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Polymeric nanoparticles for RNA delivery

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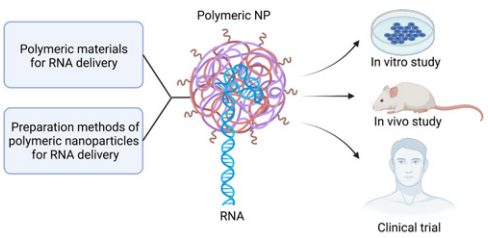
Abstract

As exemplified by recent clinical approval of RNA drugs including the latest COVID-19 mRNA vaccines, RNA therapy has demonstrated great promise as an emerging medicine. Central to the success of RNA therapy is the delivery of RNA molecules into the right cells at the right location. While the clinical success of nanotechnology in RNA therapy has been limited to lipid-based nanoparticles currently, polymers, due to their tunability and robustness, have also evolved as a class of promising material for the delivery of various therapeutics including RNAs. This article overviews different types of polymers used in RNA delivery and the methods for the formulation of polymeric nanoparticles and highlights recent progress of polymeric nanoparticle-based RNA therapy.

Graphical Abstract



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Key Points

- Cationic polymers are the most frequently used type of polymers for RNA delivery owing to their ability to complex with and condense the anionic RNA molecules and to induce endosomal escape.
- Cationic polymers often induce various degree of cytotoxicity, which could be controlled via modification of polymer properties such as charge density, molecular weight, architecture, biodegradability as well as introduction of copolymers.
- Non-cationic polymers offer better compatibility, but they usually require covalent conjugation with RNA molecules or coformulation with other positively charged species to improve RNA loading.
- Stimuli-responsive polymers change their physicochemical characters in response to endogenous or exogenous stimuli, enabling delivery of RNA in a more specific and controllable way.
- Different methods can be applied to formulate RNA-loaded polymeric nanoparticles (e.g., solid polymeric nanoparticles, polymeric micelles, and polymersomes).
- RNA-loaded polymeric nanoparticles have been explored in diverse biomedical applications, such as cancer, cardiovascular, autoimmune and infectious diseases.

Introduction

After decades of research, the once-simple notion that RNA is merely a passive carrier of genetic information from DNA to protein has been revolutionized. With the discovery of various RNA molecules and their diverse functions in regulating gene expression, RNA is now appreciated as the key mediator in virtually every gene expression pathway (Lieberman, 2018). What comes with those great discoveries is the tremendous potential of using RNA as therapeutics in that it allows for drugging many targets that were previously considered "undruggable" by conventional medicines. RNA therapy could also eliminate the risk of insertional mutagenesis compared to DNA-based gene therapy. Another advantage of RNA-based therapy is that it only needs to enter the cytosol to function and does not require further trafficking to the nucleus. To date, several RNA drugs, including antisense RNA, RNA aptamer, small interfering RNA (siRNA), and the most recent messenger RNA (mRNA)-based COVID-19 vaccines, have been successfully translated to the clinics and dozens more are in different phases of clinical trials, offering new routes to treating many previously untreatable diseases (Kim, 2020).

While RNA therapy holds great promise, one of the greatest challenges is to safely deliver therapeutic RNA to the right location of the right cell. RNA molecules are hard to penetrate cell membranes by themselves because of their highly negatively charged nature and relatively bulky size. In addition, they are susceptible to degradation by the ubiquitous ribonucleases (RNases) presented in the body, though recent advances in chemical modification of RNA can enhance their stability to some extend (Boo and Kim, 2020; Kormann *et al.*, 2011). Viral vectors such as the adeno-associated virus (AAV)-based ones have shown impressive RNA transfection efficiency (Tomar *et al.*, 2003), but their use is associated with safety concerns of the immunogenicity-related toxicity (Schott *et al.*, 2016). Delivery of chemically modified RNA directly through conjugation with targeting ligand is another promising way, manifested by the FDA-approved Givosiran, a siRNA drug conjugated with N-acetylgalactosamine (GalNAc) for targeting the hepatocytes (Scott, 2020). However, whether this strategy can be extended to other larger and more fragile RNA such as mRNA and to extrahepatic tissues remains to be seen. Nanoparticle-based technology represents a major force in RNA delivery owing to its multiple advantages, including the ability to shield encapsulated RNA molecules from harsh exterior environments (e.g., RNases), reduced risk of immunotoxicity relative to viral vectors as well as a number of ways to enhance targeting and facilitate cytosolic delivery of the RNA cargos. So far, lipid nanoparticles for RNA delivery have achieved great clinical success marked by the first FDA-proved RNA interference (RNAi) drug (Patisiran) and the newly approved mRNA COVID-19 vaccines developed by Pfizer/BioNtech (BNT162b2) and Moderna (mRNA-1273). Polymeric nanoparticles, on the other hand, are another class of nanomaterials in the forefront of pharmaceutical research for the delivery of RNA therapeutics. Merits of polymeric nanoparticles such as their vast tunability in physicochemical properties and robustness allow for greater degree of engineering to meet the delivery challenges of specific diseases. While polymeric nanoparticle-based RNA therapy has yet to reach the clinics, many encouraging results from preclinical and clinical trial (**Table 1**) studies have highlighted the great potential of this

Table 1 Polymeric vectors for RNA delivery in clinical trials

Name	*Polymeric vector*	*Active cargo*	*Administration route*	*Intended disease*	*Clinical trial Identifier & Phase*
CALAA-01	Cationic cyclodextrin-based nanoparticle	siRNA	Intravenous infusion	Solid tumors	NCT00689065 Phase I (terminated)
SNS01-T	PEI-based nanoparticle	siRNA and DNA plasmid	Intravenous infusion	Relapsed or refractory B cell malignancies	NCT01435720 Phase I/II
siG12D-LODER	Miniaturized PLGA matrix	siRNA	Local implantation	Locally advanced pancreatic cancer	NCT01676259 Phase II

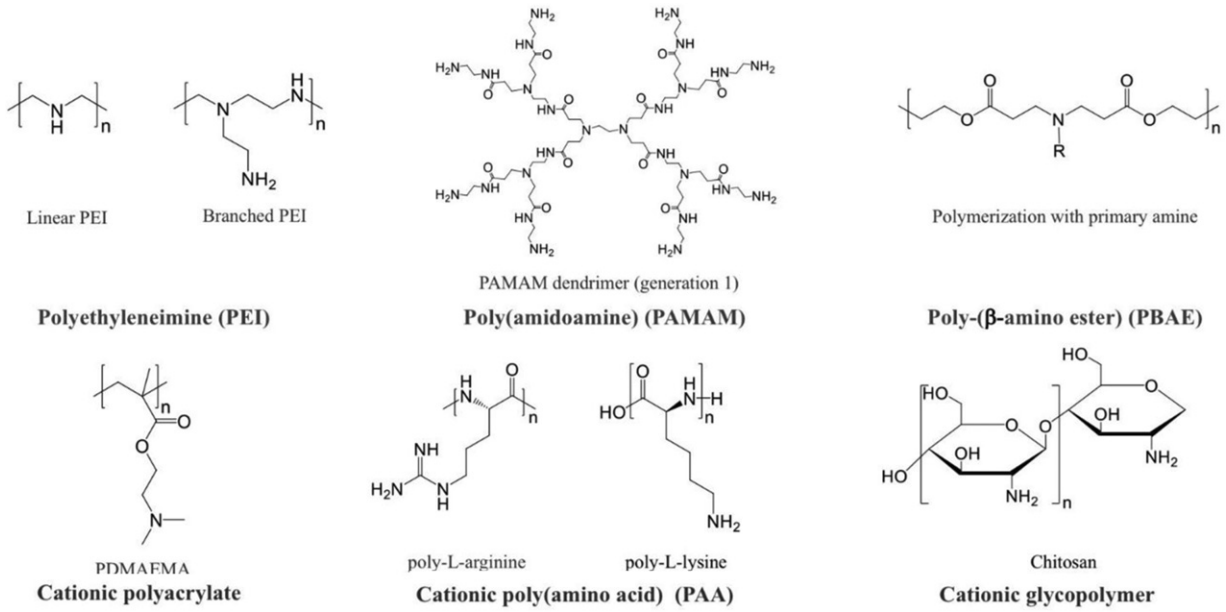

Fig. 1 Representative structures of the cationic polymers for RNA delivery.

technology. This article overviews different types of polymers used for RNA delivery and the common methods for fabrication of polymeric nanoparticles, as well as examples of polymeric nanoparticle-based RNA therapy.

Polymeric Materials for RNA Delivery

A range of polymers have been exploited for RNA delivery and they generally serve as vectors to confer protection to the RNAs against premature degradation and facilitate the cytosolic delivery of RNAs to the target cells. These polymers can be roughly divided into two categories: cationic polymers and non-cationic polymers depending on the charges they carry at the physiological conditions. Cationic polymers (**Fig. 1**) are the most frequently used type of polymers for RNA delivery owing to their ability to complex with the anionic RNA molecules via electrostatic interactions and facilitate the cytosolic delivery of RNA through a number of mechanisms (Pack *et al.*, 2005). However, cationic polymers often display higher in vitro and in vivo toxicity compared to non-cationic polymers, and thus the development of non-cationic vectors for efficient RNA delivery is of great interest. Stimuli-responsive polymers or so-called "smart polymers" are a special class of polymers that can change their physicochemical properties in response to endogenous (pH, enzyme, redox environment, etc.) and/or exogenous (electromagnetic radiation, ultrasound, etc.) stimuli. The use of stimuli-responsive polymers has been pursued as a way to improve RNA delivery specificity and efficiency. Here we summarize the commonly adopted cationic, non-cationic, and stimuli-responsive polymers for RNA delivery.

