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TRENDS

Long noncoding RNAs: from genomic junk to rising stars in the early detection of cancer

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Abstract

Despite having been underappreciated in favor of their protein-coding counterparts for a long time, long noncoding RNAs (lncRNAs) have emerged as functional molecules, which defy the central dogma of molecular biology, with clear implications in cancer. Altered expression levels of some of these large transcripts in human body fluids have been related to different cancer conditions that turns them into potential noninvasive cancer biomarkers. In this review, a brief discussion about the importance and current challenges in the determination of lncRNAs associated to cancer is provided. Different electrochemical nucleic acid-based strategies for lncRNAs detection are critically described. Future perspectives and remaining challenges for the practical implementation of these methodologies in clinical medicine are also discussed.

Keywords Long noncoding RNA · Tumor biomarkers · Liquid biopsy · Electrochemical biosensors · Molecular diagnosis

Emerging players in cancer research: long noncoding RNAs

Cancer is a major public health problem globally, constituting one of the main causes of morbidity and mortality, both in the economically developing and developed countries. In 2015, there were 8.8 million deaths from cancer, which corresponds to almost one in six deaths worldwide [1]. In addition to the enormous human costs, the economic impact of cancer is significant and ever-increasing, with an estimated value of US $ 1.16 trillion in 2010 [2].

The generic term cancer embraces a large and complex group of diseases that are characterized by the abnormal growth of cells beyond their usual limits, being able to invade adjacent regions of the body and spread to other organs. To identify how much the cancer has grown and how far it has spread at the time of diagnosis, different stages of cancer are commonly used: stage 0 means the cancer is still located in the place it began, stage 1 when spread a little into nearby tissues, stages 2 and 3 indicate larger cancers that have grown deeply into nearby tissues and lymph nodes, and stage 4 cancer, also referred to as metastatic cancer, involves distant spread (into other organs or parts of body) thereof.

Most cancers are detected when symptoms become apparent; however, the benefits of medical therapy are more limited in advanced cancers. Conversely, an early detection of cancer (stage 0 and 1 cancer) usually provides a high chance of being treated and cured. Tissue biopsy is the standard method for cancer diagnosis and detection, but it is painful and risky for patients as well as costly. Moreover, it does not favor incipient cancer detection neither allows the follow-up of the disease (the conduct of multiple biopsies is unfeasible).

In order to detect presymptomatic tumors and relieve the discomfort of patients from traditional biopsy, the analysis of circulating biomarkers shed from the tumor cells into the biological fluids has become an enticing alternative. This new diagnostic concept was termed “liquid biopsy,” and its minimally invasive nature and the few risks associated favor the repeated sampling at different times and consequently allow tracing changes in biomarkers during the course of the disease and its treatment. Although blood is the bodily fluid commonly assessed, the most suited one for carrying out the liquid biopsy will depend on the tumor type, and other sources of

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information such as urine, saliva, and cerebrospinal fluid can be exploited as well.

Cancer-derived biomarkers that circulate in the accessible biological fluids mainly include circulating tumor cells (CTCs) and macromolecules such as proteins and nucleic acids. Protein-based tests have been extensively explored, although they have not always reached the sensitivity and selectivity needed in clinical diagnostics applications. Another strategy consists in identifying mutations of the genes implicated in oncogenesis (cancer genes); nevertheless, these molecular alterations are not tissue-specific and additional parameters are required to pinpoint the tissue of origin. Furthermore, some cancer-related mutations can come with aging even in individuals who do not develop cancer throughout their life.

There is, therefore, an urgent need for new biomarkers for reliable and efficient detection of the early signs of cancer, especially in tumor types that are usually diagnosed at later stages (such as pancreatic and lung cancer), as well as those tumors whose current biomarkers are altered not only when malignant proliferation of cells occurs but also when benign lesions are developed, as is the case with prostate-specific antigen (PSA) in prostate cancer. In this scenario, circulating transcriptome has raised great expectations among researchers.

The deciphering of the human genome brought along important milestones, some of which were as striking as that most of the genomic DNA is transcribed into RNA (at least 90%), albeit the protein-coding genes account for less than 2% of the entire human genome. These results clashed with the trend to consider proteins as the main players in cellular functions and RNA as a simple intermediary in the protein synthesis machinery. The question that subsequently arose from these findings was: does noncoding human transcriptome have biological significance or conversely is it a mere "tourist" in the evolutionary path? Although it was initially dismissed as simply transcriptional noise, in-depth transcriptome exploration has pointed to the need to reconsider this concept (Fig. 1). In this vein, the discovery of microRNAs, short noncoding RNAs ranging from approximately 18 to 25 nucleotides in length, whose expression levels have been related to the onset of several diseases [3], has defied the central dogma of molecular biology.

More recently, with the progress in RNA sequencing technology, a new category of noncoding transcripts has come into the spotlight, the so-called long noncoding RNAs, abbreviated as lncRNAs. Long noncoding RNAs constitute a large and heterogeneous subset of cellular transcripts, lacking protein-coding capability, with a length above 200 nucleotides. They are involved in a wide repertoire of biological processes [4, 5] and it is scarcely surprising, therefore, that their alterations have been associated with multiple human diseases, such as neurodegenerative [6] and psychiatric [7] diseases, as well as cancer [8]. In the context of cancer, lncRNAs can function as oncogenes or tumor suppressors. The former could cause normal cells to grow out of control and turn into cancer, while the latter protect them from degenerating into cancer cells, that is, lncRNAs could be great allies or frontal enemies of cancer development. Consequently, lncRNAs might be used as diagnostic tools or therapeutic targets [9]. This review, however, focuses on the employment of these transcripts for cancer diagnosis and prognosis.

