RNA nanomedicines: the next generation drugs?
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RNA therapeutics could represent the next generation personalized medicine. The variety of RNA molecules that can inhibit the expression of any mRNA using, for example, RNA interference (RNAi) strategies, or increase the expression of a given protein using modified mRNA together with new gene editing strategies open new avenues for manipulating the fate of diseased cells while leaving healthy cells untouched. In addition, these therapeutic RNA molecules can maximize the treatment of diseases and minimize its adverse effects. Yet, the promise of RNA therapeutics is hindered by the lack of efficient delivery strategies to selectively target these molecules into specific cells. Herein, we will focus on the challenges and opportunities of the delivery of therapeutic RNAi molecules into cancer cells with special emphasis on solid tumors. Solid tumors represent more than 80 percent of cancers and some are very challenging to treat, not merely due to physiological barriers but also since the tumor microenvironment (TME) is a complex milieu of accessory cells besides the cancerous cells. In this review, we will highlight various limiting factors to successful delivery, current clinical achievements and future outlook focusing on RNAi therapeutics to the TME.

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RNAi discovery subsequently led to the development of potential novel therapeutic strategies utilizing small antagonist RNA molecules such as small interfering RNA (siRNA), microRNA (miR), small hairpin RNA (shRNA) or anti-miR (antimagomirs) of which, siRNA and miRs have shown promising outcomes in clinical trials [2,3]. RNAi molecules targeting mRNA transcripts have expanded and could streamline drug discovery and development beyond protein therapeutics. In addition, RNAi does not need to be directed to the nucleus unlike DNA-based gene therapy and its effect is comparatively more robust.

Despite the utopia, the delivery of RNAi molecules to their site of action poses challenge due to physiological barriers: degradation by serum RNases, rapid renal clearance and immunostimulation, in addition to cellular barriers, to efficiently and safely cross the hydrophobic plasma membrane. Once the delivery systems are across lipid bilayer, another set of intracellular barriers such as endosomal escape following endocytosis and competing with endogenous RNAi pathways need to be addressed [4,5]. Nanocarriers have to be devised in a manner, which not just overcome these hurdles but are potent, easy to formulate, cost-effective and scalable to be translated from bench to the clinics. This review will cover only non-viral delivery strategies with special emphasis on solid tumors and their unique microenvironment.

Chemical modifications at the RNA level
To bypass the first barrier, chemical modifications of therapeutic RNA molecule such as substitution at 2'-OH ribose or alteration of the phosphodiester backbone have been suggested [6**,**7**] resulting in enhanced stability and lowered TLR7/8 mediated immune response. Yet, the dose in which inhibition is achieved is often high enough to induce off-target effects and saturation of the silencing machinery [4]. Also, cellular delivery and decreased potency of RNA molecules post modification remains an addressable concern.

Nanocarriers modalities
Nanocarriers (NCs) offer safe and effective delivery platforms for the negatively charged and small (approximately 21 nucleotides) siRNAs without the need of additional chemical modification, reduced risk of off-target effects
and enhanced cellular delivery bypassing the membrane barrier. Furthermore, their biodegradability, minimal toxicity and physicochemical characteristics lend the therapeutic payload with excellent pharmacokinetic and biodistribution properties in vivo[8**]. Particulate NCs such as micellar or liposomal delivery systems are undergoing rapid state-of-art progress to achieve more sophisticated and specific cellular targeting with respect to tumor supporting cells [9*,10]. Here we enumerate some of the aspects with respect to the NCs design, which hold importance for efficient delivery of therapeutic RNAi.

**Systemic barriers**

**NCs specificity**

Passive tumor targeting can be achieved by exploiting tumor physiology, which due to neovascularization and poor lymphatic drainage leads to accumulation of NCs in tumor vicinity; known as Enhanced Permeability and Retention (EPR) effect [11]. Passive targeting alone has shown improvement in distribution, absorption and excretion profiles of RNA molecules. But with expanding realm of tumor heterogeneity and difference in solid tumor physiology, more specific approaches are being employed. Active targeting for instance, score way better than passive in terms of higher cargo delivery, specificity and minimal off-target effects [12]. With the approval of many cancer-associated antibody (Ab fragments) aptamers, peptides and other natural ligands, it is doable to develop siRNA formulations with targeting moieties, which enable them to home specifically towards cancer cell over-expressing surface receptors [13]. Cholesterol conjugated siRNA and endosomolytic polymer co-injection showed dual advantages of liver-specific delivery, which lead to a potent and durable silencing of target gene [14].

