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Strategies to deliver RNA by nanoparticles for therapeutic potential

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ABSTRACT

The use of a variety of RNA molecules, including messenger RNA, small interfering RNA, and microRNA, has shown great potential for prevention and therapy of many pathologies. However, this therapeutic promise has historically been limited by short *in vivo* half-life, lack of targeted delivery, and safety issues. Nanoparticle (NP)-mediated delivery has been a successful platform to overcome these limitations, with multiple formulations already in clinical trials and approved by the FDA. Although there is a diversity of NPs in terms of material formulation, size, shape, and charge that have been proposed for biomedical applications, specific modifications are required to facilitate sufficient RNA delivery and adequate therapeutic effect. This includes optimization of (i) RNA incorporation into NPs, (ii) specific cell targeting, (iii) cellular uptake and (iv) endosomal escape ability. In this review, we summarize the methods by which NPs can be modified for RNA delivery to achieve optimal therapeutic effects.

1. Introduction

Ribonucleic acid (RNA) exists in multiple forms with various nucleotide chain lengths and functions that have essential roles in both health and disease (Kim, 2020). Messenger RNA (mRNA; responsible for protein translation), antisense RNAs (asRNA; block protein translation) including microRNAs (miRNA; regulate post-transcriptional gene expression), and small interfering RNA (siRNA; silence gene expression) have all been explored and successfully applied as vaccines, protein replacement therapies, and immunomodulatory agents in many diseases, including cancer and atherosclerosis (Lin et al., 2020). Considering that only ~15 % of proteins are considered targetable for therapy using small molecule drugs, RNA therapy represents an alternative treatment option with several advantages (Dammes and Peer, 2021). RNA is easier to design and scale up than protein-based drugs (Guevara et al., 2019; Lin et al., 2020), and the recent explosion of RNA research has informed novel methods to chemically modify RNA, leading to decreased costs associated with synthesis (Jasinski et al., 2017).

However, naked RNA molecules have shown a lack of therapeutic efficacy for many reasons. RNA has a short half-life due to nuclease degradation (Orlandini von Niessen et al., 2019) and reticuloendothelial clearance (Valle et al., 2020). They also show limited cellular internalization due to their negative charge (Wadhwa et al., 2020) and/or potentially long nucleotide chain length depending on the sequence used (Park et al., 2021) (Fig. 1). Furthermore, dose-limiting toxicity can preclude RNA for therapeutic use as it can induce an immune response *via* stimulation of proinflammatory cytokine production (Freund et al., 2019). Finally, the lack of RNA thermal stability hinders clinical translation potential (Park et al., 2021).

Additionally, even upon cell uptake, RNA can be sequestered by endosomes, posing a significant barrier to inducing a therapeutic response. For example, only 1–2% of siRNAs that enter cells escape the endosome into the cytosol where they can induce mRNA degradation or block translation (Kim, 2020). Although some of these issues can be solved by direct chemical modification of RNA, specific and effective delivery to the pathological site is still lacking (Wadhwa et al., 2020). Insufficient targeting results in RNA accumulation in indiscriminate cell types, thereby decreasing therapeutic efficacy, increasing the dose required to induce an adequate response, and potentially inducing adverse side effects at off-target sites (Kim et al., 2020).

In order to overcome these limitations, nanoparticles (NPs) have been extensively used as delivery tools (Lei et al., 2020), with some formulations approved for use by the United States Food and Drug Administration (FDA). NP use for RNA delivery has been remarkably

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successful in recent years, with dozens of formulations currently in clinical trials, and two effective vaccines combatting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection currently under Emergency Authorization Use (EUA) in the United States and worldwide (Chung et al., 2020; Park et al., 2021). There are multiple reasons for this recent success: 1) Non-toxic and biodegradable components that show low immunogenicity can be used to create biologically compatible NPs for clinical use and drug delivery. 2) NPs have the distinct advantage of multifunctionalization, which is the ability to incorporate multiple therapeutic and/or diagnostic moieties on one particle, due to their large volume-to-surface area ratio (Ho et al., 2021). This can allow for combination therapy using multiple small molecule drugs, antibodies, and/or nucleic acids, along with specific cell targeting using peptides or proteins. The inclusion of labelling moieties, e.g., fluorescent markers or radioactive molecules, is particularly useful when completing cell uptake studies *in vitro* and biodistribution studies *in vivo* (Sato et al., 2020a,b). 3) NPs can also be scaled up efficiently and relatively cheaply with less potential for contamination than viral vectors (Wadhwa et al., 2020). 4) NP incorporation of RNA can prolong its half-life, facilitating increased circulation time *in vivo* (Yoshinaga et al., 2019) and increase its safety profile by shielding it from biological interactions until it is delivered to the intended site of action (Halbur et al., 2019). 5) Careful modification of NP size can also bypass macrophage clearance and enhance cellular uptake of RNA (Xu et al., 2018). 6) Furthermore, NPs have been shown to improve RNA stability. For example, when mRNA was loaded in lipid-like NPs incorporating sucrose, mannitol or trehalose, delivery efficiency was maintained *in vitro* and *in vivo* following storage for at least three months (Zhao et al., 2020). 7) Additionally, functional mRNA delivery with NPs can be effective, inducing protein expression within 1 h of administration (Li et al., 2019) and continuing for up to 48 h (Yasar et al., 2018).

Nanomedicine is a broad field encompassing NPs of vastly different size, shape, and material composition, and there is no "one size fits all approach" when it comes to NP use for RNA delivery (Formicola et al., 2019). Many reviews have discussed the various types of NPs proposed to deliver RNA for therapy (Ickenstein and Garidel, 2019; Schlich et al., 2021; Shen et al., 2019) or detailed the clinical applications of specific RNA NP combinations (Charbe et al., 2020; Li et al., 2019; Lin et al., 2020). However, there is a lack of discussion regarding the methods and approaches that can be used to incorporate RNA into NPs for successful delivery. Here, we detail the major physicochemical modifications that are required to optimize NPs for RNA delivery. This includes an analysis of the stability and efficiency of different techniques for RNA incorporation into NPs. We also examine targeting strategies that prevent off-target accumulation of RNA NPs, thereby increasing drug delivery and subsequent therapeutic efficacy and reducing side effects. Finally, we present methods to create RNA NPs that are efficiently taken up by cells and subsequently released from endosomes for therapy.

2. NP materials and methods of RNA incorporation

The most common materials used for NP formulation in RNA delivery are lipids and polymers (Uchida et al., 2020). Considering the wide variety of materials available, NP formulations should be chosen and altered based on the RNA type to be delivered. For example, NPs containing higher levels of phospholipids and polyethylene glycol (PEG) and lower levels of cholesterol and ionizable lipids have been shown to be more effective for mRNA release than for siRNA release (Hajj and Whitehead, 2017). For mRNA delivery, lipid NPs with a lamellar lipid phase, and faceted and multilamellar structures exhibit increased gene transfection (Eygeris et al., 2020). For siRNA delivery, the replacement of cholesterol with sphingomyelin in solid lipid NPs decreased NP size to 22 nm and enhanced gene silencing activity (Yusuke Sato et al., 2020). In a similar characterization study, a library of cationic multivalent peptide-functionalized polymers was synthesized by simple step-growth polymerization of L-lysine and cystine using 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC) as a coupling reagent. These polymers were tested for maximal RNA delivery following attachment via electrostatic interactions. Numerous factors improved the nanoformulation, including the incorporation of triethylene glycol (TEG) for increased stability and removal of aliphatic side chains which are trapped by endosomes (Yang et al., 2020).

Alongside the NP formulation itself, the method of RNA incorporation can affect the therapeutic outcome. As such, in this next section, we

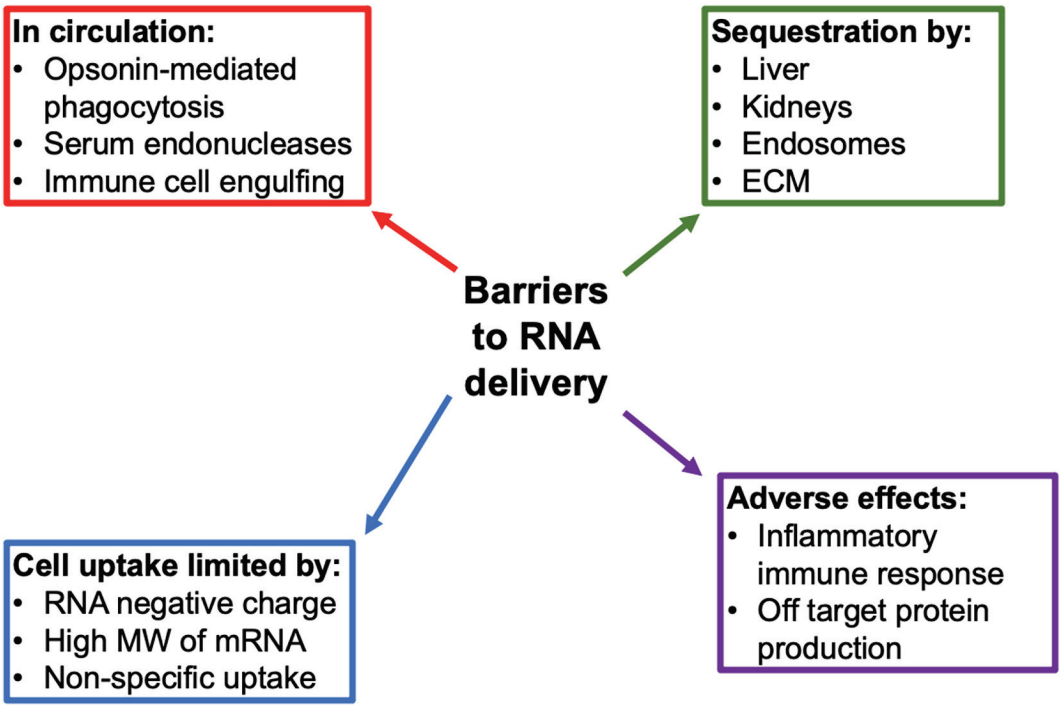

Fig. 1. Barriers to RNA delivery for therapeutic use. ECM: extracellular matrix, MW: molecular weight.