Cationic Polymers

Poly (ethyleneimine) and its derivatives

Polyethyleneimine (PEI) is among the earliest and most widely studied cationic polymers for gene delivery, including the delivery of RNA. It has high gene transfection efficiency and is often referred to as the gold standard for non-viral gene transfection (Lungwitz *et al.*, 2005). PEI can be in either linear or branched structures and its positive charge is conferred by numerous amine groups separated by short alkyl spacers, which lead to very high positive charge density within its structure. The linear PEI can be synthesized via ring-opening polymerization of 2-ethyl-2-oxazoline followed by acid-catalyzed hydrolysis, resulting in a linear structure with only secondary amines in the polymer backbone and primary amines in the terminal. The branched PEI is synthesized by ring-opening polymerization of aziridine, which gives rise to the branched architecture with primary, secondary and tertiary amines at a nearly 1:2:1 ratio (Bahadur and Uludağ, 2016; von Harpe *et al.*, 2000). It has been reported that the degree of polymerization, branching as well as charge density of PEI strongly affected its transfection efficiency, (Godbey *et al.*, 1999; Abdallah *et al.*, 1996; Wightman *et al.*, 2001) but a concrete structure-function relationship has yet to be concluded. The high RNA transfection efficiency of PEI could be attributed to (1) strong positive surface charge that is able to condense RNAs into cationic nano-complexes, which promotes interaction with the negatively charged cell membrane, facilitates cellular endocytosis and also protects RNAs from enzymatic degradation; (2) large number of amine groups that can be protonated at decreased pH in endosomes, which induces the "proton sponge" effect and causes an influx of counter ions into the endosomes, eventually leading to endosomal rupture and release of RNAs to the cytoplasm (Lungwitz *et al.*, 2005; Nimesh, 2012). Although PEI shows high RNA

transfection efficiency, it is accompanied by pronounced toxicity and adverse effects arising from cation-induced membrane destabilization, nonspecific protein interaction, and immune response, limiting its application and translation to the clinics (Lv *et al.*, 2006). Low-molecular-weight PEIs were reported to transfect siRNA and mRNA with improved toxicity profiles, (Urban-Klein *et al.*, 2005; Werth *et al.*, 2006; Rejman *et al.*, 2010) however, their transfection efficiency was often compromised and biocompatibility concerns persisted.

To alleviate the toxicity of PEI, PEI derivatives or hybrids were designed and synthesized to improve the biocompatibility and/or biodegradability while maintaining the transfection capability. PEI hybrids constructed from less-toxic low-molecular-weight PEIs (0.8–1.8 kDa) and biodegradable linkers such as ester, imine, or disulfide bonds have shown comparable gene transfection efficiency to that of high-molecular-weight PEI (25 kDa) but with significantly reduced toxicity (Kim *et al.*, 2005; Wang *et al.*, 2016; Gosselin *et al.*, 2001). PEGylation of PEI is another common way to improve the biocompatibility of PEI, however, this can also decrease the surface charge of PEI and negatively affect its ability in RNA condensation, cell uptake, and subsequent endosomal escape (Mishra *et al.*, 2004). Tuning the length and density of PEG molecules on PEI is key to achieve the optimal balance of biocompatibility and performance for specific systems (Mishra *et al.*, 2004; Tang *et al.*, 2003). Anderson *et al.* have demonstrated that 7C1, a PEI-lipid hybrid synthesized by the ring-opening reaction between C_{15} epoxide-terminated lipids and PEI (0.6 kDa) at a 14:1 molar ratio, could enable systematic delivery of siRNA to lung endothelia when formulated into nanoparticles with lipid-PEG and induce robust gene silencing at very low doses in both rodents and non-human primates (Dahlman *et al.*, 2014; Khan *et al.*, 2018). Cyclodextrin grafted or cross-linked PEIs were also used to form nanoparticles for in vivo siRNA/mRNA delivery with mitigated toxicity (Li *et al.*, 2013; Shen *et al.*, 2014; Wang *et al.*, 2017; Tan *et al.*, 2020). In addition, a proprietary linear PEI derivative named JetPEI® is available commercially, which allows in vitro and in vivo transfection of various RNAs in a relatively safe manner (Urban-Klein *et al.*, 2005; Höbel and Aigner, 2013). Notably, SNS01-T, an in vivo-JetPEI-based nanoparticle formulation for codelivery of siRNA and plasmid targeting EIF5A, has entered phase I/II clinical trials (NCT01435720) for treatment of B-cell lymphoma (Craig *et al.*, 2014).

Poly (amidoamine)

Poly (amidoamine) (PAMAM) is common in the dendrimer form and used as a tunable vector for RNA delivery for its unique properties such as defined architecture, highly branched spherical structures, and low polydispersity. Sometimes referred to as "starburst polymers," PAMAM dendrimers were first synthesized in 1985; the name was coined due to their structure of symmetric branching from a core molecule (Tomalia *et al.*, 1985). PAMAM dendrimers are radially composed, with a core of an aliphatic molecule containing primary amines, usually ethylenediamine, ammonia, or cystamine (Tomalia *et al.*, 1985; Araújo *et al.*, 2018). N- (2-aminoethyl) acrylamide branching units are then covalently bound to the core, layer by layer; these layers are called "generations" (Abbasi *et al.*, 2014). The PAMAM dendrimer is synthesized through a well-established two-step process for the addition of the branching layers, in which the core molecule undergoes a Michael addition with methyl acrylate, followed by the amidation of the resulting terminal esters with excess ethylenediamine (Abbasi *et al.*, 2014). The presence of high-density amine groups on the surface of PAMAM dendrimer enables electrostatic interactions and complexation with negatively charged RNA, while also facilitating endosomal escape through the "proton sponge" effect (Biswas and Torchilin, 2013).

Optimization of the RNA/dendrimer complex can be achieved through changing the number of generations (i.e., branching layers), adjusting the N/P (number of amino groups in PAMAM/number of phosphate groups in RNA) ratio, and modifying the primary amine terminal groups. As the number of generations increases, the interactions between the dendrimer and the RNA become stronger, and the structure becomes more stable, spherical, and densely packed (Shen *et al.*, 2007; Jensen *et al.*, 2011). This compact globular structure contains cavities in which the RNA can be encapsulated (Araújo *et al.*, 2018). Increasing the N/P ratio has also been observed to increase the stability and uniformity of the RNA/PAMAM dendrimer complex (Shen *et al.*, 2007). Highly branched PAMAM shows good transfection efficiency but also displays pronounced toxicity due to the strong cationic charge (Kim *et al.*, 2014). The acetylation (i.e., neutralization) of the terminal amines has been shown to reduce the cytotoxicity induced by the strong positive charge of these surface functional groups, as well as promote the intracellular release of siRNA from the PAMAM dendrimer; however, too much acetylation was shown to also reduce the buffering capacity, which decreased the transfection efficiency (Waite *et al.*, 2009). A careful balance of these parameters is key to achieve optimal performance. Rossi *et al.* reported the strong gene-silencing efficacy and low cytotoxicity of a generation 5 PAMAM dendrimer for systemic delivery of a cocktail of dicer substrate siRNAs (dsiRNAs) and effective suppression of HIV-1 infection in a humanized mouse model (Zhou *et al.*, 2011). Peng *et al.* demonstrated a generation 4 PAMAM dendrimer with a flexible triethanolamine core and arginine-decorated terminal branches for successful siRNA delivery to prostate cancer models both in vitro and in vivo, maintaining a low toxicity profile for a range of N/P ratios (Liu *et al.*, 2014a; Liu *et al.*, 2009). By conjugating lipid tails to a head group of PAMAM via click chemistry, the same group later developed an amphiphilic dendrimer that is able to self-assemble into nanosized micelles in the presence of siRNA, enabling robust siRNA delivery and gene silencing in a range of primary and malignant cells, as well as an effective cancer therapy in murine models (Yu *et al.*, 2012; Liu *et al.*, 2014b; Dong *et al.*, 2018).

