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Review article

MicroRNA delivery through nanoparticles

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ABSTRACT

MicroRNAs (miRNAs) are attracting a growing interest in the scientific community due to their central role in the etiology of major diseases. On the other hand, nanoparticle carriers offer unprecedented opportunities for cell specific controlled delivery of miRNAs for therapeutic purposes. This review critically discusses the use of nanoparticles for the delivery of miRNA-based therapeutics in the treatment of cancer and neurodegenerative disorders and for tissue regeneration. A fresh perspective is presented on the design and characterization of nanocarriers to accelerate translation from basic research to clinical application of miRNA-nanoparticles. Main challenges in the engineering of miRNA-loaded nanoparticles are discussed, and key application examples are highlighted to underline their therapeutic potential for effective and personalized medicine.

1. Introduction

MicroRNAs (miRNAs) are non-coding endogenous RNAs composed of short nucleotide sequences, 20–24 nucleotides, that act in the post-transcriptional regulation of gene expression [1]. As soon as miRNA activity was confirmed in humans [2,3], significant attention was generated on their clinical application, because of their impact on critical cellular activities, such as metabolism [4], differentiation/proliferation [5,6] and programmed cell death [6]. Several studies have described the influence of miRNAs on the onset and progression of diseases, including cancer [7–9], neurodegenerative disorders [10,11], cardiovascular diseases [12–14], and other pathologies [15–17]. Such studies have led to research that focused on identifying changes in miRNA expression between normal and diseased states, resulting in miRNAs being proposed as diagnostic markers or therapeutic targets [18–20]. However, despite the excitement generated by miRNAs, their efficacy is limited by poor targeting ability, short circulation time and off-target effects of naked miRNA-based agents. To overcome these barriers, miRNA-loaded nanoparticles (NPs) have been proposed [21–23], by virtue of NPs ability to shield the loaded agent from the external environment, thereby reducing inactivation or degradation, and enhancing circulation time and targeted accumulation [24]. Additionally, the use of miRNAs for therapeutic purpose carries distinctive advantages over traditional gene therapy that involves the delivery of larger molecules, such as mRNAs, or DNA. Firstly, miRNAs have a broader range of therapeutic targets, since a single miRNA can simultaneously regulate a multitude of mRNAs. Furthermore, miRNAs in the form of miRNA inhibitor and miRNA mimic can regulate both the expression and the repression of multiple genes, while the activity of siRNAs and mRNAs is limited to repression or upregulation of one specific gene, respectively. miRNAs also have a small size, which may facilitate their encapsulation into NPs with high efficiency, their delivery into cells and their ability to act at the cytoplasmic level [25,26].

Herein, we discuss the preparation, characterization and application of NPs for delivering miRNA-based agents, which include miRNA mimics (miR mimics) or miRNA inhibitors/anti-miRNAs (miR inhibitors/anti-miRs) and focus on the advantages of NP-mediated methods as compared to naked miRNA delivery. Key examples of miRNA-loaded NPs for specific disease settings are presented, including their applications in tissue engineering (TE), cancer treatment, cancer immunotherapy and neurodegenerative disorders.

2. Physiological relevance of microRNAs (miRNAs)

MiRNAs are proposed to act primarily by binding to the 3' untranslated

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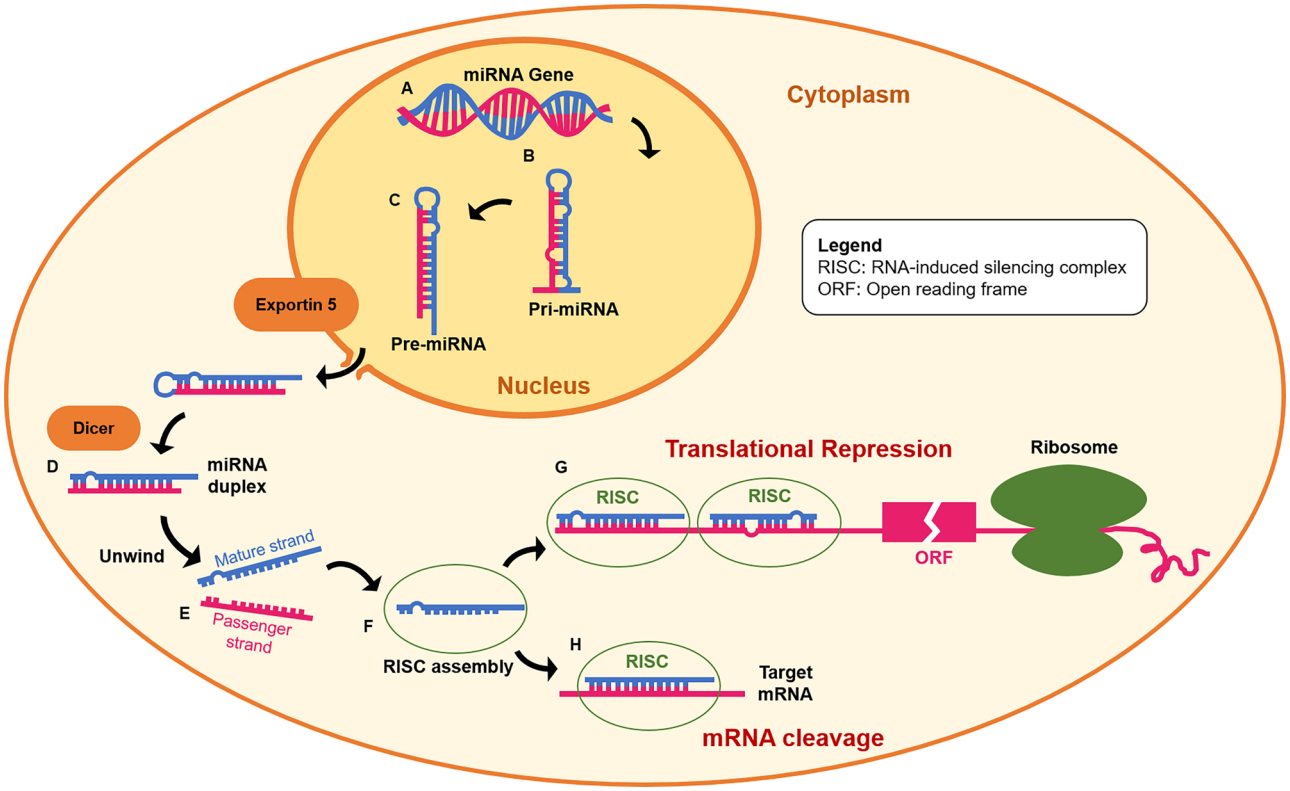

Fig. 1. Endogenous production of miRNA and their mechanism of regulation of gene expression: (A) genes encoding for miRNA are transcribed and (B) miRNA precursor (pri-miRNA) form double-stranded structures. Pri-miRNAs are further transformed into (C) pre-miRNAs that are exported by Exportin into the cytoplasm to be (D) condensed into miRNA duplexes. (E) miRNA duplexes unwind to form the passenger and guide strands. The mature strand stays in the miRNA-induced silencing complex (miRISC), forming a (F) temporary asymmetric RISC assembly. Finally, miRNAs bind target mRNAs, thereby inducing (G) translational repression or (H) degradation of target mRNAs. This figure is adapted from https://www.sigmaaldrich.com/life-science/functional-genomics-and-rnai/mirna/learning-center/mirna-introduction.html.

regions (UTRs) of messenger RNA (mRNA) without requiring perfect base pairing [27–29]. As such, one miRNA can simultaneously regulate several genes, while a single mRNA can be repressed by several miRNAs [25,26]. As shown in Fig. 1, miRNA formation begins in the nucleus with the DNA transcription of primary miRNA precursor (pri-miRNA) [30]. The transcribed pri-miRNAs are further processed to form ~70 nucleotide hairpin structures (pre-miRNAs), that are exported to the cytoplasm to be condensed into 20–24 nt double-stranded miRNA duplexes [31]. These duplexes then enter the miRNA-induced silencing complex (miRISC) where they unwind to form a mature strand and a passenger strand. The miRISC retains the mature strand and releases the passenger strand which is degraded [32]. miRISC binds target mRNAs, thereby inducing either interference with protein synthesis [33,34], or degradation of the target mRNA [35,36].

Depending on the target miRNA expression, miRNA therapy can take the form of: miRNA inhibition therapy [37,38], or miRNA replacement or reinforcement therapy (Fig. 2) [39,40]. In the former approach, an anti-miR or miRNA inhibitor is used, consisting of a single-stranded oligonucleotide with a complementary sequence to mature miRNA. In the latter approach, miR mimics that have an identical sequence as the endogenous mature miRNA are introduced into cells to provide an exogenous source of additional miRNAs.

3. Design criteria of nanoparticles (NPs) as efficient delivery vectors for miRNAs

Despite the progressively increasing knowledge of miRNA ability to mediate biological processes both *in vitro* and *in vivo*, critical hurdles related to miRNA delivery should be overcome to promote their clinical application [41,42]. Naked miRNAs are poorly taken up by cells due to their negative charge [43], undesired off-target [44] or on-target [45] effects, short half-life in systemic circulation [46,47], and limited stability in blood due to their rapid degradation or inactivation by nucleases that are abundantly present in the blood stream [48,49]. Herein, the main advantages and disadvantages of the different strategies to overcome the challenges of efficient miRNA delivery are discussed.