discuss the main methods used: encapsulation within the NP, adsorption to the surface of pre-formed NPs (Fig. 2), and a combination of these methods using the "layer-by-layer" (LbL) approach. Regardless of the method used, free, unincorporated RNA molecules should be removed, although this step is often omitted from the methods in the literature.

2.1. Encapsulation

Similar to the FDA-approved liposome Doxil® (Zhao et al., 2018), RNA molecules are often embedded in lipid- and/or polymer-based NPs. This method, known as encapsulation, can be achieved by multiple methods, most commonly nanoprecipitation or thin lipid film hydration (TLFH) (Fig. 3). The use of cationic components can enhance complexation and improve encapsulation efficiency (Lilavivat et al., 2012; Pattni et al., 2015). In nanoprecipitation, the NP components (lipid and/or polymer) are dissolved in an organic solvent and added to RNA in an acidic aqueous solution while stirring using a magnetic stir bar and stir plate. Hydrophobic and electrostatic interactions between the polar, water soluble, and hydrophilic domains drive the formation of NPs containing RNA in their core which remain suspended in the aqueous solution following evaporation of the organic solvent (Cullis and Hope, 2017; Salvage et al., 2015). TLFH involves dissolving lipids in an organic solvent, which is then evaporated under argon or nitrogen. A lipid film forms, which can be further dried under vacuum and subsequently rehydrated in an aqueous solution containing the RNA molecule, most commonly water or phosphate buffered saline (PBS) (Magro et al., 2018; J. Wang et al., 2020). Notably, vortexing and sonication during rehydration can produce heterogeneous, large multilamellar vesicles (Evers et al., 2018). As such, many researchers use extrusion to control NP size polydispersity (Chen et al., 2018; Szoka and Papahadjopoulos, 1980). However, extrusion is not typically appropriate for NPs with a monolayer, as it can alter their conformation, making them rod-shaped instead of spherical (Chen et al., 2019).

The main advantage of NP encapsulation is the physical protection of RNA from degradation in the serum, thereby extending their half-life *in vivo* (Thomas et al., 2018). NP encapsulation can also condense RNA molecules, thereby overcoming the limited cellular uptake associated with larger molecules, and increase their stability by protecting them from degradation *in vivo* (Workman and Flynn, 2009). Furthermore, siRNA has been shown to remain encapsulated within PEGylated liposomes following incubation in human serum, indicating the stability of this delivery method (Buyens et al., 2009). Examples of successful applications of RNA encapsulation within NPs, are detailed below.

2.1.1. Examples of NPs encapsulating therapeutic RNA

Encapsulation has been used for RNA delivery for multiple therapeutic applications, including siRNA treatment of cancer (Wang et al., 2013; Xu et al., 2014) and mRNA vaccines against Zika virus (Pardi et al., 2017), human immunodeficiency virus (HIV)-1 (Pardi et al., 2017b), H1N1 influenza, Ebola, and Toxoplasma gondii (Chahal et al., 2016). Notably, the only RNA NP formulations that are FDA-approved, or have EUA, use encapsulation to incorporate RNA into lipid-based NPs. Alnylam's Onpattro®, FDA-approved in 2018, is used to treat polyneuropathy associated with hereditary transthyretin amyloidosis (hATTR) (Urits et al., 2020). The formulation consists of the pH sensitive ionizable lipid DLin-MC3-DMA (MC3), cholesterol, 1,2-distearoyl-sn--glycero-3-phosphocholine (DSPC), and a PEG lipid encapsulating siRNA targeting transthyretin, a protein that is predominantly generated by the liver and can be mutated to cause hATTR (Agency, 2018; Buschmann et al., 2021).

In 2020, two vaccines against coronavirus disease 2019 (COVID-19) were produced by Moderna Therapeutics and Pfizer-BioNTech and authorized by the FDA for emergency use. The Moderna vaccine consists of lipid NPs with the ionizable lipid sphingomyelin-102 (SM-102), cholesterol (for stabilization), PEG (to reduce non-specific interactions) and phospholipids (for structure and promotion of intracellular release) that can deliver mRNA-1273 encoding the spike protein of SARS-CoV-2, the pathological virus that causes COVID-19, enabling an effective immune response against the disease (Park et al., 2021). Pfizer-BioNTech uses a similar formulation for their vaccine, replacing SM-102 with the ionizable lipid ALC-0315 from Acuitas and incorporating the mRNA BNT162b2 also encoding for the SARS-CoV-2 spike protein but stabilized by two prolines (Buschmann et al., 2021; Khurana et al., 2021). The benefits of NP-mediated delivery of encapsulated RNA, by protecting RNA from degradation and facilitating cellular uptake, is highlighted in these widely used formulations.

Despite the advantages of RNA encapsulation within NPs, RNA encapsulation can induce large increases in NP diameter which can reduce cellular uptake (Zhang et al., 2021). Certain NP components can also limit the effectiveness of RNA encapsulation. For example, it has been shown that if PEG is present on the inner surface of the inner layer of the liposome bilayer, siRNA encapsulation can be physically inhibited

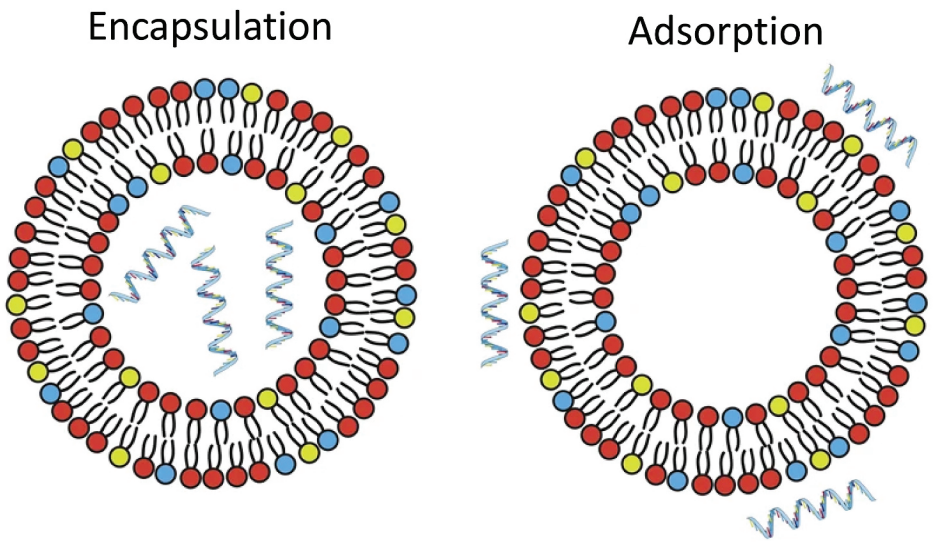

Fig. 2. Schematic of RNA that is encapsulated within NPs (left) vs. adsorbed on the surface of NPs (right), as shown with the example of liposomes. These methods can be used with NPs consisting of a variety of materials, including lipids and polymers. Adapted from (Blakney et al., 2019) under the terms of the Creative Commons CC BY license.

