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Target-responsive DNA/RNA nanomaterials for microRNA sensing and inhibition: The jack-of-all-trades in cancer nanotheranostics?☆

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ABSTRACT

microRNAs (miRNAs) show high potential for cancer treatment, however one of the most significant bottlenecks in enabling miRNA effect is the need for an efficient vehicle capable of selective targeting to tumor cells without disrupting normal cells. Even more challenging is the ability to detect and silence multiple targets simultaneously with high sensitivity while precluding resistance to the therapeutic agents. Focusing on the pervasive role of miRNAs, herein we review the multiple nanomaterial-based systems that encapsulate DNA/RNA for miRNA sensing and inhibition in cancer therapy. Understanding the potential of miRNA detection and silencing while overcoming existing limitations will be critical to the optimization and clinical utilization of this technology.

Contents

1. Introduction

1.1. The history behind miRNAs: how, why, when and where?

In 1998, Fire and Mello observed for the first time that double-stranded RNA (dsRNA) was the main cause of sequence-specific

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inhibition of protein expression in *Caenorthabditis elegans*, which they called "RNA interference" (RNAi) pathway [1]. It became evident that RNAi is critical to the control of post-transcription gene silencing in widely dispersed eukaryotic forms from yeast, fungi, plants, and animals [2,3]. To date, four major types of noncoding RNAs have been identified as RNAi effectors: small interfering RNAs (siRNAs), microRNAs (miRNAs), piwi-interacting RNAs (piRNAs) and long intervening noncoding RNAs (lincRNAs) [4,5]. microRNAs are small endogenous noncoding RNA molecules (20–23 nucleotides) derived from imperfectly paired hairpin RNA structures naturally encoded in the genome [6] that act specifically as triggering molecules to control translational repression or mRNA degradation. They regulate 10–30% of all protein-coding genes, targeting amino acid coding sequences [7], as well as promoters of gene expression [8] and long-non-coding RNAs [9,10]. This remarkable machinery involved in gene regulation processes is evolutionarily conserved and involved in many biological processes such as cell proliferation, differentiation, apoptosis, metabolism, development, aging and cancer. Moreover, miRNAs are key players in reinforcing molecular networks, acting as "genetic buffers", minimizing the noise of floppy cellular transcription regulation [11,12].

miRNA biogenesis arises in the cell's nucleus and encompasses numerous RNA processing steps (Fig. 1). miRNA coding genes are generally transcribed by RNA polymerase II (Pol II) (from polycistronic transcripts, or introns of protein-coding genes) within the nucleus, producing large (several thousand bases in length) capped and polyadenylated pri-miRNA transcripts. These pri-miRNA transcripts are processed by the RNase III enzyme Drosha to generate ~70–90 nt long precursor miRNA (pre-miRNA). pre-miRNAs present an imperfect stem-loop hairpin structure and are transported from the nucleus into the cytoplasm by the exportin 5 enzyme. After Dicer processing the precursor miRNA is transformed into a transient 22 nt mature double stranded (ds) miRNA (miR:miR duplex). The Dicer also processed the unwinding of these miRNA duplexes and promotes the incorporation of one strand of the duplex into a miRNA-associated RNA-induced silencing complex (miRISC) and a multi-protein complex that includes Dicer and proteins from the Argonaute family. The mature miRNA guides RISC to target mRNAs and or proteins, promoting their degradation [13,14]. In animals, partial complementarity (in Mammalia, the miRNAs rarely have a perfect complementarity with their targets) between mature miRNA and mRNA leads to an endonucleolytic cleavage, catalyzed by the human Ago2 in the RISC. Translational repression occurs without endonucleolytic cleavage, contrary to their "close relative" siRNA.

The mechanism described above is the accepted pathway for miRNA biogenesis; however recent studies have reported several alternative pathways, which depends on cell type, organism and biological contexts [15,16].

1.2. miRNAs: the jack-of-all-trades or the master of none?

miRNAs' small size and lack of poly-A tails may have kept them out of the spotlight for decades, however it is thought that the human genome may contain up to one thousand miRNA genes, which could regulate one third of our protein-coding genes. Actually, there is more to miRNA than meets the eye. Almost 40 years after the discovery of RNA, there is a growing evidence that these small endogenous dsRNAs are just as potent as many transcription factors and can regulate the expression of a specific gene, and hence a protein. The concept is simple: miRNAs recognize a complementary sequence encoded in a specific messengerRNA (mRNA) and bind to it, interfering with the correct translation of the mRNA sequence, impairing protein production.

Of course ascribing the silencing of genes solely to the existence of miRNA is short-sighted. In reality, these major players need a battery of enzymes to meet their ends like Drosha and Dicer, whose major role is to recognize and cleave mRNA [17]. Contrary to plant miRNAs whose sequences match with a great precision to their complementary RNA targets, animal miRNAs are far more promiscuous. The binding procedure is far less accurate and demanding as miRNAs can bind to as many as one hundred different sequences. This may sound economical and cost-effective nature-wise, however their use in laboratory

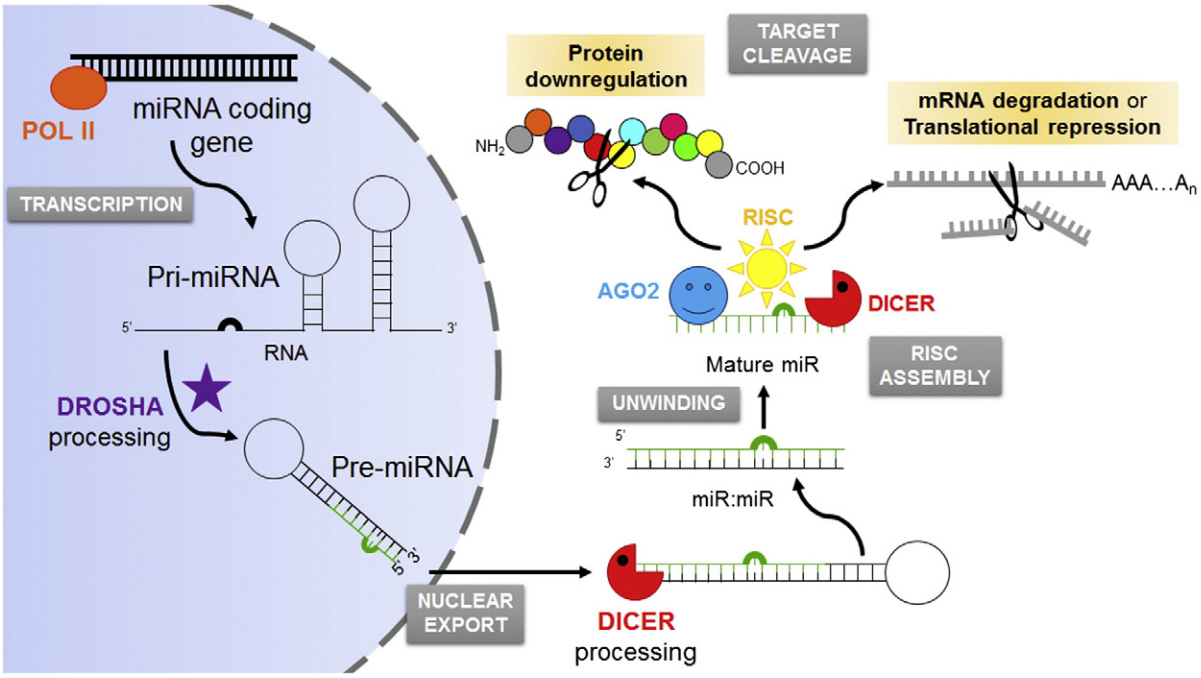

Fig. 1. miRNA biogenesis and mechanism of action. miRNA coding genes originate in the nucleus. RNA polymerase II (POL II) produces pri-miRs, transcribed from miRNA genes. Pri-miRNAs are then processed and catalyzed by DROSHA (RNase III type endonuclease) that processes pri-miRNAs to pre-miRNAs. After nuclear exportation (via Exportin5), DICER processes pre-miRNAs into 20-bp miR:miR duplexes. After miRNA duplex unwinding by the DICER, one strand is selected to function as mature miR and loaded into the RISC (RNA-induced silencing complex) and to the Argonaute family (specially, AGO2). The partner miR strand is degraded. The mature miR may lead to mRNA degradation or translational repression, as well as protein downregulation.

makes things far more tricky and delicate, as unique miRNA may regulate numerous genes and be involved in many cellular pathways and the fact propensity of animal miRNAs to degradation makes them difficult to use as diagnostic biomarkers or for therapeutic applications.

miRNAs are gaining recognition as critical regulators of many biological processes, emerging as therapeutic targets for treating disease and yet there are significant challenges to clinical utility in particular in delivering these compounds effectively. We will review the state of nanomaterials technology for miRNA and its role in translating miRNA into the clinic.