Although it remains unclear whether deregulated expression of lncRNAs is the cause or the consequence of the disease, many studies have demonstrated the clinical relevance of the altered expression profile of lncRNAs in cancer. Accordingly, their expression signatures could successfully be used for cancer diagnosis, and even prognosis and recurrence. A large number of such investigations have been carried out with samples of tumor tissues, but the relationship between circulating lncRNAs and cancer has also been explored, finding substantial changes in the abundance of certain lncRNAs between cancer patients and healthy individuals. Intriguingly, several lncRNAs have been reported to be downregulated in cancerous tissues, while upregulated in body fluids (mainly in serum) and also the opposite case. This apparent inconsistency, described for different lncRNAs and different types of cancer, remains an open question, since the mechanisms of lncRNAs secretion are not fully understood. Different explanations have been proposed [10], although it

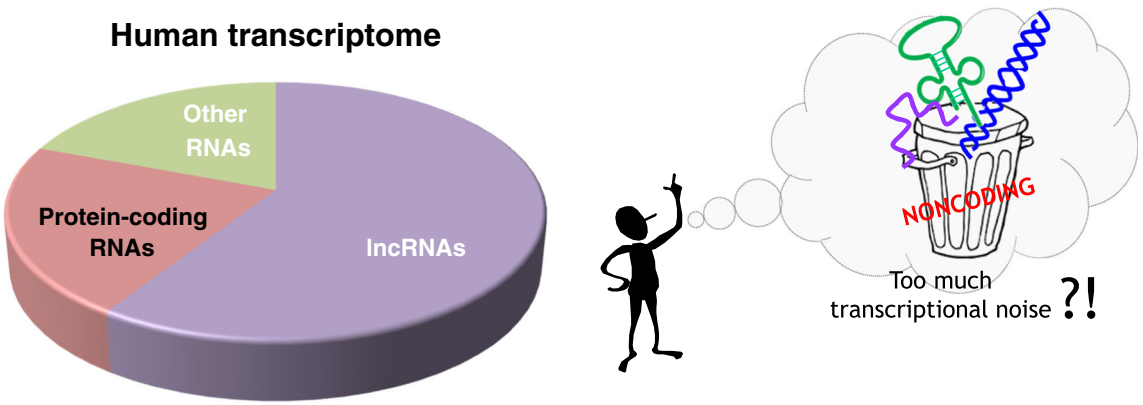


Fig. 1 The coding potential of human transcriptome shows how the genomic heterogeneity of lncRNA darkens that of coding transcripts (around 60% of lncRNA genes versus 20% protein-coding genes). Among this, almost 10% are identified as cancer-associated lncRNA. Adapted from reference 5

cannot be ruled out that the levels of circulating lncRNAs may be affected by other concomitant disease changes, besides cancer [11].

The collection of cancer-associated lncRNAs is in constant growth, and the new identified sequences are included in different existing databases containing lncRNA data (https://www.lncipedia.org; http://genome.igib.res.in/lncRNome; http://www.lncrnadb.org; among others). Some examples of lncRNAs with demonstrated diagnostic potential are reflected in Table 1 adapted from reference 12. Among all of them, just PCA3, also known as DD3, has been recommended for human use (it has been approved by the US Food and Drug Administration (FDA) [13]). This lncRNA, overexpressed in prostate cancer, has been employed for the development of a prostate cancer diagnosis assay in urine with improved results when compared with traditional PSA blood tests [14].

Nucleic acid-based biosensors for cancer-related lncRNA monitoring

In order to routinely implement the analysis of circulating RNA in clinical medicine and in particular in cancer management, there is an urgent need not only to identify reliable and efficient circulating biomarkers, as it is the case for numerous long noncoding RNAs, but also to develop accurate devices for their detection. The detection must achieve high sensitivity and selectivity, to minimize false negative and false positive results very dangerous in the clinical area. These devices must also be fast, cost-effective, and easy to use in the detection of clinically relevant circulating transcriptome. Electrochemical sensing strategies, also known as electrochemical biosensors, have the potential to tackle this challenging issue and great strides have been made in this field [15].

Biosensors are analytical devices in which a biological recognition element and a transduction system are in direct contact so as to allow the conversion of the molecular recognition event between the biosensing element and a target compound into a measurable signal generated by the transducer. With the purpose of obtaining an optimum biosensing device, different factors should be considered in order to hone its analytical performance. The biomolecular recognition reaction employed to sense RNA is the hybridization event, i.e., the pairing of complementary strands of nucleic acids according to the Watson-Crick base pairing rules, which exhibits a great selectivity. It involves the use of nucleic acids as biorecognition elements, DNA or RNA sequences, called capture probes, attached to a solid support, for the sequence-specific interaction with the target strand.