Both miR mimics and anti-miRs have been delivered *in vitro* using commercially available transfection agents, such as DharmaFECT™ and Lipofectamine™ [50–52], or by electroporation [53,54]. Alternatively, chemical modifications can be introduced to miRNAs to augment stability and allow carrier-free *in vivo* delivery of modified anti-miRs and miR mimics which are also known as antagomiRs [55] and agomiRs [56], respectively. For example, in the case of anti-miRs, *in vivo* silencing of endogenous miRNAs has been enhanced by integrating locked nucleic acids (LNA) or peptide nucleic acids (PNA), as reviewed elsewhere [57]. As an alternative to chemical modification, anti-miRs and miR mimics have been encapsulated into NPs. Due to their favorable transport properties, NPs have been reported to improve the *in vivo* delivery of miRNA agents; NPs protect their payload and enhance target specificity, [58] thus limiting adverse effects and improving therapeutic outcomes, as illustrated in Fig. 3 [59].

The main challenges in miRNA delivery with nanocarriers are related to their low encapsulation efficiency [60], and the need for cell targeting, as shown schematically in Table 1 [61,62]. Because of their high affinity with water, miRNAs quickly diffuse into the water phase when nanoprecipitation or emulsion-based preparation methods are used, resulting in low encapsulation efficiency [60]. NPs improve the tissue distribution and site-specific localization of miRNA. However, the degree of enhancement is generally poor. As such, numerous studies have focused on modifying the NP surface with ligands for specific receptors on target cells, thus facilitating NP uptake by receptor-mediated endocytosis and reducing the required dosage and side effects of treatment [21,63,64].

Moreover, colloidal stability of NPs in complex physiological media is demanded for cell-targeted delivery of miRNAs [65]. After administration, NPs should ideally circulate until they reach the desired site, and should be designed to undergo endosomal escape in order to guarantee the proper interaction between the miRNA and its intra-cellular target (for example by exploiting the proton sponge effect) [66,67]. However, circulation time depends on NP interactions with the biological microenvironment that could lead to their fast clearance. Specifically, once NPs are exposed to body fluids, their surface is covered by plasma proteins [68,69], resulting in masked surface ligands, non-specific uptake and reduced stability. There are

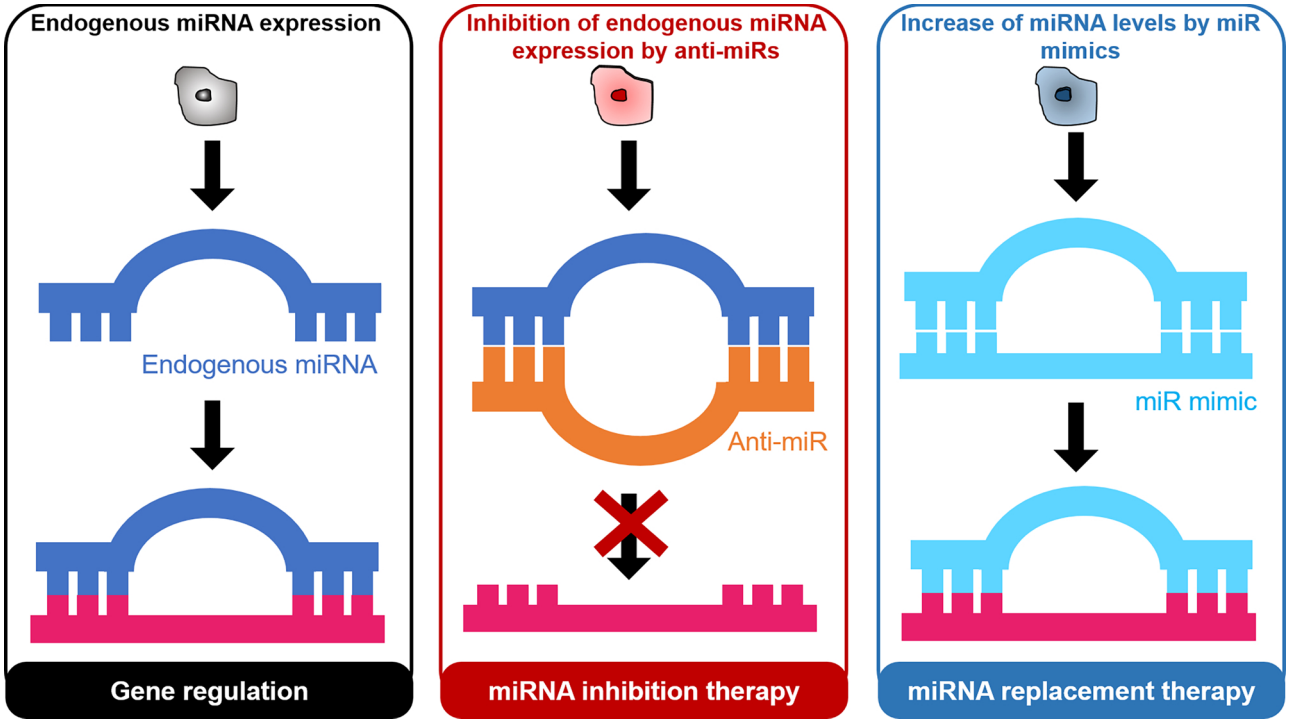

Fig. 2. (A) In a normal tissue state, expression of endogenous microRNAs (miRNAs) allows for gene regulation. In the treatment of certain diseases, (B) inhibition of specific endogenous miRNAs is achieved through miRNA inhibition therapy with anti-miRNAs (anti-miRs). (C) Alternatively, miRNA replacement therapy utilizes miR mimics to provide an exogenous source of additional miRNAs.

different factors affecting NP circulation half-life, sequestration by the mononuclear phagocyte system (MPS) and biodistribution, including surface charge and hydrophobicity, size and shape [24]. Previous studies showed that neutral particles are less subjected to opsonization than highly charged particles especially if positively charged (cationic) [70,71]. Similarly, high hydrophobicity is related to a higher likelihood of clearance, which can be reduced by modifying the surface with polyethylene glycol (PEG), or by surface-camouflaging strategies, resulting in enhanced circulation half-life [72–74].

Importantly, the disease setting crucially determines the physical and biological barriers that the NP must overcome in addition to the basic hurdles that already impede miRNA delivery [41]. Based on these considerations, different strategies can be developed to prepare NPs that can effectively deliver miRNA to the target cells.

4. Methods to prepare miRNA-loaded NPs

Various preparation techniques, such as single or double emulsions, nanoprecipitation, and interfacial polymerization, have been employed for the preparation miRNA-loaded NPs. The selection of the most appropriate method is influenced by the constituent material and the desired surface characteristics [81]. Emulsion-based methods are the most commonly used to prepare miRNA-loaded NPs. These methods utilize high-speed homogenization or ultrasonication [82]. In the single-emulsion version, an oil-in-water (o/w) emulsion is formed by homogenizing or sonicating a polymer solution into an external, surfactant-containing, water phase. The double-emulsion technique, typically used to encapsulate hydrophilic payloads, utilizes two emulsification steps to obtain water-in-oil-in-water (w/o/w) or oil-in-water-in-oil (o/w/o) emulsions [81,83].

Emulsion methods have been used to prepare monomethoxy(polyethylene glycol)-poly(D,L-lactide-*co*-glycolide)-poly(L-lysine)-lactobionic acid (mPEG-PLGA-PLL-LA) [77], PLGA and poly(glycerol adipate-*co*-ω-pentadecalactone) (PGA-*co*-PDL) NPs for miRNA delivery [84]. For instance, miR-99a-loaded mPEG-PLGA-PLL-LA NPs have been obtained *via* the double emulsion method. For this purpose, miRNA is dissolved in water and subsequently dropped into a PLL-LA solution in dichloromethane, followed by sonication. The w/o/w emulsion was then dropped in water containing Pluronic-F68 and sonicated to obtain a w/o/w double emulsion. A reduction in the surface charge from 25 mV for blank NPs to 3 mV for miRNA-loaded NPs was taken as evidence of successful miRNA loading. The authors also demonstrate 80% of sustained payload release at 132 h, suggesting extended duration for the interactions between miR-99a and target genes.

Polymer NPs can be formed *via* nanoprecipitation, by dropwise addition to water of a polymer solution in a water-miscible solvent, causing its rapid displacement [81,85,86]. For instance, miRNA-loaded PLGA/chitosan (PLGA/CS) NPs with 150–180 nm size have been prepared *via* the nanoprecipitation method by dropwise addition of PLGA solution into a water solution of CS and miR-34 s, in the presence of Poloxamer surfactant [75]. This method could achieve miR-34 entrapment efficiency (EE) between 50% and 95%, depending on the amount of miR-34 in the formulation, and controlled release up to 48 h.

Unconventional techniques, such as combination of click chemistry (CC) and controlled radical polymerization (CRP), electrospinning, and particle replication in non-wetting template (PRINT), can potentially be used to prepare miRNA-loaded NPs because of their versatility, ease of implementation and low cost. Although these techniques have not been used to prepare miRNA-loaded NPs, we advocate that they are advantageous methods, since harsh temperatures, extreme pH conditions or potentially-toxic solvents are not required. Combination of CC and CRP can allow the preparation of NPs with controlled size and improved stability, as shown with DNA-conjugated NPs [87–89], as well as *in vivo* active cell recognition, as demonstrated by Koo et al. with dibenzylcyclooctyne (DBCO)-modified liposomes that were able to bind the azide groups expressed on cancer cells [90]. PRINT is known to generate particles with precise structure through the use of elastomeric molds of predefined shape and size, into which a polymer solution containing the drug is poured. NPs obtained by PRINT have been used in mice studies to investigate specific tumour cell uptake [91], and to test the treatment of brain metastasis when loaded with docetaxel [92].