**Toxicity and immune response**

In order to facilitate complexation/loading/encapsulation of negatively charged RNAs, positively charged components are incorporated into NCs. Those include: cationic lipids (e.g. DOTAP, DOTMA, DC14); ionizable lipids (e.g. DLinDMA, DLin-KC2-DMA, DLin-MC3-DMA) or cationic polymers (e.g. chitosan, polyethyleneimine (PEI)) [15,16*,17]. These cationic compounds can efficiently condense RNA molecules and are taken up by cells, escaping the endosomes and inducing gene silencing in the case of siRNAs. In vivo, cationic-NCs can induce immune response (either by interacting with Toll-like receptor (TLR4) or by interacting with other charge recognizing intracellular proteins [18*,19]) and are marked and eliminated by mononuclear phagocytic system (MPS). This can be overcome by incorporating hydrophilic coats such as polyethylene glycol (PEG) [4] or hyaluronan (HA) [12,20], which makes them less recognizable by the MPS and in turn increase circulation time. NCs not only offer protection to RNA from serum degradation, they prevent RNA exposure in vivo from mounting an immune response.

**Intracellular barriers**

**Endocytosis and endosomal escape**

NPs internalization into the cell may end up in either getting trapped and degraded in lysosome or delivering the cargo into the cytosol. Endocytosis via caveosomes can enhance the chances for RNA delivery into the cytoplasm due to their neutral internal pH and avoidance of lysosomal trafficking [4]. For bypassing clathrin-mediated endocytosis, usage of cationic aminolipid such as The DLin-DMA family of ionizable lipids, which possess ionizable amine headgroups (at lipid pKₐ of 6–7) can be effective. As the pH decreases in endosomes, the protonated headgroups interact with endosomal membrane thereby rupturing them and releasing the cargo into the cytosol [21]. Other strategies such as utilizing the ability of particles to behave as proton-sponge leading to endosome swelling and rupture [22] or escaping endocytic-recycling pathways, for example, Niemann-Pick Type C1 to improve siRNA delivery by LNPs have been investigated [23].

**Saturation of endogenous RNAi machinery**

Irrespective of the presence or absence of target, transfection with RNAi molecules-specifically shRNA, is accompanied by cellular toxicity due to perturbation of miRNA pathway and is dose-dependent [24]. Saturation or exhaustion of endogenous RNAi machinery can be bypassed by using sequence with minimal off-target effects [25]. In one study [26], the active number of RISC molecules per mammalian cell was estimated around 10³ – 10⁴, hence RNAi molecules preferably below this number should be employed.

**The challenging physiology of solid tumors**

Solid tumors represent about 80% of all types of cancers, which are not only hard to penetrate but are heterogeneous pool of cancer cells constantly modifying the microenvironment to assist their proliferative endeavor. Following chemotherapeutic intervention, the dose received by the cells in solid tumor varies as a function of distance from blood vessel axis — with the farthest cells receiving least oxygen, nutrition and therapeutic dose [27]. Poor lymphatic drainage further leads to low convection due to build-up of high-interstitial fluid pressure (IFP) [28,29]. Nanomedicine transport following systemic injection mainly occurs by extravasation, which includes diffusion and convection and poses multiple barriers in the form of dense interstitial matrix and physicochemical properties of the NCs [30].

Several approaches have come to fore to prime tumors to enhance delivery [31]. One method is by normalizing vascular dimensions such as photoinmunotherapy [32] that can reduce IFP & improve interstitial transport of therapeutics within core areas. Similarly, enzymatic degradation of extracellular matrix (ECM) and apoptosis induction with certain chemotherapy drugs [33] can loosen up the tumor mass to alleviate solid stress, which can
further enhance blood flow and convection currents improving extravasation of NCs as well as modifying TME [34].