With the purpose of achieving higher levels of affinity and selectivity (ideally specificity) for the binding between the RNA target and the capture DNA probe, synthetic nucleic acid analogues such as locked nucleic acids, peptide nucleic acids, and morpholinos have been developed to replace traditional DNA probes. Locked nucleic acids (LNAs) are RNA mimics where the ribose sugar is locked by a 2′-O,4′-C methylene

Table 1 lncRNAs associated with various types of cancer. Adapted from reference 12

Type of cancer	lncRNA	
	Upregulated in cancer (oncogenic)	Downregulated in cancer (tumor suppressor)
Brain tumor	H19, MALAT1	MEG3
Leukemia	HOTAIRM1, LUNAR1, XIST, NEAT1, UCA1, MALAT1, CCAT1	DLEU1, DLEU2, BGL3
Lung cancer	MALAT1, ANRIL, HOTAIR, CCAT1, PVT1, H19, TUG1	MEG3
Breast cancer	HOTAIR, ANRIL, ZFAS1, HOTAIRM1, MALAT1, PVT1	GAS5, TERRA
Liver cancer	HULC, HEIH, HOTTIP, HOTAIR, MALAT1, ZFAS1, MEG3, ANRIL	MEG3
Gastric cancer	H19, CCAT1, ANRIL, MALAT1, TUG1, PVT1, CCAT1, ZFAS1	FER1L4, TERRA, MEG3
Pancreatic cancer	H19, HOTAIR, HOTTIP, HULC, PVT1, LincRNA-RoR, CCAT1	TERRA
Ovarian cancer	H19, XIST, NEAT1, HOTAIR, PVT1	MEG3
Renal cancer	H19, MALAT1	GAS5, MEG3, NBAT1
Bladder cancer	UCA1, TUG1, H19	MEG3
Colorectal cancer	CCAT1, HOTAIR, HOTAIRM1, MALAT1, H19, KCNQ1OT1, T-UCRs, ZFAS1	UCA1
Prostate cancer	PCAT1, PAC3, PCGEM1, MALAT1, HOTTIP	GAS5

bridge, thus providing enzymatic cleavage resistance. The enormous thermal stability of the resulting duplex upon introducing an LNA monomer in the capture probe is such that LNA-modified oligonucleotides instead of pure LNA sequences are recommended.

Peptide nucleic acids (PNAs) are a class of DNA analogues where deoxyribose-phosphate backbone is replaced by a derivative of glycine to which the corresponding nucleobases are linked. Their uncharged nature is the cause of the enhanced stability of PNA-RNA complexes with respect to that for the DNA-RNA counterparts, and it opens the door to new biosensing approaches [16]. However, as neutral compounds, PNAs possess poor water solubility and they tend to self-aggregate within the aqueous solution. The inclusion of an aspartic acid unit at both ends provides global negative charge that gives rise to high sensing layer quality, enabling efficient capture of long RNA [17].

Moreover, although not yet applied to the detection of long RNA molecules, morpholinos are synthetic DNA analogues with a neutral backbone of morpholine rings reported as interesting alternatives to PNAs. They exhibit higher solubility in aqueous solutions and fewer limitations on length synthesis than PNAs [18, 19].

To discuss the challenges to be faced and the possible strategies for lncRNAs recognition and signal transduction, it is important to bear in mind the structural and biological properties of these interesting transcripts.

Their definitively demonstrated presence in human biological fluids seems, however, to be incompatible with their nature as RNA molecules, which are easily degraded by endogenous and exogenous ribonucleases (RNases). Nevertheless, their remarkable stability in the bloodstream has been attributed to their capability to be incorporated into cell-derived microvesicles (exosomes), thus protecting them from the action of RNases [20]. Moreover, as lncRNAs are single-stranded nucleic acids, no thermal, chemical, or enzymatic denaturation step is required to facilitate their interaction with the surface-tethered capture probe.

Regarding their large size (longer than 200 nucleotides up to 10 kb), it negatively impacts on their diffusion through the solution, thus hampering the development of fast analyses. To circumvent this issue, Kelley's group has used rough nanostructured microelectrodes (NMEs) by spreading their high degree of surface nanostructuring over a larger size (from 5 to 100 μm) to achieve the contact between the surface-attached probes and a large portion of sample volume, thus facilitating the interaction of the slow-diffusing targets and the sensing layer, and resulting in hybridization times of less than 1 h for mRNA [21].

Furthermore, as a result of their long length, lncRNAs present a strong intramolecular secondary structure that hinders their interaction with the surface-bound capture probe. In this respect, a physical (random) or chemical (precisely controlled) RNA fragmentation pretreatment could be of great help. Likewise, the use of PNAs or morpholinos as capture probes would allow, thanks to their neutral charge, performing the hybridization with the target RNA sequences at low ionic strength and, consequently, the intramolecular folding of the lncRNA analyte would be substantially reduced.

The scarcity of cancer-associated lncRNAs in body fluids of early-stage cancer patients (they may be present only at a femtomolar level) makes their monitoring a very challenging task. Their significance in cancer has been researched mainly by RNA sequencing [22]; nevertheless, this method is very laborious and remains expensive to be implemented as routine molecular testing. Alternatively, highly sensitive long RNA detection strategies with the capability of point-of-care testing can be achieved by combining electrochemical nucleic acid biosensors with different amplification approaches, here categorized into three groups: signal amplification, target amplification, and target recycling amplification approaches.

Signal amplification

A widely used strategy to enhance the analysis sensitivity and push down the limit of detection consists in increasing the signal to noise ratio. In this context, different signal amplification approaches have been developed in combination with electrochemical biosensing [23]. Among them, those based on nanostructures and nanomaterials are of particular interest. Indeed, besides providing an improvement in the electron transfer efficiency, nanomaterial-functionalized surfaces hold suitable biocompatibility as well as an enlarged active surface area, thus becoming effective substrates for sensing layer construction. In particular, nanotextured electrodes obtained by controlled growth of 3D gold nanostructures and subsequent coating with a thin layer of palladium exhibited improved sensitivity for the detection of bacterial long transcripts owing to their capability to accommodate more oligonucleotide probes. This results in enhanced ability to capture the target sequence, when combined with the electrochemical reporter system $[Ru(NH_3)_6]^{3+}/[Fe(CN)_6]^{3-}$ [21].

