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Advancing nucleic acid delivery through cationic polymer design: non-cationic building blocks from the toolbox

Ilaria Porello, Nina Bono, Gabriele Candiani and Francesco Cellesi *

Polymers used for the delivery of nucleic acids (NAs) typically possess ionizable, cationic moieties enabling their electrostatic interactions with negatively charged NAs and form stable polyplexes. However, non-cationic building blocks have been harnessed to design cationic polymers with enhanced delivery of DNA/RNA to tissues, cells, and subcellular compartments while remaining stable in biological fluids. By customizing the chemistry of these functional groups, we can improve cell targeting behavior, uptake, endosomal escape, non-toxicity, and transfection efficiency. Additionally, the physicochemical properties, such as the loading capacity, complexation ability, size and morphology, biodegradability, pH sensitivity, and amphiphilicity, can be adjusted based on the specific application. This review summarizes the role of non-cationic moieties in various biomedical contexts, from therapeutic interventions to gene editing. By unpacking and critically summarizing the existing literature, this review provides valuable insights into the rational integration of these building blocks for designing more effective nanovectors to deliver NAs.

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1. Introduction

Delivery of nucleic acids (NAs) into mammalian cells represents a ground-breaking advancement in molecular medicine,[1] holding significant promise for genetically treating various diseases, including inherited disorders, certain types of cancer,[2] and specific viral infections (*i.e.*, COVID-19 vaccines).[3–5] Additionally, it is pivotal in basic research because it allows the exploration of cellular mechanisms and the production of specific proteins.[6]

By definition, gene-based therapy is the deliberate modulation of gene expression in target cells accomplished by introducing exogenous NAs such as DNA,[7] RNA (mRNA, small or short interfering RNA (siRNA), short hairpin RNA (shRNA),[8,9] microRNA (miRNA)),[10] antisense oligonucleotides (ASOs),[11] and CpG oligodeoxynucleotides or oligoribonucleotides (CpG-ODNs or CpG-ORNs, respectively).[12–14] Given the large size and the negative charge of these macromolecules, which limit their ability to freely cross the cell membrane (*i.e.*, at physiological pH they are both anionic, resulting in electrostatic repulsion), and their limited extracellular stability, the intracellular delivery of NAs is typically achieved using physical/mechanical methods[15–17] or mediated by vectors.[18–21]

Physical and mechanical methods, such as electroporation,[22] sonoporation,[23] magnetofection,[24] optoporation,[25] gene gun,[26] and microinjection,[27] drive naked NAs into the cytosol or the cell nucleus to achieve successful gene transfer. Despite their potential and the attention garnered from scientists, the limitations of these technologies make them less attractive than others for NA transfer purposes. Indeed, they often cause toxicity and are not very effective when used *in vivo*. A straightforward way to deliver NAs into cells relies on the use of gene delivery carriers (vectors), categorized as viral and non-viral. Engineered viral vectors, wherein the therapeutic gene cassette replaces a portion of the viral genome, currently stand as the most extensively utilized carriers in gene therapy, owing to their natural ability to enter host cells to give high transduction efficiency.[18,28,29] While significantly advancing the field of gene therapy, viral vectors come also with several drawbacks, including carcinogenesis, immunogenicity, broad tropism, limited DNA packaging capacity, and challenges in vector production.[30–33] Non-viral gene delivery (*i.e.*, a process called transfection) holds promise in addressing many of these limitations, especially when it comes to safety. For instance, synthetic vehicles are generally less immunogenic as compared to viral vectors, and patients lack pre-existing immunity, as is the case with certain viral systems. This results in non-viral vectors that are safer and better tolerated by the human body, enabling repeated administration in patients affected by diseases requiring long-term treatment, such as chronic conditions, without causing an immunological response or toxic accumulation in off-target organs. Non-viral vectors also offer

Department of Chemistry, Materials and Chemical Engineering "G. Natta", Politecnico di Milano, Via Luigi Mancinelli 7, 20131 Milan, Italy.
E-mail: francesco.cellesi@polimi.it

the potential to deliver larger genetic payloads and are typically easier to synthesize in a more reproducible manner than their viral counterparts.[21,34,35] In most cases, non-viral vectors rely on the use of cationic lipids (CLs),[7,20] cationic polymers (CPs),[21,36–38] or copolymers based on the combination of cationic monomers with hydrophobic and hydrophilic ones.[39–41] These macromolecules self-assemble with anionic NAs to form particle-like complexes (lipoplexes and polyplexes for CLs and CPs, respectively, or micelleplexes for amphiphilic CPs) ranging in hydrodynamic diameter from tens of nanometres to a few micrometres. The small size eases their penetration through the cellular membrane and subsequent NA internalization into cells. Unfortunately, non-viral vectors still possess lower transfection efficiency than their viral counterparts. On the other hand, they can be deliberately designed to encompass multiple functionalities, allowing them to navigate and overcome diverse biological barriers encountered *in vitro* and *in vivo* throughout the gene delivery process.

In this context, structure–activity relationship (SAR) studies of non-viral carriers have paved the way for improving their effectiveness and creating customized, functionalized architectures[42] with specific physicochemical features that ease intracellular delivery and precise cargo release at the target site.[43,44]

2. Challenges in nucleic acid delivery

To maximize the effectiveness of non-viral gene delivery carriers, their transfection efficiency must be improved.[45,46]

Regardless of the *in vivo* administration route (*e.g.*, inhalation, intramuscular injection, intravascular injection), non-viral complexes shall inevitably face the extracellular environments that blunt the fraction of nanovectors that reach the target therapeutic site. The optimal delivery vehicle should be neither cytotoxic nor immunogenic, protect the payload from nuclease degradation during the delivery process, and mediate the intracellular delivery of NA cargo. Further requirements include the control of the pharmacokinetics, distribution, and accumulation at the target site.[47]

The biological barriers that polyplexes encounter depend on the route of administration.[48] Although local delivery may help avoiding some hurdles faced with systemic delivery, the former typically requires deploying more complex techniques that pose other limitations. Furthermore, local delivery may only be useful in diseases where the pathology is restricted to known, accessible sites, such as certain solid cancers or traumatic injuries, so systemic administration is preferred. If limited to systemic administration, a successful carrier shall: (i) have prolonged plasma circulation and be able to cross the vessel wall, (ii) be internalized by cells passing through the plasma membrane, (iii) escape the endosome and release NAs intracellularly, and, in the case of the DNA cargo, (iv) enter the nucleus. A schematic representation of these *in vivo* hurdles is depicted in Fig. 1.

Within the circulatory system, various determinants, including serum proteins and blood cells, can hinder the biological activity of the particles, leading to their rapid clearance by the reticuloendothelial system (RES) and/or degradation before reaching the target. In such instances, the complexes are swiftly cleared from circulation by the mononuclear phagocyte system (MPS), which results in the accumulation of complexes in off-target organs, such as the spleen and the liver, contributing to their distribution into healthy organs.[49] Opsonization and recognition by immune cells, such as mononuclear phagocytes, pose a significant barrier to viral and non-viral delivery, as these professional phagocytic cells engulf the delivery particles before eliciting their effect.[50,51]

Afterward, the particles must cross the endothelium to exit the bloodstream and reach the target organ, tissue, or cell(s).

Once the delivery particles reach the target cells, they next encounter another significant barrier, namely the plasma membrane. Complexes often bind to the cell membrane through electrostatic interactions before entering the cell.[52] Depending on their physicochemical features, complexes are internalized through passive (*i.e.*, fusion) or active (receptor-mediated) transport. Endocytosis, defined as the process by which cells internalize substances through membrane-bound vesicles, is the primary mechanism responsible for the internalization of non-viral particles into cells. Regardless of the endocytic pathway (clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CvME), or micropinocytosis),[53] internalized polyplexes ultimately become trapped in endosomes. Therefore, another major hurdle to successful gene delivery is the release of the NA from the endosome before it undergoes degradation at the lysosomal level.[54] This implies that the endosomal escape should be essential for efficient transfection. Different mechanisms have been suggested for polyplexes to escape the endosomal compartment. These rely on the proton sponge theory, and polyplex- or polymer-mediated membrane destabilization (for comprehensive reviews, refer to ref. 55 and 56).

According to the proton sponge theory, most polycations function as a proton buffer and their protonable groups cause osmotically-induced membrane burst.[57] Upon endosome maturation, the membrane-bound V-ATPase proton pumps actively translocate H^+ into the endosomal lumen. The polymers with high buffer capacity can thus bind H^+, thereby counteracting endosome acidification. In an attempt to lower the pH, the proton pumps translocate even more H^+ into the endosomal lumen, which goes along with the entry of Cl^- ions to maintain the charge balance and leads to an increase in ionic concentration and an influx of water to maintain osmolarity. This process results in an increase in osmotic pressure that induces endosome swelling and eventually the release of the genetic cargo into the cytosol.[58–62]

In contrast to the proton sponge theory, which assumes that there is no interaction between polyplex and endosomal membrane, other studies support the theory of membrane permeability and nanohole formation.[63,64] These theories explain the endosomal escape of CP-based vectors and differentiate between hole formation induced by an intact polyplex and through intercalations of free polymer with the endosomal