1.3. miRNAs in cancer: oncomiRs versus tumor suppressors

Despite a quarter of century of rapid technological advances, cancer is still a major cause of mortality in the modern world, with more than 10 million new cases every year. In fact, around one in three people will be diagnosed with cancer throughout their lifetime [18]. Although we have been generating a solid and complex knowledge, revealing cancer as a disease intimately involved in the dynamic change of the genome, some continue to argue that the search for the origin and treatment of this disease will continue throughout the next quarter of the century, requiring far greater insight than we have now [19]. Cancer treatment is improving and extending patients' survival worldwide. However, traditional cancer treatment protocols lack selectivity towards cancer cells and hence adversely affect adjacent healthy tissue. Additionally, inhibition of growth and development of blood cell lineages may ultimately lead to increased susceptibility to secondary infections. Still, many clinical chemotherapeutic and radiotherapeutic regimes are not very effective, due to chemo- and radio-resistance, and is patient and tumor type-dependent. Therefore, there is an urgent need for superior and more effective cancer therapies [20].

Cancer gene therapy is receiving increasing attention and could represent an attractive approach for novel treatment or prevention of many diseases where conventional clinical procedures have poor efficacy. There are two general abnormalities in cancer cells to take into account: i) they exhibit deregulation of the cell cycle resulting in uncontrolled growth; and ii) they are resistant to death as result of abnormalities in one or more proteins that inhibit apoptosis [21]. Among these, abnormally expressed microRNAs (miRNAs) have been increasingly considered as triggers of tumor development. Abnormal gene expression can be regulated at either the transcriptional or post-transcriptional level, where miRNAs are available for sequence-specific gene silencing and regulation [22]. In fact, the significance and power of the so-called oncomiRs in the tumorigenic process have been gaining momentum in cancer research [23,24].

It is well-known that abnormal miRNA expression is intimately associated with cancer establishment and progression, having a fundamental role in cancer and in the establishment of metastasis as activators or suppressors [25]. Consequently, the use of miRNA tumor suppressors or their oncomiRs might serve as useful therapeutic molecules. In fact, the combinatorial miRNA therapeutics using both tumor suppressors and oncomiRs inhibitors will probably follow, as the therapeutic potential of specific miRNAs is discovered. Moreover, targeting a specific coding gene with multiple miRNA tumor suppressors may enhance the therapeutic effect and thus, reducing the occurrence of resistance mechanisms. This is the beauty of miRNAs — the possibility of multi-targeting, even within a single pathway. Though natural (or artificial) single point mutation in the untranslated region of an oncogene may impair the specific binding of a particular miRNA, the combination of several miRNAs targeting the same gene sequence would decrease the probability of mutation-induced resistance. The same happens for the simultaneous targeting of upregulated miRNAs using antagomiRs, as well as the replacement of lost tumor suppressor miRNAs [26]. The specific role of the most common miRNAs and type of cancer in which are deregulated is depicted in Table 1.

However, the utility of miRNAs in cancer diagnosis and treatment will strongly depend on the careful design of an ideal vector, capable of fulfilling the delivery of a therapeutic gene to as many cells as possible in a highly specific way, achieving the appropriate levels of gene expression [22,27]. The highly effective vectors render serious doubts about safety for animal or human usage and others known as safe are not efficient enough.

Table 1
miRNAs involved in cancer and metastasis (Adapted from [25,28–31]).

Function	miRNA	Deregulation in tumors
OncomiRs	miR-182	Upregulated in breast, colorectal cancers
	miR-221	Upregulated in glioblastoma, hepatocellular carcinoma, prostate, thyroid cancers
	miR-1323	Upregulated in breast cancer, neuroblastoma
	miR-107	Upregulated in acute myeloid leukemia, colorectal cancers
	miR-130	Upregulated in hepatocellular carcinoma, non-small cell lung, chronic myeloid leukemia, pancreatic, breast cancers
	miR-135b	Upregulated in highly invasive non-small-cell lung, colon/colorectal cancers
	miR-150	Upregulated in breast, lung, gastric cancers
	miR-20a	Upregulated in cervical, prostate, thyroid, lymphoma, breast cancers
OncomiRs; metastasis activator	miR-155	Upregulated in solid tumors, thyroid cancer, leukemias and lymphomas
	miR-10b	Upregulated in metastatic breast cancers
	miR-21	Upregulated in breast, lung, prostate, glioblastoma, colon/colorectal, gastric, pancreatic, liver, oesophageal and cervical cancers
Tumor and metastasis suppressor	miR-126	Downregulated in breast, lung cancers
	let-7 family	Downregulated in lung, bladder, gastric, kidney, liver, pancreatic, ovarian, melanoma, prostate cancers
		Upregulated in acute myeloid leukemia, retinoblastoma, uterine, colon cancers
	miR-106	Upregulated in breast, prostate, pancreatic, colorectal cancers
Tumor suppressor	miR-205	Downregulated in breast cancers
	miR-491	Upregulated in cervical cancers, oral squamous cell carcinoma
	miR-133b	Downregulated in gastric, bladder, prostate, colorectal cancers
	miR-7	Downregulated in glioblastoma, neuroblastoma, pancreatic, breast cancers
	miR-16	Dowregulated in gastric, breast cancers
	miR-29	Downregulated in breast, non-small-cell lung cancers
	miR-31	Upregulated in thyroid, breast cancers
	miR-33a	Upregulated in chemoresistant osteosarcoma, lung cancers
	miR-34a	Upregulated in breast, neuroblastoma, prostate, gastric cancers
	miR-122a	Downregulated in hepatocellular carcinoma
	miR-124a	Downregulated in lung, breast cancers, glioblastoma
	miR-128	Downregulated in neuroblastoma, glioblastoma, prostate, lung cancers and acute lymphocytic leukemia
	miR-141	Downregulated in pancreatic, colon cancers
	miR-143	Downregulated in cervical cancers
	miR-145	Downregulated in bladder cancers

2. miRNA sensing

As modification in miRNA profiles are intimately linked to different states of cancer and can be used as important biomarkers for cancer diagnostics, many technologies have been developed to detect the presence and changes in miRNA expression levels [32–34]. Presently, most of the miRNA sensing studies are using techniques such as Northern blot for miRNA expression patterns. Many different approaches have been exploited to profile mature miRNA expression, including deep-sequencing, quantitative PCR, in-situ hybridization or oligonucleotide microarrays. Nevertheless, these methodologies are relatively insensitive, time-consuming and labor intensive. It is extremely challenging to detect small RNAs (most of the times in low concentration) with standard techniques [35]. So it is imperative to develop new techniques which allow an easy monitoring of miRNA expression in a variety of biological sample types with substantially more sensitivity than Northern analysis.

The application of nanomaterials as platforms for optical and electrochemical sensing of miRNA has been gaining reputation in the field. Table 2 summarizes the latest types of nanomaterials-based miRNA sensors for cancer diagnostics, according to their methodology principle, surface modification, targeted miRNA and detection limit.

2.1. Plasmonic/optical-based miRNA sensors

The majority of DNA/RNA nanosensors are based on Watson–Crick base-pairing between complementary sequences. Although the binding of miRNAs to their targets depends largely on multiple factors (thermodynamic characteristics; seed sequence in hybrid formation) most of the optical miRNA sensors are based on pure DNA or RNA oligonucleotide hybridization with the targeted miRNA. These applications (see Table 2) rely on optical detection and usually use a large number of different types of fluorescent reporters, including organic dyes, fluorescent proteins or inorganic nanostructures, such as quantum dots or gold nanoparticles [63]. In fact, quantum dots are exceptional substitutes as fluorescence labels with narrow, tunable, symmetrical emission spectra, and high quantum yields [64]. This is particularly important in samples where miRNAs need to be enriched due to low concentration, once the high quantum emission of these nanostructures qualifies them for direct detection applications [63].

Gold nanoparticles have also been extensively used in miRNA detection using specially thiolated-DNA probes to hybridize with mature miRNAs, coupled to an avidin–biotin detection system. An inexpensive and easy alternative to fluorescence or radioactivity-based assays exploits the high affinity of thiol group to gold surface and the use of thiol-linked ssDNA-modified gold nanoparticles for colorimetric detection of gene targets [65,66]. These systems are simple and efficient and can even diagnose a single nucleotide mismatch between miRNA members.

Graphene oxide (GO) 2D nanostructures (with excellent thermal, electronic and mechanical performances) have also been successfully applied to miRNA detection due to their distance-dependent fluorescence quenching skills. They provide highly sensitive and specific signal, with detection limits comparable to surface-plasmon resonance (SPR) and fluorescence methodologies [63]. The optical-based methods using nanomaterials usually have a detection limit range from 0.1 aM (0.1E−18 M) to 1 pM (1E−12 M) (see Table 2). One of the most limitations of optical-based nanomaterials is that may produce high background signal due to quenching inefficiencies [67].

2.2. Electrochemical miRNA sensing: labeled miRNA versus label free

Electrochemical methods have found numerous applications in sensing miRNA molecules, due to the development of speed, simplicity, high sensitivity and low detection limits (see Table 2). Moreover, electrochemical methods allow for miRNA sensing without enzymatic amplification. Typically, the detection and quantification of the miRNA target are based on measuring the change in voltage in the presence of a specific target miRNA [63]. In all electrochemical techniques, miRNA hybridizes to a complementary probe, which is immobilized on the surface of an electrode or nanoparticle [67]. These methods are particularly important to reduce the amounts of sample required and are usually fast and very sensitive and are divided into indirect (targeting labeled miRNA) and direct methods (label-free).