Poly (β-amino ester)

Poly (β-amino ester) (PBAE) was firstly prepared in 1983 and gained popularity as a class of gene transfection agent after a demonstration by Langer's lab in 2000 (Galli *et al.*, 1983; Lynn and Langer, 2000). PBAE is notable for its facile biodegradability under physiological conditions, which endows it with low cytotoxicity compared to other cationic polymers such as PEI (Lynn and Langer, 2000; Cordeiro *et al.*, 2019). It degrades via hydrolysis of the ester bonds in the polymer backbone, yielding small

molecular-weight derivatives and nontoxic β-amino acids (Cordeiro *et al.*, 2019; Lynn *et al.*, 2001). The cationic amino groups in the amino acid monomers can electrostatically complex with negatively charged RNAs and in the meantime provide the necessary buffering capacity for endosomal escape (Cordeiro *et al.*, 2019). The synthesis of linear PBAEs is straightforward and involves the simple one-step Michael addition of a primary amine or bis (secondary amine), and a diacrylate (Anderson *et al.*, 2003). This reaction requires no solvents, catalysts, or protecting groups, and it does not generate side products. It has been observed that an increase in the molecular weight of linear PBAE correlates with an increase in both siRNA transfection efficiency and toxicity (Eltoukhy *et al.*, 2012; Green *et al.*, 2007). The increased toxicity likely results from the slower degradation of higher molecular weight PBAE. To address this issue, low-molecular-weight linear PBAEs were quaternized in order to increase their surface charge density. The electrostatic interactions between PBAE and siRNA were successfully strengthened while maintaining low cytotoxicity (Liu *et al.*, 2018). Hydrophobicity has also been shown to play a crucial role in determining the efficacy of PBAE nanoparticles in siRNA delivery; further tuning and modification of linear PBAE are necessary to balance these effects on siRNA delivery (Tzeng and Green, 2013; Dosta *et al.*, 2018). End-cap (or end-chain) modifications of PBAE with groups such as cystamine or other oligopeptides can also impact the silencing efficiency of siRNA (Tzeng *et al.*, 2012; Dosta *et al.*, 2015). Tzeng *et al.* evaluated an array of end-modified PBAE nanoparticles and found that cystamine-terminated ones generally resulted in the most knockdown by siRNA, achieving 91% knockdown efficiency in hard-to-transfect human mesenchymal stem cells 20 days after transfection (Tzeng *et al.*, 2012). Stephan *et al.* fabricated piperazine-capped cationic PBAE (PBAE-447) nanoparticles with electrostatically anchored targeting ligands for gene delivery via conjugating the targeting ligands to anionic polyglutamic acid (PGA) (Smith *et al.*, 2017). This nanoparticle system was later used to specifically deliver mRNA to immune cells, demonstrating applications such as in situ reprograming of T cells and macrophages for cell-based immunotherapy in animal models (Moffett *et al.*, 2017; Zhang *et al.*, 2019; Parayath *et al.*, 2020).

In addition to linear-structured PBAE, branched/hyperbranched and hybrid PBAEs have also been developed for RNA delivery. Highly branched PBAEs (HPAEs), despite their high molecular weight, have demonstrated low cytotoxicity and much higher transfection efficiencies than linear PBAEs as well as commercial transfection reagents due to their three-dimensional architecture, multifunctional terminal groups, and the potential for further optimization through structural modification (Gao *et al.*, 2016; Zhou *et al.*, 2016a,b; Huang *et al.*, 2015). To address the challenges associated with HPAE synthesis, a simple one-pot process involving an "amine (A2) + triacrylate (B3) + diacrylate (C2)" Michael addition approach was developed by Wang *et al.* using the triacrylates as branching monomers (Zhou *et al.*, 2016a). Patel *et al.* (2019) demonstrated the effective use of hyperbranched PBAEs to achieve nebulized delivery of mRNA selectively to lung epithelium in an animal model, while avoiding local or systemic toxicity. Lipid-PBAE hybrid nanoparticles have been developed to improve the serum stability of PBAE (Kaczmarek *et al.*, 2016; Eltoukhy *et al.*, 2013). For example, PBAE terpolymers were co-formulated with PEG-lipid to increase serum stability of PBAE nanoparticles via hydrophobic interactions, and the resulting nanoparticles were shown to successfully deliver mRNA to the lungs by intravenous administration in mice (Kaczmarek *et al.*, 2016).

Cationic polyacrylates

Cationic polyacrylates are prepared through cationic modification of the side chains in polyacrylates. The most often used cationic polyacrylate is poly (2-N,N-dimethylaminoethyl methacrylate) (PDMAEMA), which can be facilely synthesized through atom transfer radical polymerization (ATRP) or reversible addition-fragmentation transfer (RAFT) of DMAEMA monomers (Xu and Yang, 2011). One advantage of PDMAEMA is that the macromolecular structure is highly controllable during polymerization, allowing for the optimization of parameters (e.g., the choice of co-monomers) that may affect the transfection efficiency, biocompatibility, and other key characteristics (Zhu *et al.*, 2010; Dubruel and Schacht, 2006). PDMAEMA has a pK_a of 7.5 due to the tertiary amines present on DMAEMA monomers; thus, it is partially protonated at physiological pH and may promote endosomal escape via the "proton sponge" effect (van de Wetering *et al.*, 1999). PDMAEMA and its derivatives have been investigated in RNA delivery both as homopolymers and, more frequently, as subunits in block copolymers. The block copolymers were usually designed to include endosomolytic and lipophilic components in the polymer backbone to increase stability, facilitate cargo release and enhance transfection of siRNA and mRNA (Cheng *et al.*, 2012, 2017; Nelson *et al.*, 2013; Truong *et al.*, 2013). For example, Monteiro at al. developed an influenza virus-inspired diblock copolymer with the first block of poly (2-dimethylaminoethyl acrylate) (PDMAEA) for siRNA complexation and the second block composed of membrane fusion units that can be exposed in endosomes after degradation of the PDMAEA. The PDMAEA undergoes a slow, pH-independent self-degradation via a self-catalyzed process to yield benign, less toxic, acrylic acid-based byproducts, enabling "timed release" of the siRNA cargo (Truong *et al.*, 2013, 2011).

Cationic poly (amino acid)s

Poly (amino acid)s (PAAs), also known as polypeptides, are synthetic or naturally-derived amino acid chains of variable length and sequence. Cationic PAAs often contain positively charged lysine, arginine, or histidine residues; common examples are poly-L-lysine (PLL) and poly-L-arginine (PLA). PAAs can be obtained through solid-phase and automated peptide synthetic procedures (Palomo, 2014). The positive charge allows for complexation with RNAs through electrostatic interactions, as well as interaction with the cell membrane to promote cellular internalization (Tai and Gao, 2017). Of note, polyarginine peptides and arginine-rich peptides are the most commonly used type of cell-penetrating peptides and they have been directly conjugated to siRNAs for enhanced gene silencing (Milletti, 2012; Kumar *et al.*, 2007). PLL copolymers are one of the widely employed vectors for gene delivery, including siRNA (Liu *et al.*, 2012), miRNA (Jin *et al.*, 2012), and mRNA (Miyazaki *et al.*, 2020). Like that of PEI, the

cytotoxicity of PLL is associated directly to its molecular weight, where the increase in cationic charge density is thought to induce membrane destabilization leading to cytotoxicity (Martin and Rice, 2007). Copolymerization with other non-cationic monomers or modification of the cationic amino groups in PLL is common strategies to mitigate toxicity and introduce additional functionality (Ulkoski *et al.*, 2019). Poly-ICLC (Hiltonol®), an immunostimulant formulation in clinical trials, contains PLL as the stabilizer. Poly-ICLC is used as a synthetic dsRNA viral mimic in vaccines to agonize Toll-like receptor-3 and activate innate as well as adaptive immunity against HIV (NCT02071095) and cancer (NCT02423863, NCT04525859) (Saxena *et al.*, 2019; Kyi *et al.*, 2018). Similar to PLL, poly (amino acid)s derived from arginine and histidine have also been investigated for RNA delivery (Zhao *et al.*, 2012; Langlet-Bertin *et al.*, 2010).

Cationic glycopolymers

Glycopolymers are polymers based on carbohydrates. Chitosan is a cationic glycopolymer derived from the deacetylation of chitin, a biopolymer naturally found in the exoskeletons of many crustaceans, insects, and fungi (Venkatesan *et al.*, 2014). Chitosan is biodegradable and used as food additives currently, and it has been designated by the FDA as Generally Regarded As Safe (GRAS) biomaterial (Garcia-Fuentes and Alonso, 2012). Commercial chitosan has randomly distributed N-acetylglucosamine and glucosamine units due to incomplete deacetylation, and the deacetylation degree greatly affects the properties of chitosan, including its water solubility, conformation, and gene transfection efficiency (Ragelle *et al.*, 2013). The glucosamine groups on chitosan can be protonated in slightly acidic conditions, which allows complexation with the nucleic acids into nanoparticles. Generally, chitosan of high molecular weight and high deacetylation degree is associated with better RNA transfection efficiency. Liu and co-workers systematically studied the effect of polymer properties on chitosan/siRNA nanoparticle formulation and gene silencing efficiency (Liu *et al.*, 2007). They found that chitosan/siRNA formulation prepared with low molecular-weight (9–12 kDa) chitosan showed almost no silencing effect in vitro while the silencing efficiency increased to 45–65% when formulating with 65–170 kDa chitosan. The highest gene silencing efficiency of ~80% was achieved using chitosan of 114–170 kDa and a deacetylation degree of 84%, however, the silencing effect was nearly absent with 170 kDa chitosan of 54% deacetylation degree due to a lack of stability of the resulting chitosan/siRNA complex. Similar to chitosan, poly (glycoamidoamine) (PGAA) that contains repeating carbohydrate residues and amine units has also been used for the delivery of different types of RNA. For example, Reineke *et al.* spearheaded the development of multiple PGAAs that could form stable polyplexes with siRNA and enable effective siRNA-mediated gene silencing in a U-87 glioblastoma cell line (Smith *et al.*, 2011; Sizovs *et al.*, 2013). Dong *et al.* (2016) further modified PGAA with lipids and developed PGAA-brush nanoparticles for systemic delivery of siRNA as well as mRNA to hepatocytes in mice. Other cationic glycopolymers used for RNA delivery include cationic modification of dextran (Raemdonck *et al.*, 2009), cyclodextrin (Singh *et al.*, 2019), cellulose (Kim *et al.*, 2020), and hyaluronic acid (Han *et al.*, 2009). CALAA-01, the first siRNA experimental drug that entered clinical trial (NCT00689065), was a targeted, cationic cyclodextrin-based polymeric nanoparticle for the treatment of solid tumors. In this system, the cyclodextrin also functioned as a host for anchoring adamantane-conjugated PEG and PEG-transferrin ligands through the strong host-guest interaction between cyclodextrin and adamantane (Davis *et al.*, 2010; Zuckerman *et al.*, 2014). However, CALAA-01 was later terminated after phase I trial due to toxicity issues.