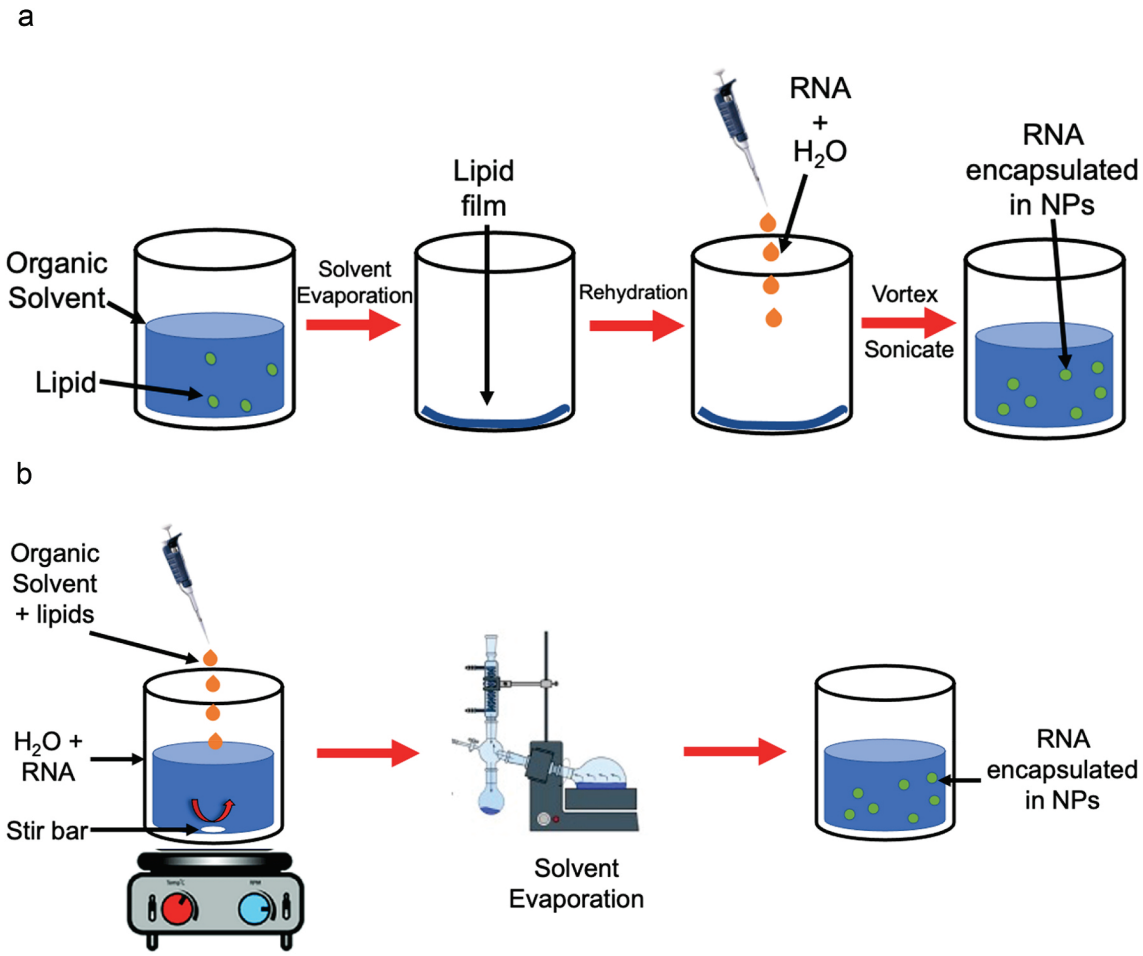

Fig. 3. Schemes showing common methods of RNA encapsulation in lipid- and/or polymer-based NPs. **a)** Thin lipid film hydration. Lipids are suspended in an organic solvent, which is then evaporated under nitrogen flow. Further evaporation can be carried out under vacuum to ensure solvent evaporation. The resulting lipid film is rehydrated with an aqueous solution containing RNA, and then vortexed and sonicated to produce NPs containing RNA in their core. **b)** Nanoprecipitation. An organic solvent containing NP components (lipids and/or polymers) is added dropwise to an aqueous solution containing RNA while stirring. The solvent is evaporated under nitrogen or by using a Rotavapor, leaving an aqueous solution with NPs containing RNA in their core.

as there is no space remaining for the RNA to exist. To avoid this without excluding PEG usage, post-insertion of PEG-lipids can be carried out by slowly adding a PEG-lipid solution to liposomes encapsulating RNA (Nosova et al., 2019). Furthermore, RNA release can be a challenge in these systems as the encapsulated RNA is not released from NPs that do not readily disassemble at the target site (Yasar et al., 2018).

2.2. Adsorption

Given the disadvantages of encapsulated RNA in terms of limited release and potential size increases, RNA incorporation into NPs by other methods has been explored. The most common alternative to encapsulation is RNA adsorption to the NP surface. RNA is strongly negatively charged, and hence, RNA can be adsorbed onto the surface of preformed cationic NPs through electrostatic interactions or combined with NP components to form lipoplexes or polyplexes in a relatively facile manner. Adsorption is normally carried out *via* room temperature or 4 °C incubation of pre-formed NPs with RNA while shaking (Chen et al., 2018). Incubation time varies according to publication but one study based on asRNA binding to PLGA NPs showed that a length of incubation up to 60 min did not affect the size or zeta potential of the NPs, possibly due to maximal absorption rapidly occurring with the first 15 min of incubation (Nafee et al., 2007). However, differences in adsorption rates between different RNA and NP types should be considered when establishing a protocol for formulation, in order to optimize the length of incubation time for optimal RNA adsorption. For example, double stranded RNA cannot uncoil and expose its bases which led to slower adsorption rates to gold NPs compared to single-stranded molecules (Li and Rothberg, 2005). This study also showed that long single-stranded RNA adsorbed more slowly, possibly due to secondary structure formation or limitations in flexibility related to increased length. Adsorption ability has also been related to NP size, with the amount of adsorbed RNA decreasing with increased mesoporous silica (MPS) NP size, from 249 μg/mg of MPS on 50 nm NPs, to 93.2 μg/mg of MPS on 300 nm NPs due to differences in surface area-volume ratio (Hikosaka et al., 2018).

A major advantage of surface adsorption of RNA is that it can reduce the overall charge of cationic NPs, which are known to induce cytotoxicity. Adsorption of both single and double-stranded RNA to gold NPs decreased their net charge density and prevented NP aggregation (Li and Rothberg, 2005). In a direct comparison, self-amplifying RNA decreased the positive charge of lipid NPs when adsorbed to, but not encapsulated in, the NPs (Blakney et al., 2019). This cation quenching is used as an indication of successful RNA adsorption to the NP surface.

Another advantage of NP RNA adsorption is that it does not significantly change the NP size as demonstrated with asRNA and chitosan-coated PLGA NPs (Nafee et al., 2007), and ribosomal RNA and copolymer-based NPs (Hernández et al., 2019). In fact, mRNA adsorption has actually been shown to reduce the size of cationic or ionizable lipid/polymer based NPs due to compaction and condensation following

electrostatic interactions between the RNA molecule and flexible NPs (Blakney et al., 2019).

2.2.1. NP materials to facilitate RNA adsorption

The electrostatic mechanism of adsorption limits the materials that can be used for NP formulation, precluding those that are anionic at physiological pH, such as PLGA and phosphatidylserine (Kimura et al., 2021). Cationic lipids, including 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), dimethyldioctadecylammonium (DDA), N-[1-(2, 3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), and DC-cholesterol are commonly used to facilitate RNA adsorption (Guevara et al., 2020; Gómez-Aguado et al., 2020). Although DOTAP NPs have a similar charge to DDA NPs, the former are larger, possibly due to their less rigid conformation, and are therefore less likely to be taken up by cells (Anderluzzi et al., 2020). Such changes in size can limit the use of certain cationic elements in NP formulation and requires investigation of alternative methods to increase the cationic charge of NPs for RNA attachment, as detailed below.

NPs with insufficient positive charge can be coated with cationic elements, such as polyethylenimine (PEI) to promote electrostatic interactions. Cationic lipids have been used to coat calcium phosphate NPs to successfully deliver siRNA against programmed cell death protein (PD)-1 and programmed death ligand (PD-L)1 in order to increase the toxicity of tumor-infiltrating lymphocytes (Wu et al., 2019). Dimethyl-di-octadecyl-346 ammonium (DDAB), a cationic surfactant widely used in NP formulation, has been incorporated into liposomes using TLFH to increase their positive charge and deliver anti-miRNA 155 delivery *in vivo* to treat acute kidney injury (Chen et al., 2018).

Chitosan, a cationic linear polysaccharide derived from the exoskeleton of shellfish, has been used to coat PLGA NPs in order to increase their zeta potential from −10 mV to 17.1 mV (Nafee et al., 2007). In this study, Nafee et al. showed that increased chitosan concentration on the NP surface directly correlated with increased zeta potential measurements. The coating enabled successful adsorption of an asRNA inhibiting telomerase to the NP surface and subsequent uptake by A549 human lung carcinoma cells. However, it should be noted that the chitosan coating also increased the NP size, a factor which may influence the extent of cellular uptake.

Notably, RNA adsorption onto an anionic molecule has also been attempted using human serum albumin (HSA) NPs (Wen et al., 2017). HSA is anionic at physiological pH due to its isoelectric point (pI) of 4.7. However, Wen et al. adjusted an HSA solution to pH 4.0, under which conditions the plasma protein becomes cationic. The HSA was then complexed with RNA and heated at 75 °C for 15 min. This method led to the formation of 110 nm NPs incorporating RNA that showed high uptake in HeLa cells.