Finally, recent advances in microfluidics have enabled the synthesis of libraries of NPs by rapid mixing of droplet streams controlled by micro pumps into silicon or glass microchannels [93]. These micro-reactors provide homogeneous conditions for NP nucleation and growth, resulting in higher reproducibility [94]. The microfluidic technology has been used to engineer small interfering RNA-loaded NPs (siRNA-loaded NPs) [95], but to the best of our knowledge, no evidence of their use to prepare miRNA-loaded NPs has been reported.

5. Selection of the biomaterial for miRNA encapsulation

The choice of the biomaterial that constitutes the NP is of paramount

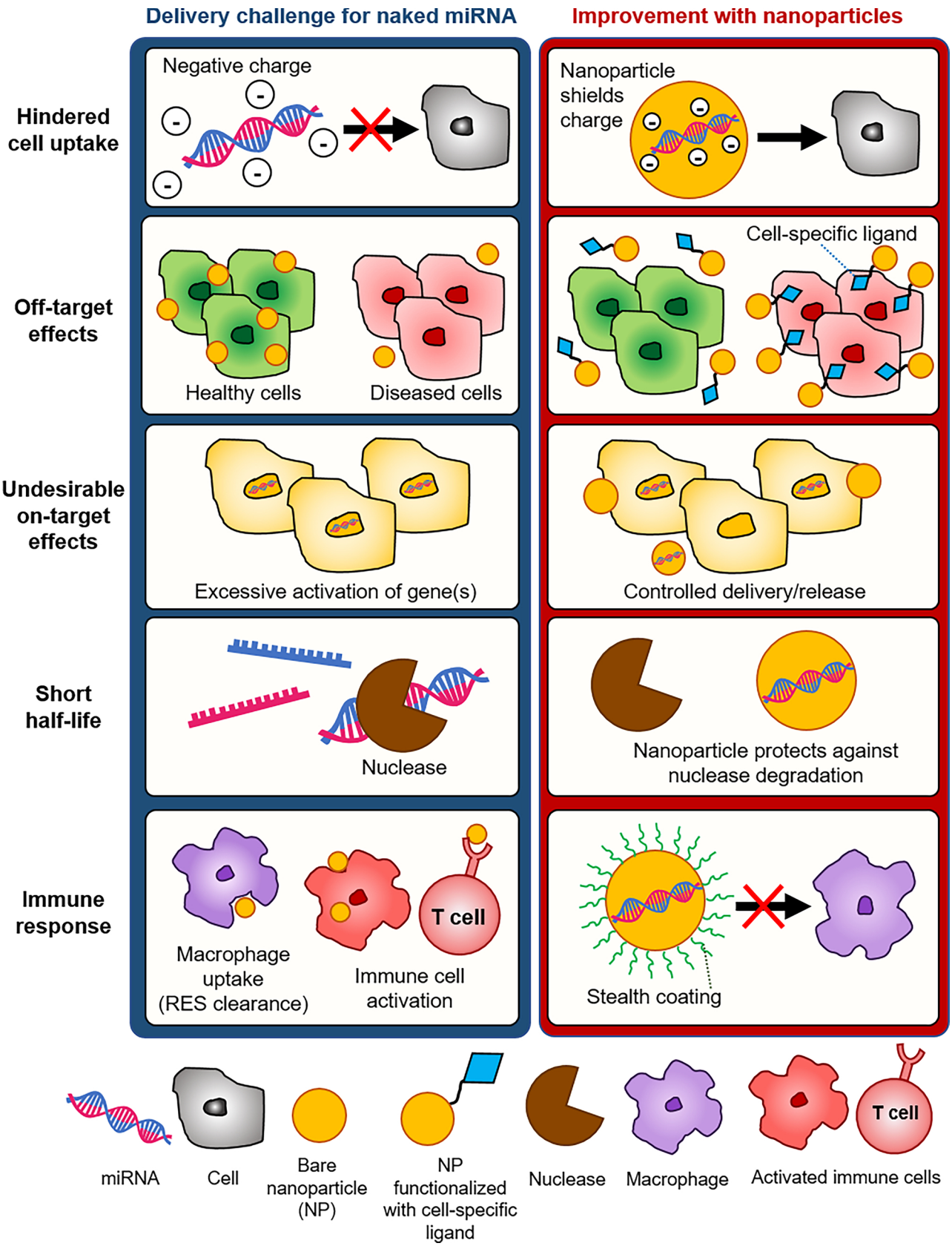

Fig. 3. Key challenges of miRNA delivery *in vivo*. The challenges of delivering naked miRNAs include hindered miRNA uptake by cells due to negatively charged groups of miRNAs, undesirable off-target or on-target effects, short miRNA half-life under physiological conditions and unfavorable immune response. NPs encapsulate miRNAs, thus shielding charge groups and allowing their uptake by cells. Functionalizing NPs with cell-specific ligands allows NPs to deliver miRNAs to specific cells, thus reducing off-target effects. NPs allow for controlled miRNA release, avoiding excessive activation of multiple gene targets. NPs also increase the half-life of miRNA *in vivo*, by protecting the payload from degradation. Finally, addition of a stealth coating around NPs prevents their clearance by the reticuloendothelial system (RES) and avoids unfavorable immune cell stimulation.

Table 1
Main general design criteria for miRNA-loaded nanoparticles (NPs).

NP requirements for *in vivo* delivery	Delivery challenges	Suggested improvements	Reference(s)
High encapsulation efficiency	miRNA water solubility and negative charge.	miRNA complexation with positively charged molecules prior to nanoprecipitation or emulsification.	[71,75,76,77]
Colloidal stability	Protein corona formation and possible aggregation.	Decoration of NP surface with anti-fouling molecules.	[72,73,74]
Cell targeting	miRNA off-target effects.	Surface functionalization of NPs with specific ligands for cell targeting.	[41,78,79]
Cargo release in cytoplasm	miRNA degradation in low pH of endosome.	Use of materials with proton-accepting groups, which enable miRNA complexation and protection from degradation (*i.e.* proton sponge effect).	[66,67]
Controlled and sustained release, and increased half-life	Fast NP degradation rate and burst-release.	Control degradation and/or trigger miRNA release with stimuli-responsive materials (e.g. containing pH-sensitive histidine-, tertiary amine-, and sulphonamide groups; or nitroimidazole or azobenzene groups for hypoxia-driven disassembly).	[80]

importance, as it dictates the choice of the preparation technique as well as the final properties and structure of the NP [96,97]. With the exciting advent of stimuli-responsive biomaterials, that change their properties upon modification of external conditions (including temperature or pH), multi-functional NPs have been developed for an expanding range of therapeutic applications (Table 2) [98,99].

5.1. Synthetic polymers

The use of synthetic polymer NPs for nucleic acid delivery has been extensively investigated, because of their efficient cargo release within the cell cytoplasm and their ease of manufacturing and functionalization with targeting molecules [41]. Among synthetic polymers, poly(ethylene imine)s (PEIs) [100,101], PLGA [75,76], poly(ε-caprolactone) (PCL) [102], and polyurethanes (PUs) [103,104] have been used for miRNA delivery.

PEI, both in linear and branched form, is a polycationic polymer. The PEI repeating unit consists of a secondary amine group and one ethylene spacer and is commercially available in different molecular weights, having different release efficiency and biocompatibility [105,106]. PEI chains are positively charged, depending on the protonation degree of secondary amines as a function of the pH. The positive charge allows electrostatic complexation with nucleic acids [107]. Due to its proton acceptor behavior, PEI exhibits weak-base buffering properties, reducing nuclease activity [108], and protecting nucleic acids from degradation within endosomal vesicles. As a proton acceptor, PEI also causes the “proton sponge effect”, leading to endosomal lysis and consequent release of nucleic acids into the cytoplasm [66,67,109].

PEI has been shown to deliver miR-145 and miR-33a, and allow the assessment of the *in vivo* anti-tumour activity of these miRNAs in a mouse model of colon carcinoma [101]. Local or systemic administration of PEI-miRNA complexes could result in reduced tumour growth compared to controls, indicating that such NPs represent a promising strategy for miRNA replacement therapy in cancer treatments [101]. PEI has also successfully delivered miR-145 *in vitro* to metastatic breast cancer cells [100], and *in vivo* in a xenograft colon cancer models [63].

However, while PEI NPs show efficient miRNA encapsulation and delivery, their high positive charge density and non-biodegradability can affect cell viability, thus limiting their use [108]. In this context, NPs that are surface-modified or based on blends of PEI and PEG have been proposed to reduce toxicity and improve biodistribution [110]. For instance, Zhang et al. have used branched PEI-PEG NPs to deliver miR-145 to prostate cancer [79]. The PEG chains were surface-modified with poly-arginine showing enhanced uptake in prostate cancer *in vivo,* reduction of tumour growth and increased survival.

Biocompatible and biodegradable synthetic polyesters, such as PLGA and PCL have also been investigated for nucleic acids delivery, because of their lower toxicity and biodegradability as compared to PEIs [111–113]. PLGA is a biocompatible and non-cytotoxic polymer, known to degrade by hydrolysis, generating lactic and glycolic acid monomers, which are metabolized through the Krebs cycle [111]. By varying the ratio between the constituent monomers, lactic and glycolic acid, the degradation rate of PLGA NPs can be tuned in the range from several months to years [111,114]. Low molecular weight polymers with higher glycolide content are more hydrophilic with a shorter degradation time. In contrast, PLGA NPs with higher lactic acid content have a hydrophobic nature and degrade more gradually.