Non-Cationic Polymers

Poly (ethylene glycol)

Poly (ethylene glycol) (PEG) is perhaps the most widely used polymer in pharmaceutical products, either by conjugation to the active ingredients or as excipients (Kolate *et al.*, 2014). The covalent attachment of PEG to oligonucleotides has been adopted for decades to improve the biopharmaceutical properties of oligonucleotides, exemplified by Pegaptanib, a PEGylated aptamer approved by the FDA in 2004 for the treatment of macular degeneration (Lu and Zhang, 2018). Traditionally, PEGylation has been used to create a large hydration layer that sterically blocks other biomacromolecules (e.g., proteins) from binding with the conjugated drugs, resulting in improved drug solubility, stability, and retention profiles. However, the increased steric hindrance could also weaken the specific interactions between the drug and its receptors. Recent studies by Zhang *et al.* have identified that PEGylation of oligonucleotides with certain brush PEG architectures can effectively shield the oligonucleotides from proteins or degrading enzymes without compromising their binding affinity to complementary targeting sequences (Lu *et al.*, 2015; Lu *et al.*, 2016). The bottle brush-architectured PEG consists of a main polymer backbone attached with a number of PEG side chains. Tuning the local PEG density is accomplished by controlling the number of PEG side chains and their molecular weight. It is believed that the intermediate PEG density provided by brush PEG architecture is key to achieve the desired performance. The same group further demonstrated that siRNA conjugated with the bottle brush-architectured PEG polymer (size ~30 nm) via a thiol-cleavable disulfide linker (termed pacRNA$_{Clv}$) could significantly prolong the in vivo blood circulation of siRNA, enhance siRNA nuclease stability and increase siRNA cellular uptake, resulting in robust RNA interference in the tumor after systemic administration (Wang *et al.*, 2019) (**Fig. 2**(A)).

Polyesters

The most prominent feature of polyesters, such as polylactic acid (PLA), poly (lactic-co-glycolic acid) (PLGA), and polycaprolactone (PCL), is their biodegradability and biocompatibility. These polymers undergo hydrolysis in physiological

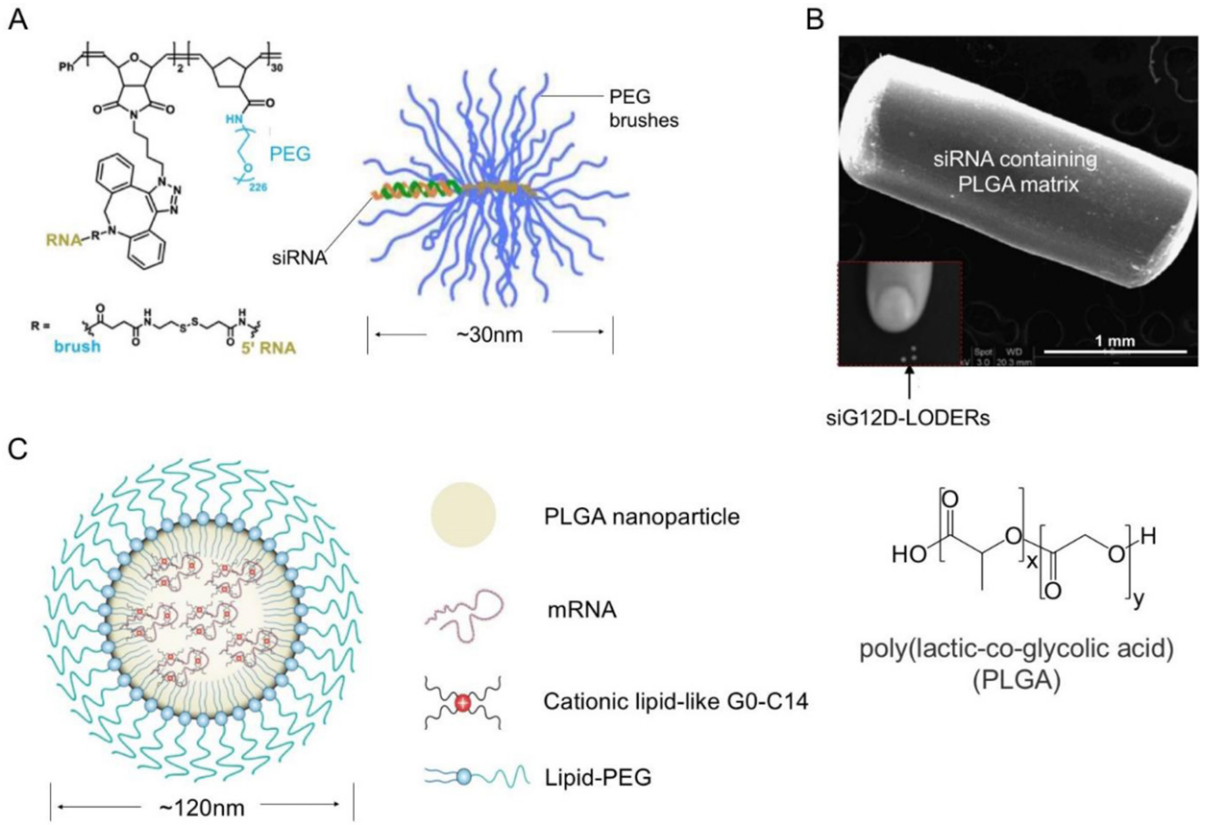

Fig. 2 (A) Structures of the bottle brush-architectured PEG nanoparticle for siRNA delivery through conjugation. (B) SEM and photo of the PLGA-based siG12D-LODERs. (C) Schematic of the lipid-PLGA hybrid nanoparticle for systemic mRNA delivery to tumors. Adapted with permission from Wang, D., *et al.*, 2019. Bottlebrush-architectured poly (ethylene glycol) as an efficient vector for RNA interference in vivo. Science Advances 5, eaav9322. Khvalevsky, E.Z., *et al.*, 2013. Mutant KRAS is a druggable target for pancreatic cancer. Proceedings of the National Academy of Sciences of the United States of America 110, 20723–20728. Islam, M.A., *et al.*, 2018. Restoration of tumour-growth suppression in vivo via systemic nanoparticle-mediated delivery of PTEN mRNA. Nature Biomedical Engineering 2, 850–864. Available at: https://doi.org/10.1038/s41551-018-0284-0.

conditions and their degradation products can be safely metabolized and eliminated by the body (Anderson and Shive, 1997). These polymers have entered the clinics for a long time as biomaterials of medical devices and tissue engineering (Zhao *et al.*, 2018). Currently, polyester is among the few synthetic polymers that are being tested in clinical trials for drug delivery purposes, (Kamaly *et al.*, 2012; Kim *et al.*, 2004) which is due in part to its excellent safety profiles. Delivery of nucleic acids, including RNAs, by polyesters is also investigated widely. Silenced LTD developed a miniaturized drug implant composed of PLGA polymer matrix and siRNA against KRAS oncogene for treatment of solid pancreatic cancer. This drug delivery system, named local drug eluter (LODER™), can be implanted into solid tumors using a 19-gauge endoscopic ultrasound needle and enables a prolonged release of siRNA as well as silencing effect in vivo for 155 days (Khvalevsky *et al.*, 2013) (**Fig. 2**(B)). siG12D-LODER™ is now in Phase II clinical trials (NCT01676259) for advanced pancreatic cancer patients undergoing chemotherapy (Varghese *et al.*, 2020).