In addition to polymers and proteins, cationic peptides with arginine-rich sequences (Lee et al., 2015), including the protamine family derived from fish sperm, have been extensively used to promote RNA cell uptake *via* co-incubation *in vitro* (Amos, 1961) and by incorporation into NPs to enhance RNA attachment. Aside from lipid based NPs, protamine-based complexes are the most used delivery tools for mRNA applied in clinical trials, in part due to their added benefit of protecting RNA from degradation *via* condensation (Gómez-Aguado et al., 2020), and have also been incorporated into liposomes for enhanced RNA attachment (Hoerr et al., 2000). For example, Wang et al. incubated protamine with mRNA encoding herpes simplex virus1-thymidine kinase, an anti-cancer suicide gene. This complex was then incubated with preformed DOTAP/cholesterol liposomes, resulting in efficient mRNA loading (7.6 μg mRNA/μmol liposome). Systemic administration of this formulation significantly inhibited tumor growth in a H460 xenograft mouse model of lung carcinoma, indicating that protamine complexation on NPs is an effective way to deliver functional mRNA (Wang et al., 2013).

2.2.2. Examples of NPs with adsorbed therapeutic RNA

Adsorption to many NP types has consistently been shown effective at delivering siRNA (Cui et al., 2017; Huang et al., 2018; Leng et al., 2020; Li et al., 2016; Xie et al., 2016), mRNA (Grabbe et al., 2016; Leng et al., 2020) and miRNA (Chen et al., 2018), and inducing a therapeutic effect *in vivo*. For example, multifunctional polymeric NPs incorporating PEG, chitosan-polyamine and lipoic acid produced by nanoprecipitation was used to adsorb siRNA targeting Enhancer of Zeste Homologue 2 (EZH2). *In vitro,* these NPs exhibited intracellular drug release in luc-A549 cells and protected therapeutic siRNA from degradation by nucleases in serum for 4 h. *In vivo,* these NPs were administered intravenously (IV) five times every other day to an orthotopic lung tumor mouse model using luc-A549 cells. This treatment led to successful downregulation of EZH2 mRNA and protein in the tumor, thereby inhibiting tumor growth and metastasis development compared to PBS control (Yuan et al., 2017). Similarly, cationic liposomes consisting of DDAB, cholesterol and DSPE-PEG were created using TLFH. Pre-made liposomes were then incubated with anti-miRNA-155 for 12 h at 4 °C to facilitate adsorption, and centrifugation was used to remove free RNA. Mice with acute lipopolysaccharide-induced kidney injury were treated with one dose of the NP-RNA formulation or PBS and sacrificed after 12 h. Compared to the control, the NP-RNA formulation significantly decreased miRNA-155 expression and inflammatory cell infiltration in the kidneys (Chen et al., 2018). These studies demonstrate that adsorption is a feasible approach for RNA attachment to NPs and subsequent administration *in vivo* for therapy.

Though adsorption has been shown to overcome some of the pitfalls of encapsulation as an RNA incorporation approach, there are still limitations to this approach. Most notably, NP material choices are limited as a cationic charge is essential to facilitate electrostatic interactions with anionic RNA. Potential materials that can be used to overcome this obstacle and successfully facilitate RNA adsorption to NPs are discussed in section 2.5.

2.3. Layer-by-layer approach

Some formulations exploit electrostatic adsorption in order to encapsulate RNA molecules using a LbL approach, where sequential immobilization of RNA and cationic lipids are used to create a multilayered NP. A NP core is usually incubated with the desired component to create another layer. After centrifugation to remove any free molecules, incubation is repeated with another component until multilayer particles are produced. This method combines the ease of simple adsorption with the benefit of using multiple layers to optimize and increase RNA loading to the NP, while also protecting the RNA from degradation (Gu et al., 2017). This method has been successfully used to attach siRNA to gold NPs surrounded by layers of PEI (Fig. 4) (Elbakry et al., 2009). LbL NPs also successfully prevented both luciferase and green fluorescent protein (GFP) mRNA degradation, which are common models used to assess mRNA delivery capability. These NPs had low toxicity and high transfection rates *in vitro* in dendritic cells (DCs) (Lacroix et al., 2020). Two types of LbL NPs have been used to deliver miRNA-34a to triple-negative breast cancer cells. Spherical PLGA cores surrounded by alternating layers of poly-L-lysine (PLL) and miRNA-34a successfully suppressed four target genes *in vitro*, resulting in reduced cell proliferation (Kapadia et al., 2020). Negatively-charged gold NPs were also coated with alternating layers of PLL and miRNA-34a and exhibited cellular uptake, target gene inhibition and suppression of cell proliferation (Goyal et al., 2018). Furthermore, this LbL formulation was more effectively internalized than simple NPs consisting of the miRNA bound to PLL. These studies highlight the wide variety of NP types and surface layers that can be exploited for RNA incorporation and therapeutic delivery.

The major disadvantage of the LbL approach is its complex methodology compared to simple encapsulation or adsorption. Furthermore, a stable NP core that can be centrifuged is essential to ensure removal of

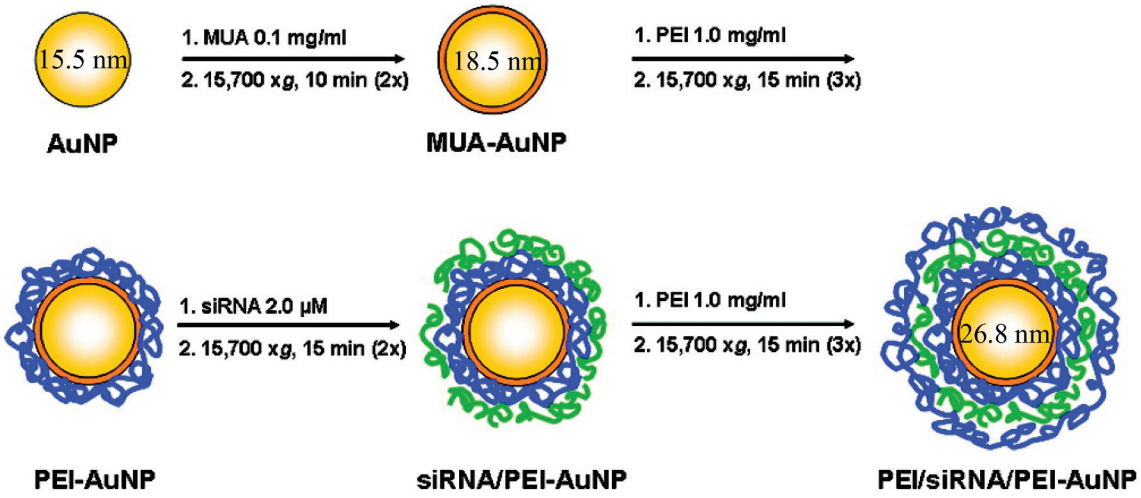

Fig. 4. Scheme indicating the layer-by-layer formation of NPs for siRNA delivery, adapted with permission from (Elbakry et al., 2009). Copyright 2009 American Chemical Society. Gold NPs (AuNP) were coated with 11-mercaptoundecanoic acid (MUA) to facilitate binding of the subsequent layers. Poly(ethylene imine) (PEI; molecular weight = 25 kDa) was then added to create a positive surface for siRNA to be attached. Finally, an outer layer of PEI was added to protect the siRNA from premature release or degradation in the serum. The hydrodynamic diameter of the NPs at some steps are indicated in nanometers. 1. indicates the compound and its concentration when added to form the next layer, and 2. indicates the centrifugation settings used to remove any unbound material after each 30 min incubation.

free molecules at each step of layer attachment. Additionally, the material choice for each layer must be precise to enable electrostatic binding of RNA. This complexity may limit the translatability and reproducibility of this approach.

2.4. Other approaches for RNA attachment

A slightly different encapsulation approach was achieved in a study using peptide amphiphile micelles by incorporating covalent binding of miRNA (Chin et al., 2020). Thiolated miRNA-145 was conjugated to DSPE-PEG(2000)-maleimide and the resulting conjugates were used to form micelles *via* TLFH. This covalent bond protected miRNA from nuclease degradation but was readily cleaved at cytosolic glutathione levels after cell internalization *in vitro*. This system was successfully applied to a mouse model of atherosclerosis, where lesion growth was reduced by 49 %. Similar approaches have been used to create polymer conjugates *via* terminal nucleotide modification of siRNA to facilitate coupling to PEG, poly(lactic-co-glycolic acid) (PLGA), hyaluronic acid (HA), and dextran, among others, which are then used to make NPs (Hong and Nam, 2014). Thiolation of RNA molecules, including siRNA targeting the *bicoid* gene, for attachment to silver or gold NPs has also been indicated as an effective way to facilitate stable RNA loading and subsequent delivery (Aali et al., 2020; Yamankurt et al., 2020).

2.5. Choosing the best method for RNA incorporation

The decision to use a specific RNA loading method must consider the biological barriers that the NP will encounter. For example, encapsulation may not be the optimal choice for inhalable formulations if the overall charge of the final product is positive. The negatively charged airway surface liquid may interact with such positively charged NPs, causing them to disassemble or aggregate over time (Yıldız-Peköz and Ehrhardt, 2020). Additionally cationic NPs tend to be cytotoxic, though the overall positive charge may be shielded by the attached RNA (Lin et al., 2020). Differences in RNA loading, in terms of physical clustering on the NP surface or in terms of absolute quantity of RNA incorporated, may be achieved depending on which method is used and should be taken into account when designing the NP delivery vehicle (Corti et al., 2020; Cox et al., 2019).