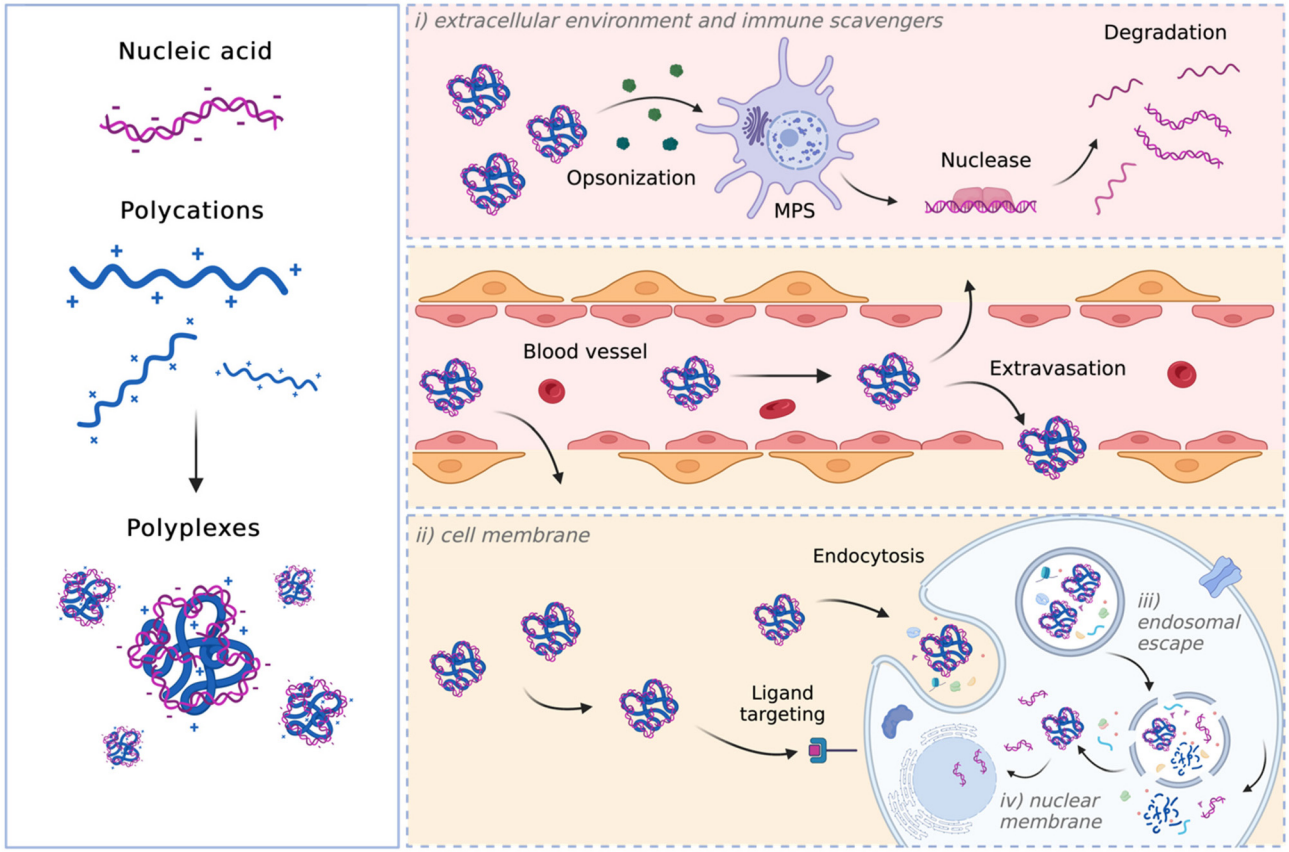


Fig. 1 Schematics of the extracellular and intracellular barriers encountered by non-viral vectors: (i) the extracellular environment and immune scavengers (*e.g.*, MPS), (ii) the cell membrane, (iii) endosomal escape, and (iv) the nuclear membrane (image created with Biorender.com).

membrane. In both cases, the endosomal compartment remains undamaged.[65]

In the first case, once entrapped within an endo-lysosomal compartment, the polyplexes will be protonated due to the activity of the membrane-bound ATPases. The polyplex with increasing charge density directly interacts with the phospholipids of the inner membrane leaflet. This interaction leads to membrane destabilization and a local loss of membrane integrity, thus forming nano-holes in it. The vector can escape the endo-lysosomal compartment through such holes.[65,66] On the other hand, the free polymer-mediated escape theory is based on the unceasing exchange of the polymer chains between and to the polyplexes and the environment. Free polymer chains intercalate within the membrane of endo-lysosomal compartment, leading to membrane destabilization and a local loss of membrane integrity. The polyplex escapes the endo-lysosomal compartment because of the polymer-induced membrane destabilization,[67] releasing the genetic cargo into the cytosol.

It is worth noting that while RNAs elicit their function in the cytosol, DNA molecules need to enter the nucleus. Hence, the intranuclear transport of DNA is another issue in the way of successful delivery.

Relying on the aforementioned considerations, an ideal NA vector shall: (i) be cationic to interact with NAs and give rise to positively charged complexes, which show enhanced NA protection and high affinity for the negatively charged cell surface; (ii) demonstrate buffer capacity to enable the proton-sponge effect; (iii) be biodegradable,[68] meaning that they should be promptly eliminated from the body after the delivery of NAs. This behavior prevents significant accumulation in the body and minimizes potential organic damage, especially after repeated administrations.[69]

In the case of CPs, all these behaviors depend upon the structural and chemical features of the polymer, encompassing charge density, hydrophobicity/hydrophilicity, architecture, and molecular weight (M_w).[70] In this regard, non-cationic building blocks have been utilized to endow CPs with improved DNA/RNA delivery behavior to tissues, cells, and subcellular compartments, while retaining stability in biological fluids.

Through the customization of the chemistry of these functional groups, we can enhance cell targeting behavior, uptake, endosomal escape, non-toxicity, and transfection efficiency. Furthermore, physicochemical properties such as loading capacity, complexation ability, size and morphology, biodegradability, pH sensitivity, and amphiphilicity can be tailored based on specific applications. This review comprehensively explores the role of non-cationic moieties in various bio-

medical applications, ranging from therapeutic interventions to gene editing. The rational incorporation of these building blocks for designing more effective CPs for NA delivery will be discussed hereafter.

With ongoing advancements in polymer chemistry, the invention and refinement of new polymerization techniques, along with the synthesis of novel functional monomers and post-functionalization methods, have enabled the constant development of polymeric nanomaterials for non-viral NA delivery. For a comprehensive overview of the state of the art of the synthetic methods used in these polymerization and post-functionalization techniques, we refer the reader to this more specialized literature.[71–74]

3. Cationic polymers as gene nanocarriers: an overview

CPs are commonly used in gene delivery applications[36,75–77] due to their high packaging capacity, structural flexibility, and the ability for further functionalization.[78,79] By adjusting their chemical composition,[80,81] it is possible to control the release of cargo and ensure that CPs are degraded and eliminated from the body.[82,83] CPs are made up of repeating units that contain (primary, secondary, and/or tertiary) amino groups as well as amidines, guanidines, and triazine moieties, which are responsible for their electrostatic interactions with the anionic NA phosphates to form polyplexes.[21,84–86]

CPs employed for gene delivery applications are typically classified based on their origin as either synthetic or nature-derived polymers, and both categories are either commercially sourced or synthesized *ad hoc*.[77,87]

3.1. Synthetic CPs

Synthetic polymers present precisely controlled chemical structures and appreciable ease of functionalization,[88] as we can select *a priori* the building blocks that we consider the most suitable for their synthesis. As a result, they can ensure high batch-to-batch reproducibility and notable chemical versatility.[89] The commercially sourced synthetic CPs most widely used in transfection include: (i) poly(ethyleneimine) (PEI),[21,90–93] (ii) poly(amido amine) (PAMAM),[94–97] (iii) poly (β-amino ester) (PβAEs),[98–100] (iv) poly[2-(dimethylamino)ethyl methacrylate] (PDMAEMA),[70,86,101,102] and (iv) poly(L-lysine) (PLL),[103] which repeating units structures are reported in Table 1.

3.1.1 PEI. PEI is a water-soluble polycation composed of repeated ethyleneimine building blocks.[104] PEI is used in both linear (*l*PEI) and branched (*b*PEI) forms.[105] *l*PEI is synthetized by cationic ring-opening polymerization of cyclic imino-ethers followed by acid- or base-catalyzed hydrolysis, whereas *b*PEI is obtained by chemical polymerization of ethyleneimine in the presence of both hydrochloric acid and sulphuric acid.[104,106] *b*PEI at a M_w of 25 kDa is considered the gold standard polymer vector for *in vitro* cell transfection.[107]

Although PEI offers several advantages, such as low cost, ease of preparation and modification, and good transfection efficiency, its application is limited by drawbacks, including its high positive charge density, which can lead to cytotoxicity in high M_w (HMw) polymers.[81,104,108,109] When comparing the cytotoxicity of equal concentrations of different PEIs (*i.e.*, 800 Da *b*PEI, 25 kDa *b*PEI, and 20 kDa *l*PEI), *b*PEIs exhibited the highest toxicity. This heightened toxicity is attributed to the elevated density of methylene charge in the structure of *b*PEIs, leading to a greater destabilization of the cell membrane.[110,111] On the other hand, experimental evidence shows that *l*PEI and *b*PEI with equivalent M_ws exhibit comparable transfection efficiencies. Conversely, they demonstrate distinct NA complexation abilities, regardless of the M_w.[77] Specifically, *l*PEI exclusively possesses secondary amines, whereas *b*PEI incorporates primary, secondary, and tertiary amines, depending on the polymerization degree.[112] These structural differences account for the buffering capacity of *b*PEI across a broad pH range and, consequently, contribute to its "proton-sponge" behaviour. This effect, in turn, facilitates the escape of polyplexes from endo/lysosomes.

3.1.2 PAMAM. PAMAM polymer is mainly explored in its dendrimeric architecture.[113,114] Several modification methods are currently used to add terminal functionalization to the structure.[115,116] Similar to PEI, PAMAM is a biostable (*i.e.*, non-biodegradable) CP. Due to the high number of secondary and tertiary amines, PAMAM exhibits robust condensation and proton buffering capabilities, resulting in effective transfection.[81,117,118] Nevertheless, various issues, such as complex synthesis, limitations in mass production, biostability, and some cytotoxicity, altogether hamper the clinical translation of PAMAM dendrimers.[116]

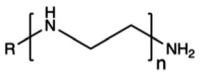
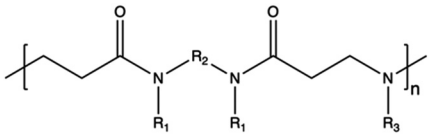
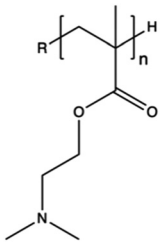
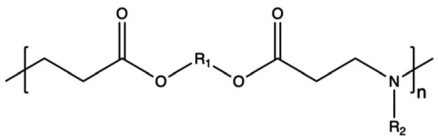
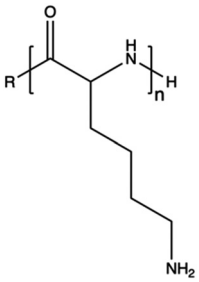
3.1.3 PDMAEMA. PDMAEMA is a water-soluble CP prepared by several controlled or uncontrolled polymerization methods.[119] PDMAEMA has been widely used due to its buffering capacity, reduced toxicity, pH- and thermo-responsiveness.[90,120]

3.1.4 PβAEs. PβAEs are biodegradable and water-soluble CPs synthesized through a one-pot Michael addition,[121] involving the reaction of primary amine or bis(secondary amine) with different acrylate or diacrylate compound.[85,122] The hydrolysis of backbone ester bonds under physiological conditions[123] ensures its biodegradability.[124,125] PβAEs exhibit high gene delivery efficiency and lower cytotoxicity compared to PEI and PLL,[126] making them an appealing alternative.

3.1.5 PLL. Owing to its remarkable capacity to condense DNA, PLL stands as one of the most utilized *in vitro* and *in vivo* CPs for gene delivery purposes.[36] The synthesis of PLL involves the conversion of an ε-amine-protected L-lysine monomer to *N*-carboxy-(*N*-benzyloxycarbonyl)-L-lysine anhydride, followed by ring-opening polymerization utilizing a primary amine initiator.[127] Control of the M_w is attainable through specific feed ratios of monomer-to-initiator.