The labeled miRNA methods usually use a chemical tag that is bound to the target miRNA sequence, which upon miRNA hybridization to the probe will produce an amplification-signal to detect and quantify the levels of expression. Voltametric and electrocatalytic nanoparticles (specially made of ruthenium and osmium oxides) are frequently applied [68] and they readily bond to the hybridized miRNA and have high catalytic capacity [63]. Direct methods (using label-free miRNAs) are faster than indirect methods and are less prompt to bias. Most of the nanomaterials used are based on nanowires of silicon or polymer materials (e.g. polyaniline). As such they can be formed into supramolecular networks with high electrochemical capacities, that depend on electrochemical redox state, humidity, pH, the presence and type of anions in solution, and temperature [63]. miRNA detection and quantification occur through changes in the environment conductivity and charge of the formed-nanomaterials network by the presence of negatively charged phosphate groups from miRNAs.

Another interesting system is the nanopore-based sequencing techniques, which use a nanoscale pore in a membrane between two electrolytes and voltage to drive molecules through the pore. Changes in the ionic current through the nanopore as single molecules (for example, miRNAs) pass through it can be monitored [69,70] (see Fig. 2). These systems can be used for direct detection of circulating cancer miRNA biomarkers in plasma and tissue from mice [62] and cancer patients [61]. Although nanopore-based sequencing techniques have the potential to be direct this technology is not yet reasonable to apply in any practical application, especially due to the difficulty in the procedure for the fabrication of the nanopore, requiring highly qualified personnel and specific equipment [63,67].

The electrochemical-based methods using nanomaterials usually have a detection limit range from 0.32 aM to 2 pM (see Table 2). The main disadvantages of electrochemical-based methods are in some cases complicated manufacturing of the devices (some are not even portable!), the inability of multiplexing and the cost [63].

3. miRNA therapeutics

miRNAs have emerged as master regulators in the expression of genes and as critical therapeutic agents in numerous disease states, such as cancer, inherited diseases, autoimmune disorders, heart diseases and in the regulation of the nervous system [28,71]. One of the most remarkable premises in miRNAs is that they are many times up-regulated in certain diseases. Therefore, the usage of specific blockers (i.e. antisense approaches) of the overexpressed mature microRNAs may be useful as therapeutic strategies to block their expression. Numerous methods have been used to inhibit miRNA repression of protein expression; however, each one is associated with RISC complex disruption. The most common method is the use of single-stranded antisense oligonucleotides sequences – antagomiRs (when cholesterol conjugated) – that are specifically designed to hybridize to corresponding mRNA and inhibit its function by disruption of ribosome assembly. Nevertheless, these antisense oligos possess weak intrinsic binding affinities and are readily degraded by nucleases. Besides, they are only designed to bind mature miRNAs and not messenger RNA, preventing the degradation of mRNA, and promoting its translation. In fact, in order to achieve effective miRNA silencing levels, the miRNA inhibition therapy needs that antisense oligos to carry chemical modifications [72]. These chemical modifications rely specially on the backbone chemistry and sugar moieties. In fact the phosphorothioate backbone

Table 2
Summary of the latest nanomaterial-based miRNA sensors used in cancer diagnostics according to the type of NP, type of assay and nanoparticle's surface modification, targeted miRNA and type of cells/samples, detection limit and explored methodology principle.

Method	Type of NP	Assay/surface modification	Target miR/cells/samples	Detection limit	Comments [Reference]
Plasmonic/ optical-based	Gold	Lateral flow nucleic acid; anti-avidin antibody	–	5E−18 M	Ultra-sensitive; fast (70 min); simple; convenient; suitable for point-of-care [36]
		Thiol–DNA hairpin-Cy3	miR-21; HCT-116 (colorectal carcinoma)	–	Nanobeacons for efficient sensing of miR-21 in living cells [37]
		Sandwich assay; thiol–DNA; avidin–biotin	miR-122a; miR-128; mouse liver; brain tissue	10E−15 M	Simple; reliable; amplification free; high specificity to discriminate mismatch of the target miRNA [38]
	Silver	Cytosine-rich oligonucleotide	–	~2E−09 M	High specificity; cost-effective; easy to synthesize; low detection levels [39]
	Magnetic (cobalt ferrite)	miRNA molecular beacon	miR-124a	–	miRNA-specific molecular beacon imaging of mature miRNA and detection of neuronal differentiation patterns [40]
	Quantum dots	Two-stage isothermal exponential amplification reaction;	let-7a; let-7b;let-7c; miR-21	0.1E−18 M	Isothermal nature; rapid and efficient amplification; near-zero background noise [41]
		Thiol–ssDNA	miR-21	10E−15 M	Excellent selectivity; distinguish single-base-mismatched, random nucleic sequences, pre-miRNA and mature miRNA [42]
		Biotin and oligo-DNA probes immobilized on glass slides; streptavidin on QDs	11 miRs from leaf and root of rice (*Oryza sativa* L. ssp. *indica*) seedlings	0.4E−15 M	Oligonucleotide microarray; good reproducibility; consistent with northern blot [43]
	Graphene oxide	GO fluorescence quenching; isothermal strand displacement polymerase reaction	miR-16; let-7a; let-7e;	2.1E−15 M	Simple; highly sensitive; selective multiple miR detection [44]
		Cyclic enzymatic amplification method; DNA probes	let-7a; let-7e;let-7i	~9E−12 M	Rapid; direct detection in biological samples; without any sample pre-treatment [45]
		PNA probes	miR-29	~1E−12 M	High specificity; quantitative monitoring miRNA levels in living cells [46]
		Single-stranded probe	miR-126	~3E−15 M	Rapid; sensitive; selective [47]
	Hyaluronic acid-based	Molecular beacons	miR-34a	–	In vitro and in vivo optical imaging allow the measurement of miR expression levels; selective recognition [48]
Electrochemical Labeled miRNA	Gold	Voltammetric; biotinylated miRNA	miR-182; glioma patients	10E−15 M	Highly reproducible; selective (sequence specificity down to a single nucleotide mismatch); time consuming [49]
	Ruthenium oxide	Polymerization of 3,3′-dimethoxybenzidine; miRNA-templated deposition of an insulating poly(3,3′-dimethoxybenzidine) film	let-7a; let-7b; let-7c; let-7d; let-7e; let-7f; let-7g	6E−15 M	No cross-hybridization between pre-miRNA and mature miRNA; sparse cross-hybridization among closely related miRNA even at single-base-mismatched level system [50]
		Voltammetric; miRNA strands as templates	let-7a; let-7b; let-7c; let-7d; let-7e; let-7f; let-7g; let-7i	2E−15 M	Minimal background; enhanced specificity and sensitivity.; fast [51]
	Osmium oxide	Electrocatalytic nanoparticle tags; oligonucleotide capture probes	miR-106; miR-139; let-7b; let-7c; HeLa cells	80E−15 M	Simple; efficient; easily extendable to a low-density array of 50–100 electrodes [52]
Electrochemical Label-free miRNA	Gold	Chronoamperometry; LNA and biotin–hairpin DNA barcodes	miR-21; BEL-7402 (human hepatocarcinoma)	60E−15 M	Excellent selectivity; high sensitivity; efficient; complicated; time consuming [53]
		Surface plasmon resonance technology; DNA/RNA antibody-based assay; thiol-DNA oligos	miR-122; mouse liver tissues	2E−12 M	Very fast; sensitive [54]
	Quantum dots	Voltammetric; LNA-molecular beacon probes	miR-16	0.32E−18 M	Ultra-sensitive; requires combining rolling circle amplification; time consuming [55]
	Carbon nanotubes	Interpenetrated network of carbon nanotubes; electroactive poly(JUG-co-JGA) polymer	miR-141; prostate cancer	8E−15 M	High sensitivity; label-free; reagentless; copolymer electroactivity is enhanced by the presence of nanotubes [56]
		ssDNA–FAM probes	let-7a	3.5E−12 M	RNase H and DNase I activities monitored based on the fluorescence changing of the DNA probe [57]
	Silicon nanowires	Direct hybridization assay; PNA probes	let-7b; let-7c; Hela cells	1E−15 M	Label-free; rapid; high sensitive; detection fully matched vs mismatched miRNA sequences [58]
		Hafnium oxide dielectric-based transistor; DNA probes	miR-10b; miR-21	1E−15 M	Highly sensitive and selective; time consuming [59]
	Polymer nanowires	Polyaniline polymer; PNA probes	let-7b; let-7c	5E−15 M	Simple; highly sensitive; uses chemical ligation and amplification for signal read-out [60]
	Nanopores	Protein-nanopore-based sensor; ssDNA probe	miR-155; let-7a; let-7b; let-7c	0.7E−09 M	Selective; direct quantification of cancer-associated miRs in blood; discrimination of single-nucleotide; low detection levels [61]
		Probe:microRNA duplex-viral protein p19	miR122a; rat liver	~16E−15 M	Uniform, robust and well-defined solid-state membrane for nanopore production; detection from biological tissue [62]

Cy3 — cyanine dye; QDs — quantum dots; GO — graphene oxide; PNA — peptide nucleic acid; FAM — fluorescein.