Multiple studies have been carried out comparing the efficacy of RNA delivery when adsorbed to or encapsulated within NPs. siRNA adsorbed to preformed PLGA NPs was shown to be more effective than encapsulation by producing NPs that were more monodisperse, smaller than 100 nm, and with high RNA association (Ceylan et al., 2020). Furthermore, the adsorbed formulation was able to silence the target GPR-87 gene in HEK 293 T cells >40 times more effectively than the encapsulated siRNA, possibly due to the limited release of encapsulated RNA. In a study directly comparing a LbL NP incorporating mRNA and mRNA adsorbed to pre-made NPs, both formulations showed low toxicity *in vitro* in DCs, and complete protection of mRNA degradation in serum. Although both formulations showed successful transfection of model GFP and luciferase mRNA, the LbL approach had superior efficiency, emphasizing the importance of comparing multiple approaches for each RNA type (Lacroix et al., 2020). However, it is important to note that adsorption alone is often a simpler and faster process than LbL or encapsulation, so the advantages and disadvantages must be carefully weighed before choosing an approach for any given NP-RNA combination.

Conversely, although adsorption has been extensively shown to stabilize and protect RNA from degradation, one study found that surface bound siRNA degraded rapidly (1 h) in serum compared to siRNA encapsulated in dendrimer-based NPs, which was stable for >24 h (Raval et al., 2019). Additionally, a recent study directly compared four cationic NP types (liposomes, solid lipid NPs, polymeric NPs, and nanoemulsions) in their ability to deliver an mRNA vaccine when the mRNA was either encapsulated or adsorbed in order to evaluate which incorporation method was best for *in vivo* applications (Anderluzzi et al., 2020). All formulations contained DOTAP or DDA. The authors show that mRNA encapsulated within DOTAP polymeric NPs and liposomes along with DDA liposomes, evoked the highest antigen expression *in vitro* in BHK cells compared to NPs with mRNA adsorbed to the surface, potentially by more effectively preventing RNA degradation by nucleases, though this hypothesis was not further tested by the authors. The superior efficacy of encapsulated RNA was verified *in vivo,* with mRNA encapsulated in DOTAP polymeric NPs showing the highest efficacy in BALB/c mice. Similarly, self-amplifying RNA encoding HIV-1 Env gp140 as a model antigen was either adsorbed or encapsulated in solid lipid NPs (Blakney et al., 2019). The authors found that RNA was equally protected from RNAse degradation *in vitro* and both methods resulted in functional delivery and antibody protection *in vitro* and *in vivo*. Based on the literature, the differences in efficacy of adsorption vs. encapsulation as RNA incorporation methods into NPs vary broadly depending on the

combination of RNA type and NP formulation used, indicating that multiple approaches should be attempted in the initial characterization of such delivery vehicles to optimize each formulation for RNA therapy.

Examples of methods used for RNA loading into NPs are provided in Table 1. However, it should be noted that many publications do not include sufficient information to determine initial RNA concentrations or final RNA loading amount. Specifically, many studies report the initial amount of RNA used for loading but do not consider the unincorporated RNA in the final nanoformulation. The discrepancy between actual vs. reported RNA amount can hinder translational potential and must be more precisely reported for reproducibility. In addition, the terms "encapsulation" and "adsorption" are often misused in the literature. Many authors describe RNA as being encapsulated in NPs, but their methods and characterization results indicate that they are in fact adsorbed on the surface following incubation with pre-formed NPs. When reporting techniques and methods of RNA incorporation in NPs and characterization of the resulting complexes, nomenclature clarity along with standardized methods of RNA loading will be essential to apply these technologies toward the clinic.

3. Targeting

The most successful NP-based therapies used in the clinic, Doxil® and the COVID-19 vaccines from Pfizer-BioNTech and Moderna Therapeutics, do not use active targeting strategies. Doxil® relies on the enhanced permeability and retention (EPR) effect, a hypothesis postulating that NPs tend to accumulate in tumor tissue due to alterations in blood and lymph flow and dysregulation of the endothelial lining of tumor cells (Shi et al., 2020). In addition, current COVID-19 vaccines under EUA do not target a specific cell type. Instead, mRNA translation occurs in local cells, including epithelial cells, adipocytes, myocytes, fibroblasts, keratinocytes, and immune cells, at the intramuscular injection site (Blakney et al., 2021; Iavarone et al., 2017; Tan et al., 2020) or are cleared to the liver, where translation occurs. The resulting proteins are then released into the circulation to interact with immune cells that produce antibodies (Guevara et al., 2020). Generally, vaccines do not require targeting, as mRNA translation of antigens can occur in any cell type and subsequently induce antibody production in local immune cells.

Most RNA therapies, however, will require delivery to a specific cell type for therapeutic effect, particularly for protein replacement therapies. Based on this requirement, a staggering number of non-targeted RNA NPs in clinical trials have failed due to insufficient delivery to the target site (Herrera et al., 2018). Therefore, NP modification to enable specific targeting should be considered for RNA NP formulations. (Chu et al., 2016; Huang et al., 2016; Tietjen et al., 2017). It has been established that nonspecific expression/suppression of protein production by RNA can have detrimental effects, as shown with factor VII, factor IX, and interleukin-12 (Dammes and Peer, 2021). For this reason, targeted NPs have been employed to ensure precise delivery of RNA therapeutics to the desired cell type in a variety of disease states, e.g., to tumor cells for cancer treatment (Fig. 5) (Ho et al., 2021). It is important to note that targeting can be affected by both the disease and age states, due to dysregulation of specific receptors and/or biological barriers (Magro et al., 2018). Therefore, it is essential to use *in vitro* and *in vivo* models incorporating as many relevant variables as possible, including pathology and aging, when validating targeted RNA nanotherapeutics. Here we describe some targeting moieties that have been used for specific delivery of NPs incorporating RNA therapeutics, along with modifications in NP structure and administration route for targeted RNA delivery. Although the list of potential targeting agents and modifications is extensive, we highlight some of the most common strategies used, along with recent, novel, and promising contributions to the field of RNA NP targeted delivery primarily developed in the last five years.

Table 1
Examples of NPs and methods used for RNA loading.

NP Components	Type of RNA	μg of RNA/ mg of NP	Method of Incorporation	Reference
Gold	siRNA	7×10^8	Adsorption	Yamankurt et al. (2020)
CTAB	HIV TAR RNA, U4 snRNA	55.5	Encapsulation	Workman & Flynn (2009)
DOTAP, cholesterol (1:1 ratio), DSPE-PEG, DSPE-PEG-AA	siRNA	103.4	Encapsulation	Wang et al., 2013, Xu et al. (2014)
ionizable cationic lipid (proprietary to Acuitas), phosphatidylcholine, cholesterol, PEG-lipid (50:10:38.5:1.5 ratio)	mRNA	50	Encapsulation	Pardi et al., 2017, Pardi et al., 2017
Stöber silica	RNA	333	Adsorption	Hikosaka et al. (2018)
DOTAP, DOPC, cholesterol (2:1:3 ratio)	siRNA	92.6	Adsorption	Wu et al. (2019)
Human serum albumin	RNA	33	Adsorption	Wen et al. (2017)
DOTAP, cholesterol (1:1 ratio), DSPE-PEG, DSPE-PEG-AA	mRNA	215.5	Adsorption	Wang et al. (2013)
mPEG-bPEI-PAsp(DIP-BzA), simvastatin	siRNA	92.6	Adsorption	Huang et al. (2018)
DSPC, POPG, Cholesterol) (7:2:1 ratio)	siRNA/ miRNA (combined together)	66.2	LbL	Gu et al. (2017)
Gold, PEI	siRNA	24.4	LbL	Elbakry et al. (2009)
PLGA, PLL	miRNA	56.6	LbL	Kapadia et al. (2020)
Gold, silica, PLL	miRNA	35.4	LbL	Goyal et al. (2018)
PLGA	siRNA	8.5×10^{-4}	Encapsulation	Ceylan et al. (2020)
DDA bromide, DOTAP	Self-amplifying RNA	1.2×10^4	Encapsulation and adsorption	Blakney et al. (2019)