Under physiological pH conditions, all primary ε-amino groups of PLL are protonated, resulting in a structure lacking buffering capacity for endosomal escape.[128] Typically, only

Table 1 Common synthetic CPs used as vectors for gene delivery

Polymers	Acronym	Repeating unit	Ref.
Poly(ethylenimine)	PEI		21 and 90–93
Poly(amido amine)	PAMAM		94–97
Poly[2-(dimethhylamino) ethyl methacrylate]	PDMAEMA		37, 70, 102 and 130
Poly(β-amino esters)	PβAEs		98–100
Poly(L-lysine)	PLL		103 and 127–129

PLLs with a M_w > 3 kDa can effectively condense DNA into stable complexes, underscoring the importance of the primary amine count. Despite the efficient condensation achieved by HMw PLL, these polymers often exhibit relatively high cytotoxicity.[129]

3.1.6 Charge adjusting functionality. Nitrogen-based cationic groups such as imidazolium, guanidinium,[100,131] and heterocycles[132–135] are commonly integrated into synthetic polymers. The type of cationic center influences the pK_a, thus affecting the electrostatic interactions with the NA, and the endosomal escape, which are crucial for efficient gene delivery. Guanidinium is particularly attractive due to its ability to form hydrogen bonds with phosphate anions and guanine, thereby enhancing NA interaction.[100,136,137] Alternatively, melittin holds promise as a suitable unit for integration into synthetic CPs,[138] as it can be modified with maleic anhydride to introduce protected lysine residues, which can subsequently be deprotected at acidic pH.[139] This peptide is toxic to mammalian cells, but conjugation with various CPs significantly mitigate its cytotoxicity, facilitating endosomal release, enhancing NA loading, and improving transfection efficiency.[138,140,141] Few polycations with non-nitrogenous charge centers (such as phosphorus and sulfur heteroatoms) are also reported in the literature, although their chemical instability in biological environments and the limited number of synthetic pathways hamper their use for gene delivery.[142]

3.2. Natural polymers

In contrast to synthetic CPs, nature-derived polymers have gained prominence because of their remarkable biocompatibility, bioactivity, and cell-mediated hydrolytic degradation.[143,144] However, it remains challenging to accurately characterize and identify the chemical structure and composition of polymers obtained from natural resources. Therefore, such CPs are characterized by intrinsic batch-to-batch variability which complicates the development of new gene-delivery formulations.[145,146] Furthermore, the high bioactivity of these natural polymers often triggers a robust immunogenic response.[143] Natural CPs are typically sourced from two primary natural origins, namely proteins, biogenic polyamines, and polysaccharides.[147] Chitosan (CS), a polysaccharide comprising randomly distributed β-(1-4)-linked D-glucosamine and *N*-acetyl-D-glucosamine, has been widely employed in gene delivery. This is due to its cationic nature, low toxicity, biodegradability, and minimal immunogenicity.[148,149] However, CS also has some weaknesses, such as poor solubility under

neutral pH conditions and lower NA complexation ability compared to other CPs.[150,151] To address these issues, chemical modifications can be used to enhance the performance of CS, such as the formation of trimethyl CS.[152] The reactive groups on the glucosamine units allow polysaccharides to undergo various chemical modifications,[153] so that the resulting engineered derivatives can be customized to display specific biomedical functions.

Spermine, a naturally occurring polyamine, assists in compacting cellular DNA within eukaryotic cells.[154] However, on its own, it exhibits poor NA condensation, limited transfection efficiency due to its low M_w (~200 Da), and poor endosomal escape capability, despite its good proton-buffering capacity.[155,156] Studies have demonstrated that polymerizing spermine increasing its M_w can be advantageous for delivering siRNA.[157] For instance, synthesis of spermine-based PβAEs revealed to be successful in obtaining polymers more biocompatible than 25 kDa *b*PEI and able to encapsulate and deliver siRNA more efficiently.[158]

4. Non-cationic building blocks for polycation modification

To date, various hybrid polycationic structures, integrating carefully selected functionalities, have been devised to enhance delivery efficiency, thus overcoming the biological barriers, biocompatibility, and interactions with target cells.[159,160]

For example, modifying the surface properties of polycations through neutralization or hybridization with other biodegradable polymers or reducible groups can mitigate their toxicity,[69,161,162] while enhancing *in vivo* bioavailability.[163,164]

On the other hand, enhanced biodegradability is a beneficial feature that facilitates the effective unpacking and release of loaded therapeutic genes in the cytosol.[145,165] In this light, crosslinking or conjugating non-biodegradable polycations with stimuli-responsive functionalities can trigger carrier disruption. This, in turn, facilitates the controlled release of the cargo and subsequent elimination of the polymeric material from the bloodstream.[58] Also, amine groups within the polymer chains can be utilized to anchor specific moieties and ligands to targeted particular organs and cell sites, limiting potential off-target delivery.[166] The following sections highlight the most promising and updated strategies for incorporating non-cationic moieties, monomers, and functional groups into polycations, endowing them with specific behaviour. Table 2 showcases key examples of commonly employed functional unit structures.

4.1 Anti-opsonization functionality

4.1.1 Poly(ethylene glycol) chains. In recent years, poly (ethylene glycol) (PEG) has been widely used as a stealth coating in drug delivery systems, owing to its ability to improve stability, lengthen circulation time, and reduce non-specific cellular uptake of nanoparticles.[167–173] Indeed, PEG prevents protein adsorption on polyplexes, thereby reducing opsonization[174] while shielding the surface from the action of enzymes and antibodies that may induce degradation and clearance.[175] Despite these features, PEGylated vectors may exhibit shortcomings that reiterate the PEG dilemma.[176,177]

PEG can induce an adverse immune reaction through the production of anti-PEG antibodies that, when present in high concentrations, lead to the rapid clearance of PEGylated complexes.[115]

Taking PEI as an example, PEG grafting onto the CP was shown to decrease the overall surface charge density, thereby affecting the cellular uptake, intracellular trafficking, and endosomal escape of PEI-based nanoparticles.[176] Moreover, PEGylation can decrease the transfection efficiency of non-viral gene delivery systems by reducing the non-specific interactions between the cell and polyplexes.[178] On the other hand, PEGylation increased the blood circulation time of PEI-based complexes,[179] by reducing the interactions between polyplexes and the serum proteins.[148,149,180,181]

In this light, alternative surface modification strategies, including the use of different charge-shielding polymers, conditional removal of PEG, biomimetic surface modifications, and platelet membrane cloaking techniques, could potentially overcome the limitation of PEG.

4.1.2 Zwitterionic polymers. Recently, zwitterionic polymers have garnered significant attention as attractive alternatives due to their superhydrophilicity, biocompatibility, and antifouling properties.[182–187] In contrast to PEG, which binds water molecules through hydrogen-bond interactions, zwitterionic molecules can create stronger hydration shells[115,188] through ion–dipole interactions with denser and tighter adsorbed water molecules.[189] This results in a more effective protection from non-specific protein adsorption.[190–192]

Nonetheless, zwitterionic materials can also mitigate immune response and provide high stability to the nanovectors and prolong circulation time,[193] thereby preventing the undesired long-term accumulation of nanocarriers in the RES.[115,194] In literature, the development of CPs functionalized with zwitterionic moieties was primarily accomplished through the polymerization of 3-[[2-(methacryloyloxy)ethyl]-dimethylammonio]propionate (CBMA) and 2-methacryloyloxyethyl phosphorylcholine (MPC).[195] Several studies have reported enhanced gene delivery efficacy and biocompatibility of PEI-based vectors following this functionalization.[196,197]

MPC derivatives containing a phosphorylcholine are highly water soluble[198] and can undergo polymerization through various radical polymerization techniques.[199] This allows for the production of cationic MPC copolymers with desired properties suitable for gene delivery purposes.[200–203] In a work by Bhuchar *et al.*, water-soluble CPs incorporating MPC and 2-aminoethyl methacrylamide hydrochloride (AEMA) with block and random architectures were synthesized by the reversible addition–fragmentation chain transfer (RAFT) method.[203] As expected, the incorporation of MPC polymer segments in CPs significantly improved their biological performances. Polyplexes based on P($AEMA_{26}$-*b*-MPC_{27}) were assessed in Hep G2 cells, and they were identified as optimal

Table 2 Examples of structures of non-cationic functional units used to modify CPs. R indicates the attachment site of the functional unit to the polymer chain. Where the attachment site is not explicitly mentioned in the unit depicted, it is integrated into the CP structure *via* co-polymerisation of the vinyl group

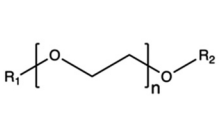
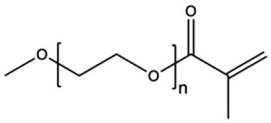
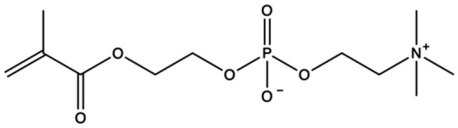
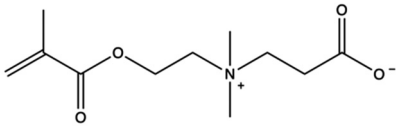
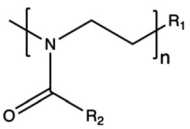
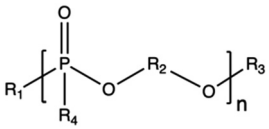
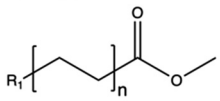
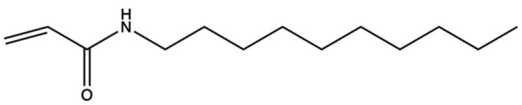
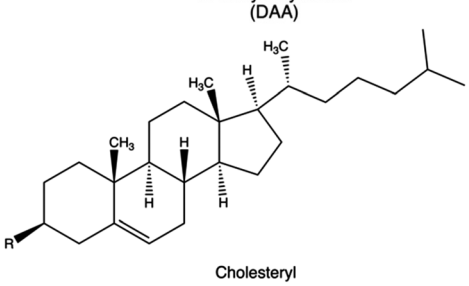
Property	Category	Monomers or functional units	Ref.
Anti-opsonization	PEG chains	 Poly(ethylene glycol) (PEG)  Poly(ethylene glycol methyl ether) methacrylate (PEGMA)	174–176, 180, 222 and 223
	Zwitterionic compounds	 2-methacryloyloxyethyl phosphorylcholine (MPC)  3-[[2-(methacryloyloxy)ethyl]-dimethylammonio]propionate (CBMA)	182, 184–187, 189, 191 and 192
	Poly(oxazoline)s	 Poly(oxazoline)s (POx)	204, 205, 207, 210, 211, 214 and 219
	Poly(phosphonate)s	 Poly(phosphonate)s	224–227
Advancing membrane-permeation	Alkyl chains	 Fatty acids  N-Decylacrylamide (DAA)	228–231
	Cholesterol derivatives	 Cholesteryl	232 and 233