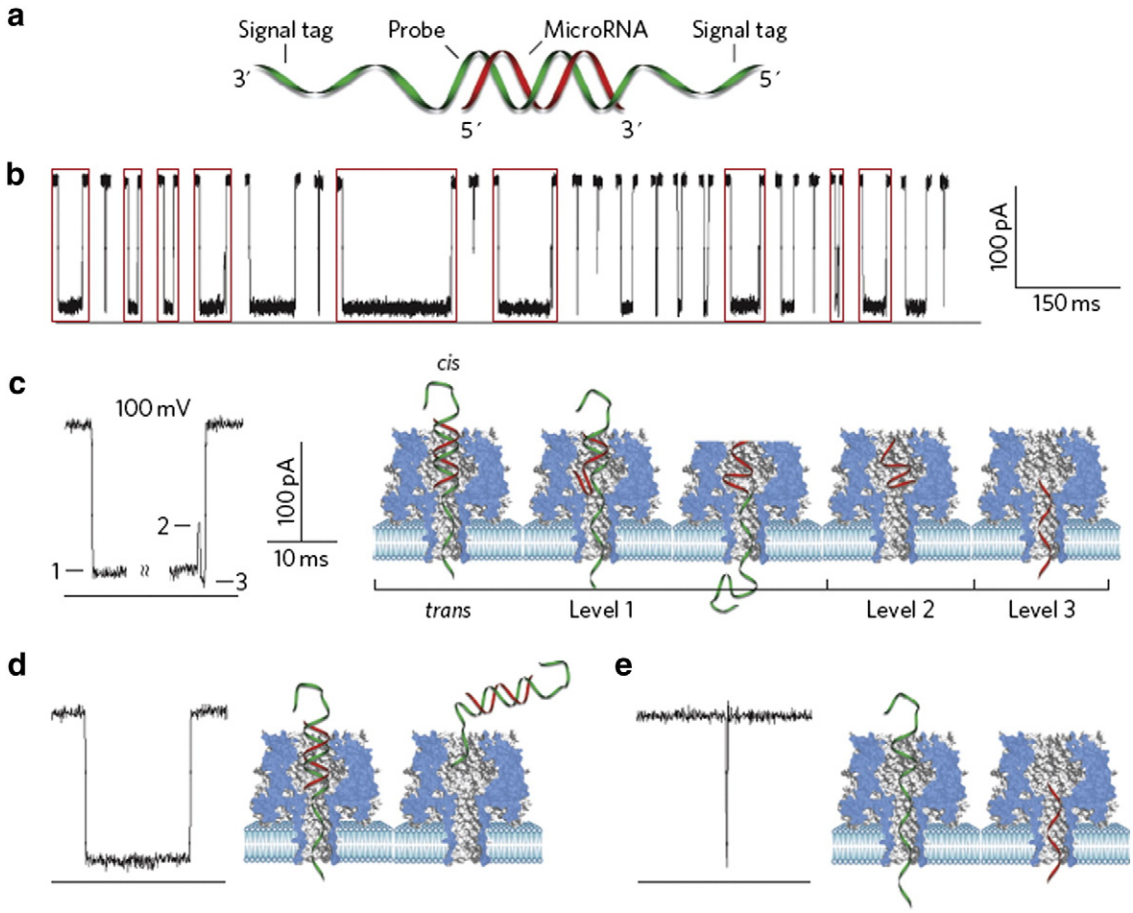

Fig. 2. Capturing single microRNA molecules in the nanopore. (a) Molecular diagram of a microRNA (red) bound to a probe (green) bearing signal tags on each end. (b) Sequence of nanopore current blocks in the presence of 100 nM miR-155 and 100 nM P155 in the cis solution. Red boxes represent the multi-level current pattern. (c) A typical multi-level long block (from b) at þ100 mV generated by the miR-155-P155 hybrid. Right panel: diagram showing the molecular mechanism of hybrid dissociation and translocation. Level 1: trapping of the microRNA-probe hybrid in the pore, unzipping of the microRNA from the probe and translocation of the probe through the pore. Level 2: unzipped microRNA residing in the pore cavity. Level 3: translocation of the unzipped microRNA through the pore. Lower panel: multi-level blocks at þ150 and þ180 mV. Increasing the voltage reduced the duration of Levels 1 and 3, which supports the above mechanistic model. (d) A single-level block (from b) generated by a trapped miR-155-P155 hybrid that exited the pore from the cis entrance without unzipping and translocation. (e) A spike-like short block generated by the translocation of unhybridized miR-155 or P155 from the cis solution.
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modification is, to date, one of the most widely used modifications. However, chemical modifications are too expensive or inadequate [73].

DNA plasmids, carrying the sequence for the miRNA coding gene, are typically used as substitutes of the RNA antisense oligos in order to overcome some of the instability problems.

In cases where the miRNA expression is downregulated, exogenous mature miRNAs (known here as miRNA mimics) may be delivered to restore their expression and promote normal cell homeostasis, by a miRNA replacement therapy approach. However, the exogenous administration of single stranded miRNA mimics falls short of expectations. In fact, endogenous double stranded miRNAs have a 100 to 1000 fold higher potency compared to the exogenous ones [72].

Targeting ectopically miRNAs may provide a vital diagnostic or therapeutic strategy for human cancer in the near future. In fact, both the antisense inhibition as the mature miRNA replacement therapies are valuable and have been considered as potentially treatment approaches (Fig. 3) [74]. In any case, choosing the appropriate vehicle/vector to increase the delivery and the specificity of the miRNA seems to be one of the most important aspects influencing the activity of the miRNA.

There is then a paradox in the field: while unmodified miRNAs and siRNAs are profound regulators of transcription and post-transcriptional signaling their use and manipulation are limited by their physicochemical property. Unmodified RNA oligos have i) extremely short half-lives (seconds to minutes) when delivered into the bloodstream due to renal clearance (because of their small size), ii) are negatively charged, making it difficult to cross the cell membrane, iii) weak protection against action by serum ribonucleases, iv) poor chemical stability, v) need for targeted delivery to specific tumor cells so as not to disrupt normal cells, and last but not least vi) difficulty in silencing multiple targets simultaneously, so as to avoid development of resistance to therapeutic agents [75]. Thus, the efficient delivery of RNA antisense and siRNA oligos or mature miRNAs is a huge bottleneck in miRNA delivery progress. Nanotechnology and nanoparticles (NPs) in particular can overcome some of these drawbacks offering a protective environment to RNA oligos and an efficient and biocompatible delivery vehicle for intracellular gene regulation.

3.1. Vectors for miRNA delivery

All of therapeutics must meet the same main challenge — developing a system capable of circulating in the blood stream undetected by the immune system and able to recognize the desirable target and signal it for effective drug delivery or gene silencing. In the past, modified viral vectors were found to be extremely efficient in gene delivery; however they are prompt to an extensive immune response impairing most of the proposed viral systems. Besides viral vectors, miRNA delivery in vitro is usually achieved using cholesterol conjugated 2′-O-methyl antisense oligonucleotides — the so-called locked nucleic acid (LNAs)

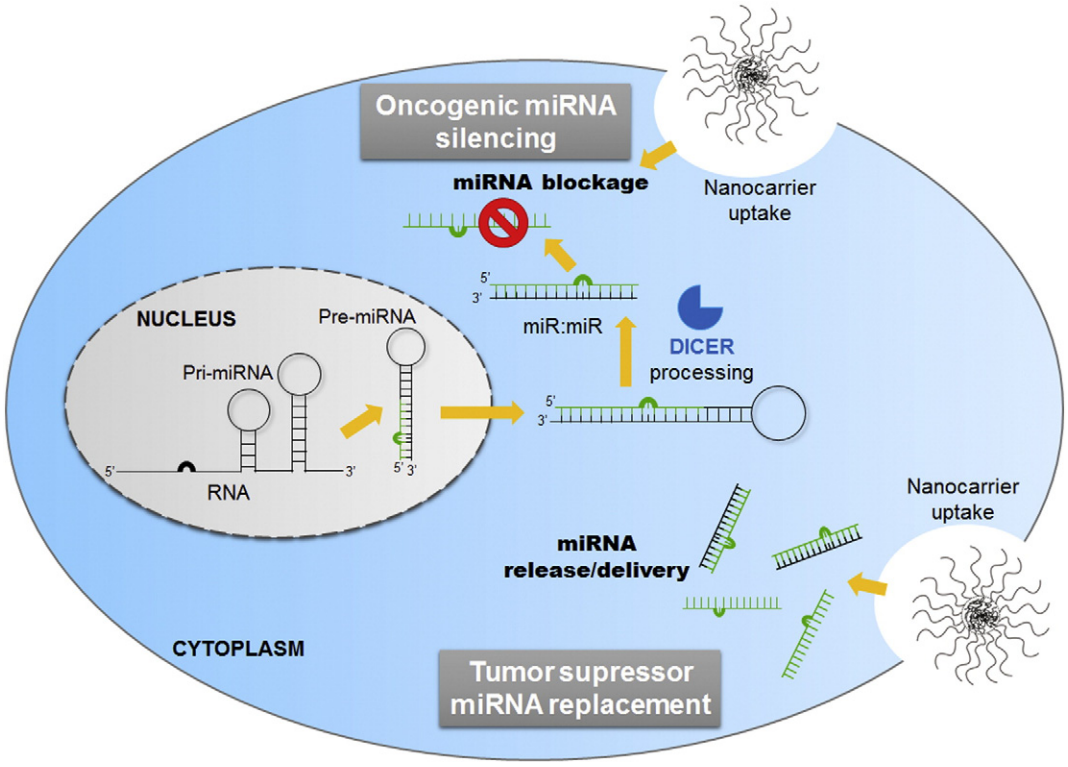

Fig. 3. miRNA inhibition strategy versus miRNA replacement therapy. When a miRNA that is up-regulated intimately contributes to disease progression such as cancer, oncogenic miRNA inhibition therapy can be used to steric hindrance the miRNA expression. Contrary to this approach, if an miRNA is downregulated, the miRNA release/delivery may took place in order to balance miRNA expression levels, via a tumor suppression miRNA replacement strategy with an oligonucleotide that mimics the same sequence as the endogenous mature miRNA.

based antisense oligos. These LNAs are oligos with multiple miRNA binding sites that are recognized as competitive inhibitors or oligos complementary to miRNA binding sites [76].