Nanomaterials have been also employed as carriers for loading numerous electrochemical mediators and natural redox enzymes to further amplify the electrochemical signal. In this context, Pt-Pd bimetallic nanodendrites/nanoflower-like clusters were adsorbed onto graphene oxide nanosheets to enhance the catalytic efficiency towards hydrogen peroxide in cooperation with the enzyme horseradish peroxidase also adsorbed. The use of target-specific detection probes modified with such labels in a sandwich-type genosensor enabled the detection of the long noncoding RNA HULC (highly upregulated in liver cancer), whose overexpression has been related to hepatocellular carcinoma, at low picomolar levels [24]. Nevertheless, this detection limit would likely be insufficient for early cancer diagnosis, when considering that these studies

were performed with a short mock target, i.e., a synthetic 38mer DNA sequence, and poorer performances would be anticipated, when challenging this biosensor with clinical specimens.

There is another subset of signal amplification techniques that take advantage of nucleic acid polymerization and/or hybridization to improve the assay sensitivity, among which are signal-mediated amplification of RNA technology (SMART) and hybridization chain reaction (HCR). SMART [25] involves two DNA probes of different lengths. The longer one contains a single-stranded T7 RNA polymerase promoter sequence at its 5′ end, while its 3′ end is blocked, thus preventing its elongation. As a result, the longer probe will act as a template and the shorter one will be extended. In the presence of the target, and only in its presence, a three-way junction (3WJ) structure is formed between probes and target. Then, a DNA polymerase elongates the shorter probe, leading to a dsDNA that includes an active T7 polymerase promoter site. It allows T7 RNA polymerase to produce multiple copies of RNA to be subsequently detected via a hybridization-based assay. Since the target sequence is not amplified but a surrogate, SMART is based on target-dependent signal amplification (Fig. 2A). The main strength of this approach is the capability of easily detecting different targets since the RNA sequence responsible for the signal could be the same. It is, therefore, possible to maintain the hybridization-based detection design, and only minor adjustments in the template and extension probes would be required for each particular target.

HCR is an enzyme-free method proposed in 2004 [26]. It consists of a hybridization process in which two species of DNA hairpins with a particular structure (short loop and long stem) take part alternately forming a supersandwich structure. The presence of the target sequence or a surrogate triggers a chain reaction of hybridization events between both hairpins, leading to the formation of DNA duplexes that grows until the hairpin supply is depleted. Since it does not require enzymes and its scheme is relatively simple, HCR amplification has often been used in combination with other amplification strategies, as will be seen later.

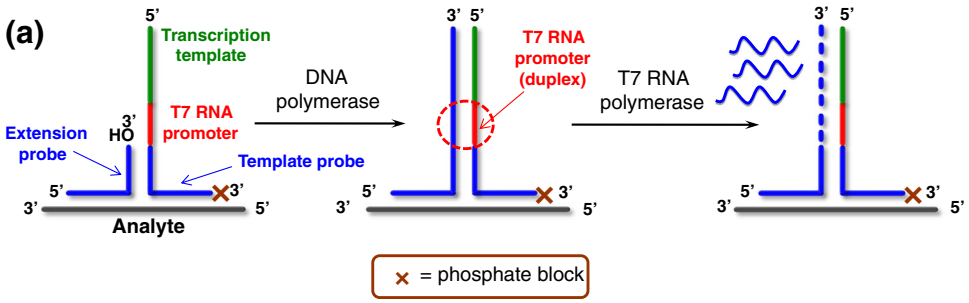
Target amplification

Target amplification strategies allow target nucleic acid fragment multiplication with the aim of attaining a target amount sufficiently high to be detected with a hybridization-based sensor.

Since its invention more than three decades ago, polymerase chain reaction (PCR) has been the most extensively used nucleic acid amplification technique. It relies on a series of repeated thermal cycles in which the starting DNA copies are exponentially replicated by using just a heat-resistant DNA polymerase and a pair of primers, yielding millions of DNA copies within 1–2 h [27]. When RNA is the starting material, this should be firstly converted into its complementary DNA sequence by a reverse transcriptase enzyme (an RNA-dependent DNA polymerase). Then, the resulting ssDNA molecules serve as a template for conventional PCR amplification that proceeds in the usual way, generating dsDNA molecules that are ultimately quantified. This technique is referred to as reverse transcription polymerase chain reaction (RT-PCR). However, to facilitate surface hybridization process, an excess of one primer can be used so that after consuming the limiting primer, single-stranded products are linearly generated. This strategy, denoted as RT-asymmetric PCR, has been recently implemented to assess differential expression of two human long noncoding RNAs in a glioblastoma cell line and healthy brain tissues, finding a good agreement with qRT-PCR results [28], which are typically taken as the reference for the validation.

Nevertheless, the need for temperature cycles and precise control thereof for proper PCR operation requires sophisticated instrumentation, making its integration with biosensing devices difficult. More recently, nucleic acid amplification techniques that run at a constant temperature have been reported to facilitate the design and development of biosensing platforms with the required sensitivity [29]. Among the different isothermal amplification techniques described, those that work at cellular temperatures (≈ 37 °C) are particularly convenient, since they require less energy, which translates into simpler instrumentation and therefore adequate for use at the point of need.