3.1. Types of targeting moieties

3.1.1. Peptides as targeting moieties

Targeting is commonly achieved *via* incorporation of peptides that specifically bind to a receptor that is highly expressed on the target cell compared to off-target cells. The most common method for peptide incorporation into RNA delivery vehicles exploits maleimide-thiol reactions *via* conjugation of lipids/polymers or NPs with an attached maleimide to peptides containing a free cysteine. This covalent binding is generally accepted to be stable *in vitro* and *in vivo* (Wang et al., 2017). For example, two peptide sequences (REKA, VHPKQHR) targeting fibrin and vascular cell adhesion protein (VCAM-1) respectively were incorporated into micelles for targeted delivery of anti-miRNA to treat atherosclerosis. This peptide-based targeting successfully promoted specific uptake by macrophages and inflamed endothelial cells, and facilitated the functional inhibition of miRNA-33a and miRNA-92a (Kuo et al., 2014). Angiopep-2 peptide was effectively used to target low-density lipoprotein receptor-related protein (LRP), which is overexpressed on both the blood brain barrier (BBB) and glioblastoma multiforme (GBM) cells. This dual targeting by a single peptide promoted BBB crossing and specific tumor delivery of TGF-ß siRNA (Qiao et al., 2018). Tumor-associated macrophages have been targeted using a-peptide, a scavenger receptor B type 1 targeting peptide, and M2pep, an M2 macrophage binding peptide, to deliver siRNA against

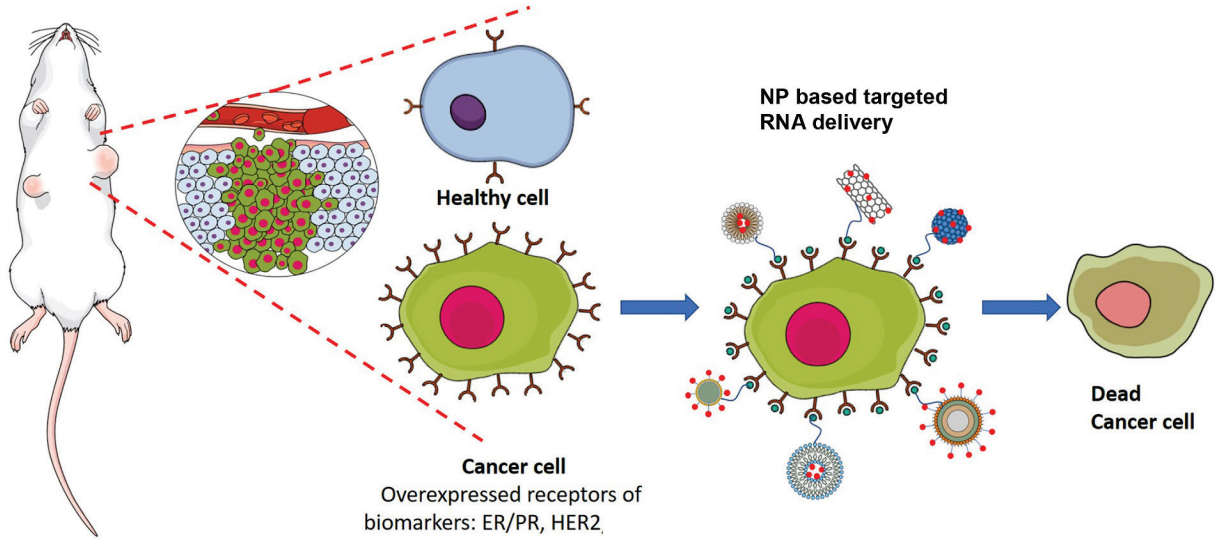

Fig. 5. Scheme showing specific targeting of cancer cells by NPs, adapted with permission from (Liyanage et al., 2019). Cancer cells can be specifically targeted as they overexpress receptors such as the estrogen receptor and progesterone receptor (ER/PR) and human epidermal growth factor receptor (HER2) compared to healthy cells. Ligands for these receptors can be attached to NPs containing RNA, facilitating their preferential uptake and therapeutic effects in cancer cells vs. healthy cells.

anti-colony stimulating factor-1 receptor. This formulation eliminated macrophages by 52 % from melanoma in mice (Qian et al., 2017). The same M2pep was used to deliver gold NPs with anti-vascular endothelial growth factor (VEGF) siRNA to treat lung cancer in mice (Conde et al., 2015). Vascular smooth muscle cell plaque targeting has been achieved using micelles incorporating CCR2-binding peptides for delivery of miRNA-145 (Chin et al., 2020). This platform prevented lesion growth by ~50 % in a mouse model of early-stage atherosclerosis. The wide variety of biological peptides identified, along with the extensive use of phage display technology to identify synthetic peptides that preferentially bind to specific receptors, makes peptide incorporation a versatile and comprehensive targeting strategy.

3.1.2. *Proteins as targeting moieties*

Whole proteins, including antibodies, can also be applied for cell targeting, although their effective use is limited by their size. The transferrin receptor (Tfr) is commonly used for specific delivery due to its upregulation in multiple disease states. For example, transferrin was conjugated to PEI for selective siRNA delivery to activated T cells in the asthmatic lungs of mice (Xie et al., 2016). Apolipoprotein A-I (ApoA-I) has been used for liver targeting of siRNA to treat hepatitis B-infected mice (Kim et al., 2007).

Monoclonal antibody incorporation on the NP surface has been used for targeted delivery of RNA to leukocytes (Peer et al., 2008), DCs (Zheng et al., 2009), and $CD4^+$ T cells (Ramishetti et al., 2015). Peer et al. covalently attached anti-$ß_7$ integrin mAbs to hyaluronan on unilamellar vesicles which encapsulated protamine-condensed siRNA targeting cyclin D1. This antibody incorporation facilitated $ß_7$ integrin specific leukocyte uptake of NPs, thereby inducing an anti-inflammatory effect in mice with dextran sodium sulfate-induced colitis (Peer et al., 2008). siRNA has also been embedded in polyelectrolyte layers coating a polymeric core, with an outer layer of CD20 antibodies conjugated to HA to facilitate dual targeting of blood cancer cells (Choi et al., 2019). This system successfully suppressed B-cell lymphoma (BCL)2 protein expression and cell proliferation *in vitro* and *in vivo*. These studies demonstrate that whole proteins can be used for targeting, despite their larger size compared to peptides.

3.1.3. *Other targeting moieties*

Other targeting moieties have shown some success *in vitro* and *in vivo*. Dual targeted chitosan NPs were developed to deliver siRNA targeting P21-activated kinase 1, which is overexpressed in many cancers, to hepatocellular carcinoma cells (Zheng et al., 2020). The targeting ligands used were: 1) Lactobionic acid, the galactose of which binds the asialoglycoprotein receptor. This receptor is overexpressed on the hepatocellular carcinoma cell membrane. 2) Glycyrrhetinic acid, which binds to the glycyrrhetinic acid receptor. This receptor is overexpressed on hepatocytes, particularly hematoma cells. These NPs enhanced both targeting and cell uptake in BALB/c nude mice bearing Hep3B tumors compared to non-targeted NPs. Folate conjugation to NPs has also been used to deliver functional siRNA to GBM, as folate receptors are commonly overexpressed in cancer cells in order to promote DNA replication (Yoo et al., 2021). Additionally, receptors for mannose, a sugar monomer, on DCs have been targeted *in vivo* in the development of anti-cancer vaccines (Mockey et al., 2007; Perche et al., 2011; Pichon and Midoux, 2013), as such targeting has been shown to increase internalization into the target cell (Pei et al., 2021).

3.2. *Modifying NP structure for targeting*

Targeting can also be achieved by altering the NP structure itself. For example, lipid NPs incorporating the helper lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were shown to preferentially interact with ApoE and thereby accumulated in the liver, while those with DSPC targeted the spleen (Zhang et al., 2020). The DOPE formulation doubled firefly luciferase mRNA delivery to the liver and increased liver transfection three-fold in mice. The DSPC-based NPs showed a similar increase in delivery of the same mRNA as well as Cy3-siRNA. Interestingly, in vaccine development, it has been shown that a cationic charge is essential to retain NPs at the injection site, prolonging interaction with innate immune cells and subsequent success of mRNA transfection and translation (Henriksen-Lacey et al., 2010). This knowledge can be used to develop more effective vaccines by using encapsulation to maintain a cationic surface charge on NPs.

3.3. *Administration routes for targeting*

NP administration route, which has often been shown to determine NP localization, can also be used to optimize RNA therapeutic targeting. RNA NPs are most frequently injected IV in preclinical studies, with injection site varying from the tail vein in mice for systemic administration to subretinal injection for targeted treatment of monogenic

retinal degeneration (Patel et al., 2019) or glaucoma (Dillinger et al., 2018). In a comprehensive study, the most efficient renal localization occurred when NPs were administered IV in the tail vein, compared to retro-orbital vein injection, oral gavage or injection in the subcutaneous flank or intraperitoneal regions of mice (Williams et al., 2017). The same study showed that dosage affected targeting ability, with the administration of a decreased NP dose corresponding to decreased non-specific organ accumulation. The use of alternative administration routes not only offers hope for more targeted and effective RNA delivery, but could also allow for safer, more convenient treatment and better patient compliance (Eek et al., 2016).