For in vivo miRNA delivery, the most traditional procedure is the administration of naked miRNAs, which results in liver accumulation. In fact, the ischemic injury or tumor vasculature delivery is found to be disappointing and challenging. Consequently, the use of nanomaterials for miRNA strategies is paving the way for miRNA sensing and delivery [72].

The success of a gene therapy vector depends on three factors, 1) choosing an appropriate therapeutic gene, 2) the delivery of the therapeutic gene to as many diseased cells as possible, and 3) the accomplishment of a suitable level of gene expression. Several different approaches which preserve biocompatibility, selectivity and efficiency have been developed based on nonviral lipids or protein carriers, including cholesterol, metal NPs, liposomes, antibody promoter fusions, cyclodextrin NPs, fusogenic peptides, aptamers, biodegradable copolymers, and polymers [77]. Positively charged cationic liposomes and polymers, such as polyethylenimine (PEI), are currently the two major carriers used to complex with negatively charged oligonucleotides for systemic delivery [72,78].

3.2. Nanomaterials-mediated delivery: the necessity in cancer nanotheranostics

Cancer therapy is not a one-size-fits-all scenario. It must be adapted to each patient in a highly specific and selective way [79]. In fact, advances in cancer diagnosis and therapy have not been as effective as for other chronic diseases, and effective detection methods only exist for some types of cancer [19]. Thus, the main challenge is to find new and more effective diagnostic agents for the monitoring of predictive cell molecular changes that are involved in tumor development. The key to the efficient and ultimately triumphant treatment of cancer is early and accurate diagnosis [80].

It's here that nanotechnology can be exploited for cancer theranostics (combination of diagnostics and therapy in one system using the benefits of nanotechnology) via the development of diagnostics systems such as colorimetric and immunoassays, and in therapy approaches through gene therapy, drug delivery and tumor targeting systems [81–83]. The use of nano-sized materials for diagnostics and therapy purposes is a promising field as more and improved techniques are becoming available with increased sensitivity at lower costs, when compared to the traditional methods. An inspiration to science from the time of Faraday, today nanomaterials and nanoparticles are being used for an ever-growing number of biological applications. The high surface-to-volume ratio and size-dependent optical properties, of the nanoparticles make them interesting materials for their use in biomedical applications and are extremely attractive for cancer nanotheranostics [84].

When referring to cancer therapy, targeting and localized delivery are of utmost importance in enhancing the therapeutic effect and decreasing undesirable distribution to healthy organs and tissues. Multifunctional intelligent nanomaterials may potentiate the development of personalized cancer therapy based on the individual's biological information within the tumor (biomolecular profiling) [85]. In fact, nanomaterials are increasingly used as imaging agents and drug delivery vehicles because their tunable physical and chemical properties offer unique opportunities for developing new approaches to diagnose, prevent and treat diseases. The possibility to combine in one single device chemical and physical properties (i.e. the bioactivity of a given compound bound to fluorescence, enabling self-tracking) and magnetism properties (allowing cell separation, heat generation), makes nanoparticles unique multipurpose tools [82,86,87].

Although there are technical challenges associated with the therapeutic application of nanomaterials, the integration of therapy with diagnostic profiling has accelerated the pace of discovery of new nanotechnology methods. The development of a safe, efficient, specific and nonpathogenic vehicle for gene delivery is highly attractive

[88,89]. This integration is fuelling biopharmaceutical research and development efforts by expanding and validating the pool of potential therapeutic targets, straining the already serious bottlenecks in drug discovery industry [90].

Further research into the fundamental mechanisms of gene therapy using nanodevices could unveil new dimensions of nanoparticle-mediated gene silencing that will have profound implications for understanding gene regulation, and which could also affect the development of functional genomics and therapeutic applications.

3.3. Inorganic vs organic DNA/RNA nanomaterials for cancer therapy

Suitable inorganic (quantum dots — QDs, gold, silica and magnetic nanoparticles) and organic (liposomes, lipids, dendrimers, micelles) nanomaterials can be used as nanovectors for miRNA silencing and delivery (see Fig. 4). In fact, the nanodelivery of therapeutic nucleic acids is highly desirable and constitutes an important challenge to gene therapy. The unique physico-chemical properties of both inorganic and organic nanomaterials show great potential of application in biomedicine for the development of sensing platforms and therapeutics [91]. Recent advances in approaches using nanomaterials functionalized with oligonucleotides have made them an attractive tool for DNA/RNA based therapy, where antisense RNA technology and miRNA sensing in particular show a significant potential for gene regulation in therapeutics and in diagnostics [92,93].

Although the delivery of miRNAs by nanomaterials is recent and mostly used for in vitro applications, their use as miR vehicles has huge advantages. In fact, oligonucleotides such as anti-miRNA, antagomiRs, synthetic miRNA mimics and mature miRNAs can be conjugated to nanomaterials, protecting from nuclease degradation when compared to free/naked oligos. Moreover, immune stimulation and/or suppression associated with DNA/RNA nanomaterials are far more reduced than with the naked oligos [75]. Nevertheless, the size, charge, surface chemistry and hydrophobicity of the nanomaterials dictate their interaction with the immune system [94]. And the interaction of nanomaterials with the immune system determines the therapeutic efficiency of the delivered miRNA. Although numerous polymers can be used to avoid immunostimulation, PEGylation is still the most commonly used system to bypass the recognition of the phagocytic immune cells, increasing the circulation and lifetime inside the body by avoiding opsonization [95].

There are almost 100 reports published for the delivery of miRNAs in cancer using inorganic and organic nanomaterials; Table 3 describes some of the most interesting and successful systems, in terms of type of NP, surface modification, targeted miRNA, type of cancer, target cells/organs/organisms and explored methodology.

One of the most used inorganic nanoparticles for miRNA delivery in cancer is the gold nanoparticles (AuNPs) as they possess astonishing optical and electronic properties, well-regulated morphology and size dispersion, great stability and biological compatibility and easy surface functionalization. The main characteristics include the electrical, chemical, and optical properties. The optical properties of AuNPs are significant because its adsorption and emission of the wavelength are within the visible range of light and because of their size- and shape-dependent properties [121,122].

Concerning miRNA silencing and delivery systems using AuNPs, the majority uses the delivery of miRNAs that mediate efficient knockdown in functional reported gene assays (luciferase and GFP) and also apoptosis-inducing miRNAs, in vitro only. The only study reported so far concerning the use of AuNPs for the detection in living cells at the same time as oncomiR inhibition occurs was reported by Conde et al. (see Fig. 5). The authors reported a gold nanoparticle-based nanobeacon (Au-nanobeacon) as an innovative theranostic approach for the detection and inhibition of sequence-specific miRNA in vitro [37]. The proposed method allows real-time detection of the beacon's signal while yielding a quantifiable fluorescence directly proportional to the level of gene silencing [37,123–125].

A theranostics system using aptamers and molecular beacons for the detection and inhibition of miRNAs at the same time was also reported using conjugated magnetic fluorescence (MF) nanoparticles. The authors described selective systems that simultaneously target cancer tissue, image intracellular expression of a specific miRNA and treat miRNA-involved carcinogenesis [100].

Not only with gold or iron oxide nanoparticles, but also with the other inorganic nanoparticles (QDs, silica, graphene), the utmost common miRNA therapeutic approaches in cancer are the inhibition of oncomiRs (see Table 3). Silica NPs with cationic surfaces and modified/