Table 2 *(Contd.)*

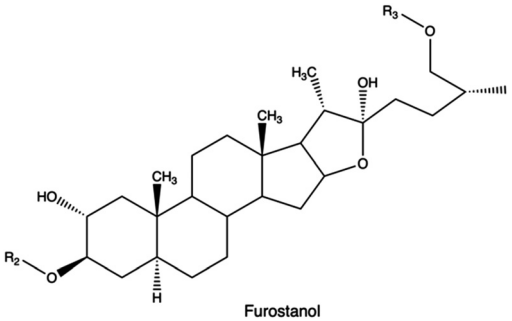
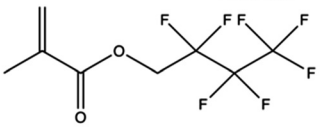
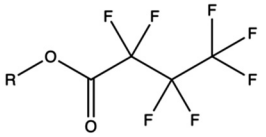
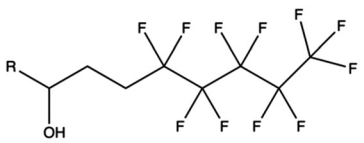
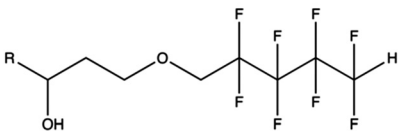
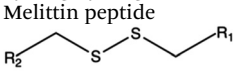
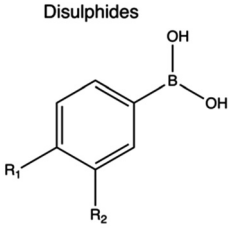
Property	Category	Monomers or functional units	Ref.
	Saponin derivatives	 Furostanol	234–238
	Fluorine derivatives	 Heptafluorobutyl methacrylate  Heptafluorobutyrate   Fluoroalkanes	239–246
	Penetrating peptides	Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Gln Transactivator of transcription (TAT) peptide Arg-Gln-lle-Lys-lle-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-Gly-Gly Penetratin peptide Gly-lle-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Lue-lle-Ser-Trp-lle-Lys-Arg-Lys-Arg-Gln-Gln Melittin peptide	247–252
Bioreducible	Disulphide bonds	 Disulphides	253–259
	Boronic acids	 Phenylboronic acid derivatives	260–262

Table 2 *(Contd.)*

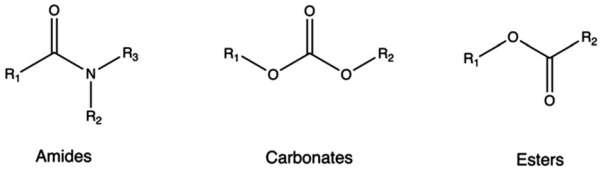
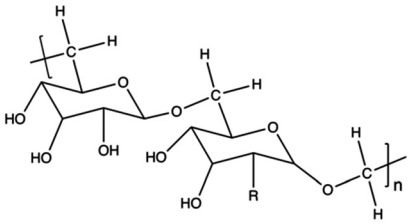
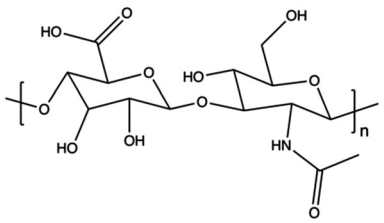
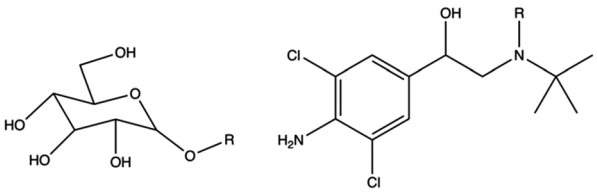
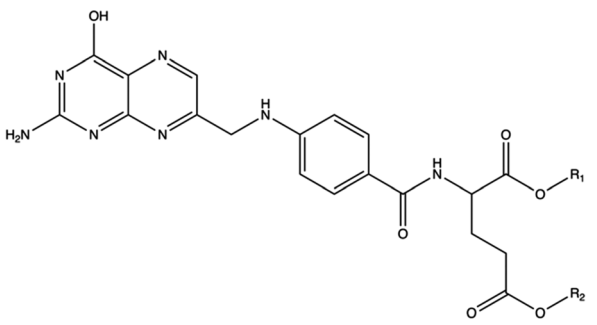
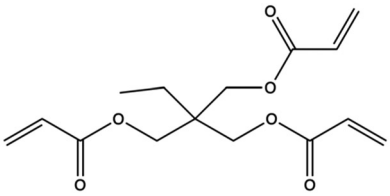
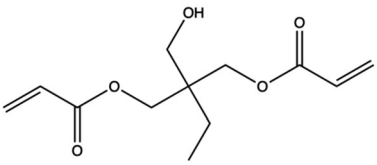
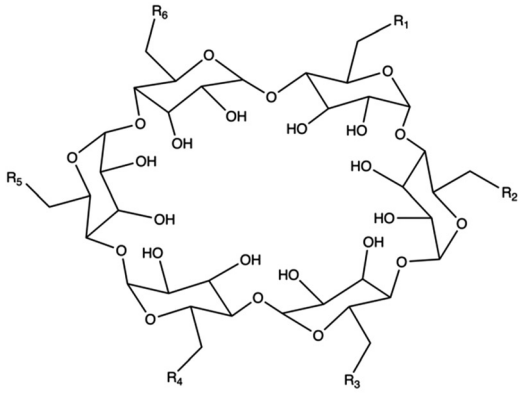
Property	Category	Monomers or functional units	Ref.
Biodegradable	Synthetic	 Amides Carbonates Esters	263–267
	Polysaccharides	 Dextran (DEX)	268–271
		 Hyaluronic acid (HA)	
Active targeting		 Mannose Clenbuterol	272–278
		 Folate (FA)	

Table 2 *(Contd.)*

Property	Category	Monomers or functional units	Ref.
Branching		 Trimethylolpropane triacrylate	279–284
		 Trimethylolpropane dimethacrylate	
		 Beta-cyclodextrin	

MPC copolymers due to their sustained low protein activation ability, combined with enhanced gene expression.[203]

In another study, a small library of zwitterion-modified PEIs was synthesized by grafting (in different ratios) 1,3-propanesultone or β-propiolactone onto hydrophobically modified (10 kDa and 25 kDa) *b*PEI (H-PEIs) *via* ring-opening addition reactions. The zwitterion moieties were introduced to H-PEIs *via* the reaction of amines producing *N*-sulfopropylated H-PEI (H-PEIs-S) and *N*-carboxyethylated H-PEI (H-PEIs-C), respectively. Evidence indicates that the zwitterionic modification conferred reduced cytotoxicity and mitigated nonspecific protein adsorption in the nanocarriers. However, no improvement was found in the overall siRNA to cancer cells (*i.e.*, HeLa-Luc and A549-Luc cells). These results suggest that achieving an optimal balance between transfection efficacy and cytotoxicity may be dependent on the appropriate cationic to zwitterion components ratio.[115]

4.1.3 Poly(oxazoline)s. Poly(oxazoline)s (POx) have garnered considerable interest for their ability to confer stealth properties to nanocarriers, enhancing circulation time and pharmacokinetics, and reducing nonspecific interactions with biological components.[204–209] POx exhibit minimal immunogenicity, high hydrophilicity, and thermo-responsive properties, depending on the specific substituents.[204,205,210,211] This versatility makes them competitive alternatives to PEG, with the additional advantage of potential side-chain functionalization.[204–207,212,213] POx block copolymers of various architecture proved to be well tolerated by mammalian cells and possess physicochemical properties similar to PEG, including non-ionic character, solubility in hydrophilic and lipophilic solvents, and main chain flexibility.[210,214,215] Several studies have explored the use of PEI-*graft*-poly(2-ehhyl-2-oxazoline) copolymers for DNA delivery. The copolymers varied in composition, PEI content (ranging from 5% to 84%), molar mass (from 8 to 40 kDa), and chain architecture (linear or comb-like). They consistently demonstrated significantly reduced cytotoxicity compared to pristine PEI, along with similar efficient binding and compaction of DNA.[211,216–218] In

a recent study by Haladjova *et al.*, gene vector systems derived from two series of random copolymers based on poly(2-methyl-2-oxazoline) (PMetOx) and PEI were investigated.[211] PMetOx-*co*-PEI copolymers formed small, well-defined, and colloidally stable polyplexes with DNA, regardless of the overall degree of polymerization and PEI content.[211] Cook *et al.* reported the synthesis of hyperbranched poly(ethylenimine-*co*-oxazoline) (PEI-*co*-POx) using AB2 thiol–yne photo-addition chemistry.[219] This method enables the production of dendrimer-like structures with well-defined branching patterns, where PEI contains only secondary amines. Hyperbranched PEI-*co*-POxs were observed to complex GFP plasmid DNA, forming small positively charged particles. In contrast, linear forms tended to form larger aggregates. Moreover, differences were noted in the *in vitro* cytotoxicity: linear PEI-*co*-POx exhibited reduced toxicity compared to *b*PEI, and hyperbranched PEI-*co*-POx showed even lower toxicity compared to the linear counterparts. Delivery of pDNA encoding GFP revealed that PEI-*co*-POx copolymers with high percentages of ethylenimine units exhibited transfection efficiencies slightly lower than 25 kDa *b*PEI. Considering this, alongside their lower toxicities, hyperbranched PEI-*co*-POx emerges as a promising candidate for further non-viral gene delivery studies.[219]

4.1.4 Poly(phosphonate)s. Poly(phosphonates)s, and more specifically poly(phosphoester)s (PPEs), have emerged as a promising and appealing alternative to PEG among biocompatible and degradable polymers known to enhance the *in vivo* performance of pharmaceuticals. Their chemical structure is highly versatile, allowing for precise modulation of their degradation products and timing through tailored chemistry.[220,221]