Recombinase polymerase amplification (RPA) is an isothermal nucleic acid amplification method particularly appealing due to its exponential nature and its simple reaction scheme [30]. In this method, a recombinase protein associates with ssDNA primers giving rise to recombinase-primer complexes that examine the double-stranded DNA template for homologous sequence. Once identified, the recombinase-primer complex invasion occurs, causing the two strands of the DNA duplex to separate locally. The displaced strand is stabilized by single-stranded binding proteins, thereby avoiding the rehybridization of the dsDNA template. Subsequently, the recombinase disassembles leaving the 3′-terminus of the primer free to be extended by the action of a DNA polymerase enzyme (Fig. 2B). One of the most prominent aspects of RPA technology is its high reaction speed, even superior to that of PCR. In this regard, given the absence of thermal cycles, amplification kinetics is conditioned by the activity of the enzymes that work in concert, and RPA has been reported to be faster than other isothermal exponential variants such as loop-mediated isothermal amplification (LAMP) or helicase-dependent amplification (HDA) that occur at 60–65 °C [29]. Here again, RNA targets should be previously retro-transcribed into DNA by a reverse transcriptase and the generated DNA strand, complementary to the RNA target, is subsequently amplified by RPA. The resulting


(a)
5'
Transcription template
3'
HO
T7 RNA promoter
Extension probe
Template probe
5'
3'
3'
5'
Analyte
DNA polymerase
3' 5'
T7 RNA promoter (duplex)
5'
3'
3'
5'
T7 RNA polymerase
3' 5'
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3'
3'
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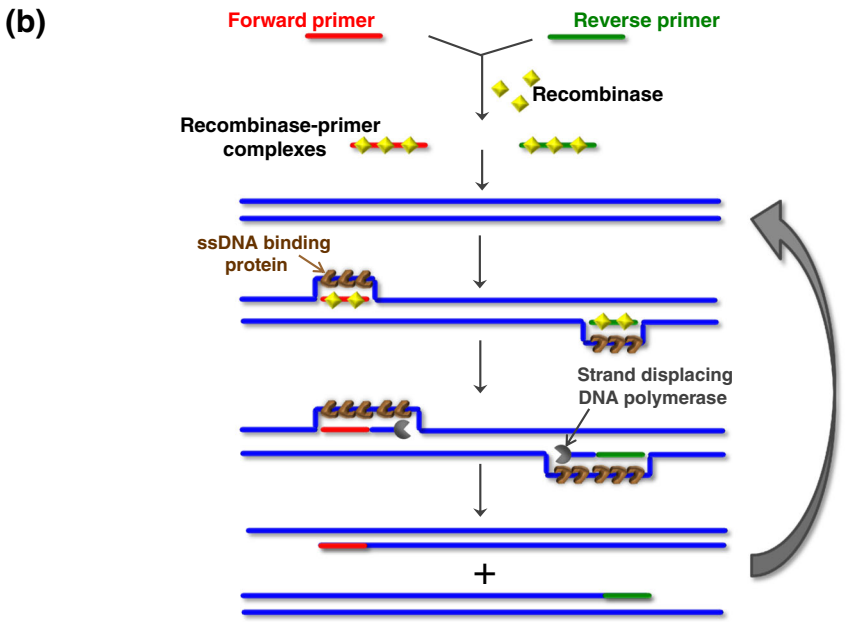
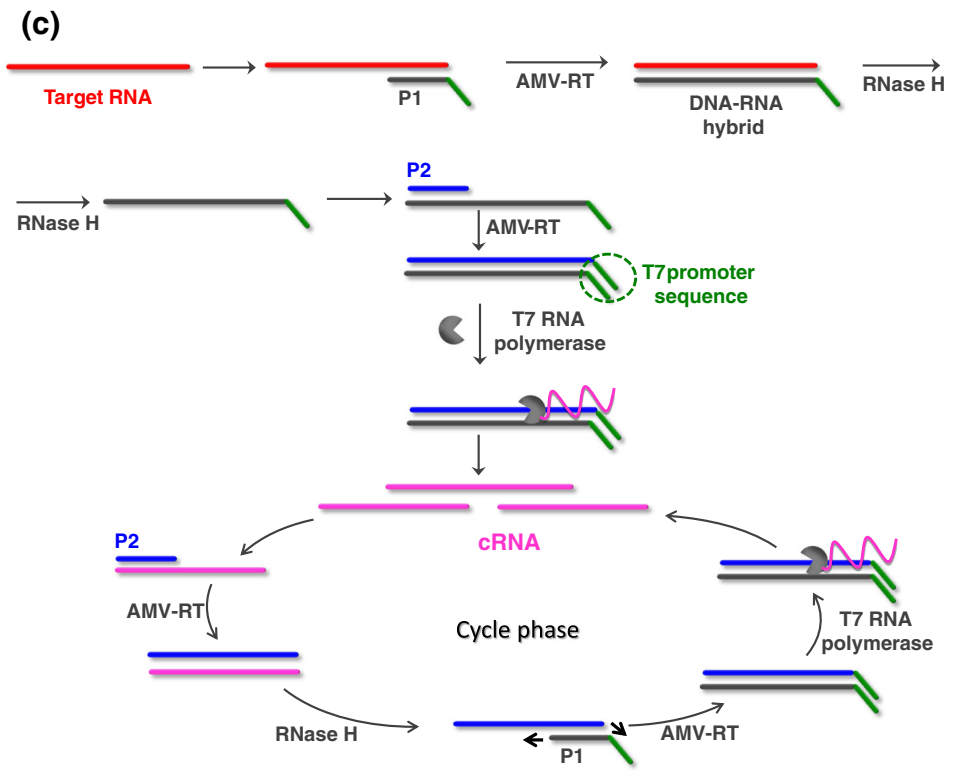
× = phosphate block