MiRNA encapsulation is made difficult by the negative surface charge of PLGA NPs [115,116], which reduces complexation efficiency with the phosphate groups of miRNAs. This limitation can be overcome by using cationic compounds combined with PLGA. For instance, CS, a cationic polymer, has been shown to promote the retention of miRNAs inside PLGA NPs [75]. Protamine sulfate (PS) coating of PLGA NPs has also been used to enhance complexation of miRNAs on the NP surface [76]. In another study, Wang et al. have generated PLGA-PEI NPs to co-deliver miR-542-3p and doxorubicin (DOX) in triple negative breast cancer cells [117]. PLGA-PEI NPs could be covalently grafted with hyaluronic acid (HA) to be specifically internalized by cancer cells, thus enhancing their cytotoxicity as assessed *in vitro* on breast cancer cells.

PCL has attracted high attention for the preparation of biocompatible NPs for nucleic acid delivery. NPs composed of PCL are promising for their high colloidal stability, quick cellular uptake, low toxicity *in vitro* and *in vivo*, and controlled release of their drug cargo. The degradation product of PCL is 6-hydroxyhexanoic acid, which is a natural metabolite in the human body [118]. Liu et al. have prepared PEG-peptide-PCL NPs by the double emulsion method to co-deliver miR-200c and docetaxel [102]. A gelatinases-cleavable peptide was selected to facilitate delivery after NPs internalization by tumour cells. In addition, spermidine has been used to complex with miR-200c during NP preparation, achieving about 85% transfection efficiency *in vitro*.

Among synthetic polymers, PU-based NPs have shown high biocompatibility combined with versatile physical and chemical properties [119]. Cationic PU-short branch PEI nanocomplexes have been developed by Chiou et al. as vehicles for miR-145 delivery to inhibit the stem cell niche in the brain tumour [103]. The authors demonstrate successful miR-145 transfection (nearly 90%) and efficient knockdown of miRNA targets, Sox2 and Oct4, *in vitro*. Intracranial injection of PU-PEI-miR-145 nanocomplexes could reduce tumour growth by synergizing with chemoradiotherapy. Furthermore, such nanocomplexes show a therapeutic effect on lung adenocarcinoma model [104], with decreased metastases, inhibited epithelial-to-mesenchymal transition (EMT) *in vitro*, increased survival and delayed tumour growth in mice [104].

5.2. Natural polymers

Among natural polymers, materials that can be obtained from animal or vegetal sources, CS and HA are promising carriers for nucleic acids [120–122]. CS is obtained from chitin and is non-cytotoxic, non-immunogenic, and highly biocompatible [123,124]. At acidic pH, the protonated amino groups of CS rapidly interact with opposite-charged molecules, such as siRNA or miRNA with high loading efficiency [22,125]. In spite of these advantages, CS NPs offer limited control over the delivery of nucleic acids because of their strong interaction with the loaded agent [126–128], which results in inefficient unpacking of the complex in the cytoplasm [127]. To overcome these drawbacks, hydrophobic moieties have been conjugated to CS to weaken the polymer/nucleic acid interaction and enhance cytoplasmic drug delivery. Lipid chains, bile acids or negatively charged polymers, such as PLGA, have also been combined with CS to facilitate miRNA release [129–131].

HA is a highly hydrophilic anionic natural polysaccharide, that can be recognized by HA receptors on cells [132,133]. By virtue of its chemical versatility, HA has been extensively used to prepare NPs for the delivery of cytostatic drugs, proteins, polynucleotides, immunomodulators and imaging agents. HA covalently grafted to cationic polymers or lipid molecules has been proposed to deliver polynucleotides. For example, HA chemically linked to PEI and PEG has been used to release miRNA and DOX [117,134]. HA has also been successfully used to modify the surface of cationic polynucleotide-loaded NPs [135]. However, although cationic polymers efficiently form complexes with nucleic acids due to charge interactions [129], their positive charge contributes to their uptake by the MPS with subsequent rapid elimination and toxicity [136]. For this purpose, several authors have proposed surface-modifications with HA to mask the positive charge of

Table 2
Applications and therapeutic effects of nanoparticles (NPs) for delivering microRNAs (miRNAs) and/or drugs in tissue engineering (TE) and cancer.

NPs	miRNAs	Application	Therapeutic effect	Stage	Ref
CS	agomiR-199	Tibia regeneration	Osteogenic differentiation of MSCs and bone regeneration	Pre-clinical Bone defects on Sprague–Dawley rats	[56]
Hyaluronan sulfate	miR-21	Myocardial infarction	Reduced inflammation and reduced cardiac fibrosis	Pre-clinical Myocardial infarction in C57BL/6 mice	[159]
PEI-PLGA	miRNAs targeting COX1 & COX2	Flexor tendon adhesions	Reduced inflammation and support for tendon healing	Pre-clinical Tendon injury in chicken	[160]
PLGA	miR-26a	Calvarian bone regeneration	Increased osteoblastic activity	Pre-clinical Bone defect in C57BL/6 mice	[161]
Polyketal	miR-106b, miR-148b and miR-204	Myocardial infarction	Improved cardiac function and reduced infarct size	Pre-clinical Myocardial infarction in C57BL/6 mice	[162]
Fe_3O_4-PEI	Let-7a	Glioblastoma	PI3K and RAS downregulation	In vivo proof of concept (sub cutaneous breast cancer in nu/nu mice)	[144]
Gold	miR-182	Brain cancer	Reduced tumour burden and increased survival	Pre-clinical Intra-cranial tumours on SCID mice	[163]
Gold	miR-145	Breast cancer, prostate cancer	miR-145 expression recovery	In vitro	[78]
HA-CS	miR-34a and doxorubicin (DOX)	Triple negative breast cancer	Increased cell sensitization to DOX	In vitro	[117]
Liposomes	miR-101 and DOX	Hepatic carcinoma	Reduced tumour growth	In vivo proof of concept subcutaneous tumour in BALB/c nude mice	[23]
Liposomes	Anti-miR-21	Glioblastoma	Inhibition of miR-21 expression	Pre-clinical Intra-cranial tumours in C57BL/6 mice	[164]
PEG-lipids	miR-122	Hepatic carcinoma	Reduced tumour growth	In vivo proof of concept Flank model of HCC in nude mice	[158]
PCL-PEG	miR-200c and docetaxel	Gastric cancer	Tumour growth inhibition	In vivo proof of concept Flank model in Balb/C mice	[102]
PEI-PEG	miR-145	Prostate cancer	Tumour growth inhibition	Pre-clinical Intra-peritoneal tumour in nude mice	[79]
PEI	miR-145 and miR-33a	Colon carcinoma	Reduced tumour growth	In vivo proof of concept subcutaneous tumour in nude mice	[101]
PEI	miR-145	Metastatic breast cancer	Reduced cell proliferation *in vitro*	In vitro	[100]
PLGA	miR-99a	Hepatic carcinoma	Tumour growth inhibition	In vivo proof of concept subcutaneous tumour in nude mice	[77]
PLGA-PEG	miR-7 and paclitaxel (PTX)	Ovarian cancer	Increased cancer cell apoptosis and sensitization to PTX *in vitro*	In vivo proof of concept subcutaneous tumour in nude mice	[165]
PLGA-PEI-HA	miR-145	Colon carcinoma	Tumour growth inhibition	In vivo proof of concept subcutaneous tumour in nude mice	[63]
PU-PEI	miR-145	Lung tumour	Reduced tumour growth and prolonged survival	Pre-clinical Intra-bronchial tumours in nude mice	[103,104]
PU-PEI	miR-145	Brain tumour	Reduced tumour growth and prolonged survival	Pre-clinical Intra-cranial tumours in nude mice	[104]
Silica	Anti-miR221 Temozolomide	Glioma	Increased cancer cell apoptosis *in vitro*	In vitro	[146]
Silica	miR-34a	Neuroblastoma	Tumour growth delay and apoptosis and reduced vascularization	Pre-clinical Retro-peritoneal tumours in CB-17/SCID mice	[147]

cationic NPs, lipid complexes or liposomes [135]. For instance, HA has been electrostatically attached to the surface of positively charged liposomes [137,138] and calcium phosphate NPs [139], as well as chemically bound to lipids on the NP surface. Liang et al. have used HA-NPs loaded with miR-145 to target colon cancer cells through their overexpressed CD44 receptors. Real-time qPCR and western blot analyses revealed enhanced accumulation and reduced tumour growth [63], suggesting the potential therapeutic success of utilizing such NPs in the clinic.

5.3. Inorganic NPs

Due to their biocompatibility, controllable size and morphology, several inorganic materials have been proposed in nanomedicine, including gold, calcium phosphate, silica and iron oxides [140,141]. Firstly, iron oxide-based NPs have been proposed for imaging purposes, as contrast agents for magnetic resonance imaging (MRI), in thermal therapy, as well as in drug and gene delivery. The combination of magnetic NPs with cationic compounds is needed to achieve high miRNA encapsulation [142]. Streptavidin-coated magnetite (Fe_3O_4)-based NPs have been modified with biotin-bound miR-335/PEI complexes to deliver the miRNA cargo to human mesenchymal stem cells (hMSCs), achieving enhanced target gene knockdown as compared to a PEI-miR strategy [143]. Yin et al. have used zinc-doped Fe_3O_4 NPs to deliver let-7a miRNA to glioblastoma cells by magnetofection [144]. Loading of let-7a miRNA into NPs has been achieved *via* a layer-by-layer approach, using branched PEI as polycation. These complexes showed high cellular uptake (at nearly 98%) and downregulation of the let-7a targets, PI3K and RAS.