In addition, their water-solubility, anti-biofouling properties, cytocompatibility, and stealth effect[227,285] explain the recent attention to developing novel synthetic methodologies and biomedical applications for PPEs.[221] Indeed, numerous PPE-based drug delivery systems, PPE-containing nanomaterials for surface protein adsorption,[286] and gene delivery nanocarriers[225,287] have been examined. Notably, the interactions between hydrophilic PPEs and blood proteins have been widely explored.[266,288] Schöttler *et al*. demonstrated that nanocarriers modified with either PEG or PPE exhibit reduced protein adsorption when incubated in human plasma compared to unmodified particles. Furthermore, mass spectrometric analysis of the protein corona formed on the particles' surface post-plasma incubation revealed a similar protein profile for both PEGylated and 'PPEylated' particles.[227] One of the first relevant study demonstrating the effectiveness of using PPE for gene delivery was proposed by Wang *et al.*[289] The cationic copolymer was synthesized from poly(1,2-propylene phosphonate) by the Atherton–Todd reaction with spermidine side chain (PAA-SP). PPA-SP was able to efficiently condense plasmid DNA and formed complexes at N/P ratio ≥2. It offered notable protection to DNA against nuclease degradation and showed lower cytotoxicity than PLL and *b*PEI in cell culture. PPA-SP mediated efficient gene transfection in several cell lines, and the transfection protocol was optimized in HEK293 cells where PPA-SP/DNA polyplexes yield a luciferase expression level similar to that given by complexes made of PEI/DNA or based on TransFast™ commercial reagent.[289] Previous studies demonstrated that amphiphilic cationic block copolymers of PEG, poly(ε-caprolactone) and poly(2-aminoethyl ethylene phosphate) ($mPEG_{45}$-*b*-PCL_{30}-*b*-$PPEEA_{15}$) are capable of binding siRNA to form a micelle/siRNA complex (micelleplex).[224] In another work, Mao *et al.* confirmed the *in vivo* efficiency of systemic administration of these micelleplexes for cancer therapy, by targeting the acid ceramidase (AC) oncogene, which plays a key role in cancer development.[225,290] They investigated the knockdown in BT474 breast cancer cells and the inhibitory effect of micelleplexes administration on tumour growth. The micelleplexes obtained significantly reduce the expression of either a luciferase reporter gene or the endogenous acid ceramidase gene in cell culture, with acid ceramidase knockdown resulting in a significant increase in cell apoptosis. Importantly, this delivery system does not activate the innate immune response and is promising for systemic delivery of siRNAs for cancer therapy.[225,287]

4.2 Advancing membrane-permeation functionality

4.2.1. Hydrophobic chains. The idea of hydrophobically modifying CPs to enhance their transfection efficiency, reduce their cytotoxicity, and improve their colloidal stability has also been exploited.[291] Indeed, some CPs are particularly suited for hydrophobic modifications,[228] owing to the presence of primary, secondary, and tertiary amines. Given that the cell membrane is a phospholipid bilayer, the incorporation of hydrophobic moieties such as aliphatic chains or aromatic rings into polymers can improve their interaction with cell membranes.[292–294] Esterification of alkane chains has been found to increase hydrophobicity, thus promoting cellular uptake. However, (too) long alkane chains can weaken the electrostatic interactions between CPs and cell membranes due to increased steric resistance and reduced charge density. As a result, polymers functionalized with medium-length alkane chains are more effective gene delivery vectors.[134,295] In this regard, Candiani *et al.* investigated the effect of lipophilic tails of variable length tethered to cationic nanovectors. The transfection efficacy progressively increases using chains ranging from C_8 to C_{14}, while it significantly diminishes for longer chains (C_{16}, C_{18}).[296]

Linear alkyl chains of fatty acids including acetate, butanoate, hexanoate, myristate, and butyric anhydrides are the most commonly used hydrophobic groups as they increase colloidal stability and biocompatibility of the resulting nanovectors.[228–230,297] However, the ultimate effect is significantly influenced by factors such as the degree of acetylation. Using *b*PEI as an example, an acetylation degree of 25% of primary amines was found to give the best overall transfection performaces.[297] Similar results were observed when *b*PEI was functionalized with an acetylation degree of 57%, significantly improving cellular uptake performance compared to pristine *b*PEI 25 kDa.[125] Conversely, a higher degree of acetylation was found to have a negative effect.[126]

Based on this observation, it can be concluded that a lower degree of functionalization is associated with better transfection performance. This may be attributed to the fact that acylation conceals the free amine groups of PEIs, thereby reducing their natural steric hindrance. The effect is significantly influenced by factors such as the degree of substitution, the position of the alkyl group, and the chain length. Similar observations rises from the copolymerization of HMw brushed poly (spermine acrylamides) (P(SpAA)) with *N*-decylacrylamide (DAA) monomers employed to increase the hydrophobicity of the resulting CPs.[231] Different copolymers with varying ratios of cationic SpAA and hydrofobic DAA units were synthesized to investigate the impact of different hydrophobic fractions on siRNA delivery in presence of mucous. All the copolymers showed an improved cellular uptake in lung cells, and a ratio of 43/57 cationic to hydrophobic monomer subunits resulted in a better cellular uptake with respect to Lipofectamine and induced a ten-fold higher uptake than *b*PEI polyplexes under similar conditions.[231]

4.2.2. Fluorine derivatives. Recent studies have shown promising advantages in incorporating fluorinated groups into the structure of the vectors to facilitate their penetration of biological barriers,[298] including the cellular membrane.[108,109,299] Fluorinated polycations exhibit enhanced hydrophobicity and lipophilicity attributed to the low polarizability and surface energy of the C–F bond. This facilitates self-assembly and phase separation in solution, promoting cell uptake through membrane fusion or endocytosis.[300] By increasing the hydrophobicity and lipophilicity of PEI, fluorination was employed to improve its NA delivery efficiency while mitigating its cytotoxicity.[301] Experimental evidence indicates that fluorinated polymers preserve their transfection performances even in the presence of serum, and this is attributed to their ability to counteract protein adsorption, promoting polyplex stability.[108,109,302–304] In a study where fluorinated PDMAEMAs were obtained *via* RAFT copolymerization, the resulting polyplexes exhibited significant higher DNA release and transfection efficiency in 293T and HeLa cells compared to polyplexes formed by the pristine PDMAEMA.[305] In confirmation of the beneficial role of fluorine modification on the gene transfection ability, Zhang *et al.* functionalized *b*PEI with alkanes, cycloalkanes, and fluoroalkanes and carried out a throughout comparison.[109] Among them, the polymer functionalized with fluoroalkanes exhibited high hydrophobicity and lipophilicity promoting effective interaction with the cell membrane and facilitating endocytosis.

Alternatives fluorinated moieties commonly used to functionalize PAMAM, PPI, PEI, PLL, and other polymers are anhydrides, epoxides, ethyl esters, acyl chlorides, carboxyls, and acrylates.[298]

4.2.3. Cell-penetrating peptides. Cell penetrating peptides (CPPs), such as lysine–histidine, TAT sequence, penetratin, trasportin, and melittin, have been used to design smart polycationic gene delivery systems with improved cell internalization and endosome escape efficiency.[306–308] CPPs are small amphipathic peptides (formed by 5 to 30 amino acid residues) capable of overcoming the cellular membrane *via* passive diffusion or endocytosis.[309] Moreover, they can facilitate endosomal escape due to their unique membrane-disturbing property, attributed to their relatively rigid secondary structure, which can interfere with cellular membrane integrity.[310,311] Pan and colleagues synthesized a mannosylated carrier by tethering low M_w (LMw) PEI with protamine (LMWP) (Man-PEI5k-LMWP) to deliver plasmid tumour necrosis factor-related apoptosis-inducing ligand (pTRAIL) to colorectal cancer cells. CPP insertion into the Man-PEI5k/pTRAIL complex significantly increased the transfection efficiency and inhibition efficacy of HCT116 both *in vitro* and *in vivo*.[312]

4.2.4. Other approaches. Other compounds, including cyclic hydrophobic molecules, such as cholesteryl chloroformate, dexamethasone mesylate, and saponin, have been used for hydrophobic modification of CPs. Using a LMw PEI as an example, the conjugation with cholesterol and PEG was found to enhance the uptake of the resulting complexes.[232,233]

Saponins, which are glycosides found in plants and marine organisms comprised of a sugar residue attached to a steroidal or terpenoid aglycone,[234] can function as a transfection enhancing agent. This is attributed to their membrane-permeabilizing function[235] and ability to impart endosomal escape capabilities.[236,237,313]

In a study Hasanzadeh *et al.*, the bi-functionalization of LMw PEI with fluorine atoms and saponin residues resulted in a versatile and efficient delivery vehicle with reduced cytotoxicity. The authors utilized phenylboronic acid (PBA) as an ATP-responsive cross-linker for saponin-PEI conjugation. Complexes made of PEI-PBA-SAP-F and CRISPR/Cas9 system displayed good stability in serum and excellent gene editing ability in HEK 293T-GFP cells.[238]

4.3 Triggered disassembly

The incorporation of covalent bonds susceptible to degradation under physiological conditions presents an appealing approach to mitigate polymer toxicity, facilitate cargo release, and enhance transfection.[314]

In the realm of biodegradable polymers, we distinguish between those that are inherently biodegradable (*i.e.*, undergo spontaneous degradation) and stimuli-responsive ones, which experience triggered degradation in response to specific stimuli.

In the latter case, the modification involves introducing (bio)degradable (cross)links into the polycationic backbone or side chains, such as ester, imine, amide, carbamate, ketal, or disulfide bonds, enabling the creation of bio-reducible CPs.[315] These nanomaterials undergo degradation into low M_w fragments through cleavage driven by chemical stimuli, such as pH variation, reactive oxygen species (ROS), adenosine triphosphate (ATP), and redox signals.[316–319] As a result, the CPs effectively mitigate the issue of cytotoxicity while preserving the gene transfer potential of the pristine polymers.[68,235–237,320]

It is worthy of note that some stimuli, *e.g.* those driven by pH variations occurring when moving towards different intra-

cellular environments (*e.g.*, from cytosol to endosomes) or related to specific cellular activity, can be exploited to achieve the desired effect. This is evident when targeting the highly acidic tumor microenvironment.[321] In this context, pH-responsive nanocarriers have been synthesized by incorporating ionizable pH-responsive functional groups, such as carboxylic acids, or acid-unstable covalent bonds, such as hydrazones, ester, orthoester, acetal, and cisacotinyl bonds.[322–324] The resulting nanocarriers exhibit advantages in terms of cytotoxicity, biodegradability, cellular uptake, and NA release.[147,325–327]

A series of di-amine monomers containing stabilized orthoester groups were synthesized to give rise to an array of copolymers with unique pH-responsive properties.[328] Moreover, Qi and co-workers obtained nanovectors based on pH-responsive branched polycations through the amino-epoxy ring-opening reaction of acid-sensitive monomers with impressive pH-responsive degradability in an acidic endosomal compartment (pH 5.0) and low-to-negligible cytotoxicity.[329] This promoted the release of plasmid payload within the cytosol, with a subsequent increase in the transfection efficiency with respect to the non-pH-responsive counterparts.

