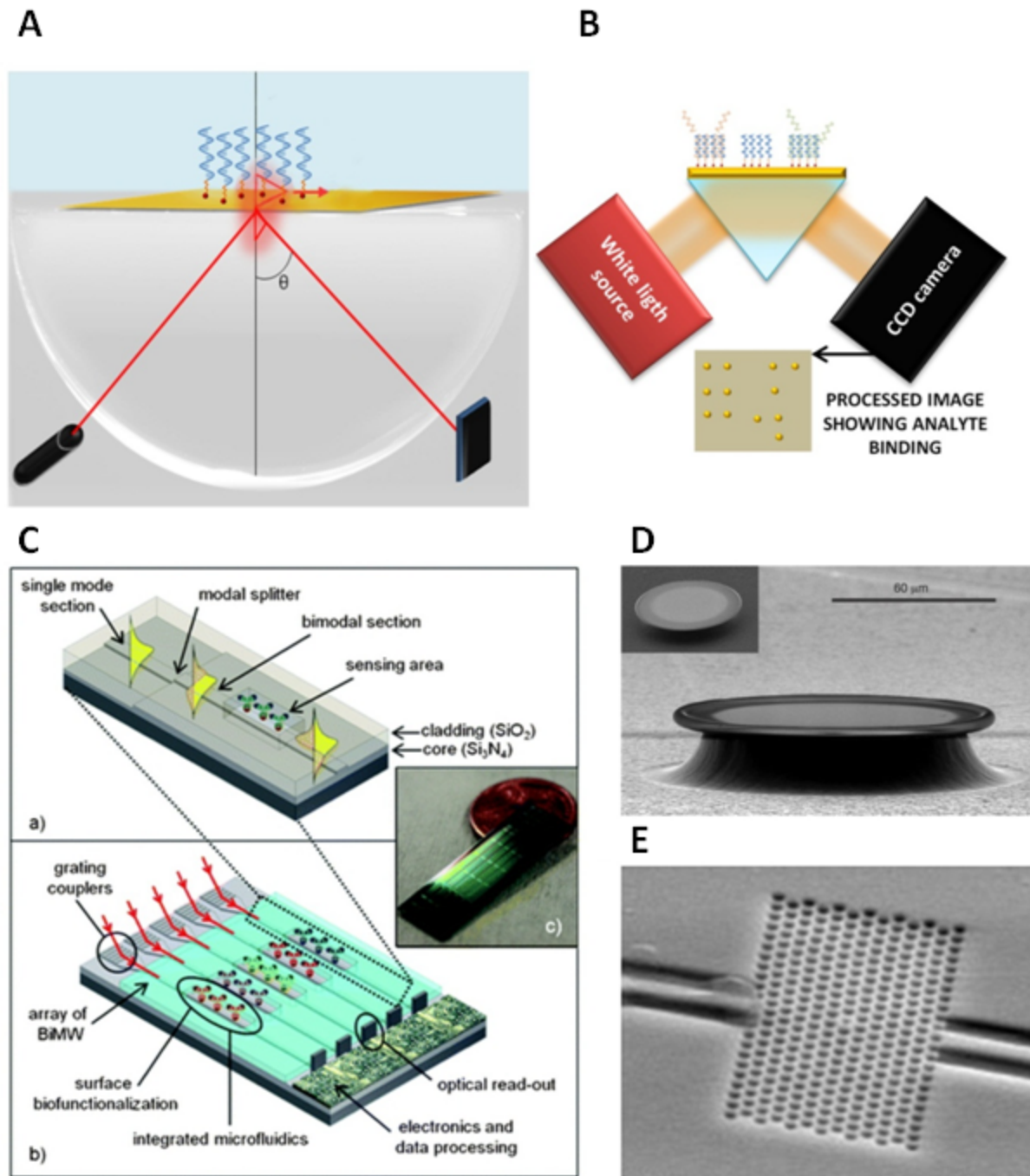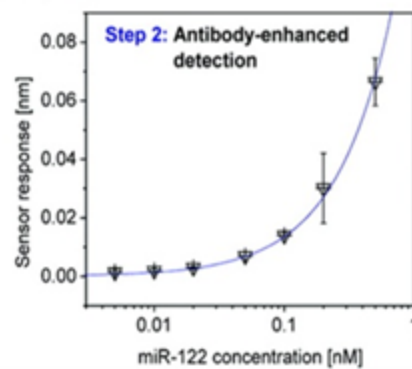
Figure 1