Due to a lack of specific interaction with RNAs, polyester by itself often has low RNA entrapment efficiency when formulated into nanoparticles. Modification of polyesters has been employed to increase their complexation with RNAs through, for example, grafting the side chains of polyesters with amino or other cationic groups. Usually, aliphatic polyesters with functional side chains are synthesized by polymerization or copolymerization of monomers containing protected functional groups via polycondensation, ring-opening polymerization (ROP), or enzymatic polymerization routes (Seyednejad *et al.*, 2011). For instance, polyesters bearing ene and epoxide side chains were formed by polycondensation of trimethylolpropane allyl ether (TPAE) and diacid chlorides, allowing further chemical modifications of the polyester side chains with amino and lipid molecules for tuning the physiochemical properties of the polyesters (Yan and Siegwart, 2014). Siegwart *et al.* demonstrated using these functional polyesters for selective delivery of siRNA and mRNA to the lung after systemic administration (Yan *et al.*, 2016a; Yan *et al.*, 2017a,b). Coformulation with other cationic molecules is another viable way to improve the RNA entrapment and transfection efficiency of polyester-based nanoparticles. One study revealed that simply complexing siRNA with cationic spermidine before entrapment into sub-200 nm PLGA nanoparticles could improve siRNA loading efficiency by over 40 times, and a single topical application of these siRNAs containing PLGA nanoparticles to mouse female reproductive tract resulted in effective and sustained gene silencing of the female reproductive mucosa for at least 14 days (Woodrow *et al.*, 2009). In addition, cationic lipids have also been incorporated into polyester nanoparticles to

increase the RNA loading and transfection efficiency. For example, 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), a cationic lipid frequently used in cationic liposomes for gene transfection, was shown to significantly potentiate the transfection efficiency of siRNA loaded PLGA nanoparticles when formulated into the PLGA matrix (Jensen *et al.*, 2012; Colombo *et al.*, 2015). With insights from a high throughput screening of lipid-like molecules (lipidoids) for gene silencing (Love *et al.*, 2010), Xu *et al.* utilized the ring-opening reaction between 1,2-epoxytetradecane and generation 0 of PAMAM to yield a lipidoid termed G0-C14, which drastically enhanced the siRNA entrapment after coformulation into PLGA nanoparticles, allowing for nanoparticle-mediated systemic siRNA delivery to tumors and efficient gene silencing in cancer cells (Xu *et al.*, 2013; Zhu *et al.*, 2015). The cationic lipid-like G0-C14 also aided effective entrapment of bulky mRNA molecules into PLGA nanoparticles, which enabled systemic delivery of PTEN and P53 mRNA into tumors for restoration of lost tumor suppressors (Islam *et al.*, 2018; Kong *et al.*, 2019) (**Fig. 2**(C)).

Stimuli-Responsive Polymers

The active therapeutics entrapped in polymeric nanoparticles have to be released from the polymer matrix in order to fulfill their desired biological effect. Conventional drug release mechanisms from polymeric nanoparticles rely largely on drug diffusion, osmotic pumping, and polymer erosion/degradation (Kamaly *et al.*, 2016). In contrast, nanoparticles based on stimuli-responsive polymers allow the release of their cargos in response to changes in their physical, chemical, or biological environment, enabling drug release in a more controlled manner. Such stimuli may trigger changes in the physicochemical properties of the polymers (charge switching, chemical bond breaking, etc.) leading to further nanoparticle structural change that facilitates releasing of the active payloads. The stimuli can be classified as either "endogenous stimuli" or "exogenous stimuli": endogenous stimuli are stimuli that arise from the chemical or biological changes of the site of interest itself within the body, such as changes in pH and redox status, whereas "exogenous stimuli" are those manipulations from outside the body, such as application of light irradiation or ultrasound. By integrating two or more stimuli-responsive properties, polymers with multi-responsiveness are also achievable.

Endogenous stimuli-responsive polymers

Endosomal escape is a critical challenge for nanoparticle-mediated RNA delivery. A variety of pH-responsive polymers have been developed to take advantage of the acidic endosomal environment (pH 4.5–6.5) (Hu *et al.*, 2015) and facilitate the escape of nanoparticles from endosomes to the cytoplasm (Xu *et al.*, 2016; Yu *et al.*, 2011). For example, Stayton *et al.* designed and synthesized a class of pH-responsive diblock copolymer containing a block of cationic dimethylaminoethyl methacrylate (DMAEMA) for siRNA complexation and a second block of DMAEMA, propylacrylic acid (PAA), and butyl methacrylate (BMA) for endosomal escaping (Convertine *et al.*, 2009; Convertine *et al.*, 2010; Manganiello *et al.*, 2012). At physiological condition (neutral pH), the second block exhibits ampholytic nature with both positively-charged DMAEMA and negatively-charged PAA masking the hydrophobic BMA, while under the acidic condition in the endosomes, protonation of PAA along with a concurrent increase in positive charge from DMAEMA effectively switch the polymer from a hydrophilic polyampholyte to a hydrophobic polycation that can disrupt the endosomal membrane and facilitate endosomal escaping for efficient cytosolic delivery of siRNA. A similar pH-responsive copolymer called VIPER (virus-inspired polymer for endosomal release) was constructed using a DMAEMA-based cationic block and a poly (2-diisopropylaminoethyl methacrylate) (p-DIPAMA)-based pH-responsive block conjugated with melittin, a membrane-lytic peptide. VIPER can self-assemble into nanoparticles at physiological pH with melittin buried within the nanoparticle hydrophobic core but undergoes a conformational change and exposes the membrane-disrupting melittin peptide when the p-DIPAMA is transitioned from hydrophobic to hydrophilic under the lower pH endosomal environment. This virus-like endosomal escape mechanism has been successfully applied to deliver plasmid and siRNA to the tumor and lung in mice (Cheng *et al.*, 2016; Feldmann *et al.*, 2018). The bulky size and high susceptibility to nuclease degradation have made efficient delivery of mRNA especially challenging compared to siRNA and DNA. To address the specific challenges in mRNA delivery, Waymouth *et al.* developed a novel type of charge-altering releasable transporters (CARTs) based on oligo (carbonate-*b*-α-amino ester)s that function initially as polycations to complex, protect and deliver mRNA into cells and later degrade into neutral small molecules in early endosomes under cytosolic pH (McKinlay *et al.*, 2017; Haabeth *et al.*, 2018). This type of self-immolation polymers works by intramolecular rearrangement upon partial deprotonation of the initial ammonium cations inside the cells, resulting in simultaneous polymer disassembly, charge neutronization, endosomal escaping and mRNA releasing. CART polymers have been shown very effective for in vivo delivery of mRNA to animal models via multiple administration routes and have been applied in areas such as mRNA cancer vaccine (Haabeth *et al.*, 2018) and immunotherapy (Haabeth *et al.*, 2019). Further optimization of the CART polymers has also been made by incorporation of lipid domains and using CART based on cationic oligo (serine esters) (McKinlay *et al.*, 2018; Benner *et al.*, 2019). Moreover, various pH-responsive polymers with ultrahigh pH sensitivity have been synthesized to exploit the slightly acidic tumor microenvironment (pH 6.5–6.8) for enhanced RNA delivery (Yang *et al.*, 2012; Saw *et al.*, 2019; Xu *et al.*, 2017). For example, Wang *et al.* developed a tumor acidity-sensitive PEGylated anionic polymer containing a pH-liable amide bond that can degrade under the slightly acidic condition of the tumor microenvironment and subsequently expose the positively charged amino groups (Yang *et al.*, 2012). This initially anionic pH-responsive polymer was used to coat cationic PEI/siRNA nanoparticles so that nanoparticles were protected by PEGylation during circulation but underwent de-PEGylation after entering the tumor microenvironment due to charge repulsion between the newly formed positively charged polymer ligand and the cationic PEI/siRNA core, leading to enhanced tumor cell uptake and gene silencing efficiency.

The highly reductive intracellular environment is another endogenous stimulus commonly exploited for designing responsive polymers to facilitate rapid release of RNA payload into the cytosol. High concentration (mM range) of intracellular glutathione (GSH) accounts for a key part of the reductive intracellular environment, whereas extracellular GSH is usually much lower (μM range) (Cheng *et al.*, 2011). This inspired construction of bio-reducible polymers containing disulfide linkages that can be cleaved by intracellular GSH through thiol-disulfide exchange reaction. The disulfide linkage has been incorporated into the backbone of a number of cationic polymers, such as PEI, (Xia *et al.*, 2013; Dai and Zhang, 2019) PBAE (Kozielski *et al.*, 2013; Yin *et al.*, 2011) PDMAEMA (Lin *et al.*, 2013), and PAMAM (Nam *et al.*, 2015) for fabrication of redox-responsive polymeric nanoparticles for RNA delivery. Farokhzad *et al.* developed a class of L-cysteine-based biodegradable poly (disulfide amide)s (PDSAs) via a facile and rapid one-step polycondensation reaction of L-cystine ester and versatile fatty diacids (Wu *et al.*, 2015). The resulting PDSAs and corresponding nanoparticles are redox-hypersensitive with controllable hydrophobicity, degradation rate, and redox response that are tuned by the alternation of the fatty diacid structure. Nanoparticles based on this type of PDSA were later co-formulated with cationic lipidoid G0-C14 to systemically deliver siRNA (Xu *et al.*, 2018) and mRNA (Kong *et al.*, 2019) for cancer therapy in animal models.

Upregulation of reactive oxygen species (ROS) parallels many diseases and ROS-responsive polymers have been designed to release RNA specifically at the diseased site in response to upregulated ROS (Wilson *et al.*, 2010; Zheng *et al.*, 2019, 2018). ROS responsiveness can be imparted to polymers via two major mechanisms, namely ROS-induced non-cleavable hydrophobic-hydrophilic transition and ROS-induced cleavage of chemical bond (Ye *et al.*, 2019). Polymers containing chalcogen elements (e.g., thioether, selenium, tellurium) can be readily oxidized by ROS and form covalent bonds with oxygen atoms, which enable hydrogen bonding formation with water molecules and thus achieve the hydrophobic-hydrophilic transition. In other cases, ROS can cleave polymers containing chemical structures like thioketals, vinyldithioethers, phenylboronic acids/esters (PBAs/PBEs), and proline oligomers. For example, Murthy *et al.* developed a polymer PPADT composed of ROS-sensitive thioketal linkages and co-formulated it with the TNF-α–siRNA and cationic lipid DOTAP into thioketal nanoparticles (TKNs) (Wilson *et al.*, 2010). The TKNs are stable to acid-, base- and protease-induced degradation and remain intact in the gastrointestinal tract after oral administration, but specifically release TNF-α–siRNA to sites of intestinal inflammation in response to the abnormally high levels of ROS, leading to effective silencing of proinflammatory TNF-α mRNA in intestines and protection from ulcerative colitis in mouse models.