Silica NPs and mesoporous silica NPs (MSPs) are silica-based nanostructures which possess high biocompatibility and stability [145]. For these reasons, MSPs have received great attention in clinical applications. MSPs have been used to co-deliver anti-miR-221 and Temozolomide (TMZ) to treat drug-resistant glioma cells [146]. Silica NPs carrying a tumour suppressor miRNA miR-34a have been developed by Tivnan et al. to specifically inhibit neuroblastoma growth [147]. GD2-antibody could further be conjugated on the NP surface to enhance specific targeting, delaying tumour growth, enhancing apoptosis and reducing vascularization *in vivo*.

Calcium phosphate (CaP) NPs are inexpensive, nontoxic, bioresorbable and easily synthesized nanocarriers. They have been widely used as gene carriers for 40 years, using DNA-CaP co-precipitation as an efficient method to introduce nucleic acids into cells; they remain stable in the endosomal compartment and are rapidly degraded in the mildly acidic lysosome environment, causing the vesicle to burst, releasing their content in the cytoplasm [148]. Banik et al. have shown that large molecular weight gene therapeutics, such as plasmid DNA can successfully interact with CaP-NPs in a spontaneous and cooperative manner, without NP intercalation with DNA base pairs [148]. However, miRNAs might have poor interaction because of low spatial charge density. Accordingly, long-chain miRNAs (lc-miRNAs) have been developed to enhance encapsulation efficiencies into PEI-CaPNPs [149]. Lc-miRNAs have been prepared by adding thiol groups on both the 3' and the 5' miRNA end, thus obtaining longer chains with higher encapsulation efficiency.

Gold NPs (Au-NPs) have been used in drug delivery by virtue of their biocompatibility, ease of functionalization, and tunable size and shape [150–152]. The surface of Au-NPs is typically functionalized with thiol or amino groups to enable miRNA entrapment. Ghosh et al. have developed a non-toxic gold-based formulation to obtain excellent miRNA cellular uptake through endocytosis and intracellular delivery [153]. This formulation was based on miRNA/ Au-NPs-S-PEG, with a ratio of 1-10-0.5, respectively. Cystamine functionalization of Au NPs can also be used for miRNA complexation, whereas PEG can prevent NP aggregation and miRNA degradation. Ekin et al. have used Au-NPs to deliver and recover the expression of miR-145 (a tumour suppressor gene) into prostate and breast cancer cells, where miR-145 is usually downregulated [78].

5.4. Lipid-based NPs

Lipids are the main components of the cell membrane, thus making lipid-based NPs (LNPs) capable of interacting with the membrane, promoting cellular uptake of their contents. LNPs are composed of a lipid bilayer that contains the miRNA in the aqueous core or, in the case of multi-lamellar liposomes, between lipid bilayers [154]. Typically, miRNA-loaded LNPs are made with a mixture of cationic lipids, neutral lipids and PEG [155]. Spontaneous electrostatic interaction between cationic lipids and negatively charged miRNA results in an efficient condensation of miRNAs and in their protection from enzymatic degradation. Also, the positive charge facilitates the interaction with the opposite-charged cellular membrane [155]. However, the use of cationic lipids is commonly associated with cell toxicity, as they can disrupt cell membrane integrity, induce cytoplasm vacuolization and

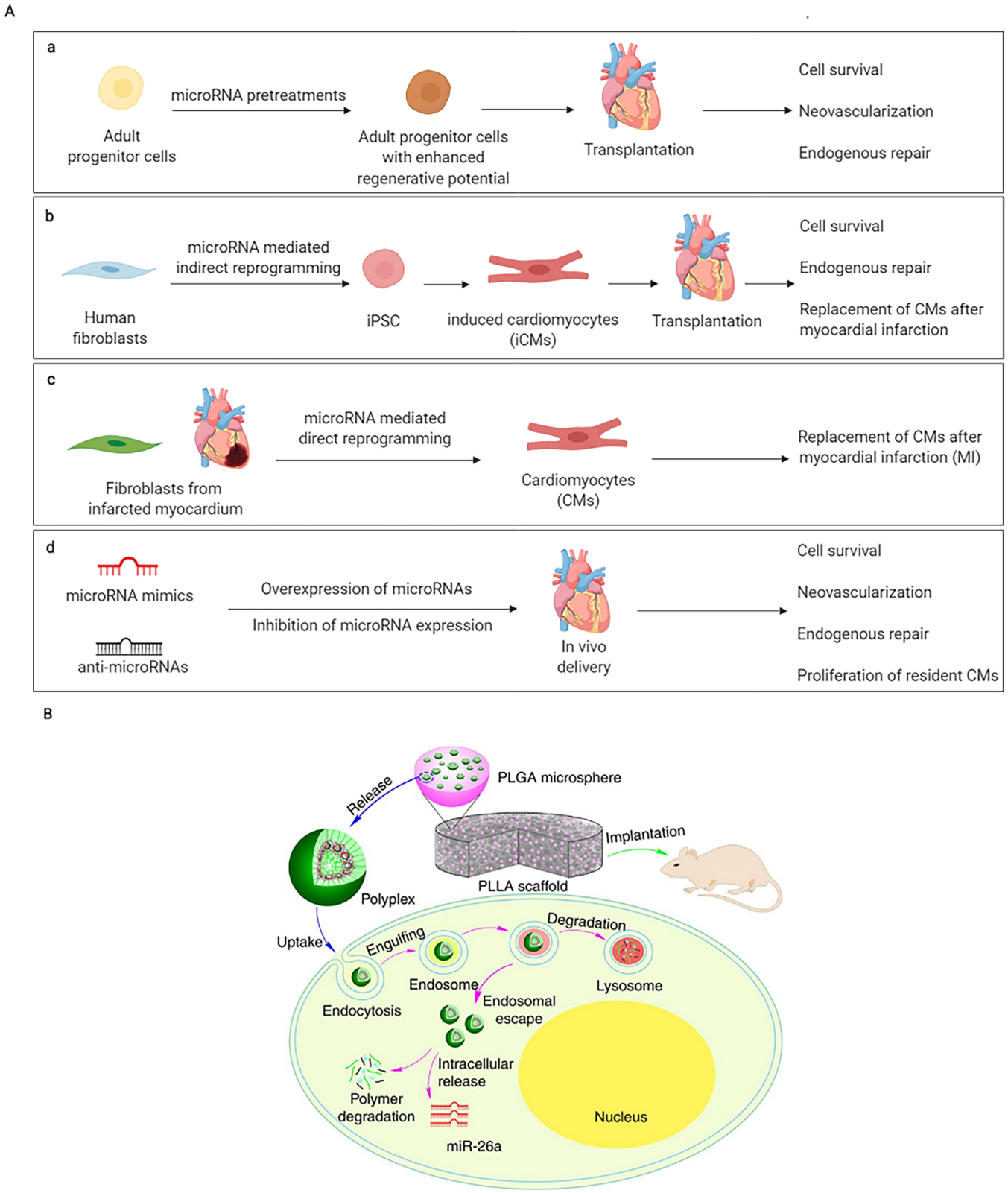

Fig. 4. microRNA (miRNA) therapeutic approaches in tissue engineering (TE). (A) Different miRNA-based therapeutic strategies for cardiac repair and regeneration after injury. miRNAs can (a) augment the regenerative capacity of adult cardiac progenitor cells, (b) reprogram human fibroblasts into iPSCs (c) directly reprogram human fibroblasts into cardiomyocytes *in vivo*, and (d) guide gene expression associated with cardiac repair. This figure was created with BioRender. (B) miRNA-based therapy to regenerate bone defects, using a cell-free 3D scaffold loaded with miR-26a-containing PLGA microspheres. Image reproduced with permission from Zhang, X. et al., Nature communications, 7 p.10376 (2016) http://creativecommons.org/licenses/by/4.0/.

reduce cell activity [156]. Furthermore, the presence of cationic lipids can result in the interaction with negatively charged serum proteins, thus inducing aggregate formation, which are subsequently eliminated by the liver and the spleen. For these reasons, several strategies to reduce the cationic charge have been attempted. For instance, combination with neutral lipids, such as cholesterol (Chol), dioleoylphosphatidyl ethanolamine (DOPE) and phosphatidylcholine (PC), known as "helper lipids", has been proposed to enhance stability and reduce toxicity of LNPs [157]. PEG modifications on the LNP surface have also been implemented to increase their half-life. PEGylated cationic/neutral LNPs have been used for the systemic delivery of liver-specific tumour suppressor miR-122 in hepatocellular carcinoma (HCC), achieving ~ 50% decreased growth after 30 days [158].

6. Therapeutic application of miRNA-based nanomedicine

6.1. miRNA-based nanomedicine in tissue engineering (TE)

MiRNAs are potent therapeutics for TE applications. In this context, miRNA-activated scaffolds can be prepared for tissue-specific miRNA replacement, or modulation (Fig. 4). Here, we report the recent results with miRNA in TE, focusing mostly on cardiac and bone regeneration.