Figure 2

A

B

White ligth source

CCD camera

PROCESSED IMAGE
SHOWING ANALYTE
BINDING

θ

C

a)

single mode
section

modal splitter

bimodal section

sensing area

cladding (SiO₂)
core (Si₃N₄)

c)

b)

grating
couplers

array of
BiMW

surface
biofunctionalization

integrated microfluidics

optical read-out

electronics and
data processing

D

60 μm

E

Figure 3

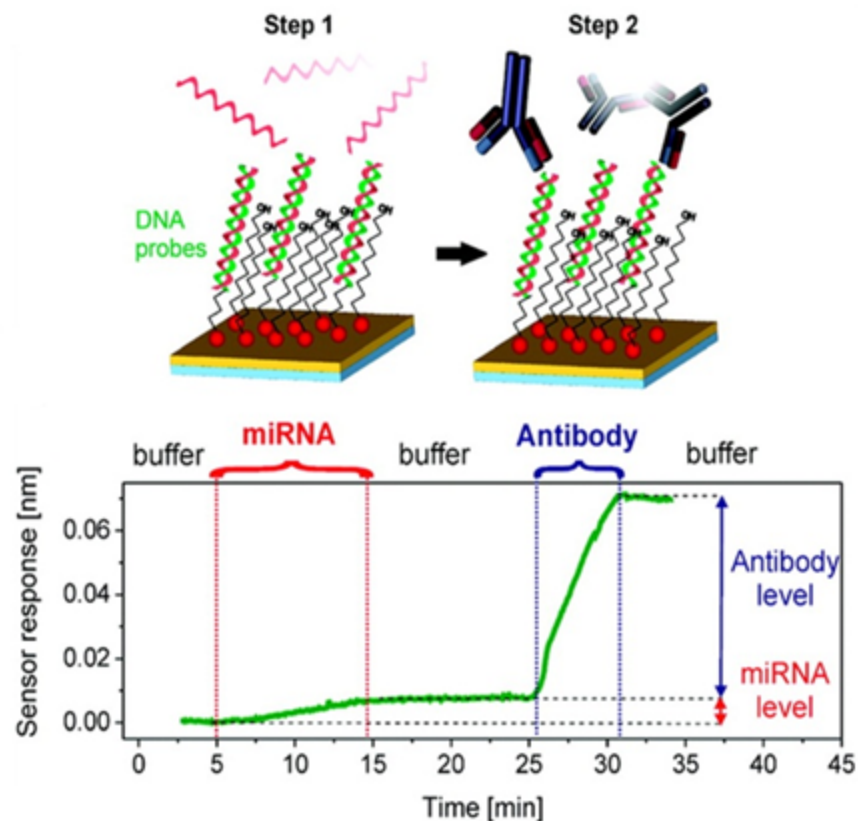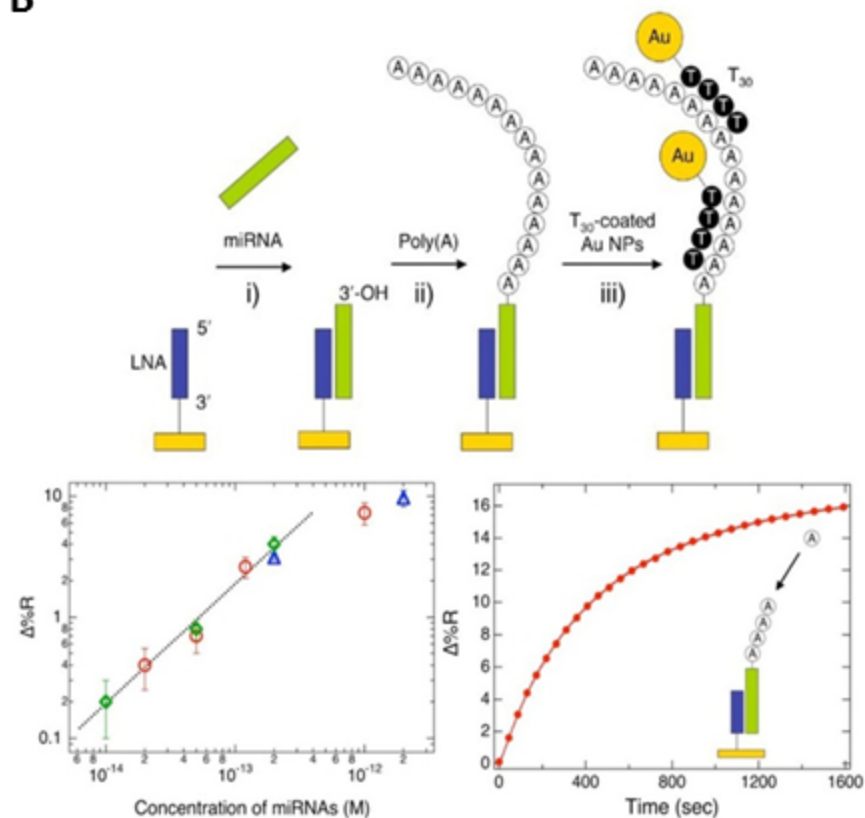# Figure 4