(b)
Forward primer
Reverse primer
Recombinase
Recombinase-primer complexes
ssDNA binding protein
Strand displacing DNA polymerase


(c)
Target RNA
P1
AMV-RT
DNA-RNA hybrid
RNase H
RNase H
P2
AMV-RT
T7promoter sequence
T7 RNA polymerase
cRNA
P2
AMV-RT
Cycle phase
T7 RNA polymerase
RNase H
P1
AMV-RT

◀ **Fig. 2** (**A**) Scheme of signal-mediated amplification of RNA technology (SMART). (**B**) Mechanism of recombinase polymerase amplification (RPA) reaction. (**C**) Principle of nucleic acid sequence-based amplification (NASBA)

methodology is known as reverse transcription recombinase polymerase amplification (RT-RPA) and it has been successfully applied to quantify the lncRNA HOTAIR overexpressed in ovarian cancer cell lines [31].

There are, however, other isothermal amplification techniques that emulate in vivo retroviral replication mechanisms to yield RNA amplicons from an RNA template. This is the case for nucleic acid sequence-based amplification (NASBA). NASBA technology involves three enzymes, avian myeloblastosis virus reverse transcriptase (AMV-RT), RNase H, and T7 DNA-dependent RNA polymerase, and two primers, primer P_1, complementary to the 3′-end of the target, including an inactive (for being single-stranded) T7 promoter sequence at 5′-terminus and primer P_2 whose sequence matches the 5′-end of the target (Fig. 2C). Primer P_1 binds to the RNA target to be subsequently extended by the reverse transcriptase AMV-RT, leading to a DNA-RNA hybrid. The RNA strand in this heteroduplex (i.e., the target) is then hydrolyzed by RNase H. The resulting ssDNA hybridizes primer P_2 and the reverse transcriptase elongates the primer, producing a dsDNA molecule bearing an active T7 RNA polymerase promoter site. T7 RNA polymerase recognizes its promoter and binds to it, transcribing multiple RNA amplicons complementary to the original target. They enter an amplification cycle where dsDNA molecules are produced, generating in turn more RNA amplicons. These ssRNA products can be captured via hybridization and detected. Despite its strengths (the amplified product is a single chain, enabling simple and fast hybridization detection with no need of a previous denaturation step), very few efforts have been made to combine NASBA technology with electrochemical readout so far [32].

Target recycling amplification

Previous approaches denoted as signal-based amplification approaches allow increasing the signal derived from a single-binding event; therefore, the binding affinity between the capture probe and the lncRNA target would restrict the improvement in sensitivity. By contrast, target recycling-based amplification strategies, in which each target molecule is involved in multiple hybridization events, could achieve superior sensitivity, since they dodge the affinity reaction stoichiometry.

Enzyme-assisted target recycling (EATR) approaches [33] use nucleases to specifically recognize the probe-target complex and catalyze the cleavage of phosphodiester bonds of the probe, leading to a measurable signal. At the same time, the target is released, thus enabling its hybridization to another probe which is cleaved as well, and this repeated cyclic process ultimately provides a greatly amplified signal. Nucleases employed for EATR assays mainly include endonucleases and exonucleases. Endonucleases recognize specific nucleic acid sequences in dsDNA and cleave either the two strands within the sequence (restriction endonucleases) or only one strand of the duplex (nicking endonucleases). This sequence specificity limits, however, their scope of application with regard to the target sequence. By contrast, exonucleases are sequence-independent nucleases that allow the development of more general schemes. They catalyze the stepwise removal of nucleotides at either 3′- or 5′-ends from ssDNA or dsDNA regardless of the sequence. Among the multiple exonucleases used in EATR approaches (Exo III, λ-Exo, RNAse H,…), duplex-specific nuclease (DSN) is of particular interest for RNA detection because of its strong preference for DNA digestion in DNA-RNA heteroduplexes, while it is practically inactive towards ssDNA and RNA [34]. Likewise, a pertinent design of the probe would expand the nuclease toolbox for lncRNA detection through target recycling amplification.

The linear nature of these amplification strategies makes them less sensitive to contamination and therefore to false positives. Nevertheless, in the case that the signal gain is insufficient, integration of two or more amplification strategies can be performed. This approach is termed as cascade amplification and it implies that the product of the upstream strategy serves as the input for the downstream strategy. A target recycling approach is usually combined with a signal amplification method.

A recent example of this strategy involves an RNase A-aided target recycling along with an HCR-based signal enhancement [35]. In the presence of the endoribonuclease RNase A, a small amount of RNA target triggers the formation of numerous DNA intermediates, IT, from an RNA primer probe anchored on magnetic nanospheres. These intermediates are subsequently entrapped onto a sensing film harboring a DNA capture probe in the presence of a helper probe. The subsequent incorporation of two detection probes functionalized with an electroactive tag, and partially complementary to each other as well as to the immobilized DNA architecture, leads to concatemer structures with multiplex redox tags per target molecule, resulting in an amplified electrochemical signal. This method was optimized for simultaneous detection of two specific RNA sequences whose expression is markedly reduced in human lung cancer cell lines (tumor suppressor lncRNAs). In order to obtain a distinguishable readout of each target, two different redox tags, ferrocene and methylene blue, were employed without recording cross-reactivity. The HCR-based strategy provided about 3.2-fold signal increase, while the amplification power of the target recycling strategy was not investigated. Evaluation of the analytical performances using long RNAs from cancerous and normal cells instead of short synthetic targets is another pending task (Fig. 3A).