For lung diseases, intranasal and inhalable RNA NPs have been exploited to treat respiratory conditions including asthma, chronic obstructive pulmonary disease (COPD), and lung cancer (Gómez-Aguado et al., 2020; Kankala et al., 2018; A. K. Patel et al., 2019; Xu et al., 2018; Xu et al., 2021). Lung cancer treatment has been investigated using NPs that co-deliver the anti-cancer drugs doxorubicin and cisplatin, and two types of siRNA to suppress multidrug resistance-associated protein (MRP)-1 and BCL2 mRNA (Taratula et al., 2011). Inhalation of this formulation led to preferential accumulation in the mouse lungs, with minimal escape into the systemic circulation and limited deposition in off-target organs. Encapsulation is particularly useful when creating inhalable formulations, as this method can protect the integrity of the RNA molecule during the drying process (Xu et al., 2018). For example, encapsulation within solid lipid NPs protected the structure and function of tumor necrosis factor (TNF)-α siRNA following thin-film freeze-drying to create aerosolizable dry powders for lung delivery to treat a variety of respiratory illnesses including asthma (Wang et al., 2021). Furthermore, mannitol can be used for inhalable RNA NP optimization, as it has been shown to enhance stability of siRNA-lipid-polymer NP complexes, possibly by replacing water as a hydrogen-bonding agent during lyophilization and acting as a barrier *via* matrix formation to prevent aggregation (Dormenval et al., 2019).

Transdermal delivery of NPs containing RNA has been achieved using multiple methods. Mesoporous silica NPs were coated with siRNA targeting transforming growth factor-ß receptor-1 (TGFßR-1) and PLL, mixed with moisturizer and topically applied on a squamous cell carcinoma tumor in a mouse xenograft model (Lio et al., 2019). Following treatment every three days for 18 days, tumor growth was not significant. This was compared to the application of a scrambled siRNA sequence which resulted in a three-fold increase in tumor size. Similarly, gold NPs with covalently attached siRNA designed to knock out epidermal growth factor receptor (EGFR), were mixed with the petrolatum-based moisturizer Aquaphor® and topically applied on SKH1-E hairless and C57BL/6J mice. This siRNA was chosen as a model due to its importance in epidermal cell proliferation, and its potential for use in cancer, where it is often overexpressed. The attached siRNA could penetrate human epidermal keratinocytes *in vitro*, and reduced EGFR mRNA levels by 52 % after three week treatment (three applications per week) (Zheng et al., 2012). Alternatively, microneedle patches consisting of a HA matrix with mesoporous silica-coated NPs have been used for TGFßR-1 siRNA delivery through the stratum corneum (Wang et al., 2020).

Along with non-invasive transdermal delivery, oral delivery of RNA has been investigated using many NP platforms, including mannose-modified trimethyl chitosan-cysteine (MTC) polymers (He et al., 2020) and lipidoid NPs (Ball et al., 2018). Iqbal et al. demonstrated the importance of surface charge when administering RNA within NPs by developing PLGA-PEG NPs coated with amine group-containing lipids with varying surface charges to deliver TNF-α siRNA. The aminated NPs, but not carboxylated or neutrally-charged ones, significantly accumulated in the inflamed colons of mice and suppressed mRNA expression and TNF-α secretion (Iqbal et al., 2018).

Although incorporation of active targeting moieties and optimization for administration route can increase the amount of RNA delivered in NPs to the target site, modifications are still required to ensure adequate cell uptake. Subsequent endosomal escape is also essential for NPs to reach the cytosol where protein translation/silencing occurs. Below, modifications to enhance both cellular uptake and endosomal escape are discussed.

4. Cellular uptake and endosomal escape

Intracellular delivery is vital for successful protein translation or silencing, and NP modifications can ensure effective penetration of the cell membrane. There are various mechanisms of cellular uptake of RNA lipid NPs, with clathrin-dependent endocytosis and micropinocytosis being the most common. NPs taken up by the latter pathway show higher efficacy (Evers et al., 2018; Gilleron et al., 2013; Wang and Huang, 2013). Clathrin- and caveolin-dependent endocytosis are both receptor-mediated, thereby allowing targeting and promotion of cellular uptake *via* appropriate ligand incorporation on the NP surface (Donahue et al., 2019). Alternatively, NPs can be internalized by immune cells such as macrophages, neutrophils, and DCs by phagocytosis (Ahmad et al., 2019; Donahue et al., 2019). In this case, NPs must bind to a phagocytic cell surface receptor, e.g., mannose receptor, before being internalized in phagosome vesicles (Donahue et al., 2019).

Along with poor cellular uptake, a major factor limiting the efficacy of NP-based RNA therapies is insufficient endosomal escape into the cytoplasm. Following internalization, only NPs that can escape the endosome are able to exert their intended therapeutic effects, making it an essential aspect of NP formulation (Donahue et al., 2019; Martens et al., 2014). In this section, we will discuss the incorporation of cell penetrating peptides (CPPs) on NPs to enhance cellular uptake, along with methods to promote endosomal escape and increase the efficacy of RNA therapeutics.

4.1. Cell penetrating peptides

Along with targeting, peptides are commonly used to promote cellular uptake. CPPs can disrupt the lipid bilayer, or enhance micropinocytosis by triggering the grouping of anionic glycosaminoglycans on the cell surface (Vallazza et al., 2015). As most CPPs are positively charged, they have the added benefit of condensing mRNA, allowing for more effective delivery. The HIV-1 transactivator of transcription derived (TAT) peptide is a well characterized and widely used CPP. When conjugated to lipid NPs, siRNA uptake increased in the mouse lung *in vivo*, knocking down p38 MAP kinase mRNA by 30–45 % after 6 h (Moschos et al., 2007). The CPP Hph1 was conjugated to PEG-siRNA with KALA peptides to increase cellular uptake and subsequent gene silencing *in vitro* (Choi et al., 2010). KL4 can mediate siRNA and mRNA cell transfection *in vitro* in A549 human lung epithelial cells and BEAS-2B human bronchial epithelial cells (Qiu et al., 2017, 2019, 2020). The cationic CPPs RALA, LAH4 and LAH4-L1 have been used to facilitate mRNA adsorption to PLA NPs and to promote cellular uptake in DCs *in vitro* (Coolen et al., 2019). The amphipathic RALA peptide has also been used in vaccine formulation to condense mRNA into nanocomplexes capable of escaping endosomes and inducing an immune response (Udhayakumar et al., 2017). If insufficient cellular uptake of RNA NPs is observed in *in vitro* studies, incorporation of a well-established CPP should be considered.

4.2. NP modifications to promote endosomal escape

After NPs are internalized through endocytosis into an early endosome, the endosome can mature from an early to late stage endosome, during which acidification can occur (Donahue et al., 2019; Martens et al., 2014). During acidification, ATP-dependent proton pumps transport hydrogen ions into the endosomes, thus lowering the pH inside the vesicle. Due to the relatively negative charge of the phospholipids comprising the outer layer of the endosomal membrane, interaction with the sudden influx of positively charged hydrogen ions causes the

endosomal membrane to destabilize (Martens et al., 2014). During this destabilization period, the NP may be able to escape from the endosome or the endosome may fuse with a lysosome (Donahue et al., 2019; Martens et al., 2014). NPs and their internal constructs may become degraded in these lysosomes, recycled in the perinuclear region, or exocytozed from the cell. Those that are capable of escaping the lysosome are then able to exert their intended therapeutic effects.

Many researchers have added surface modifications that can destabilize the endosomal membrane or have taken advantage of pH changes during endosomal maturation to enhance endosomal escape of NPs containing therapeutic RNA (Fig. 6). Such modifications are described below.

4.2.1. *Membrane disrupting modifications*

Membrane destabilizing modifications target the transformation stage from a late endosome to a lysosome. At this stage, the membrane is naturally unstable, and the endosomal membrane can be disrupted through pore formation, rupture, or fusion (Fig. 7). Late endosome instability can be exacerbated through intelligent NP design, most commonly with the use of cationic polymers and lipids to form pores or rupture the endosomal membrane (Martens et al., 2014). For example, PEI has a large number of protonable amine groups and can act as a "proton sponge" with a high buffering capacity (Boussif et al., 1995). The abundant binding of cations to the surface of PEI leads to an influx of chloride ions and water into the endosome, inducing endosome swelling and rupture (Martens et al., 2014). Through this mechanism, PEI NPs successfully delivered siRNA to treat GBM *in vitro* as well as miRNA to treat prostate cancer both *in vitro* and *in vivo* (Melamed et al., 2018; Zhang et al., 2015). While PEI can efficiently disrupt the endosome and allow RNA NP endosomal escape, it has high cellular toxicity. This has been combatted through conjugation with other polymers such as chitosan (Mainini and Eccles, 2020).

Along with their role in enhancing cellular uptake, CPPs can be used to enhance endosomal release (Ahmad et al., 2019; Donahue et al., 2019; Remaut et al., 2007). CPPs generally form a secondary amphipathic alpha-helical structure that displaces the endosomal lipid membrane in response to the low pH in the endosome/lysosome. This conformational change facilitates NP fusion to the endosome and RNA release into the cytosol (Ahmad et al., 2019; Liang and Lam, 2012). This concept was successfully applied when hemagglutinin-based NPs showed increased lysosomal escape of siRNA and increased cytotoxicity towards MCF-7 breast cancer cells (Guo et al., 2021).