Myocardial infarction (MI), a leading cause of death, consists in the irreversible loss of cardiomyocytes, cardiac ECM degradation and the formation of a fibrotic scar. [166] The discovery that miRNA treatment can induce cardiomyocyte proliferation and the direct reprogramming of fibroblasts into cardiomyocytes have raised the interest in using miRNAs as potential therapeutics in MI, as illustrated in Fig. 4A [167,168]. In spite of the excitement raised by these findings, the lack of suitable delivery vehicles for miRNAs have limited their practical application. Recently, Di Mauro et al. have generated CaP-NPs which successfully encapsulated and delivered miR-133 or miR-39-3p into murine cardiac cells without altering their biological function [169]. When administered *in vivo*, *via* retro-orbital injection, miRNA-encapsulated CaP-NPs accumulated in the left cardiac ventricle and in the liver, spleen and kidney. This non-specific uptake suggests that surface-modification of NPs for specific tissue targeting can improve the efficacy of miRNA-loaded NPs. Yang et al. have generated polyketal (PK3) NPs loaded with a combination of three miRNAs (miR-106b, miR-148b and miR-204), targeting Nox2, a ROS intermediate, with increased expression after MI [162]. Potentially, the inhibition of Nox2 expression by local treatment with NPs improved cardiac function and reduced infarct size in mouse models. Bejerano et al. also showed tissue remodeling post-MI through the delivery of miR-21 assembled into hyaluronan-sulfate (HAS) NPs [159]. After MI, prolonged macrophage recruitment to the infarct area could result in persistent inflammation and delayed tissue repair, thus inducing further cardiac damage. Also, miR-21 upregulation in peritoneal macrophages has been previously shown to promote inflammation resolution [170]. HAS-mediated delivery of miR-21 *in vivo* could elicit the natural reparative ability of macrophages, thus reducing cardiac fibrosis and cell apoptosis.

Several studies have elucidated the role of miRNAs in bone remodeling, by controlling osteoblast differentiation and inhibition through the regulation of different signaling pathways [171]. Zhao et al. have used CS-based NPs to deliver anti-miR-138 to bone marrow mesenchymal stem cells (BMSCs) to promote osteogenic differentiation [172]. Anti-miR-138 could regulate the ERK1/2 pathway, inducing osteoblast differentiation. Wang et al. have engineered miR-21-loaded HA/CS NPs (miR-21-HA/CS NPs) that could coat cell culture plates to induce osteogenic differentiation of BMSCs [173]. miR-21 has been selected because it downregulates SOX2 expression, thereby inducing the differentiation of hMSCs towards osteogenic lineages. After BMSC transfection with miR-21-HA/CS NPs, osteogenic-related genes could be upregulated compared to controls, including Collagen type I alpha1 (COL1), osteopontin (OPN), Runt-related transcription factor 2 (RUNX2), and osteocalcin (OCN). Moreover, after 14 days of culture with miR-21-HA/CS NPs, BMSCs that were subsequently cultured in an osteogenic differentiation medium could display significantly higher ability for ECM mineralization, as suggested by a higher calcium accumulation compared to controls.

As TE often requires the presence of three-dimensional (3D) constructs to favour and support new tissue ingrowth, recent approaches combining NPs with injectable hydrogels or scaffolds have also been exploited. For instance, Zhang et al. have proposed a two-stage miRNA delivery strategy to improve controlled release and transfection efficiency of miR-26a for calvarial bone regeneration (Fig. 4B) [161]. This strategy relies on the encapsulation of polyplexes carrying miRNAs into PLGA microspheres and their subsequent immobilization onto poly(L-lactic acid) (PLLA) scaffolds. This two-stage miRNA delivery could regenerate bone defects in mice by activating the osteoblastic activity of endogenous stem cells.

CS hydrochloride NPs loaded with agomiR-199-5p have been used to induce hMSC osteogenic differentiation *in vitro* through the regulation of the HIF pathway [56]. NPs could allow sustained agomiR-199-5p delivery *in vitro* for at least 21 days. Here, agomiR-loaded NPs were encapsulated into a fibrin gel and the scaffolds were implanted within a tibia bone defect in rats, allowing successful *in situ* bone tissue regeneration. In another study, Zhou et al. demonstrate a local sustained gene delivery system by using PEI-modified PLGA-NPs embedded into HA hydrogel, to deliver an engineered miRNA plasmid which targets cyclooxigenase COX-1 and COX-2 to reduce flexor tendon adhesions [160]. Administration of miRNA by PLGA NPs in hydrogel could prevent tendon adhesions by decreasing inflammatory response by directly downregulating COX-1 and COX-2.

6.2. miRNA-based nanomedicine in cancer

In cancer treatment, miRNA-based therapy can follow two strategies: (i) miRNA inhibition and (ii) miRNA replacement (Fig. 5) [174]. The first approach provides specific anti-miR oligonucleotides (AMOs) that inhibit tumour-promoting miRNAs (oncomiRs), such as miR-519a and miR-32 [175,176]. The latter strategy aims at introducing exogenous miRNAs (miR mimics) known to promote tumour suppression, such as miR-34a and let-7b [177,178]. However, in spite of the evidence of potentiated anti-cancer toxicity of combinational treatments based on anti-cancer drugs and miRNAs, clinical application is limited by practical hurdles. Preclinical trials have highlighted the difficulty of delivering naked miRNAs to tumours and the need for more efficient delivery systems [179,180].

Numerous authors have shown that chemotherapeutics and miRNAs can be encapsulated within NPs, representing an effective strategy to achieve potentiated cytotoxicity both *in vitro* and *in vivo*. For instance, miRNA-mediated regulation of the p53 network or the extracellular signal-regulated kinase (ERK) pathway has been used in synergy with cytotoxic drugs, such as DOX and Paclitaxel (PTX) [165,181]. Deng et al. have designed CS/HA NPs, exploiting the positive charges of CS to form complexes with miR-34, a regulator of the p53 pathway. DOX was co-encapsulated with miR-34 into CS/HA NPs, showing enhanced efficacy against triple negative breast cancer cells *in vitro*, as miR-34 restored the normal p53 pathway, sensitizing the cells to DOX chemotherapy [181]. Recently, nano-sized liposomes have also been prepared encapsulating DOX in combination with miR-101, a tumour-suppressive miRNA that is downregulated in HCC [182]. The effect of co-delivery could inhibit the growth of malignant HCC cells, both *in vitro* and *in vivo*, due to the synergistic effect of both agents [23].

Cui et al. demonstrate that the co-encapsulation of PTX and miR-7 into mPEG-PLGA-PLL-LA NPs [165]. miRNA-7 suppresses the EGFR/ERK pathway, which is known to drive cell proliferation and resistance to PTX [122,183]. Their developed NPs could enhance PTX release and improve miRNA efficiency, resulting in increased apoptosis of ovarian cancer cells and reduced resistance to PTX [165]. Costa and colleagues demonstrate the preferential accumulation within brain tissue of liposomal NPs that were conjugated with chlorotoxin (CTX), a glioma-specific peptide marker. CTX-NPs could efficiently deliver anti-miR-21 oligonucleotides, with no signs of systemic immunogenicity [164].

Studies on lung adenocarcinoma (LAC) have shown the presence of a small subset of cancer stem cells (CSCs) with altered miRNA expression [184]. miR-145 loaded into PU-PEI NPs showed beneficial effect on LAC metastases through the inhibition of EMT processes and improved sensitization to chemoradiotherapies, prolonging survival of mice. Abnormal expression of miRNAs has also been found in malignant brain tumours. For instance, inhibition of miRNA-21 has been used in GBM treatment to rescue tumour suppressor genes, such as PTEN and PDCD4, while miRNA-182 has been associated with favorable prognosis because of its role as a regulator of apoptosis, growth, and differentiation [185–187]. Anti-miR-21 NPs co-encapsulating DOX have demonstrated enhanced cell apoptosis and reduced tumour growth in brain tumour models [64]. Kouri et al. demonstrate gold NPs covalently functionalized with miR-182 that can selectively deliver miR-182 to brain tumours, reducing tumour size and increasing survival [163]. These studies demonstrate the extensive potential of miRNA-loaded NPs in cancer treatments.

7. Frontiers in miRNA-based therapies

7.1. Cancer immunotherapy

MiRNAs play a significant role in immune system modulation [188]. As a result, inhibiting or upregulating specific miRNAs can potentially modulate the immune response to tumours, for example by controlling macrophage polarization or by regulating their activities in the tumour micro-environment (TME) [189,190]. For instance, upregulation of specific miRNAs, such as let-7c [191,192], and miR-146a [193,194] has been shown to drive

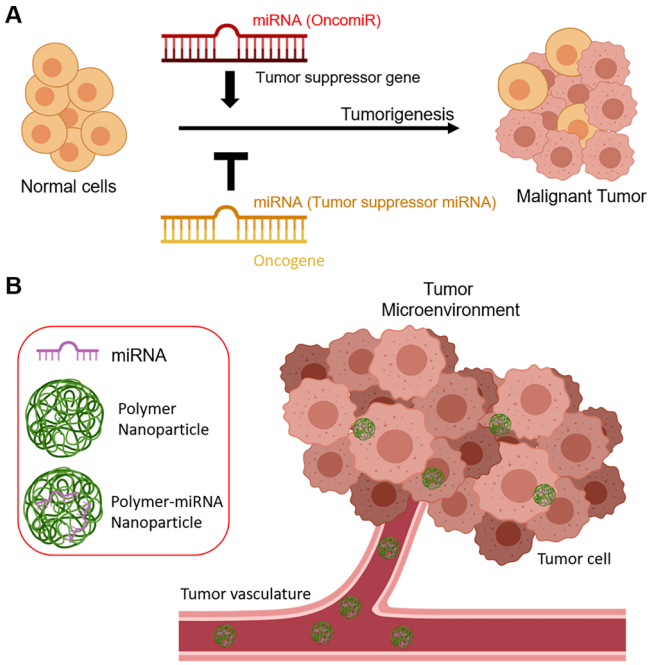

Fig. 5. (A) Roles of microRNAs (miRNAs) in cancer. Depending on the target gene, miRNAs suppress or stimulate cancer-related genes. Image modified from Oliveto, S. et al., World journal of biological chemistry, 8 (1), p.45 (2017). (B) Therapeutic approach using miRNA-loaded polymer NPs circulating in the tumour microenvironment. Images (A) and (B) were created with BioRender.