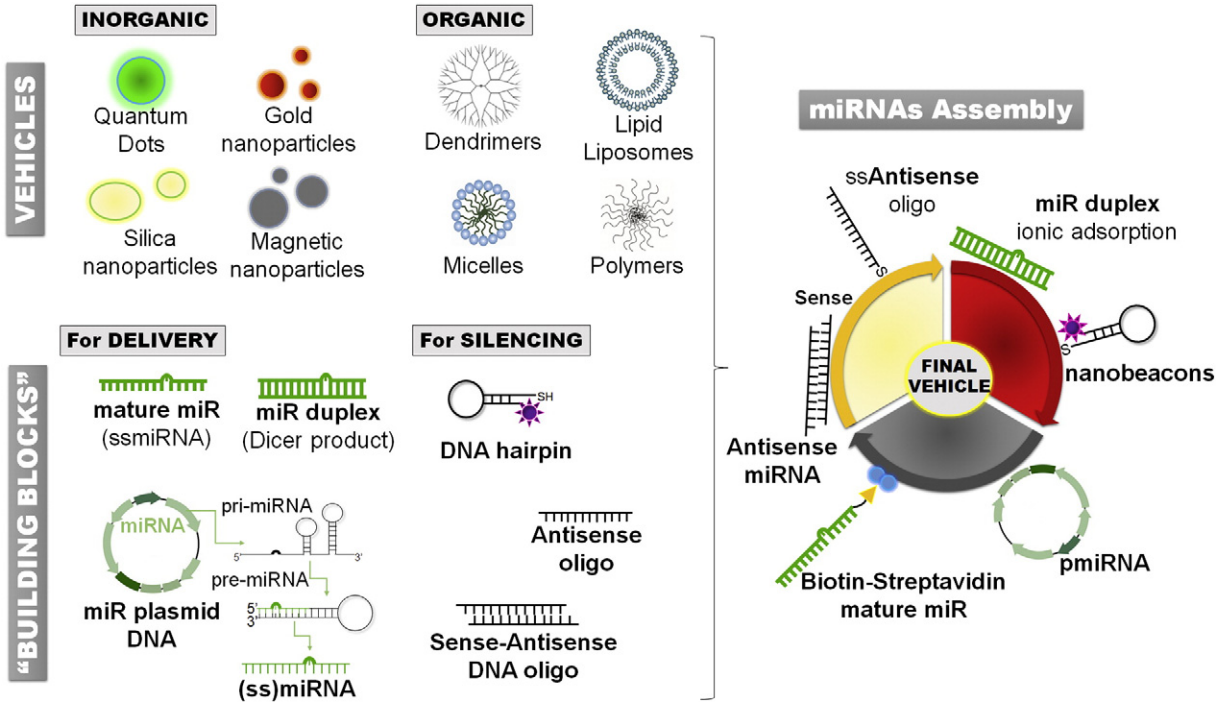

Fig. 4. Synthetic and biological "building blocks" used to construct organic and inorganic nanovehicles for miRNA delivery and silencing.

Table 3
Summary of nanomaterials used in miRNA cancer therapy (miRNA inhibition and/or miRNA replacement therapies) according to the type of NP, surface modification, targeted miRNA, type of cancer, target cells/organs/organisms and explored methodology/approach.

Vehicle	Type of NP	Surface modification	Target miRNA	Cells/type of cancer	Route administration	Approach	Comments [Reference]
Inorganic	Gold	Thiol–DNA hairpin–Cy3	miR-21	HCT-116 (colorectal carcinoma)	Cell culture	Inhibition	Nanobeacons for efficient sensing, targeting and silencing of miR [37]
		Mimic miRNA (sense-antisense miRNA duplex)	miR-205 miR-20a	PCa (human prostate cancer)	Cell culture	Inhibition and replacement	Delivery of tumor suppressor miR and silencing of oncogenic miR [96]
		Cysteamine (aminothiol cysteine)-miRNA	miR-31 miR-1323	NGP (neuroblastoma); HEYA8 (ovarian cancer)	Cell culture	Inhibition and replacement	Deliver tumor suppressive miR31 and oncogenic miR1323 [97]
		DNA antisense-modified oligos	miR-29b	HeLa (human cervical carcinoma); 293T (human embryonic kidney)	Cell culture	Inhibition	Ability of DNA Antisense-modified oligos to silence a miRNA [98]
		Thiol–miRNA–Cy5	miR-130b	Multiple myeloma cell line model (MM.1S)	Cell culture	Inhibition	Efficient knockdown in luciferase functional assay [99]
	Magnetic (iron oxide)	Aptamer and miRNA molecular beacon	miR-221	U-87MG; PC-12; F11; C6; HepG-2; SK-Hep-1; Caco-2; CT-26; TPC-1; NPA; U-2OS; HeLa; A549; PC-3; F9	Cell culture	Inhibition	Antitumor therapeutic effects by inhibiting miRNA function — successful astrocytoma-targeting theranostics [100]
		LNA anti-miR	miR-10b	MDA-MB-231 (human breast cancer); orthotopic MDA-MB-231-luc-D3H2LN tumors	Cell culture; systemic	Inhibition	Nanodrug accumulates in the primary tumor and lymph nodes; metastasis is prevented [101]
	Quantum dots	Monoclonal antibodies–(ss)miRNAs	miR-491	HepG2 (hepatocellular carcinoma); HBT3477 (human breast cancer)	Cell culture	Inhibition	Biotin streptavidin system to deliver an apoptosis-inducing miRNA [102]
	Silica	Cell surface antigen GD2 antibody-miRNA	miR-34a	Neuroblastoma mice model	Systemic	Replacement	Tumor reductions and apoptosis and anti-angiogenic activity [103]
	Graphene oxide	Poly(amidoamine) dendrimer-grafted gadolinium; epirubicin (EPI); synthetic miRNA	Let-7g	U87 (human glioblastoma)	Cell culture	Replacement	Non-viral vector for chemogene therapy and molecular imaging diagnosis [104]
Organic	Lipid-based liposomes	Pre-miR	miR-133b miR-34a	A549 (non-small cell lung cancer)	Cell culture; systemic	Replacement	Mature miR-133b expression in lung 52-fold higher than untreated mice [105]
		Single-chain antibody fragment (scFv)-miRNA	miR-34a	Lung cancer — lung metastasis of murine B16F10 melanoma	Systemic	Replacement	Survivin expression downregulation in metastatic tumor; reduced tumor load in lung [106]
		Synthetic miRNA	miR-143 miR-145	DLD-1 human colorectal cancer	Systemic; local	Replacement	Tumor-suppressive effect on xenografted tumors [107]
		2′-OMe-4′-thioribonucleoside-anti-miR	miR-122	HeLa (human cervical carcinoma); Huh-7 (human hepatoma)	Cell culture; systemic	Inhibition	Delivery to the liver and efficient inhibition of miR activity at a low dose in vivo [108]
		Synthetic miRNA	miR-34a let-7	Non-small cell lung cancer mice model	Systemic	Replacement	Anti-miRs displayed a 60% reduction in tumor area compared to control [109]
		Pre-miR	miR-107	Head and neck squamous cell carcinoma	Systemic	Replacement	Significantly retarded tumor growth by 45.2% [110]
		miR-expressing plasmid	miR-7	Lung cancer	Systemic	Replacement	Tumor regression in mouse xenograft model [111]
		Lactosylated gramicidin; anti-miR; Lac-DOPE; gramicidin A	miR-155	HCC (hepatocellular carcinoma) model	Cell culture; systemic	Inhibition	Lac-GLN as a liver-specific delivery vehicle for anti-miR therapy [112]
	Polymer-based carriers e.g. PLGA, collagen, PEI, dendrimers	Synthetic miRNA	miR-16	Prostate cancer	Systemic	Replacement	Growth inhibition of metastatic prostate tumors via multiple cell-cycle genes downregulation [113]
		Synthetic miRNA	miR-34a	HCT-116 and RKO (human colon cancer)	Cell culture; local	Replacement	Induces senescence-like growth arrest through modulation of the E2F pathway [114]
		Synthetic miRNA	miR-33a miR-145	Colon carcinoma	Systemic; local	Replacement	Efficient; biocompatible; profound antitumor effects [115]
		Synthetic miRNA	miR-135b	Lymphoma	Local	Inhibition	Antisense-based miR inhibition reduced tumor angiogenesis and growth in vivo [116]
		Synthetic miRNA	miR-21	U251; LN229 (human glioblastoma)	Cell culture	Inhibition	miR inhibitor enhanced apoptosis and decreased cell invasiveness [117]
		Synthetic miRNA; 5-FU	miR-21	Human glioma	Cell culture	Inhibition	Increased apoptosis and decreased migration ability of tumor cells [118]
	Exosomes vesicles	Antisense RNA anti-miR	miR-150	Tumor-associated macrophages	Cell culture; systemic	Inhibition	Downregulation of miR-150 and VEGF levels in vivo; angiogenesis impairment [119]
		Synthetic miRNA	let-7a	Xenograft breast cancer	Systemic	Replacement	Efficiently deliver microRNA to EGFR-expressing breast cancer [120]

Cy3 — cyanine dye; LNA — lock nucleic acid; GD2 — disialoganglioside; PLGA — poly(lactic-co-glycolic acid); PEI — polyethylenimine; Lac-DOPE — N-lactobionyl-dioleoyl phosphatidylethanolamine, a ligand for the asialoglycoprotein receptor (ASGR); VEGF — vascular endothelial growth factor; EGFR — epidermal growth factor receptor.

encapsulated with fluorescent fluorophores have been shown to be efficient gene transfection agents, protecting nucleic acids from enzymatic degradation [126].

An important finding when applying inorganic nanomaterials for miRNA is that very low concentrations of miRNA are required for target suppression [126]. Moreover, Natarajan and co-workers reported that 100 nM was the minimum miRNA concentration for maximum apoptosis induction [102].

Concerning the organic vehicles, one of the most common nanomaterials for miRNA delivery are the liposome/lipid-based and the polymeric systems, especially the use of cationic lipids (cationic head group plus hydrophobic moiety bound to a linker) since strongly attached to nucleic acid phosphate groups [126] and cationic polymers (natural polymers — peptides, proteins, chitosan; or synthetic polymers — dendrimers, poly(lactic-co-glycolic acid), poly(L-lysine)), polyethylenimine, collagen) [127].