ROS-cleavable delivery systems have been extensively reported in the literature. Several chemical groups, such as thioether, selenium, and PBA ester have been reported to be ROS responsive,[330,331] undergoing physio-chemical and polarity variation in the presence of ROS. Thioacetals, instead, undergo direct cleavage under similar conditions. In a study by Zhang *et al.*, the authors developed an array of fluorobenzene-substituted polycations with ROS-responsive thioacetal linkage *via* the diepoxide ring-opening polymerization and investigated the performances of the resulting polyplexes in terms of transfection efficiency and cytotoxicity in tumor (PC-3) cell lines. Interestingly, under higher ROS level conditions, polyplexes easily degrade while facilitating the intracellular release of DNA.[332]

In the context of bio-reducible compounds, those containing disulfide bonds and boronic acid derivatives deserve special attention for the vast number of applications in gene delivery field.

4.3.1. Disulfide bonds. Disulfide bonds are frequently incorporated into CP structures as their cleavage can be initiated by redox signals, enabling the design of bio-reducible polymers.[333] Polycations incorporating disulfide groups remain stable in an extracellular oxidizing environment. However, they undergo rapid breakdown when in a highly reducing environment typical of the cytoplasm,[334,335] since disulfides can be efficiently cleaved by intracellular reducing agents such as glutathione (GSH), which is currently the sole effective stimuli examined within the cytosol thus far.[336–338] Under these conditions, the delivery vector is able to effectively unpack and release the loaded gene.[315,339–341] GSH responsiveness is particularly evident in tumor treatments, capitalizing the high concentration of GSH in the tumor microenvironment (~2–10 mM, that is, 100–1000 fold higher than in normal tissues).[342]

Disulfide bonds have been efficiently incorporated in different CPs, including PEI, poly(amido ethylenimine) (PAEI), and poly(cystaminebisacrylamide-diaminohexane) (poly (CBA-DAH)).

In the preparation of bio-reducible PEI-based polymers, Gosselin *et al.* employed LMw PEI as the precursor, followed by cross-linking using two disulfide-containing cross-linkers, namely dithiobis-(succinimidylpropionate) (DSP) and dimethyl-3,3′-dithiobispropionimidate·2HCl (DTBP). These bio-reducible PEI-based polymers were used to prepare DNA-containing vectors that showed good transfection efficiencies in CHO cells owing to the stimulus-triggered release of the transgene *via* reductive cleavage of the disulfide bonds.[253] In another study, Liu and collaborators synthesized a disulfide-containing cross-linked PEI (PEI-SS-CLs) using click chemistry. An azide-terminated PEI was initially synthesized from a LMw PEI (1.8 kDa), which was then converted into a HMw disulfide-containing cross-linked PEI through the introduction of a dialkyne cross-linker. The results showed that the cross-linked PEI exhibited both higher gene transfection efficiency and lower cytotoxicity if compared to the gold standard 25 kDa PEI.[254]

In an alternative strategy to obtain (bio)degradable CPs, Bauhuber and co-workers employed a smart chemistry approach to synthesize a library of PEG-SS-PEI diblock copolymers, where PEG moieties linked PEI through disulfide bonds, and compare them with non-degradable PEG-S-PEI (with a sulfide bond as the linker between PEG and PEI) copolymers.[255] Interestingly, when tested at the same PEG contents, PEG-SS-PEI diblock copolymers were the most effective in transfection, highlighting the importance of the intracellular PEG cleavage in the transfection process. The same approach was employed to synthesize a novel bio-reducible targeted gene vector c(RGDyK)-poly(ethyleneglycol)-SS-PEI (RGD-PEG-SS-PEI), where a disulfide bond served as the linker for the PEG and PEI blocks. RGD-PEG-SS-PEI-based complexes carrying DNA showed higher transfection efficiency and reduced cytotoxicity both *in vitro* (in U87 cells) and *in vivo* (glioblastoma in mice) if compared with PEG-PEI complexes (no disulfide linkage).[256]

In the case of PAAs, Christensen *et al.* synthesized three types of disulfides containing PAEI from step polymerization of CBA and ethylenediamine (EDA), diethylenetriamine (DETA), and triethylenetetramine (TETA).[257] These PAEI derivatives were capable of complexing DNA to form well-defined polyplexes with higher transfection efficiency than their pristine counterparts. In particular, the PAEIs resulting from CBA and TETA exhibited even higher transfection efficiency than 25 kDa *b*PEI even in the presence of serum. Chen and colleagues synthesized reducible hyperbranched PAEIs (RHBs) through Michael addition of *N*,*N*-dimethylamino dipropylenetriamine (DMDPTA), and two bisacrylamide monomers, CBA and HMBA, at specific molar feeding ratio to finely tune the density of disulfide linkages. When employed in transfection studies, the performances of these polymers as gene delivery vectors were dependent on the disulfide content, as this

affected cytotoxicity, M_w of the degradation products, polyplex disassembly, and transfection efficiency.[258] Similarly, Wu *et al.* synthesized PAAs with a controlled disulfide linkage content and studied their effect on PAEI cytotoxicity.[259] Results indicated that the toxicity of nanovectors based on bio-reducible PAAs was decreased linearly with an increase in the disulfide content.

Interestingly, when evaluated in two cell lines, lower toxicity of bio-reducible PAEI-based complexes was observed in cells with higher levels of GSH. Jere *et al.* developed a bioresponsive reducible polyspermine (RPS) carrier composed of repeated spermine units with disulfide bridging obtained by Michael addition reaction between spermine and *N*,*N*′-cystaminebisacrylamide (CAM) in two stoichiometric ratios (2 : 1 and 4 : 1).[343] RPS showed 1.8 times more resistance to pH change than *b*PEI 25 kDa thanks to the stronger buffering capacity, and strong DNA binding ability over a range of RPS/DNA ratios, indicating potential high transfection capabilities. Moreover, RPS demonstrated remarkable atoxicity even at concentrations 20-fold greater than those at which *b*PEI was cytotoxic. The study proved that RPS-pDNA binding is significantly reduced in the presence of a reducible environment, and RPS breakdown generates small spermine-like molecules easily metabolized and cleared by the cells.[343] These results suggest that RPS might transfect cells more efficiently and safely than 25 kDa PEI.

4.3.2. Boronic acid derivatives. Boronic acid and its derivatives, especially in the form of phenylboronic acid (PBA), have been largely introduced into various polycationic structures due to the multiple and simultaneous positive effects conferred on the nanocarriers. Boronation, in fact, can significantly enhance endo/lysosome escape of polyplexes, inducing NA release in response to ATP, pH and ROS.[295,344,345] Additionally, being highly stable and atoxic, boronated CPs have the potential to serve as carriers for gene delivery.[261] The stimuli-responsiveness of PBA was exploited for developing pH and ATP-responsive vectors for the delivery of miRNA (miR146a) into androgen-independent prostate cancer (AIPC) cells, aiming to inhibit the expression of epidermal growth factor receptor. The vector consisted of an inner core, made of PEI-4-(bromomethyl) PBA complexed with miR146a (PEI-PBA/miR146a), and an outer layer, made of PEI-dimethylmaleic anhydride-cetuximab (PEI-DMA-C225), self-assembled by electrostatic interactions. When uptaken by AICP cells, the outer layer was disassembled in response to the intracellular pH (pH 6.0), releasing the inner core into the cytosol of the tumorous cells. The high ATP concentration existing in that compartment, in turn, triggered the release of the miR146a.[262]

4.4 Hydrolytically labile functionality

Biodegradable synthetic polymers, such as polyesters, polyanhydrides, polycarbonates, polyamides, and polyurethanes,[346] typically feature hydrolytically labile chemical bonds, such as esters, anhydrides, carbonates, amides, and urethanes, respectively.[347]

As a rule of thumb, it is possible to confer biodegradability to non-biodegradable CPs by introducing specific moieties onto their structures. In these instances, non-cationic, naturally-derived biodegradable polymers, including dextran, cyclodextrin, and hyaluronic acid, can be employed for this purpose.[153,348] Indeed, upon hydrolysis, these biodegradable polymers can be degraded into smaller components, which can be readily metabolized by the body.[130]

It is noteworthy that among the frequently used polycations listed in Table 1, PβAEs are notable for their degradation in both basic and acidic environments as well as physiological conditions. This degradation is facilitated by ester hydrolysis, resulting in the production of small bis(β-amino acid)s and diols, which are reported to possess relative biocompatibility without requiring further modification, thus circumventing issues related to the accumulation and toxicity.[349] In contrast, all other CPs necessitate modifications to attain biodegradable characteristics.

The two major polymerization pathways used to incorporate non-cationic biodegradable structures to polycations are: (i) step polymerization, (ii) ring-opening polymerization (ROP), and (iii) a combination thereof.[143,350–352]

Modification of LMw PEIs with linear biodegradable polyesters was an effective strategy to improve the biocompatibility and degradability in physiological conditions.[98] Indeed, the results of different studies showed that the complexes made of PEIs tethering polyester units were less cytotoxic and more effective in transfection if compared to the gold standard 25 kDa PEI.[265,353] Similar outcomes were obtained with nanocarriers composed of 600 kDa PEI copolymerized with amino alcohol esters, diglycidyl adipate, diglycidyl succinate, and diglycidyl oxalate.[341] Experimental evidence showed that, in comparison with 25 kDa PEI, these novel vectors facilitated DNA release, minimized cytotoxicity, and increased transfection efficiency.[341]

Poly(lactide-*co*-glycolide) (PLGA), polylactide (PLA), and poly (caprolactone) (PCL) are among the most widespread polyesters employed for polycation functionalization.[98,263,350]

Park and his team decorated PEI with PLGA units and achieved co-transfection in hMSCs cells by conjugating the SOX9 gene or core-binding factor alpha (cbfa-1) with small interfering RNA (siRNA) *via* ion interactions. In this way, they achieved good transfection efficiency and the desired nanocarrier biocompatibility features.[265] In the study proposed by Xiong *et al.*, the synthesis of a novel family of PEO-*b*-polyester copolymers grafted with short cationic moieties is reported.[354] Poly(ethylene oxide)-*b*-poly(3-caprolactone-*g*-polyamine) (PEO-*b*-P(CL-*g*-polyamine)) block copolymers were prepared from PEO-*b*-poly(α-carboxyl-3-caprolactone) (PEO-*b*-PCCL), then, active ester method was used to attach pendant polyamine groups (as spermine (SP)) to the polyester section by amide bond formation. The developed amphiphilic copolymers were shown to be non-haemolytic, biodegradable, and less toxic than PEI against the chosen cancer cell line. They were also able to effectively bind siRNA, self-assemble into micelles and protect siRNA from degradation by nuclease in serum. PEO-*b*-P(CL-*g*-SP) micelles were very efficient in delivering siRNA into cytoplasm by endocytosis and facilitated endosome escape after cellular uptake.[354]