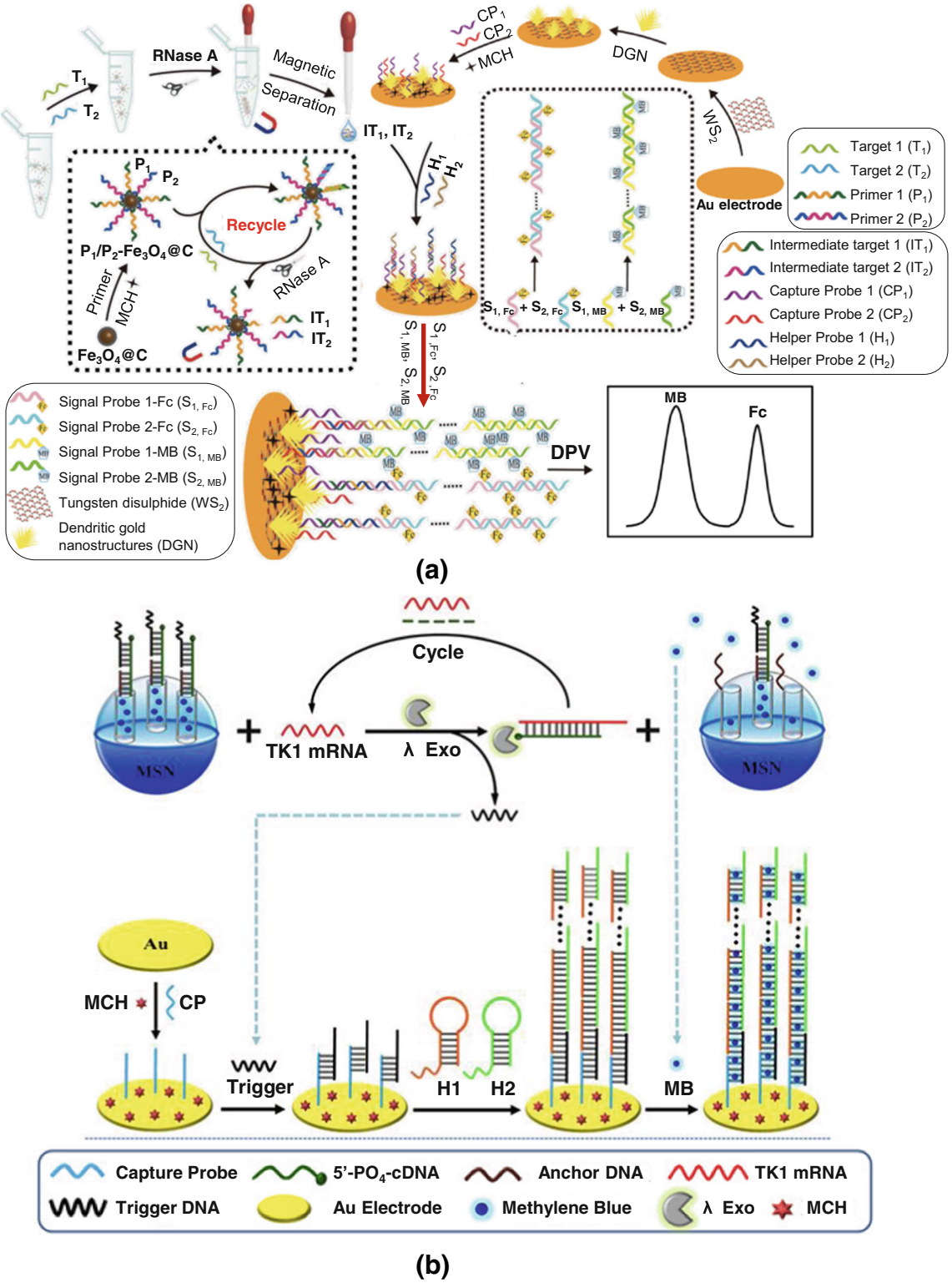


Fig. 3 (**A**) Combination of RNase A-assisted target recycling with HCR-induced signal amplification for dual determination of lncRNAs. Reprinted with permission from Biosensors and Bioelectronics, 113, Li X, Peng G, Cui F, Qiu Q, Chen X, Huang H, Double determination of long noncoding RNAs from lung cancer via multiamplified electrochemical genosensor at sub-femtomole level. Copyright (2018) Elsevier. (**B**) Design strategy of cascade signal amplification coupling target-activated HCR and electroactive cargo release. Reprinted with permission from Analytical Chemistry, 90, Cheng H, Liu J, Ma W, Duan S, Huang J, He X, Wang K, Low background cascade signal amplification electrochemical sensing platform for tumor-related mRNA quantification by target activated hybridization chain reaction and electroactive cargo release. Copyright (2018) American Chemical Society