Lately, Anderson and co-workers reported the delivery of miRNA mimics and siRNAs involved in inhibition of tumor growth to in vivo lung cancer mice model. The authors showed that the combination of miRNA replacement and siRNA targeting leads to tumor regression, improving therapeutic responses over those observed with siRNA alone [128]. A similar vehicle was reported using siRNAs/miRNAs encapsulated in a cationic lipid nanoparticles but now for liver cancer. The modified lipid encapsulating miRNA mimic was intratumorally injected and resulted in ~50% growth suppression of liver tumor xenografts [129]. The combination of both miRNA and siRNA delivery was also demonstrated recently by Choi et al. The authors developed a smart RNAi nanoplatform based on tumor-targeted and pH-responsive nanoparticles. The nanoparticles, coated with calcium phosphate (CaP) and modified with an artificial RNA receptor (Zn(II)-DPA) and anticancer drug doxorubicin (Dox), are capable of encapsulating siRNAs, miRNAs, and oligonucleotides for effective delivery of siRNA and miRNA replacement

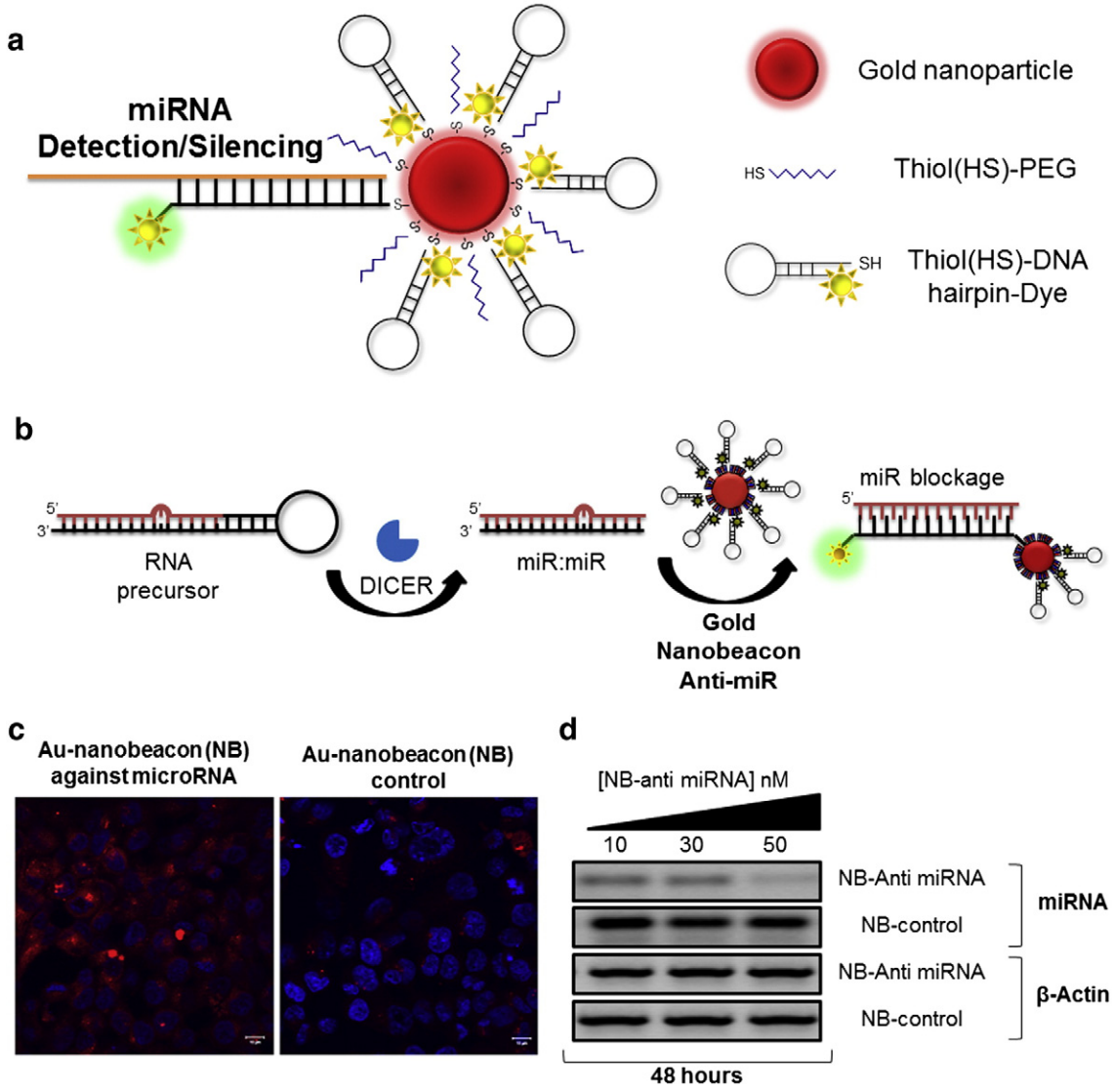

Fig. 5. (a) Smart and universal gene silencing tool based on AuNPs functionalized with a fluorophore labeled hairpin-DNA — gold nanobeacon (Au-nanobeacon). This system effectively detects and silences a specific microRNA while simultaneously signaling its action via fluorescence emission in cells. (b) Blocking the microRNA pathway via a gold nanobeacon anti-miR complementary to a specific microRNA involved in cancer progression. (c) Confocal imaging (scale bar, 10 μm) shows internalization of 50 nM anti-miR Au-nanobeacon and Au-nanobeacon control. Target recognition leads to change of anti-miR Au-nanobeacon conformation in the cytoplasm with concomitant fluorescence signal (red, Cy3) encircling the cell nuclei (blue, DAPI), showing the efficacy of miR21 detection. (d) Quantitative assessment of miRNA silencing in cells induced by 50 nM of anti-miR Au-nanobeacon for 48 h of incubation using β-actin as reference was confirmed by RT-PCR followed by agarose gel electrophoresis.
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into in vitro and into in vivo tumor-bearing mice by systemic administration [130].

Despite the difference in nanomaterial's composition and coating, the majority of the organic nanomaterials use the delivery of tumor suppressor miRNAs for efficient in vivo miRNA replacement therapy, in lung, colorectal, liver and head and neck cancers. The major inconvenience of using cationic lipid and liposomal-based nanomaterials is the toxicity issue when administered in vivo [131]. Future surface passivation with long-circulating polymers (i.e. polyethylene glycol — PEG), hyaluronic acid (reduce immunogenicity) [132], cholesterol (improve transfection, protects nucleic acids) or vitamin E (increase stability) [105], as well as the optimization of formulation to clinical doses is imperative [126].

4. Current challenges and future perspectives

4.1. Technical hurdles (design, sensitivity, stability, functionality and reliability) and limitations

At the beginning of this millennium an amazing genetic phenomenon was projected into the limelight and claimed by most as the most substantial research in years and the "gentlest art of shredding genes".

Its proposed mechanism was relatively simple and obeyed the law of common sense: introducing antisense RNA sequences into cells could inhibit the production of the protein encoded by the sense RNA strand. It was almost inconceivable that it could ever fail to succeed.

Except for a number of genes that failed to show sensitivity to antisense RNA, with sense RNA, used as a negative control in antisense RNA experiments, being as effective as antisense RNA (which seemed completely counter-intuitive), the most technical hurdles rely on the production of a delivery vehicle. In fact, only two miRNA candidates/vehicles, an inhibition therapy miR-22 antisense LNA (*Santaris Pharma*) and a replacement therapy liposome miR-34 mimic (*Mirna Therapeutics, Inc.*) have reached clinical trials [72].

Opinions are divided as to the future of gene delivery. Some continue to argue that a generic version of nanoparticle-based drug delivery vehicle would solve all hurdles [133], while others claim that a single system cannot be the answer for all diseases, as RNAi therapy approaches must consider the different cell/tissue/organ types [72].

One thing is consensual — successful miRNA or RNAi therapeutic nanoparticle must guarantee the following (adapted from [72,133–136]):

1) Be made of an *active/effector ingredient* together with *organic* or *inorganic* cores, within a range of 50 to 200 nm in diameter;
2) *Long circulation times* — not to be removed from circulation by the immune system before it reaches the desirable target (PEGylation is perhaps the most used method);
3) *Maximize* the *transport* and *accumulation* in *target* cell/tissue/organs (increasing the enhanced permeability and retention – EPR – effect) and avoid off-target trafficking preventing side effects;
4) *Outstanding pharmacokinetics* (clearance and volume of distribution);
5) An easy *separation* of the *miRNA* from the *vehicle* when reaching the target but not prior to reaching it (this fact forced an increased dose of active miRNA);
6) The necessity to develop a *robust* and *feasible manufacturing process* (large-scale production, uniform quality from batch to batch, costs, etc.…);
7) Needs of *FDA* or other regulatory *approval* (*Fact*: FDA has never approved an administered generic therapeutic nanoparticle!);
8) *Safe* and *biocompatible* (perhaps one of the most important for approval!);
9) RNAi *potency* (not all sequences work and produce an effective and long-lasting silencing or delivery);
10) Requirement for *high specificity*, specially due to the fact that miRNAs are able to regulate genes without perfect pairing (nanomaterials with cell-specific ligands that allow receptor-mediated uptake into target cells, such as tumoral cells);
11) *Feasible clinical doses* (recent IC50 ~ 30–10 μg/kg [137]);
12) To be *commercially competitive* (although successfully in clinics, they have to be competitive, which means less expensive and more effective than the available ones).
13) Obtain *strong patent protection*, for market exclusivity for the development of generic therapeutic nanoparticles beyond patent expiration.