Biodegradable CPs were also developed using polyurethanes (PUs). Hung *et al.* synthesized an array of cationic PUs containing tertiary amines in the backbone and primary, secondary, or tertiary amines on the side chain. Such PUs were used to obtain biodegradable nanovectors with limited cytotoxicity and comparable transfection efficiency with respect to PEI and PDMAEMA.[355] This experimental evidence supports the idea of using PUs combined with traditional polycarbonates (PCs) for *in vivo* gene delivery nanocarriers. In this regard, Liu *et al.* synthesized poly(ester-*co*-urethane) with 800 Da PEI on the side chain (PEU-*g*-PEI).[356] In addition to its great biodegradability, PEU-*g*-PEI exhibited higher transfection efficiency in delivering DNA in COS-7 cells as compared with pristine PEI. Furthermore, Chiou and co-workers employed PU-PEI to deliver miRNA (miR145) in brain tumors[357] and the PU-PEI-mediated miR145 delivery successfully inhibited the tumorigenesis of glioblastomas. They also employed the same PU-PEI carrier to treat lung cancer with a miR145-based therapy.[358] *In vivo* evidence showed promising results in xenograft tumors, including significantly reduced growth and metastasis, enhanced sensitivity of tumors to chemoradiotherapies, and prolonged survival of tumor-bearing mice. Furthermore, PU-PEI has been used for siRNA delivery to address metastasis in head and neck squamous cell carcinoma by targeting the two upregulated proteins, EZH2 and Oct4.[359]

Due to its high biocompatibility, low inherent cytotoxicity, and tuneable mechanical properties, PCs have emerged as an attractive alternative for gene delivery applications.[360] He and co-workers initially synthesized poly(5-methyl-5-allyloxycarbonyl-trimethylene carbonate) (PMAC) using porcine pancreas lipase (IPPL).[361] Subsequently, they converted the allyl functionality of PMAC to epoxide groups (PMAC-O), and further modified it with PEI to give rise to PMAC-*g*-PEI copolymers. When using PMAC-*g*-PEI to obtain gene delivery vectors, they showed enhanced transfection efficiency and lower cytotoxicity in 293T cells if compared to PEI-based polyplexes. Moreover, they additionally introduced 5,5-dimethyltrimethylene carbonate (DTC) into the backbone of PMAC-*g*-PEI, resulting in the preparation of P(MAC-*co*-DTC)-*g*-PEI which further affected the unpacking and endosomal escape of the loaded DNA.[362]

Dextran is a typical example of a biodegradable natural polysaccharide explored for gene delivery applications, owing to its remarkable water solubility and chemical flexibility.[363] Dextran has been employed mainly to reduce the toxicity of PEI and other non-degradable polycations.[160] Gong *et al.* reported efficient transfection and low cytotoxicity of a dextran-grafted-PEIs system, achieving miRNA transfer into osteosarcoma (OS) cells and OS-bearing nude mice.[364] In a similar approach, Wang and colleagues modified the hydroxyl groups of dextran with a reduction-sensitive disulfide linker. This modification provided initiation sites for sequential PDMAEMA polymer chain growth through ATRP, aiming to reduce the overall cytotoxicity of the resulting delivery systems and further enhance their gene-transfection efficiency into various cell lines.[365]

4.5 Active-targeting functionality

Passive, active, or endogenous targeting may be used to facilitate the intracellular delivery of NAs to the appropriate organs, tissues, and cells.[366] When active targeting is utilized, homing peptides, saccharides, antibodies, and proteins can be conjugated to polycations to bind to specific receptors and cellular compartments.[367]

Immune cells, crucial in various diseases such as cancer and autoimmune disorders, are prime targets for NA delivery. Present throughout the body, they include macrophages, dendritic cells, and lymphocytes. Delivering genetic therapeutics to these cells offers potential for anti-inflammatory treatment, immune stimulation, and immunotherapies.[367,368]

Macrophages can be targeted *via* their mannose receptor by conjugating mannose to PLL or polyaspartamide (pAsp(DET)).[272,273] Using this approach, Man-PLL polyplexes displayed increased DNA transfection efficacy, and even higher transfection performances were observed when using Man-pAsp(DET) polyplexes to transfect murine bone marrow-derived macrophages. Similarly, dendritic cells were transfected with polyplexes made of mannose-PEI conjugates for targeted pDNA delivery, resulting in very high transfection efficacy.[274]

Primary human T cells were targeted *via* their CD3 receptor by conjugating anti-CD3 antibodies to PLL.[369] In this way, pDNA polyplexes provided up to a 1000-fold increase in gene expression with respect to unmodified PLL.

Due to its intrinsic ability to accumulate nanoparticles, the liver is a crucial target for NA delivery. While a passive targeting approach may be used to accumulate the polymeric nanocarrier in this organ, specific targeting of hepatocytes might require additional synthetic efforts. This may be achieved by targeting the asialoglycoprotein receptor (ASGPR), which is highly expressed by hepatocytes.[370] For instance, Plank *et al.* designed asialoorosomucoid-modified (ASOR) PLL-polyplexes to deliver pDNA to hepatocytes *via* ASGPR *in vitro* and *in vivo*.[371]

The lung is another crucial target organ which benefits from accessibility through both local and systemic routes. Overcoming the mucosal barrier and immune surveillance of alveolar sites is essential for the effective treatment of diseases such as cystic fibrosis, COPD, asthma, and pulmonary fibrosis, often necessitating receptor-targeting strategies.[367] Lactoferrin receptor was typically targeted for the selective delivery of pDNA to bronchial epithelial cells.[372] Accordingly, common approaches for lung targeting involve the conjugation of PEI with lactoferrin. Alternatively, other strategies utilize the conjugation with (i) the anti-platelet endothelial cell adhesion molecule (PECAM) antibody to achieve efficient systemic gene delivery to the lung with minimal toxicity,[373] (ii) β2-adrenoceptor to deliver NAs into lung epithelial cells, (iii) clenbuterol to deliver pDNA and enhance gene expression in alveolar epithelial cells,[275] (iv) prostaglandin I2 analogues (Iloprost and Treprostinil).[374]

Transferrin and lectins have also been investigated for lung targeting, as they were hypothesized to play a role in the

specific nanocarrier uptake.[375–378] In particular, targeted delivery of siRNA to pulmonary T cells *via* transferrin-PEI conjugated was investigated as a potential therapy for asthma.[275,379,380]

It is well known that treating the brain *via* systemic administration routes remains challenging due to the poor accessibility of the brain through the blood–brain barrier (BBB). Therefore, brain-targeted NA delivery may be achieved by decorating polyplexes with specific ligands that facilitate NA transfer *via* receptor-mediated transcytosis, which typically controls the permeation of nutrients, proteins, or lipids across the BBB.[381]

Transferrin and lactoferrin receptors, which regulate the transport of iron across the BBB, have been investigated for targeted delivery of PEG-PAMAM-[382,383] and polymethylene polyphenylene isocyanate (PPI)-based[384,385] nanocarriers to the central nervous system.[386] Other strategies involve the conjugation with (i) retro-enantio peptide (re-TfR) to bind transferrin receptor and enhance transgene expression in TfR-rich K562 cells and Neuro2a cells,[387] (ii) low-density lipoprotein receptor 1 (LRP1), angiopep and RVG29 peptides to facilitate overcoming BBB and the brain accumulation,[381,386,388] (iii) peptide meningitis derived EPRNEEK for targeting laminin.[389]

Regarding tumor targeting, folate (folic acid, FA) is a widely employed ligand for anti-cancer agents because its target (folate receptor (FR)) is normally over-expressed in several cancer cells but rarely present on the surface of healthy cells.[390] In particular, these RPs are elevated in malignant tissues of the ovary, uterus, endometrium, brain, kidney, head and neck, and skin.[277] Liang *et al.* synthesized PEGylated PEI derivatives, where folate was conjugated on the distal end of the PEG-PEI complex (FA-PEG-PEI).[278] They tested the effects of the polyplexes on three cell lines with different expression levels of FR (*i.e.*, a FR-free hepatoblastoma HepG2 cell line, a low-level FR glioma C6 cell line, and a high-level FR 293T cell line). Compared with 25 kDa PEI, FA-PEG-PEI decreased the cytotoxicity and increased transgene expression in all the cell lines examined.[278]

Similarly, in a work proposed by Aranda *et al.*, β-ciclodextrin conjugated with *l*PEI macromolecules were decorated with FA after polyplexes formation. The resulting nanocomplex showed to be efficient vectors for gene delivery *in vivo* in a mouse model, leading to relatively high transfection levels in the lung and, especially, in the liver.[277]

4.6 Branching functionality

The topological structures of polymers play a critical role in determining their performances as gene delivery vectors. Various non-linear polymers, including (hyper)branched, dendritic, multi-arm, and brush-shaped structures (Fig. 2), often display higher NA complexation ability than linear counterparts.[142,391] Furthermore, non-linear polymer topologies have been found to affect the size and shape, charge density of the resulting polyplexes, and therefore their interactions with biological barriers and cellular compartments.[142]

Introducing branching points into the polymer chain is essential to modify polymer topology. These units are either charged (*e.g.*, tertiary amines) or neutral, and they can be incorporated during polymerization using multifunctional monomers or through post-modification methods, including crosslinking.[158,391] *b*PEI, PβAEs, PLL, PAMAM dendrimers present tertiary amines as branching points, and exhibit diverse amine groups with varying pK_a values, enhancing NA binding and buffering capacity compared to linear polycations[142,392–394] at the same M_w. However, higher charge density may increase cytotoxicity due to cell membrane disrup-

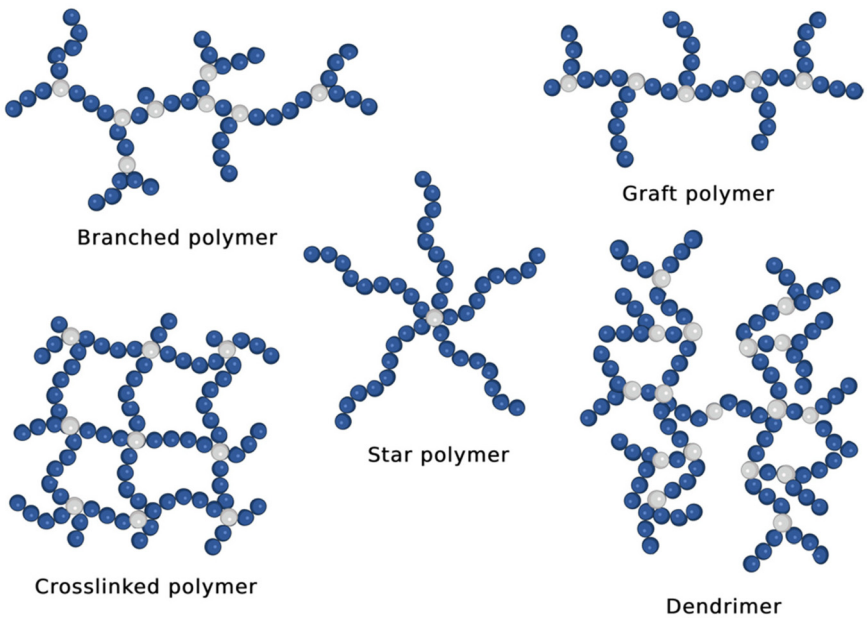


Fig. 2 Typical architectures of non-linear CPs employed for gene therapy (image created with Biorender.com).

tion.[395] Recent studies highlight the superior transfection efficiency and lower cytotoxicity of branched polymers like PβAEs, synthesized using non-ionic branching points from multifunctional acrylates *via* Michael addition of primary or secondary amines.[122,349] PβAEs synthesized by adding non-ionic trimethylolpropane triacrylate as the multifunctional monomer were able to co-deliver plasmid DNA encoding Cas9 and short guide RNA (sgRNA), respectively, to enable gene knockout following a CRISPR-mediated cleavage at one genomic site as well as gene deletion following DNA cleavage.[396] Recently, highly branched PAEs were investigated in SAR studies to highlight the effect of the polymer architecture on the gene transfection performances of the resulting polyplexes.