Figure 5

A

HG-forming strand
WC-forming strand
• 8-amino-adenine
● SH

3′   5′
5′   3′
5′
3′
5′
Target
3′
3′
5′
Vertical spacer

B

Color scale for base pair probabilities

0   1

Minimum Free Energy
(MFE) structure
-19.59 kcal/mol.

Matching region:
UGGAAUAAUUUAUCUUCUUCUUCU

C

▲ RNA-List on T15-3A-TFC-SH
✶ RNA-Control on T15-3A-TFC-SH

SPR signal (RIU)

1,0x10^{-4}
8,0x10^{-5}
6,0x10^{-5}
4,0x10^{-5}
2,0x10^{-5}
0,0

Concentration (nM)

— 10 nM RNA-List on T15-3A-TFC-SH
— 10 nM RNA-List on T15-3A-DFO-SH

SPR signal (RIU)

1,0x10^{-4}
5,0x10^{-5}
0,0

Time (s)

Figure 6

A

B

C

5′ –GGG GAGGUAGUAGGUUGUAUAGUU UGGGGC–U
3′ –UCC UUCUGUCAUCUAACAUAUCAA GUCCCG–U

U UAGGGUAUC C

10 20 30

70 60 50 40

Table 1

TYPE	SUBTYPE	NAME Long name	NAME Short name	SIZE	FUNCTION	SITE OF ACTION
Coding	Messenger RNA	messenger RNA	mRNA		Encode the information for protein production; Represent ~2% of the genomic output	Cytoplasm
Non-coding (Do not encode information for proteins; play structural or regulatory roles)	House-keeping or Structural ncRNAs	Ribosomal RNA	rRNAs	100-5000 nt depending on the subunit	Provide the machinery for protein synthesis from mRNA transcripts	Cytoplasm
		Transfer RNA	tRNAs	60-95 nt		
		Small nuclear RNA (including spliceosomal	sn-RNAs	80-350 nt		
		Small nucleolar RNA	sno-RNAs	48-250 nt	Modify ribosomal RNAs (rRNAs) by orchestrating the cleavage of the long pre-rRNA into its functional subunits	
	Regulatory ncRNAs — Long ncRNAs	Long intergenic ncRNAs including very long intergenic ncRNAs	LincRNAs and vlincRNAs	200 nt up to 1 Mb	They regulate protein expression by diverse mechanisms: - As guides and tethers for chromatin-modifying complexes - As molecular scaffolds for protein complexes that lack protein–protein interaction domains - By binding to transcription factors to preventing them from binding to their target DNA sequence - By interacting directly with other ncRNAs (e.g. microRNAs) to prevent them from binding to mRNA.	Nucleous or Cytoplasm
		Promoter associated ncRNAs	pa-RNA	200 nt to >100 Kb		
		Natural Antisense Transcripts	NATs	200 nt to >100 Kb	DNA replication interference, chromatin remodeling, transcriptional regulation, RNA masking, double-stranded RNA (dsRNA) mediated siRNA mechanism and translation interference	