The heterogeneity of enzyme activity within the body is also exploitable for designing enzyme-responsive polymers to enhance RNA delivery. Certain pathological events such as inflammation and cancer are accompanied by an up-regulation of enzyme concentrations or activities in the diseased site (De La Rica *et al.*, 2012). Enzyme-responsive polymers are most often prepared by covalent conjugation of enzyme-cleavable sequence to the polymer backbone and typical trigger enzymes include proteases, esterases, glycosidases as well as transferases (Wang *et al.*, 2018). Gly-Phe-Leu-Gly is a peptide sequence that can be cleaved by cathepsin B, a lysosomal protease overexpressed in cancer cells (Gondi and Rao, 2013). Several polymer-drug conjugates via this peptide linker have entered the clinical trials for enhanced release of chemo-drugs in tumors (Duncan, 2009). Cathepsin B-responsive polymeric vectors were also synthesized to enhance the endosomal release of siRNA to the cytosol (Rozema *et al.*, 2015). The delivery approach works by reversible masking of the cationic membrane-disruptive polymer via protease-sensitive substrates. Matrix metalloproteinases (MMPs), a family of zinc-dependent proteases that function to degrade extracellular matrices, are found to be up-regulated in various diseases including cancer (Malemud, 2006). Torchilin *et al.* reported the construction of MMP-2 responsive micelles based on a PEG-peptide-PEI-PE copolymer for systemic co-delivery of siRNA and hydrophobic drugs to the tumor (Zhu *et al.*, 2014b). The MMP-2 sensitive peptide linker allows for tumor-specific de-shielding of PEG and exposure of previously hidden PEI for enhanced tumor cell uptake. In a recent study (Zhou *et al.*, 2019), Shen and co-workers developed a γ-glutamyl transferase (GGT)-sensitive polymer that can achieve negative-to-positive charge reversal after GGT-mediated conversion of γ-glutamylamide to primary amine on the polymer side chains. In situ cationization of the drug-conjugated polymer nanoparticles by GGT, which is overexpressed on the cell membrane of tumor endothelium as well as cancel cells, was found to induce adsorption of the nanoparticles to the cell membrane and initiate adsorption-mediated transcytosis across multiple cell layers, leading to significantly augmented tumor penetration depth and treatment efficiency. This type of GGT-responsive polymer may also be exploited for delivery of RNA to solid tumors.

The immense difference between intracellular adenosine triphosphate (ATP) level and extracellular ATP level has also been utilized to design polymers that facilitate RNA delivery into cells. Phenylboronic acid (PBA), a synthetic molecule applied in RNA affinity chromatography, is able to form reversible covalent ester bonds with 1,2- or 1,3-*cis*-doils on the ribose ring (Uğuzdoğan *et al.*, 2002). Kataoka group exploited this property and synthesized a cationic di-block copolymer, poly (ethylene glycol)-*block*-poly (L-lysine), that is further functionalized with PBA for siRNA binding and self-assembly into micelles. After cellular uptake, the siRNA can be rapidly released from micelles in response to abundant intracellular ATP due to competitive binding of the ATP ribose to the PBA moiety (Naito *et al.*, 2012, 2018).

Exogenous stimuli-responsive polymers

Light is an appealing external stimulus for RNA delivery because it is safe and non-ionizing radiation with good spatial and temporal controllability. The light-responsive polymer can be engineered to promote rapid and controlled endosomal escape of RNA. Blersch *et al.* performed a high-throughput screening of light-sensitive polymeric nanoparticles for non-coding RNA transfection by creating a library of 160 polymer formulations that contain *o*-nitrobenzyl group-based photocleavable linkers (P1) in the polymer backbone (Blersch *et al.*, 2020). Using the best formulation identified (P1C7), gene knockdown efficacy with green

fluorescence protein (GFP) siRNA and ultraviolet light (UV) irradiation was five-fold higher over the control vector lipofectamine thanks to enhanced endosomal escape after UV irradiation. The P1C7 nanocarrier was further tested for the in vivo delivery of miRNA involved in skin wound healing (miRNA-150–5p) using a mouse model. Wounds treated with the miRNA via the light-responsive polymeric nanoparticles and UV irradiation healed faster and expressed lower quantities of the miRNA target gene compared to the control groups. Duan *et al.* (2020) developed a photodegradable branched poly (ß-amino ester) (BPAE-NB) by incorporating light-sensitive 2-nitrobenzene moieties into the backbone, which enabled strong siRNA condensation through its cationic polymer chains while also possessing the ability to break down into low-molecular-weight components via UV light irradiation in order to promote siRNA release and reduce cytotoxicity. Near-infrared (NIR) light is considered superior to UV light as an external trigger in that it can penetrate much deeper into tissues and also interfere less with biological molecules. Zhao *et al.* (2017) reported a siRNA delivery system comprised of an inorganic up-conversion nanoparticle coated with polymers containing UV light-sensitive cationic side chains for siRNA complexation. The up-conversion nanoparticle could convert 980 nm NIR light into UV light, which then could transfer the UV light-sensitive polymer side chains from cations to zwitterions, enabling effective siRNA release in response to NIR light irradiation.

Ultrasound, due to its safe and noninvasiveness nature, has also been applied to facilitate polymeric nanoparticle delivery of RNA cargos. High-intensity focused ultrasound (HIFU, frequency range 0.8–3.5 MHz) is particularly promising as an external trigger since it can generate a small focal point of sufficiently high ultrasound intensity to induce changes while away from the focal spot the intensity is low and harmless to the body (Manouras and Vamvakaki, 2017). The ultrasound-responsive polymeric nanoparticles are commonly achieved through the incorporation of gas bubbles that collapse transiently in response to ultrasound, resulting in a local cavitation effect that can increase cell membrane permeability and promote the release of the active cargos (Manouras and Vamvakaki, 2017). For example, Shuai *et al*. developed an ultrasound-responsive nano-system comprised of positively charged siRNA/polymer micelles complexed onto negatively charged gas-core liposomes, which showed enhanced gene silencing and therapeutic effect in mouse cancer models upon exposure to ultrasound (Yin *et al*., 2013, 2014). Of note, polymers that are intrinsically responsive to ultrasound are also available, such as those containing the HIFU-labile 2-tetrahydropyranyl methacrylate (THPMA) motif (Wang *et al*., 2009; Xuan *et al*., 2012). These polymers with intrinsic ultrasound sensitivity may be exploited for RNA delivery in the future.

Magnetic fields are another external stimulus frequently exploited for drug delivery. It penetrates deep in the tissue and barely interacts with the body, making the magnetic field one of the safest external triggers. The use of magnetic fields has been mainly centered on enhancing the accumulation of magnetic drug delivery systems in the diseased site via the application of external magnets. An obvious way to apply this to RNA delivery is via the integration of cationic polymers with magnetic nanoparticles such as superparamagnetic iron oxide nanoparticles (SPIO) (Chen *et al*., 2013; Liu *et al*., 2011; Park *et al*., 2011). Mao *et al*. demonstrated using PEI coated SPIO to deliver siRNA to inflamed joints under the guide of an external magnetic field for treatment of rheumatoid arthritis in a rodent model (Duan *et al*., 2014). Some metal-containing polymers (metallopolymers) can also exhibit magnetic properties by themselves (Yan *et al*., 2016b), and their cationic variants may be explored for RNA complexation and delivery in the future (Zhu *et al*., 2018). Moreover, the superparamagnetic property was also observed in pure organic π-conjugated polymers (Rajca *et al*., 2001), however, their application in drug delivery is currently limited by the very low temperature (below 10K) required to exhibit magnetism.

Thermo-responsive polymers can change their physical or chemical properties in response to heat. Since heat in tissue can be easily generated and controlled by means such as photothermal effect, alternating magnetic field, and focused ultrasound (Kamaly *et al*., 2016), thermo-responsive polymers are promising delivery vectors for RNA. A classic example of a thermo-responsive polymer is Poly (N-isopropyl acrylamide) (PNIPAM) invented in the 1950s (Calejo *et al*., 2013). PNIPAM is hydrated with extended chain conformation when the temperature is below its lower critical solution temperature (LCST, $\sim$32°C in water) but undergoes a reversible phase transition to a dehydrated and hydrophobic form when the temperature is above LCST, resulting in a compact and collapsed conformation that could lose 90% of its hydrated volume (Forney *et al*., 2013). The LCST of PNIPAM can be further tuned by copolymerization or grafting with other components (Roy *et al*., 2013). A number of copolymers containing PNIPAM and cationic polymers have been reported to deliver DNA plasmids, (Kurisawa *et al*., 2000; Hinrichs *et al*., 1999; Türk *et al*., 2004; Zintchenko *et al*., 2006) which theoretically should also be applicable to RNA delivery. A popular alternative to PNIPAM is Poly (*N*-vinyl caprolactam) (PNVCL), a thermo-responsive polymer with similar LCST but improved biodegradability and biocompatibility (Cortez-Lemus and Licea-Claverie, 2016). Other thermo-responsive degradable polymers include certain polyesters (Jiang *et al*., 2008), polycarbonates (Kim *et al*., 2011), and poly (ethylene oxide)/poly (propylene oxide) copolymers (also called poloxamers or Pluronics®) (Zhang *et al*., 2010). In particular, cross-linked Pluronic/PEI nanocapsules exhibiting a thermally reversible swelling/deswelling volume expansion behavior were demonstrated by Park *et al*. as efficient vectors for temperature variation-induced endosomal disruption and cytosolic delivery of siRNA (Choi *et al*., 2006; Lee *et al*., 2008).