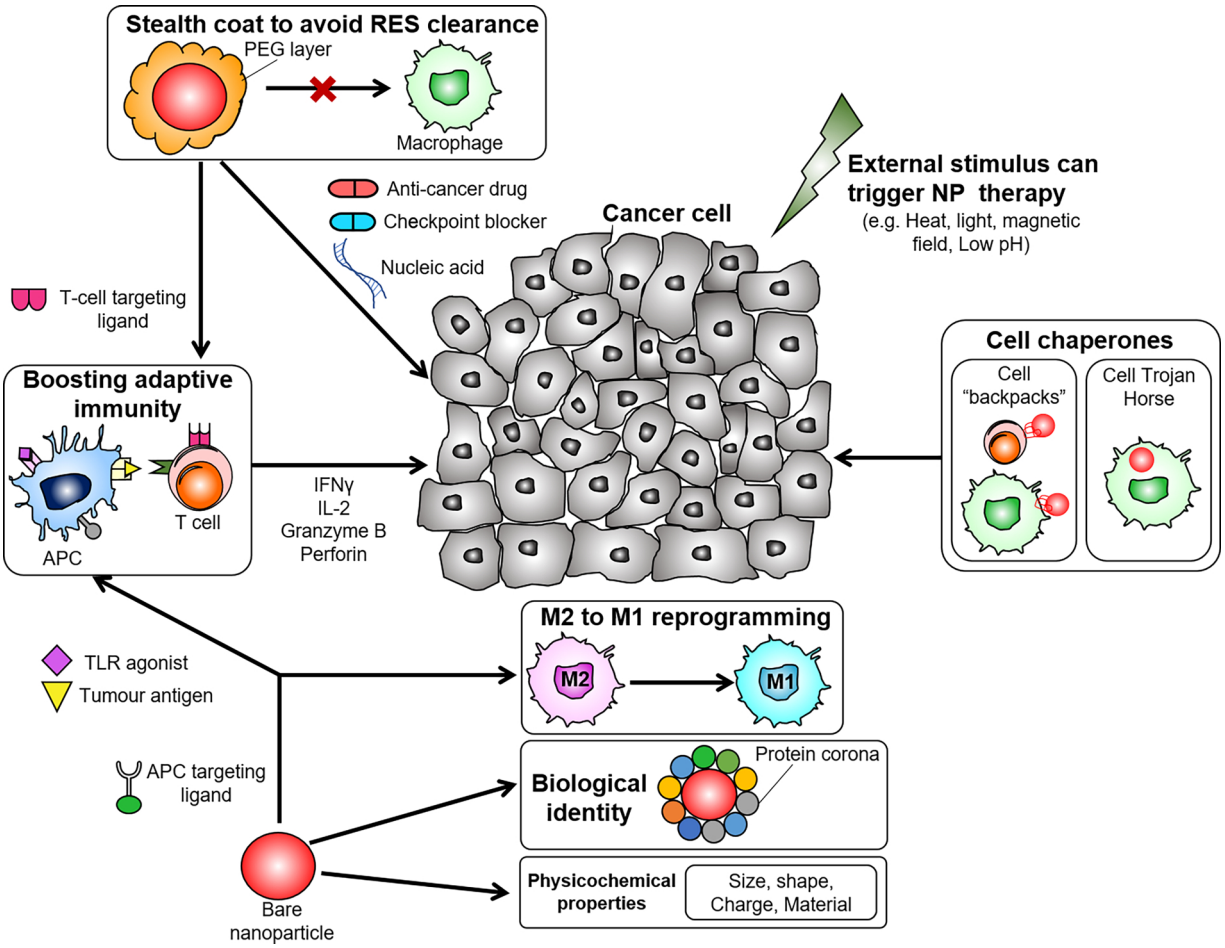

Fig. 6. NPs can be applied in cancer immunotherapy to i) enhance the activity of T cells, ii) induce macrophage reprogramming towards the tumoricidal M1 phenotype, iii) target immune cells and exploit their tumour infiltration ability to penetrate inside the tumour mass, where release can be triggered by either internal or external stimuli. Image modified with permission from Gun, S. Y. et al., Redox biology, p.101174 (2019) (published under Creative Commons attribution Licence CC-BY).

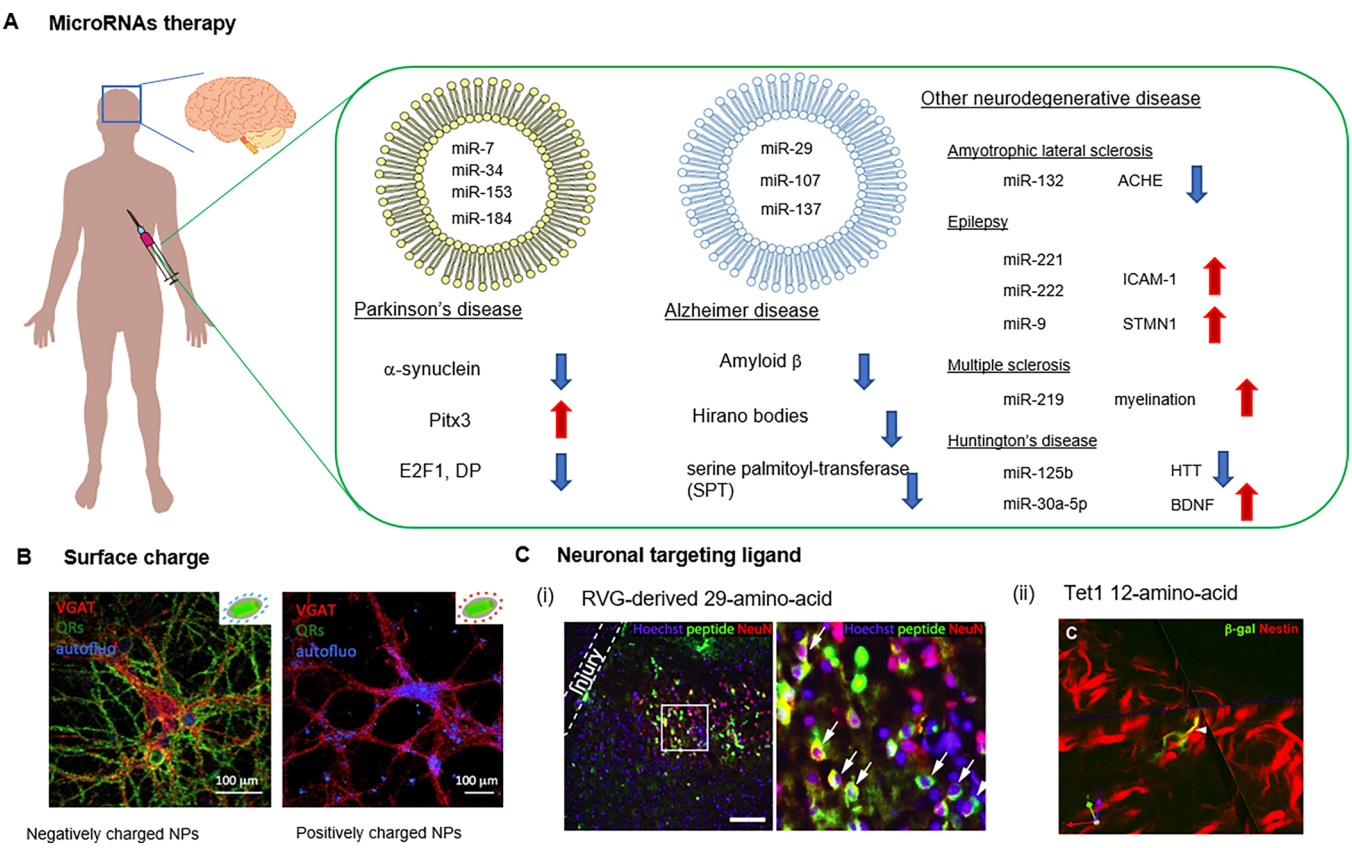

Fig. 7. (A) Representative potential microRNA-therapies for treating neurodegenerative diseases. Targeted NPs release miRNA to neurons by: (B) surface charge (reproduced with permission from Dante, S. et al., ACS Nano 11, 6630–6640 (2017), or (C) surface functionalization with specific neuronal binding ligand such as (i) RVG-derived 29-amino-acid and (ii) Tet1, 12-amino-acid (reproduced with permission Kumar, P. et al. ACS Nano 2016, 10, 8 7926–7933 and Kwon, E. J. et al., Biomaterials 31, 2417–2424 (2010), respectively).

immunosuppressive and pro-tumour activity of (pro-tumour) "M2"-type tumour-associated macrophages (TAMs). In contrast, (anti-tumour) "M1"-type macrophages, characterized by tumoricidal activity, can be induced by increased expression of a.o. miR-125b [192,195], and miR-155 [196–198]. As immunomodulation is often constrained by poor specificity, NP-based specific delivery of immunomodulatory agents has the potential to improve on-target delivery while minimizing off-target effects (Fig. 6) [199]. Although numerous studies have demonstrated the anti-tumour efficacy of NP-based immunomodulation, targeting macrophages, T cells, and dendritic cells (DCs) [200,201], there is a startling lack of studies on immunomodulation through miRNA-loaded NPs. Importantly, the design of miRNA-based NP platforms for immunomodulation may build upon existing RNA-based NP technologies or immune system targeting strategies, briefly summarized below [8,202–204].