…and overcome some of the following barriers/limitations:

a) *Cellular entry*, using targeting ligands (e.g. cell penetrating peptides, antibodies, targeting peptides, aptamers);
b) *Endosomal/lysosomal escape* of miRNA and siRNA, using fusogenic peptides or polymers and lytic (that cause lysis) lipids;
c) *Degradation* and *elimination*: crucial to have a precise control of size (optimal 50–200 nm); charge (typically highly cationic for RNA condensation but carefully needs to be considered in term of toxicity and clearance); chemical surface modification (crucial for stability and specificity) and shape (important in terms of toxicity and clearance);
d) *Renal clearance*: naked siRNA, with average diameter of less than 10 nm, is rapidly excreted from the blood compartment through renal clearance; nanomaterials chemically modified to increase the retention time of the miRNAs in the circulatory system (e.g. cationic polymers) are frequently proposed;
e) *Cytoplasmic and systemic delivery* of miRNAs versus type of administration (i.e. local, intratumoral, systemic, intra-peritoneal, deep-injection, subcutaneous);
f) *Vascular extravasation* and *diffusion* in target tissues (since nanomaterial is required to extravasate from the blood stream into the extracellular matrix, and then diffuse to reach all cells). Usually, nanomaterials with ≤400 nm in diameter can easily extravasate and accumulate in the leaky vasculature of solid tumors.

4.2. Potential solutions: local scaffolds as smart delivery vehicles?

While some disease types could benefit from systemic delivery of genes albeit following the implementation of all of the abovementioned tactics for proper targeting and transfection, the field could benefit from utilizing available technologies developed for tissue engineering and controlled delivery systems for local drug administration. Yet, miRNA delivery via biomaterial scaffolding has been poorly applied to this technology. Once miRNAs are natural players in controlling cell functions and differentiation processes, we therefore expect that miRNA delivery from scaffolds (e.g. hydrogels [138,139], biopolymers [140], dendrimers [141]) for inhibition or for replacement therapies will be highly adopted in future use not only for cancer but also for tissue engineering, while regeneration replacing or augmenting cell therapy [142]. The delivery of miRNAs from scaffolds provides a versatile method, for controlling stem cell differentiation and tissue functions and inflammation like extensively reported elsewhere [143,144], but specially to miRNA local administration to tumor milieu. A tissue-specific scaffold that locally deliver payload to the tumor with minimal inflammation and tissue toxicity, readily adjusted through manipulation of material type and formulation, would be the ideal scaffold biomaterial for local delivery or replacement of miRNAs.

Consequently, in order to build a scaffold platform to better support nanomaterials/nanoparticles for local delivery, a successful system must guarantee the following (adapted from [145]):

i. *Biodegradability*. The main goal of the scaffold is to release their cargo into the xenografted tissue and over time, replace the implanted scaffold or tissue engineered construct. The scaffold must be somehow degradable in order to release their content to the target

cells/tissues/organs. The secondary products of this degradation should be non-toxic and able to suffer normal body clearance. Usually to allow degradation within the target tissue a controlled inflammatory response and recruitment of inflammatory macrophages is required.

ii. *Functionality*. The scaffold must provide a matrix for normal cell adhesion, where they function normally and migrate on the surface and through the scaffold.

iii. *Mechanical properties*. In an ideal scenario the scaffold must have mechanical properties consistent with the anatomical site where it is going to be implanted. It must be strong enough to allow handling but most important to be attached to the target site.

iv. *Scaffold architecture*. The architecture of scaffolds is of critical importance, once it should have an interconnected pore structure and high porosity to ensure cell adhesion and penetration, as well as appropriate diffusion of their cargo within the construct and to the extracellular environment (of a tumor tissue for example). In fact, a porous interconnected structure is required to allow diffusion of products out of the scaffold, for example nanoparticles carrying antisense oligos or mature miRNAs.

v. *Manufacturing technology*. A particular engineered scaffold must be cost effective and should allow for large scale-up production in order to be clinically and commercially feasible. The development of a scalable manufacturing process is essential to ensure successful translation to clinic.

Nanomaterials have a proclivity in vivo and in vitro to bioaccumulate within various types of cells, producing several degrees of bioaccumulation in such tissues as the lymph nodes, bone marrow, spleen, adrenals, liver and kidneys. Besides, numerous studies report that nanoparticles can stimulate and/or suppress the immune responses, and that their compatibility with the immune system is largely determined by their surface chemistry [94,146]. In fact, particle size, solubility and surface modification largely determine its biocompatibility and hence applicability [94] (see Fig. 6).

There is not a simple solution to overcome the biocompatibility issue. Even though there is no any general mechanism for making nanoparticles universally 'non-toxic' to all living cells and all organisms, there are important findings that can be applied for increasing nanoparticle biocompatibility (adapted from [148,149]):

i. Using the *lowest nanoparticle dose* to get the desired response for the shortest period of time;

ii. *Nanoparticle's coating*: extremely important for stability but also for biocompatibility (a coat that completely covers the nanoparticle reactive surface may avoid complement or antibody attachment, or dissolution of the coating by cell digestion);

iii. Always *test nanoparticle/biological interactions* experimentally (almost no importance is given when testing nanomaterials in the detection of genetic damages or in identifying protein markers of toxicity, or measuring the level of oxidative stress, see example [124]);

iv. *Modify the nanoparticles*, if the biosafety test fail, in order to eliminate membrane lipid peroxidation, the generation of reactive oxygen species, acute and chronic release of inflammatory factors (and 'complement' activation), the possibility of nanoparticles becoming 'stuck' during filtration or passage through pores and fenestrations.

With all the features described above concerning nanoparticles and scaffold design in mind, it is time to combine these two products using the well-studied gene delivery via nanoparticles, now embedded in a scaffold. This combination may produce incredible two- and three-dimensional systems, which will provide essential key functions, such as (adapted from [150]):

a) *shielding* the particles from *agglomeration*;
b) furnishing a scaffold for the *attachment of functional molecular entities*;
c) precise *local* (miRNA) *delivery*;
d) *tunable release* of functional moieties;
e) *radial tunability* and intrinsic balance between *kinetics* and *thermodynamics*.
f) providing *direct control of structure* from the molecular to the macroscopic level.

Creating scaffolds and building blocks with the already optimized nanoparticle's systems will definitely provide unique tools for the creation of highly efficient catalysts, sensors, and devices, but especially for miRNA (siRNAs or antisense oligos) delivery and specific chemotherapeutic drug-delivery with stimuli-responsive surfaces and layer-by-layer construction [150].

5. Final conclusions

Over 800 human miRNAs have been discovered to date, emphasizing the importance of these effector molecules in human body and adding a new dimension to our understanding of complex gene regulatory networks. Understanding and exploiting new platforms for controlling their expression, by inhibition or replacement therapies are of urgent need. Consequently, nanotechnology and biomaterials have been gaining momentum in establishing solid knowledge in the

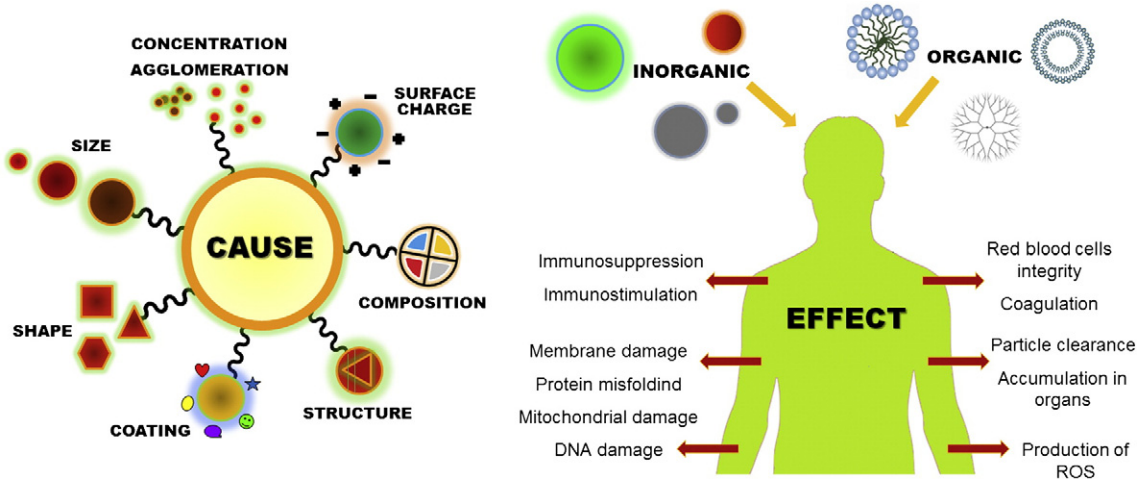

Fig. 6. Nanoparticles' biosafety versus toxicity: causes and effects. Nanoparticles biocompatibility/effects and their use in biological applications can be influenced by size, shape, solubility, composition and surface charge and modification/chemistry.
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development of new sensing and inhibition therapies using miRNAs in cancer. All the tools have been created. Incredible advances have occurred in developed new nanovehicles capable of efficiently transporting miRNAs and antisense DNA or RNA oligonucleotides, especially in the systemic administration route, although to date, the majority of miRNA vehicles including liposome-based and some nanoparticle-associated have been found to accumulate primarily in the liver, spleen and kidney. For these reasons, miRNA local delivery via biomaterials scaffolding needs added support and focus.

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