Preparation Methods of Polymeric Nanoparticles for RNA Delivery

Besides the polymeric materials themselves, formulation of the polymer and RNA into RNA-containing nanoparticles also plays a major role in the overall efficiency of RNA delivery, as this process dictates the size, surface chemistry, stability, and RNA loading efficiency of the resulting nanoparticles. Generally, polymeric nanoparticles can be prepared either by dispersion of pre-formed polymers in bulk (top-down approach) or polymerization of monomers (bottom-up approach). However, the bottom-up

approach often requires mixing of organic solvents, surfactants along with monomers and initiators, some of which can be toxic or incompatible with RNA molecules and require sophisticated purification steps afterward, thus the bottom-up approach is seldom adopted for RNA delivery. This section outlines the common strategies used to prepare water-soluble polymeric nanoparticles for RNA delivery using pre-formed polymers.

Emulsion/Solvent Evaporation Method

The emulsion/solvent evaporation method is a classical way to prepare polymeric nanoparticles from a preformed polymer. The nanoparticles are formed by emulsifying a volatile water-immiscible organic phase containing the preformed polymers with an aqueous phase containing the surfactants (emulsifiers), followed by evaporation of the volatile organic solvent through prolonged stirring and/or reduced pressure (Soppimath *et al.*, 2001). Commonly used organic solvents for this method include dichloromethane, chloroform, and ethyl acetate, while polyvinyl acetate (PVA), Vitamin E TPGS and PEGylated lipids are examples of the surfactants employed to stabilize the emulsions. The emulsification is typically carried out via high-sheer force processes such as vigorous homogenization or ultrasonication to mechanically reduce the size of emulsion droplets. There are two approaches to prepare emulsions, e.g., oil-in-water (o/w) or so-called single-emulsions, and (water-in-oil)-in water (w/o/w) or so-called double-emulsions. The single-emulsions technique requires dissolving the active ingredients (e.g., RNA) with the polymers in organic solvents, which can be challenging for the highly hydrophilic RNA or may need additional modification of the RNA to increase its solubility in organic solvents. In contrast, the double-emulsions technique allows dissolving RNA in a small amount of aqueous phase and forms water-in-oil emulsions first, then the water-in-oil emulsions are further emulsified with a surfactant-containing aqueous solution to produce aqueous soluble nanoparticles. The resulting particle size is affected by the particular emulsification technique, type, and concentration of surfactant, as well as properties of the polymer itself (Tice and Gilley, 1985).

Emulsion/Solvent Diffusion Method

This method involves emulsification of a partially water-miscible organic solvent (e.g., benzyl alcohol, isopropyl acetate, ethyl acetate) containing the polymer and RNA with an aqueous solution containing the surfactant. The phase separation and formation of oil-in-water emulsion is achieved through saturation of the aqueous phase with the partially water-miscible organic solvent. Once the emulsification is completed, a large amount of water is added to dilute the emulsion and induce diffusion of organic solvent from dispersed droplets into the surrounding aqueous phase, leading to the formation of solid polymeric nanoparticles. The organic solvent can then be eliminated by filtration or evaporation. It should be noted that the hydrophilic RNA is also at risk of diffusion out of the nanoparticles to the aqueous phase. Increasing the binding of RNA to polymers via electrostatic/hydrophobic interactions may improve the RNA entrapment efficiency.

Emulsion/Reverse Salting-out Method

This method can be considered as a modified version of the emulsion/solvent diffusion strategy. It is based on the salting-out effect, that is separation of certain water-miscible organic solvent from aqueous solution with a high concentration of dissolved salts (high ionic strength). Instead of using partially water-soluble organic solvents like those in the emulsion/solvent diffusion method, this method forms emulsions from water-soluble organic solvents (e.g., acetone or ethanol) and an aqueous phase that contains surfactants and high concentrations of salting-out agents. Typical salting-out agents are electrolytes, such as magnesium chloride or calcium chloride, but non-electrolytes like sucrose could also be used (Reis *et al.*, 2006). After preparing the oil-in-water emulsion (with RNA in the organic phase), a dilution of the salting-out agents by pure water is initiated to increase the miscibility of the organic solvent with the aqueous phase, thus allowing the organic solvent in dispersed droplets to diffuse into the external aqueous phase and eventually leading to the formation of solid nanoparticles. The salting-out agents and organic solvents can be eliminated by centrifugation or filtration in the end to purify the polymeric nanoparticles.

Nanoprecipitation Method

The nanoprecipitation method, also designated as solvent displacement or interfacial deposition method, is based on the rapid mass transfer along with the interface of two liquids with different surface tensions, a phenomenon named Marangoni effect (Bilati *et al.*, 2005). In this method, the polymer along with RNA is dissolved in a water-miscible organic solvent (e.g., acetone, acetonitrile, dimethylformamide) and subsequently added to a copious aqueous phase in a dropwise manner under moderate stirring. Because of the Marangoni effect, rapid and spontaneous dispersion of the polymer solution into the aqueous phase occurs, leading to the formation of nano-sized polymer droplets (Quintanar-Guerrero *et al.*, 1998). As the organic solvent diffuses out of the nanodroplets, the polymer precipitates in the form of solid nanoparticles. Usually, a surfactant is added to the aqueous or organic phase to increase the colloidal stability and prevent aggregation of the polymeric nanoparticles. The nanoprecipitation method is advantageous in term of its simplicity and ease of operation, however, like the solvent diffusion method, hydrophilic payloads are often entrapped with a lower efficiency than hydrophobic ones (Barichello *et al.*, 1999). Therefore, increasing the binding of RNA to polymers is important to ensure a high RNA loading efficiency. Additionally, the parameters involved in the fabrication process, such as oil/aqueous phase

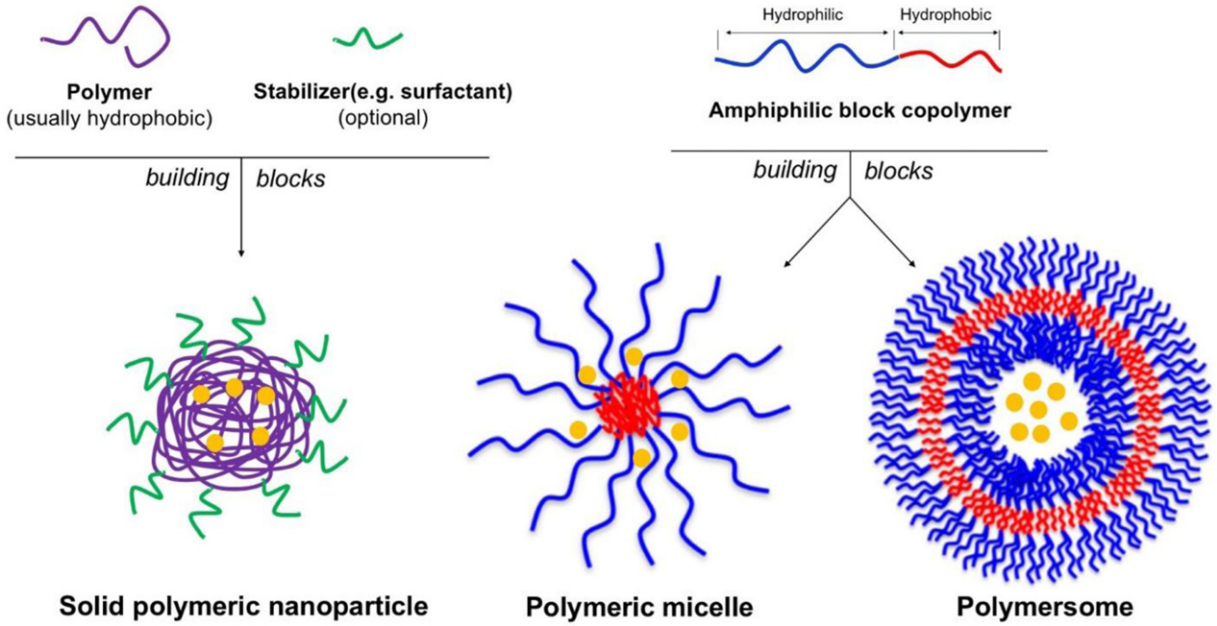

Fig. 3 Structures of solid polymeric nanoparticle, polymeric micelle, and polymersome. Yellow dots represent loaded RNA cargos. Adapted with permission from Lotocki, V., Kakkar, A., 2020. Miktoarm star polymers: Branched architectures in drug delivery. Pharmaceutics 12, 827.

ratio, organic phase injection rate, and aqueous phase agitation rate need to be optimized as they can greatly affect the size distribution as well as entrapment efficiency of the resulting nanoparticles (Reis *et al.*, 2006).