Researchers have used monocytes/macrophages as enablers of NP-based anti-cancer therapy, by virtue of their ability to naturally home to tumours. Choi et al. show that macrophages that phagocytized gold nanoshells (Au-NS) can accumulate into breast tumour spheroids, resulting in cancer cell death through photo-induced ablation [205,206]. Anselmo et al. and Doshi et al. have attached a therapeutic cargo to monocytes in the form of a "cellular backpack" and demonstrate that monocyte-associated "backpacks" could accumulate to greater degree in inflamed organs, as compared to a "free backpacks" [207,208]. Alternatively, Song et al. demonstrate the TAM-specific delivery of siRNA targeted against vascular endothelial growth factor (VEGF) and placental growth factor (PIGF), that support the pro-tumour activity of TAMs [209]. Mice being administered with siRNA-loaded NPs showed decreased TAM accumulation in the tumour microenvironment and striking reductions in tumour growth compared to control mice.

Similarly, T cells, which display cytotoxic activity against tumour cells, can be exploited to facilitate intratumoral delivery of NPs [210,211]. For example, Siriwon et al. show that CAR T cells can effectively deliver and release an antagonist against T cell activity deep within the tumour microenvironment, thus supporting effector T cell cytotoxicity [212]. NPs can also be used to deliver cytotoxic T lymphocyte molecule-4 (CTLA-4) siRNA into tumour-infiltrating T cells (TILs), to increase the proliferation and the anti-tumour activity of effector T cells [213]. Smith et al. have utilized NPs to deliver leukemia-specific CAR genes into T cells, achieving long term disease remission [214]. This strategy could potentially be used to achieve a sufficient pool of therapeutic T cells to elicit "on-demand" cancer immunity [214].

NPs-based immunotherapy can also be achieved by targeting dendritic cells (DCs). DCs present epitopes of tumour-associated antigens (TAAs) to T cells, activating T cells for their attack of TAA-presenting tumour cells [215]. Self-assembled DNA–RNA nanocapsules have been developed to that deliver tumour neoantigens to DCs, increasing the $CD8^+$ T cells and reducing the growth of colorectal tumours [8]. Warashina et al. have developed a lipid NP for DC-specific delivery of siRNA against a suppressor of cytokine signaling 1 (SOCS1). Treated DCs displayed a marked increase in cytokine production with consequent reduction of tumour growth *in vivo* [216]. Kranz et al. have further exploited the antiviral defence mechanisms of DCs, using DC-targeting RNA-lipoplexes to trigger the release of pro-inflammatory interferon-α [217].

7.2. Neurodegenerative disorders

Neurodegenerative disorders, such as Parkinson's disease (PD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), and Huntington's disease (HD), are characterized by, among other features, the progressive loss of neuronal synapses, leading to neuronal death. Recent studies have reported that abnormal miRNA expression contributes to the degradation of neurons [218], suggesting that neurodegenerative diseases can be classified as an RNA disorder [219]. miRNA-loaded NPs that regulate RNA transcripts may thus represent a form of novel therapeutics for treating neurodegenerative diseases (Fig. 7) [220].

Comparative studies of miRNA profiles in post-mortem brain tissue from PD patients and healthy controls have shown altered miRNA levels in PD patients. For instance, miR-7, miR-153, and miR-433 drive the over expression of α-synuclein, which has been associated with aberrant soluble oligomeric conformations in *in vitro* experiments [221,222]. Dysregulation of miR-34 has been

associated with the pathogenesis of PD along with altered mitochondria activity and oxidative stress [223]. In addition, several studies show reduced expression of miR-133 in PD patients, which regulates dopaminergic neuron development, differentiation and maturation [224]. miR-133 also further regulates Pitx3, a transcription factor involved in dopaminergic neuron differentiation [225].

Additionally, many miRNAs are abnormally regulated in the brains of AD patients. For instance, decreased expressions of miR-29, miR-189, and miR-9 are found in the AD brain, as evidenced by genome-sequencing and miRNA profiling. Decreased expressions of miR-298, miR-328 and miR-195 have been found in AD mouse models [226,227]. Recent studies have shown the relationship between miRNA dysregulation and extracellular amyloid β plaque (Aβ) production and aggregation which gives rise to neuro-degeneration [228]. For example, decreased expression of miR-107 may contribute toward faster disease progression by regulating beta-site amyloid precursor protein (APP) cleaving enzyme 1 (BACE1) [229]. miR-106a and miR-520c have been found to suppress the expression of APP *in vitro* [230]. miR-17 and miR-20a, along with miR-147, miR-153, miR-644, and miR-655 as well as miR-323-3p, have been found to directly target APP [231]. Additional studies have identified dysregulated miRNAs that contribute to AD pathogenesis through atypical regulation of numerous proteins, which is involved in disease progression. Downregulated miR-103 or miR-107 expression can also lead to increased cofilin 1 (CFL1) levels in AD brains, which may associate with the formation of intracellular Hirano bodies [232]. Other studies show that decreased expressions of miR-9, miR-137, miR-181c, and miR-29b-1 in AD brain can upregulate serine palmitoyltransferase (SPT), long chain subunit 1 (SPTLC1) and 2 (SPTLC2), which are the rate-limiting enzymes in the synthesis of ceramides [233].

The dysregulation of miRNA also plays an essential role in other neurodegenerative diseases. Many studies have identified ALS-relevant transcripts and pathways that are targeted by dysregulated miRNAs. For instance, miR-132 regulates acetyl-cholinesterase and polypyrimidine tract binding protein 2 (PTBP2), which influences alternative splicing of tau in ALS [234]. Wang et al. showed that miR-219 and miRNA-338 downregulation is observed in murine CNS, regulating oligodendrocyte differentiation and myelination [235]. Several miRNAs may also play a role in HD, particularly by targeting the Huntingtin gene (Htt). Sinha et al. show that miR-125b, miR-214, miR-150, miR-146a have the potential to reduce Htt aggregates [236]. The encapsulation of these miRNA in NPs may further represent a feasible strategy for more efficient delivery, thus contributing to improved therapeutic outcomes.

To date, delivery of miRNAs to the brain and to the spinal cord mainly relies on recombinant adeno-associated virus (rAAV) [237]. Miyazaki et al. showed that miR-196a delivery using AAV vector could enhance the decay of mRNA androgen receptor by silencing Elav-like family member 2 (CELF2) in mice [238]. They found that the early delivery of miR-196a by an AAV vector could ameliorate the spinal and bulbar muscular atrophy phenotype in mice. Another study showed that miR-132 delivery using AAV9 vector could recover motor function, thus regulating muscle locomotion as well as survival rate of HD model mice [239].

As AAV vectors have a limited capacity for encapsulation (4.5–5 kb) due to size limitations, non-viral vectors may substantially increase miRNA delivery efficiency to the brain, improving neurodegenerative disease treatment. Drug delivery to the brain mediated by NPs can also allow blood-brain barrier (BBB) crossing and selective targeting of neurons. For example, heat generation from iron oxide NPs has been shown to enhance BBB bypass of NPs [240]. Dante et al. showed that negatively charged (−COOH) NPs could effectively localize at the membrane of neurons and influence neural excitability [241,242]. Ligand-based targeting has been also considered for the improved delivery of NPs to brain and neurons. Kumar et al. reported that a short peptide-derived rabies virus glycoprotein (RVG) localize on neuronal cells *in vitro* through specific binding to the acetylcholine receptor [243]. Kwon et al. proposed a non-viral delivery vehicle to adult neural stem cells using a 12-amino-acid, Tet1, which could bind specifically to neuronal cells [244]. Therefore, NPs represent potential therapeutic tools in neurodegenerative disorders by virtue of their ability to deliver miRNA treatments to deeper regions of the brain.

8. Conclusions

As important regulators of cell behavior in normal and pathological conditions, miRNAs hold immense potential for clinical applications, ranging from cancer therapy to TE approaches for the treatment of bone and cardiac defects, to neurological disorders, as described in the above paragraphs [3]. Moreover, because miRNAs inhibit/promote the expression of a multitude of genes compared with mRNA and siRNA, miRNA therapy potentially carries distinct advantages for disease treatment and regenerative medicine [25,26]. Efficient and safe delivery of miRNAs is of paramount importance for their exploitation in the clinics, as naked miRNAs are susceptible to rapid degradation and traditional transfection reagents are not suitable for in-human applications [8].

Encapsulation of miRNAs into NPs can overcome these delivery challenges, resulting in enhanced targeting efficacy and reduced off-target effects of the encapsulated payload. Importantly, the design of NPs with genetic cargo has primarily focused on siRNA, DNA, plasmids or oligonucleotides, and only few have detailed work with miRNA. In this work, we discussed the advantages of nanomedicines for miRNA delivery, reviewed the recent applications of NPs and miRNAs carriers, and highlighted potential new fields of use, which can benefit from miRNA-based therapies. The development of miRNA-loaded NPs is nascent and, often, authors describe methods for preparing NPs for miRNA delivery while referring to previous protocols, which are originally developed for drugs or other types of RNA or DNA cargo. Few studies present a thorough comparison between miRNA-loaded NPs and NPs with other RNA (for example siRNA-loaded NPs) [245]. Even so, these comparative studies only investigated the physicochemical properties of the NPs or their uptake by cells *in vitro* and it remains to be validated if these findings are also relevant *in vivo*. Indeed, as crucial biochemical differences exist between miRNAs and other nucleic acids, we should be cautious to extrapolate or predict outcomes for miRNA-loaded NPs based on the findings of other DNA/RNA-associated NPs. Nonetheless, our understanding of miRNA-loaded NPs is only beginning, and the future holds several therapeutic possibilities for miRNA-loaded NPs.

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