		Transcripts derived from repetitive elements including Pseudogenes derived ncRNAs	repRNAs	200 nt to >100 Kb	influence gene and pseudogene expression, at both epigenetic and post-transcriptional levels.	
		Intronic RNAs including circular intronic RNAs (ciRNAs)		200 nt to several Kb	Epigenetic regulators of transcription in cis/in trans	
	Regulatory ncRNAs — Small ncRNAs	Small interfering ncRNAs	si-RNAs	~ 20 nt	Post-transcriptionally regulate mRNA stability and translation	Nucleous or Cytoplasm
		micro RNAs	mi-RNAs	21-25 nt	Post-transcriptionally regulate mRNA stability and translation. It can also silence genes transcriptionally.	
		Tiny RNAs	ti-RNAs	<22 nt	Regulate initiation of transcription and gene expression by controlling RNA polymerase II binding and behaviors, and modulating local epigenetic structure associated with transcription initiation, but not elongation.	
		Piwi-interacting ncRNAs	pi-RNAs	20-30 nt	Guide modification of chromatin structure in the nucleus, thereby contributing to the establishment and maintenance of distinct chromatin domains.	

Table 2

RNA type	Sample source	Method	Sensitivity	Single/Multiplex detection	Reference
miRNA	Mouse liver total RNA	SPR	0.2 nM-2 pM	Single	Sipova *et al*. [77]
	Synthetic RNA	SPRi	40 nM-4 nM	Single	Nasheri *et al.* [78]
	Synthetic RNA	SPRi	10 fM	Multiplex	Fang *et al.* [56]
	Mouse liver total RNA	SPRi	fM level	Multiplex	Fang *et al.* [56]
	Synthetic RNA	Nanoparticle-Enhanced Diffraction Gratings (NEDG)	50 pM	Single	Lee *et al.* [80]
	Synthetic RNA	SPRi	100 fM	Multiplex	Zhou *et al.* [81]
	Total plasma RNA	LSPR	23-35 fM	Multiplex	Joshi *et al.* [82]
	Human plasma samples without RNAs extraction	LSPR	fM level	Multiplex	Joshi *et al.* [82]
	Cell-line extracted total RNA	Arrays of silicon microring resonators	2 nM	Multiplex	Qavi *et al.* [83]
	Synthetic RNA	SERS	μM	Multiplex	Driskell *et al.* [84]
	Urine samples	MZI	1 fM	Multiplex	Liu *et al.* [85]
mRNA	Cell-line extracted total RNA	Arrays of silicon microring resonators	160 pM	Multiplex	Kindt *et al.* [87]
		SPR	pM level	Multiplex	Huertas *et. al* [88]
viral ssRNA 25-79 nt	Synthetic RNA	Scattering on gold nanoparticles	80 pM	Single	Griffin *et al.* [89]
triplex-forming RNAs	In-vitro transcribed RNA (96 nt)	SPR	200 pM	Single	Carrascosa *et al.* [92]
rRNA	*E. Coli* extracted total RNA	SPR	10 fM	Single	Joung *et al.* [97]
		SPRi	3.8 nM	Multiplex	Nelson *et al.* [98]