**Hypoxia and cancer stem cell**
The physical make-up of solid tumor leads to induction of genetic instability in the necrotic regions (Figure 1) characterized by hypoxia and low pH. Hypoxia induces release of chemoattractants to recruit endothelial precursor cells (EPC), immune cells mainly tumor associated macrophage-M2 type (TAM) and cancer-associated fibroblasts (CAF), which drive angiogenesis, immunoeediting and ECM remodeling respectively in favor of tumor proliferation and metastasis (Figure 1). These foci are enriched with non-proliferating cancer-stem cells (CSC) [35], which in order to survive develop chemoresistance, anoikis resistance and potential to metastasize by expressing markers for epithelial-to-mesenchymal transition (EMT) — ultimately responsible for subsequent relapse in patients [36].

Though CSCs are in constant flux due to modulating TME, they are genetically stable unlike cancer cells and specific targeting towards key molecules which affect CSC metabolism and survival rather than replicative pathways can actively target them and shift the balance against CSC survival. Additionally, signaling pathways molecules promoting stemness or cell renewal properties of CSC [39] by accessory cells such as CAF [37] or mesenchymal stromal cell [38] can be potential targets.

**Tumor microenvironment**
The complex cross talk amongst diverse key players is facilitated via autocrine and paracrine signaling functions

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*Nanobiotechnology*

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**Figure 1**

Solid tumors possess putative targets besides cancer cells which form the tumor microenvironment and are known to harbor supporting cells such as immune cells, CAFs, EPCs, and CSCs in necrotic regions which generate mainly in response to hypoxia. Many cells over-express markers which can be targeted actively through RNAi-based nanocarriers to block the cross-talk amongst various players through secretions of growth factors, cytokines and inhibitory molecules. Abbreviations: NP, RNA encapsulated active nanocarriers targeted towards cell of interest; CAF, cancer-associated fibroblasts; CSC, cancer stem cells; DC, dendritic cell; MDSC, myeloid derived stem cell; EPC, endothelial progenitor cell; NK, natural killer cell.
through secretion of growth factors, chemokines, cytokines and proteolytic enzymes [34,40]. These non-cell autonomous interactions amongst tumor environment subpopulations can enhance tumor robustness through tumorigenesis, metastasis and therapeutic resistance [41]. Colony-stimulating factor (CSF-1) secreted mainly by fibroblaststroma produce macrophages, is an important mediator and triggers several events [42]. On one hand, TNFα secretion by differentiated macrophages can lead to digestion of ECM via matrix metalloproteinase (MMP) and an influx of additional inflammatory cells; on the other end, induced monocytes and fibroblast recruit EPCs mediated by VEGF secretion to develop microvessels to support tumor growth [43]. CAFs-predominantly present at the marginal areas are responsible for much of ECM remodeling of the tumor. They enhance tumor invasiveness, metastasis and angiogenesis by secreting ECM components. Targeting ECM components (such as collagen, fibronectin etc.) secreted majorly by CAFs and stromal cells via RNAi will not merely aid in the aforementioned tumor priming, but will block the flux of communicating molecules responsible for tumor malignancy.

Several attempts have been made to interfere with the functioning of tumor supporting cells to tame cancer growth. Promising results have been achieved by delivering antisense oligonucleotide via particulate delivery system to target anti-tumor marker molecules into endothelial cells (VEGF) [9*], fibroblasts (FAP) [44], M2 macrophages (VEGF) [45] and breast cancer-stem cell (fibronectin-EDB) [10]. Other recent studies aimed at modifying TME to achieve tumor regression [46–48] and inhibiting metastasis [49]. Another point of caution is the over-use of anti-angiogenic molecules which may end up in hypoxic state of tumor favoring CSC formation [50,51**]. Hence, a fine balance needs to be ascertained in order to prevent tumor relapse due to CSC.