Polymeric Micelle

Characterized by a hydrophobic core and hydrophilic shell, polymeric micelles are nano-sized aggregates formed by the self-assembly of amphiphilic block copolymers in an aqueous solution. To prepare micelles, amphiphilic copolymers are usually first dissolved in a small volume of organic solvent and then water is added dropwise under stirring to induce the self-assembly of amphiphilic copolymers into micelles, followed by removal of the organic solvent via dialysis or evaporation. The payload such as RNA can be further loaded into the micelles by simply mixing RNA with micelles containing cationic segments. Similar to low-molecular-weight surfactants, amphiphilic block copolymers exist separately as unimers in diluted aqueous solution but start to aggregate and self-assemble into micelles when their concentration is increased to a certain level (Lotocki and Kakkar, 2020). This minimally required concentration for micelle formation is referred to as the critical micelle concentration (CMC), which is influenced by factors such as polymer type, structure, length as well as components of the aqueous medium (Ghezzi *et al.*, 2021; Owen *et al.*, 2012). Micelles are stable in a dynamic way with a continuous exchange of unimers between the micelle and the bulk phase when the unimer concentration is higher than the CMC, however, they tend to disassemble after dilution below the CMC. A lower CMC value corresponds to a micelle system with higher thermodynamic stability. Compared to micelles made of low-molecular-weight surfactants, polymeric micelles are much more stable with CMC typically at micromolar range (Ghezzi *et al.*, 2021; Owen *et al.*, 2012). Even at a concentration below CMC, polymeric micelles can retain their structure much longer than low-molecular-weight surfactant micelles, which usually disassemble in a time scale of microseconds (Ghezzi *et al.*, 2021). This is due to the fact that the long polymer chains have more points of interaction than small molecules (Owen *et al.*, 2012). Increasing the hydrophobic chain length is associated with higher micelle stability and cross-linking of the micelle core or shell can improve micelle stability as well (Lu *et al.*, 2018).

Polymersome

Analogous to the liposome, polymersomes are synthetic vesicular structures with a polymer-based bilayer membrane and an aqueous core that can accommodate hydrophilic payloads such as RNA (**Fig. 3**). Polymersomes are also formed by the self-assembly of amphiphilic block copolymers in an aqueous solution. A common method to prepare polymersomes is via the film rehydration technique, in which a volatile solvent with dissolved amphiphilic copolymers is evaporated to produce a thin film of multilayer copolymers, followed by rehydration with an aqueous solution containing the payloads to be encapsulated and vigorous agitation, sonication and/or extrusion to yield polymersomes of a narrow size distribution (Letchford and Burt, 2007). Other methods, such as electroformation, cosolvent addition, and double emulsion templating, have also been applied for the synthesis of polymersomes (Iqbal *et al.*, 2020). Due to the thick, robust, and intercalated membrane structure, polymersomes often

possess a higher chemical and physical stability than their lipid counterparts, enabling payload encapsulation in a less leaky and more controllable way. Tuning the composition and molecular weight of the copolymers allows for controlling the polymersome properties such as membrane permeability and responsiveness (Zhu *et al.*, 2014a).

Microfluidics

Microfluidic technology involves the controlling of fluids in microscale channels, which has broad applications in areas such as lab-on-a-chip, (Dittrich and Manz, 2006; Abgrall and Gue, 2007) point-of-care diagnosis, (Wang *et al.*, 2013; Zhang *et al.*, 2017) and cellular manipulation (Warkiani *et al.*, 2016; Yun *et al.*, 2013). Historically, microfluidic systems were fabricated on glass or silicon via photolithography and etching strategies derived from microelectronics manufacturing (McDonald *et al.*, 2000). Modern microfluidics is often made from poly (dimethylsiloxane) (PDMS) by soft lithography methods that are faster and less expensive, providing rapid prototyping without the need for cleanrooms for fabrication (McDonald *et al.*, 2000). The continuous variability of reaction conditions, precise control of parameters, and enhanced mixing are some of the key features that have made microfluidics attractive for the synthesis of nanoparticles (Marre and Jensen, 2010). Polymeric nanoparticles prepared by bulk synthesis tend to be heterogeneous with large polydispersity in size due to the inability to control the mixing condition of precursors. With the millisecond mixing, rapid solvent exchange, and tunable operating condition, polymeric nanoparticles synthesized by microfluidic systems displayed greatly improved controllability, homogeneity and reproducibility (Karnik *et al.*, 2008). In a comparison study, PLGA nanoparticles fabricated by nanoprecipitation in microfluidics resulted in an average size of 50 nm with a polydispersity index (PDI) of 0.1, in contrast, those produced by the bulk nanoprecipitation method had an average size of 200 nm and PDI of 0.4 (Wang *et al.*, 2014). Monodispersed polymersomes and micelles have also been produced in a controllable way using microfluidics (Shum *et al.*, 2008; Capretto *et al.*, 2013). Various microfluidic techniques, including hydrodynamic flow focusing (HFF) (Rhee *et al.*, 2011), HFF with micromixer (Hasani-Sadrabadi *et al.*, 2016), droplet-based method (Hung *et al.*, 2010), and in-fiber emulsification (Kaufman *et al.*, 2012) have been developed for the high-quality synthesis of size-controlled and cargo-loaded polymeric nanoparticles. Several microfluidic systems, such as NanoGenerator™ and NanoAssemblr®, are also available commercially for the production of polymeric nanoparticles up to the clinical scale.

Besides these common methods, other methods such as electrospraying (Kurakula and Naveen, 2021) and supercritical fluid technology (Chakravarty *et al.*, 2019) are also able to produce polymeric nanoparticles with their respective advantages, but they require special equipment and are also not often used for RNA formulation currently. Other nanoparticle formulation techniques may exist for a specific type of polymers. For example, ionic gelation is a common method used for the preparation of chitosan- or alginate-based nanoparticles through multivalent ion-mediated crossing-linking effect (Fan *et al.*, 2012; Pedroso-Santana and Fleitas-Salazar, 2020). For large-scale and clinical-stage production of polymeric nanoparticles requiring stringent batch-to-batch consistency and quality control, microfluidics may become indispensable as they not only allow for the synthesis of polymeric nanoparticles with narrow size distribution and high reproducibility, but also enable in-line characterization, feedback control, and high-throughput continuous production (Fraikin *et al.*, 2011; Gong, 2008; Wang *et al.*, 2010).

Summary

The world has witnessed the tremendous power of mRNA vaccines in helping end the COVID-19 pandemic, which very likely will spark a new round of effort for developing more RNA-based therapeutics. With our evolving understanding of the key roles that different RNAs are playing in regulating the biological processes, a safe and robust way to deliver them to the right place becomes the greatest challenge in order to translate our fundamental understandings into life-saving therapeutics. Polymeric nanoparticles are a class of promising vehicles for RNA delivery. They can provide the RNA with the necessary protection against enzymatic degradation along the path to the site of action and also be facilely engineered to display a variety of functionalities such as disease targeting, endosomal escaping, RNA releasing as well as intelligently responding to internal or external stimuli. Moreover, polymeric nanoparticles can be constructed completely biodegradable so that they are degraded into biocompatible, non-toxic, and easy-to-eliminate small molecules after fulfilling their delivery task, minimizing potential side effects associated with the delivery vector.

While significant progress has been made, many challenges still present and hinder the translation of polymeric nanoparticle-based RNA therapeutics to the clinics. The majority of polymers developed for RNA delivery are highly cationic, which are prone to induce cytotoxicity and adverse immune response if being taken up non-specifically. On one hand, strategies to improve the targeting specificity of polymeric nanoparticles should be advanced. For example, nanoparticle-mediated tissue-specific delivery of RNA has been reported for the lung, spleen, and liver, in which the tissue selectivity after intravenous administration was thought to arise from distinct protein coronas that could be recognized specifically by certain organs (Kaczmarek *et al.*, 2016; Cheng *et al.*, 2020). In this regard, studies on the relationships between protein corona identity and in vivo fate of nanoparticles may guide future designing of polymeric nanoparticles for tissue-specific RNA delivery; On the other hand, it is highly desired to develop non-cationic polymers and mechanisms that can still aid efficient RNA protection and delivery (Wang *et al.*, 2019; Yamankurt *et al.*, 2020). The large molecular weight, diverse polymer architecture, and numerous functional groups render polymeric nanoparticles highly engineerable, which permits a high degree of modification for optimization or increasing the versatility of polymeric

nanoparticles in RNA delivery; however, the complex structure and modification also pose a serious challenge in terms of batch-to-batch production of the polymeric nanoparticle vectors in a consistent and reproducible way, which is critical for clinical translation. This demands advances in polymerization techniques to ensure a high level of control over polymer properties such as their molecular weight, architecture and polydispersity as well as control over the formation of nanoparticles. Fundamental research on how polymeric nanoparticles interact with the biological system and their structure-property relationships under physiological condition are also important as these knowledge should offer guidance for designing polymeric nanoparticles tailored to specific delivery needs. With continued research and development, it is anticipated that polymeric nanoparticles will constitute a crucial member of delivery vectors to help maximize the clinical potential of RNA therapeutics.

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