4.2.2. *pH-sensitive disruption*

The acidic environment of the late endosome/lysosome can also be exploited by incorporating pH-sensitive polymers and amino acids into the NP membrane for RNA delivery. For example, poly(2-(diisopropylamino ethylmethacrylate) (PDPA) is a pH-sensitive polymer that is capable of self-assembly into NPs at neutral pH but disassemble after protonation at pH 6.51–6.85 (Jäger et al., 2015; Xu et al., 2016). Therefore, upon endosome acidification, the NPs disassemble, induce membrane instability, and release siRNA *in vitro* and *in vivo* (Xu et al., 2016). Alternatively, incorporation of arginine and lysine, which both protonate at an acidic pH, can promote endosomal escape (Mainini and Eccles, 2020). For example, decoration of apoferritin nanocages with lysine improved the anti-tumor effect exerted by siRNA delivery in 4T1 breast cancer mouse models compared to those lacking lysine (Huang et al., 2020). Similarly, arginine was added to PEI-based NPs to deliver

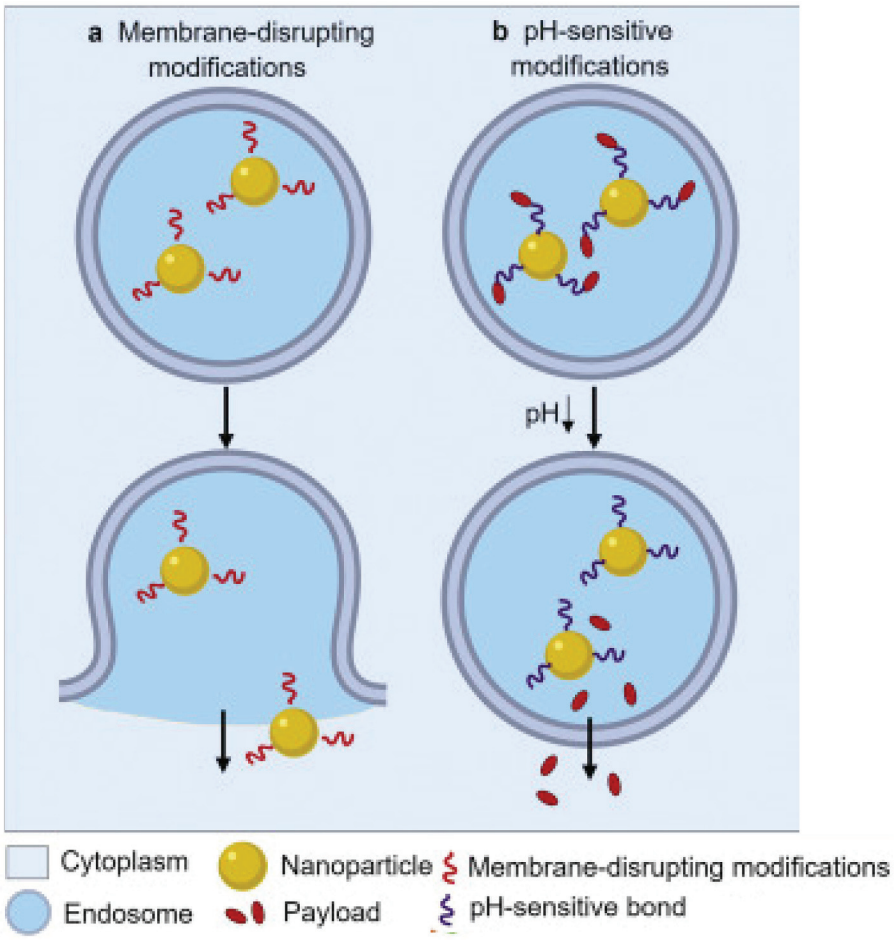

Fig. 6. Scheme showing NP modifications to enhance endosomal escape, modified with permission from (Donahue et al., 2019). **a)** Endosomal membrane-disrupting modifications on the NP can form openings by which the NP can 1) escape with its RNA cargo or 2) promote NP fusion to the endosomal membrane and release RNA directly into the cytoplasm. **b)** pH-sensitive modifications. The acidic environment in endosomes weakens the bonds that bind the NPs to the RNA cargo, thus allowing the RNA to escape the endosome.

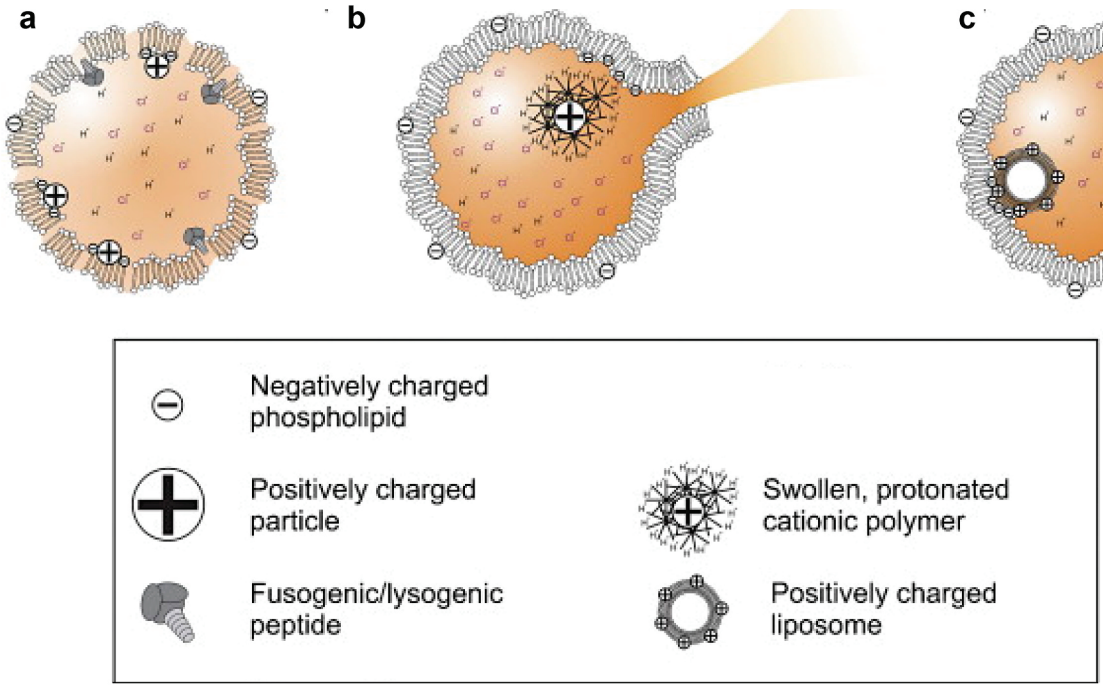

Fig. 7. Scheme displaying different types of membrane destabilization that can lead to endosomal escape, reprinted with permission from (Martens et al., 2014). **a)** Pore formation on the endosomal membrane due to cationic molecules or fusogenic peptides. **b)** Endosomal rupture due to osmotic swelling caused by an increased cationic charge. **c)** Fusion of the endosomal membrane and the NP (e.g., positively charged liposome) allowing for RNA release from the NP into the cytoplasm.

siRNA to breast cancer cells (Lu et al., 2019). While PEI itself is usually sufficient to induce endosomal escape, it's toxicity could be mitigated by the addition of arginine.

5. Conclusion

NPs have proven themselves effective and as safe delivery tools for RNA molecules *in vitro*, *in vivo,* and in clinical settings. Furthermore, they have shown impressive versatility, being applied in a wide range of pathological states as therapeutics and in preventative medicine through vaccination. As seen with the rapid development of lipid NP-based mRNA vaccines for COVID-19, RNA NP technology can be translatable and effective, and mass production of such therapies is feasible. The continued success of RNA NP therapy should continue to build on the strengths of previous successful formulations and the knowledge gathered to date.

In general, translatable RNA NP formulations should include (i) effective and stable RNA incorporation methods, (ii) appropriate targeting, (iii) sufficient cellular uptake and (iv) endosomal escape. The ease of NP multifunctionalization makes it possible to incorporate all these characteristics. The choice of NP material and modification should be optimized based on the RNA molecule to be delivered and the target site, as both factors can impact the efficacy of RNA delivery. This optimization is particularly important as the RNA construct in the NP can be easily exchanged for another, thus allowing for easy technology transfer to produce therapies for a variety of diseases. Initially, the stability and efficiency of RNA incorporation into NPs should be evaluated before application *in vitro* to identify if the specific nanocarrier chosen is appropriate for the RNA to be delivered. Furthermore, comprehensive characterization of the extent of cellular uptake at the target site and subsequent endosomal escape *in vitro* should be completed for each RNA NP complex to identify what specific modifications are essential for optimal delivery *in vitro* and *in vivo*.

As RNA molecules continue to be characterized as causative agents of disease or potential targets for therapy, NPs will offer a safe and efficient way to deliver them. The rapid increase in knowledge emerging related to NP processing *in vivo* will facilitate more advanced modifications. Utilization and optimization of the wide array of NP surface modifications available, individually or in combination, presents a great opportunity to enhance NP delivery efficacy.

Declaration of competing interest

The authors declare no competing interests.

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