Immuoediting of TME renders a tolerogenic state which is anti-inflammatory, rich in regulatory T-cells and low expression of tumor associated antigens. RNAi mediated modulation of immune cells such as dendritic cell targeting with siRNA against STAT3 led to improvement of CD8+ T-cell infiltration and tumor regression [52]. In another study, CTLA4 expressing CD8+ T-cell were targeted with CTLA4 aptamer fused with siSTAT3 and resulted in reduction in tumor growth and metastasis [53]. Extracellular communication vesicles such as exosomes released from cancer cells are known to facilitate interactions with the TME. In one study, shRNA mediated inhibition of Rab27a was found to diminish metastasis in breast cancer model due to exosome-mediated neutrophil mobilization [54].

It is important to highlight key molecules/cytokines such as TNFα, CSF-1, HIF, SDF-1 which facilitate the cross-talk between tumor cells and initially recruited CAFs, TAMs and EPCs. The dynamic way with which tumor evolves has led to development of combinatorial therapies which can disable several supporting pathways to tumor progression in one shot. For example, knockdown of key molecule secreted by many cells (e.g. MMP9 secreted by CAF, MDSC and tumor cells) or combination treatment such as knockdown of SDF-1 gene in CAF

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<td><strong>RNAi-based lead NCs under clinical trials (as in September 2015: <a href="https://clinicaltrials.gov/">https://clinicaltrials.gov/</a>)</strong></td>
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<td>SPC 3649 (Locked nucleic acid LNA-miR122)</td>
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<td>ALN-TTR02 (SNALP)</td>
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together with immune therapy to PD-1 expressing CD8\(^+\) T-cells can possibly curtail tumor propagation in early stages itself.

Active cellular targeting by ligand-based or mAb-based LNPs
In our previous work, we reported successful and selective delivery of Hyaluronan (HA) grafted-siRNA loaded lipid-based NCs in ovarian adenocarcinoma [12] and glioblastoma [16\(^*\)] in xenografted mice models of human tumors. Additionally, active cellular targeting of immune cells such as activated leukocytes [55\(^*,56\(^*\)] and specific subset of CD4\(^+\) T-cells [17] have been reported by our group with subsequent knockdown of target genes showing ultra-fine tuning of siRNA delivery to circulating immune cells in vivo with a scalable platform. Hence, NCs mediated delivery can be safely and specifically targeted to desired destination with minimum off-target effects and systemic toxicity.

Clinical translation
Unlike first generation LNP-RNAi therapeutics, clinical results of the second generation LNPs are showing improved siRNA delivery and subsequently higher knockdown of target gene, lower toxicity and lower dose at which highest response is attained [57]. Despite initial hiccups, RNAi therapeutics has gathered pace with many promising outcomes in the last 5 years [2,3,57,58]. Table 1 enlists updated state of clinical trials of different RNAi therapeutics. Different conditions ranging from Hepatitis C virus [59], Transthyretin Amyloidosis [60\(^*\)] to Glaucoma [61] have been addressed with much success, pointing to bright scope of RNAi therapeutics.

As particulate delivery systems end up accumulating mainly in liver, most hepatic disease have been worked upon with RNAi therapeutics [57]. Besides, tumor targeting has been achieved owing to active targeting and EPR effect. However, focus should also be diverted towards other topical conditions addressable such as mucosal or dermal applications.

Future outlook
Solid tumors possess putative targets besides cancer cells, which form the tumor microenvironment and are known to harbor supporting cells such as inflammatory cells, fibroblasts, stromal cells, endothelial precursor cells. This subset of cells within tumor mass is constantly modifying the tumor microenvironment to support its proliferation, migration and invasion. Many cells over-express markers, which can be actively targeted via NCs and manipulate the fate of these cells with RNAi. Within tumor subpopulations of fibroblasts, macrophages, T-cells and carcinomas that can be targeted with RNAi ‘Omic’ will play a pivotal role to find the best targets that can reduce tumor burden and ultimately can eradicate tumors.

We envision that combination therapy will gradually enter the clinic with RNAi as knocking down a single gene is not enough to overcome tumor heterogeneity. Moreover, sensitizing several genes for treatment with the gold standard chemotherapy and novel biological treatment may increase the arsenal of drugs available to the oncologist and ultimately might prove RNA as a novel therapeutic modality to treat solid tumors.

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References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:**

- of special interest
- of outstanding interest


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