Xie *et al.* propose to employ citric acid (CA) as the backbone to link spermine (SPE) for developing a novel hyperbranched polyspermine (HPSPE).[397] The resulting HPSPE was synthesized *via* a polymerization reaction between activated carboxyl groups of CA and amine groups of SPE, composed solely of endogenous compounds. HPSPE demonstrated efficient complexation of DNA into stable nanocomplexes with excellent biocompatibility profiles compared to 25 kDa *b*PEI, showing promising potential in addressing toxicity concerns associated with other synthetic materials. This is achieved while ensuring effective condensation of NAs due to the hyperbranched high M_w structure.[397]

A key structural factor, named branch unit distribution (BUD), was identified as playing an important role for transfection capability. Poly(amino esters) (PAEs) with a more uniform distribution of branch units, in fact, showed an enhanced transfection efficacy.[398] Multi-arm, or "star" polymers are typically synthesized through controlled-living polymerization techniques, which allow to vary M_w and composition, depending on the specific application.[399,400] The central core can be either a multifunctional LMw molecule or a macromolecule.[142] PDMAEMA-based star polymers as well as other types of cationic polymers based on oligoethylene imine, poly(aminoethyl methacrylate) (PAEMA), PDMAEMA-*block*-poly(poly(ethylene glycol)ethyl ether methacrylate) (PPEGEEMA), have also been used as arms in the synthesis of star polymers *via* controlled polymerization for NA delivery.[401,402]

The use of cyclodextrins (CD) as central cores has been widely explored, *via* an effective conjugation of their multiple hydroxy groups to the polymeric arms,[403–406] taking also advantage of their high biocompatibility, degradability, and possible encapsulation of other hydrophobic components in their inner compartment.[407] Liu and colleagues synthesized a new CD derivative (CD-PLLD) consisting of a β-CD core and PLL dendron arms *via* click conjugation. Using CD-PLLD as the carrier, an anticancer drug docetaxel (DOC) and pMR3-specific siRNA can be co-loaded into a single carrier. With the co-loading of the hydrophobic drug and functional siRNA, CD-PLLD/DOC/pMR3 resulted in an enhanced apoptosis and a decreased invasive capacity of the treated HNE-1 cells than CD-PLLD/DOC (solely loading with DOC) and CD-PLLD/pMR3 (solely loading with pMR3 siRNA).[408]

Xiu *et al.* synthesized β-CD core-based cationic star polymers with PDMAEMA polymers as the multi-arm part *via* an ATRP technique. The resulting polymer can effectively condense pDNA and with this rational design gene-delivery system exhibited positive gene-transfection efficiency.[409] The enhanced transfection performance revealed by star-shaped polycations obtained from a CD core was also confirmed in the work presented by Wang and collaborators. Linear poly(2-aminoethyl methacrylate) (l-PAEM) and three star-shaped PAEM polycations (s-PAEM) polymerized from β-CD at different M_w, were employed to condense pDNA. The transfection efficiency of the polyplexes was assessed in MCF-7 and COS-7 cells using pEGFP as the reporter gene.

Complexes formed by LMw-PAEMs exhibited the lowest cytotoxicity and, at a specific N/P ratio, the s-PAEMs showed significantly enhanced transfection efficiency than linear PAEMs of comparable M_w.[410]

Brush- or comb-like cationic polymers link several side chains into a single macromolecule. These macromolecular architectures may be exploited to improve the poor transfection efficiencies of oligocations, by grafting them to a common polymer backbone, thus achieving higher charge density.[411,412] Graft polymers based on PDMAEMA, PEI, PEG-*b*-PEI, oligoamines, oligopeptides, and other structures have all been explored for NA delivery,[142] with different type density and length of the polycationic grafts, as well as different controlled polymerization and post-polymerization strategies. Brush copolymers with pendant oligopeptide combs were obtained *via* free radical and RAFT polymerization of vinyl-terminated cationic oligopeptide, copolymerized with *N*-(2 hydroxypropyl) methacrylamide (HPMA).[413]

A post-polymerization of poly(glycidyl methacrylate) (PGMA) with tetraethylenepentamine (TEPA), pentaethylenehexamine (PEHA), and tris(2-aminoethyl) amine (TREN) was also conducted to generate a library of graft polymers for NA delivery.[414] The ring-opening metathesis polymerization (ROMP) of cyclooctene-oligopeptide macromonomers resulted in comb-like structures exhibiting over two-fold higher pDNA transfection efficiency in COS-1 cells compared to non functionalized polyplexes. While their efficiency was lower than that of Lipofectamine, these structures demonstrated lower cytotoxicity.[415] Tetralysine-containing comb polymers, modulated through copolymerization of a zwitterionic sulfobetaine-cyclooctene monomer, exhibited a weaker DNA binding affinity compared to the tetralysine-comb homopolymers. This resulted in a two-fold increase in delivery efficiency with SCOV3 cells.[416]

5. Conclusion and future perspectives

NAs have emerged as a new therapeutic category for preventing and treating various diseases. However, NA therapeutics must have safe, efficient, and biologically stable delivery systems to function effectively *in vivo*. Although ionizable lipid-based

nanoparticles have shown promising clinical results in the two FDA-approved COVID-19 mRNA vaccines, in the FDA-approved liver-targeting siRNA formulation Onpattro®, and liver gene editing clinical trials,[417] the restricted delivery to non-hepatic targets and tumors remains a big hurdle. Further optimization of non-viral synthetic delivery systems is thus urged. In this context, polymeric systems encompassing a number of tuneable moieties and functional groups offer a promising avenue for successful NA delivery.

Polymeric vehicles for NA delivery have advanced significantly in the last decade, addressing challenges such as stability and intracellular trafficking. Stable polyplexes feature ionizable, polycationic moieties that enable electrostatic interactions with negatively charged NAs. Nonetheless, the presence of non-cationic components has been leveraged to create polycations with improved NA delivery, stability in biological fluids, and tailored physicochemical properties. Customizing macromolecular structures and innovative designs made it possible to refine toxicity-efficiency trade-offs and enhance precision targeting. Additionally, properties such as loading capacity and stimuli responsiveness can now be tailored to the specific application. Although viral vectors currently dominate the clinical gene therapy panorama, it is expected that synthetic vectors will increasingly prevail in clinical trials in the coming decades.[418] Despite their promise, polyplex delivery systems are still in their early development stages. Indeed, according to the global clinical trial database (ref. ClinicalTrials.gov), there is only a limited number of human clinical trials involving polyplexes.[419]

This is primarily because of the layered complexity of these nanomaterials. Most polymeric delivery systems in use comprise quite complex formulations and cumbersome preparation to ensure good performance (*e.g.*, stability, target specificity, and efficient delivery). Transitioning these systems from the laboratory scale to large-scale production for clinical use requires extensive optimization and standardization. Additionally, some delivery vehicles may trigger immune response and toxicity, and lead to off-target effects.

The slow progress in clinical translation may also be ascribed to inadequate screening workflows, which rely on the insufficient, inefficient, and non-robust methods used to assess the efficacy, safety, and other critical polyplex features. As a result, it is currently very challenging to identify the most effective and safe polyplex formulations, slowing down the overall progress of bringing them to the clinic and delaying benefits to patients. To overcome this issue, it is critically important to design and leverage high throughput *in vitro* platforms, which can significantly speed up and scale up preliminary screening for factors such as efficacy, toxicity, and stability.[420–422] Similarly, high throughput *in vivo* platforms may enable the rapid assessment of how these formulations behave in complex biological systems, including their biodistribution, pharmacokinetics, and therapeutic effects. Moreover, coordinated pre-clinical studies may ensure that all necessary safety, efficacy, and regulatory requirements are met comprehensively and efficiently, providing robust and reliable data to pinpoint the best polyplex formulations for further development.

The majority of current clinical trials focus on mRNA-based therapeutics and vaccines.[423,424] Advanced polyplex design may also lead to a shift in focus toward other applications, such as the delivery of ASOs and siRNA, both of which have demonstrated the ability to silence specific genes and offer potential treatments for a wide range of diseases. Just as an example, CRISPR/Cas9 is now the gold standard for therapeutic gene editing, but we still need to enhance its delivery efficiency to fully utilize its potential.

Besides, while some NA delivery systems have demonstrated some targeting effectiveness *in vitro*, *in vivo* targeting of tissues and organs remains a significant challenge. In this regard, a deeper understanding of biological processes and barriers governing cell-nanomaterial interactions, coupled with innovations in polymer chemistry, will continue to enable the development of advanced NA delivery systems. Modular synthesis accompanied by thorough characterization is a key area for improvement, and current advancements in polymer science hold promise for widespread clinical deployment in gene therapy. In this regard, the synthetic routes should ensure scalability and adherence to good manufacturing practices (GMP).

It is expected that personalized medicine will play a crucial role in the near future. Consequently, customizing polymeric vectors to match individual patient requirements, *e.g.*, genotypic and phenotypic variations, will be essential. Polyplexes represent an exciting avenue for NA delivery and a valuable tool in the quest for effective treatments. With ongoing research, we can look forward to breakthroughs that will reshape medicine and improve patient outcomes.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflicts of interest

There are no conflicts to declare.

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