The increase in the specific electrochemical signal by cascade amplification could also be accompanied by an increase in the background signal, albeit not necessarily to the same extent. To circumvent this issue, an electrochemical genosensing platform that combines an HCR amplification on gold substrates modified with a binary DNA self-assembled monolayer, with target-responsive electroactive cargo (methylene blue, MB) release from mesoporous silica nanoparticles (MSNs) [36], has been recently proposed. Methylene blue molecules, chosen to reveal the HCR by intercalation into the long dsDNA molecules generated on the gold surface and subsequent detection by square wave voltammetry, were trapped in the pores of MSNs and sealed with a molecular gate. In this way, in the absence of the RNA target, MB molecules remain inside the MSNs, and the background signal became minimal. The molecular gate is a 3-component partial duplex DNA made up of an anchor DNA strand immobilized on MSNs via click chemistry, a 5′-PO_4 DNA strand complementary to the target RNA (5′-PO_4cDNA), and an HCR trigger DNA fragment partially complementary to the DNA probe immobilized on the gold surface (Fig. 3B). Thus, if the RNA target is present, its affinity by the 5′-PO_4cDNA induces the displacement of the latter from the nanoparticle surface as well as the departure of the HCR trigger strand. Consequently, surface-initiated HCR occurs and, since the DNA gate is opened, MB molecules are released, intercalated into the HCR products, and electrochemically detected. On the other hand, the 5′-PO_4cDNA sequences bound to the RNA target were digested by λ-exonuclease, thereby releasing the RNA target, which can provoke the aperture of more DNA gates and trigger the release of more MB molecules (target recycling amplification). The described genosensing platform exhibited a wide range of response towards a synthetic 30mer RNA sequence (from 0.1 fM to 1 pM). Likewise, it was faced to total RNA extracted from human liver hepatocellular carcinoma cells or normal human hepatocyte cancer cells recording different expression levels of the RNA target.

Besides the challenges described above, the ability to interrogate several targets at once is of utmost importance for clinical diagnosis. Indeed, in general terms, any single molecular marker tends to show limited diagnostic potential. Consequently, in order to enhance cancer diagnostic capability, panels consisting of several circulating lncRNAs have started to be reported as more efficient for the screening of certain cancer types [37–39]. The analysis of such panels of lncRNAs demands the development of multiplex tests. In this regard, electrochemical transduction has proven to be particularly suitable for multiplexed analysis at the point of need. Furthermore, solid-phase nucleic acid amplification strategies that integrate nucleic acid multiplication, hybridization, and detection on the same sensing surface have been reported as particularly convenient, not only for reducing the possibility for primer-dimer formation, but also for multi-target analysis, as a result of their space-resolved detection [40].

With the aim of comparing lncRNA expression in different clinical conditions, thus performing quantitative analysis of these cancer biomarkers, normalization with the expression of an internal RNA control to exclude any nonspecific variation is required. Housekeeping gene transcripts, i.e., transcripts of constitutive genes indispensable for basic cellular function, are typically used as endogenous controls owing to their presumed stable expression. However, expression levels of housekeeping genes can vary in given situations that have motivated a lack of consensus on the use of internal standards for relative quantification of circulating transcriptome; consequently, their practical utility should be evaluated in each case. The endogenous controls typically used to monitor the expression profile of lncRNAs are β-actin [28] and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [31].

Outlook

Cancer is one of the main challenges for the twenty-first century scientific community, and its early detection is crucial to improve the expectations of cure and survival. The current trend is the search for molecules released into the bloodstream that alert about the disease in the earliest stages, the so-called liquid biopsy. Recent studies focused on human transcriptome have revealed the presence in accessible human body fluids of long RNA sequences unable to encode proteins but with potential clinical utility as biomarkers for certain cancer types. These transcripts are referred to as long noncoding RNAs (lncRNAs) and their aberrant expression has proven to be a reliable predictor of cancer. However, their low abundance in human fluids and their large size pose important technical challenges for their detection.

As reviewed here, electrochemical nucleic acid-based sensing platforms for lncRNAs monitoring are being developed and optimized to render their analysis highly sensitive, selective, and compatible with liquid biopsy concept. They combine nucleic acids and synthetic analogues as molecular receptors with isothermal amplification strategies that make use of nanomaterials and molecular biology tools for the detection of these long oligoribonucleotide biomarkers. Up to date, just a handful of examples have been reported. They have shown good analytical performance when challenged to simplified samples (mainly in spiked buffered solutions); however, their capability for screening and quantitating lncRNAs in complex clinical samples still remains to be demonstrated.

Additional efforts should also be directed to the integration of these biosensing platforms in miniature and automated systems, demanded in clinical settings for routine analyses. Electrochemical methods are particularly suited to these purposes that foster the assessment of reduced sample volumes,

faster reaction times, and the possibility of multiplexed detection. Regarding the simultaneous detection of multiple targets, only dual approaches have been developed. Although, the need for normalizing results through an endogenous control and evaluating panels of biomarkers to draw meaningful conclusions in clinical diagnosis calls for solutions to fill this gap.

Moreover, it is worth highlighting that, similarly to other clinical tests, lncRNA analyses are susceptible to how samples are handled and stored. In order to promote assay reproducibility, normalized protocols of sample collection, lncRNA extraction, and internal control selection should be established [41]. Although a variety of highly efficient kits are commercially available for extraction and purification of total RNA from different sample types, there are some aspects to be deeply considered such as the sample volume to collect, the need for adding an anticoagulant in blood analyses and which one would be the best option, or the suitable temperature for sample storage. Likewise, the usefulness of the endogenous control should be evaluated not only considering the type of biological fluid but also the possible influence of other patient's pathologies.

Besides being an appealing option as noninvasive biomarkers for many cancer types affecting tissues or organs difficult to access, lncRNAs can be targeted for therapeutic purposes since they have demonstrated a cell- or tissue-specific expression. Depending on the modes of action of lncRNAs, therapeutic strategies can be classified into two categories: (i) modulation of their expression (transcriptional inhibition or upregulation) and (ii) modulation of their function [42]. Of particular interest is the powerful genomic editing tool CRISP due to the low possibility of off-target effects, as well as its good performance independently of the cellular location of the lncRNA [43].

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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