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Review Article

Molecular additives: all gain, no pain in gene delivery

G. Protopapa [1], F. Fruzzetti [1], I.M. Coizet, G. Dadomo, N. Bono, G. Candiani [*]

GenT_LAB, Dept. of Chemistry, Materials and Chemical Engineering "Giulio Natta", Politecnico di Milano, Via Bassini 6, Milan, 20133, Italy

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ABSTRACT

Non-viral gene delivery systems face a persistent challenge: achieving high transfection efficiency while maintaining acceptable cytotoxicity. Cationic vectors effectively condense nucleic acids into complexes and promote cellular uptake, yet their positive surface charge drives cytotoxic interactions with cellular membranes and serum components. This review examines how molecular additives, auxiliary charged molecules incorporated during complexation, address this fundamental trade-off through ternary complex formation. We systematically analyze three functional classes of additives based on their primary mechanisms. Additives enhancing transfection efficiency, including oligoamines (spermine, spermidine) and modified polypeptides (carboxymethylated poly-L-histidine), boost gene delivery by enhancing proton sponge activity, improving endosomal escape, and stabilizing nucleic acid interactions via dehydration effects and hydrogen bonding. Additives enhancing viability, comprising synthetic polyacids (polyglutamic acid, polyaspartic acid) and polysaccharides (alginate), reduce cytotoxicity by neutralizing surface charge, preventing protein corona formation, and minimizing nonspecific membrane interactions while maintaining colloidal stability. Dual function glycosaminoglycans (hyaluronic acid, heparin, chondroitin sulphate) simultaneously address both parameters by coupling electrostatic shielding with receptor-mediated cellular uptake via CD44, HARE, and RHAMM, enabling targeted delivery despite their reduced surface charge. Beyond cataloging these additives, we provide mechanistic insights into how assembly pathways, stoichiometric ratios, and molecular interactions govern complex performance. This analysis establishes rational design principles for optimizing the efficiency-cytotoxicity balance in non-viral gene delivery, demonstrating that strategic additive selection enables the development of safer and more effective vectors.

1. Introduction

Transfection, the introduction of exogenous genetic material into host cells through non-viral means, is widely applied for gene therapy in the treatment of inherited disorders, cancer, and viral infections [1], as well as in fundamental research and bionanotechnology [2]. Gene delivery now serves as a key enabling technology for CRISPR-Cas9–based genome editing [3] and nucleic acid (NA) vaccines [4,5].

For gene delivery to be effective, it is essential to introduce functional NAs into target cells to regulate gene expression. Common cargoes include plasmid DNA (pDNA), messenger RNA (mRNA), and short regulatory RNAs, including small interfering RNA (siRNA), microRNA (miRNA), short hairpin RNA (shRNA), and antisense oligonucleotides (ASOs) [6,7]. However, the delivery of naked NAs is ineffective due to their strong anionic charge at physiological pH, which limits cellular uptake, and their high susceptibility to nuclease degradation, which compromises extracellular stability [8]. These limitations have driven the development of efficient and biocompatible gene delivery systems.

To address these challenges, current NA delivery strategies may be broadly classified into physical methods and vector-based systems. Physical approaches, including electroporation, sonoporation, magnetofection, optoporation, gene gun, and microinjection, employ external stimuli to transiently permeabilize membranes, enabling NA delivery [9]. Despite their effectiveness under controlled conditions, limited *in vivo* applicability, invasiveness, and toxicity restrict their clinical translation [10]. Conversely, vector-based systems encapsulate or complex NAs into particle-like assemblies (complexes) that actively mediate cellular internalization and are classified as viral or non-viral [6]. Viral vectors, engineered viruses in which therapeutic genes replace portions of the viral genome, remain the most efficient vehicles, exploiting the natural viral infection machinery [11]. However, restricted tropism, immunogenicity, limited packing capacity, safety concerns [12], and high production costs have progressively shifted research toward non-viral carriers [13]. Among these, cationic polymers

* Corresponding author. GenT_LAB, Dept. of Chemistry, Materials and Chemical Engineering "Giulio Natta", Politecnico di Milano, Milan, Italy.
E-mail address: gabriele.candiani@polimi.it (G. Candiani).
[1] Equally contributing Authors.

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and lipids represent versatile platforms for non-viral NA delivery. Their positive charge enables spontaneous self-assembly with anionic NAs through electrostatic interactions, forming lipoplexes and polyplexes ranging from tens to hundreds of nanometers that protect NAs from enzymatic degradation while facilitating cellular uptake and intracellular trafficking [6]. In particular, among cationic polymers, 25 kDa branched polyethylenimine (*b*PEI) serves as the gold standard due to its efficient NA condensation and endosomal escape via the proton-sponge effect. More generally, non-viral vectors offer chemical tunability, scalable manufacturing, reduced safety concerns, and tailored physicochemical parameters to optimize transfection [14].

Despite these advantages, the interaction between non-viral gene delivery systems and their cellular targets remains highly context-dependent. Most *in vitro* studies employ adherent monolayer cultures using immortalized cell lines, primary cells, and tumor-derived models [15]. Immortalized cell lines are preferred for preliminary evaluations due to their high proliferative rates and short doubling times, facilitating nuclear access of exogenous DNA during mitosis. By contrast, primary cells exhibit limited proliferation and undergo senescence, resulting in very low transfection efficiency (TE) and requiring high DNA doses [16]. Differences in membrane composition, endocytic activity, metabolic state, and stress tolerance critically influence both TE and cytotoxicity of carriers. Consequently, formulations exhibit markedly different performances across cell types: systems ensuring efficient NA delivery in one context may prove suboptimal or toxic in another [14]. This variability demonstrates that distinct cell types impose specific constraints, necessitating application-specific optimization rather than universal formulations [17].

This need for flexible, context-adaptable optimization has prompted increasing interest in molecular additives as versatile molecules to modulate the performance of non-viral gene delivery systems. These auxiliary charged molecules are introduced during complexation to fine-tune the properties of the resulting complexes without requiring chemical modification of the primary vector. By participating in electrostatic interactions with NAs, the carrier, or both, additives influence key physicochemical features such as condensation, charge distribution, stability, and buffering capacity, thereby modulating cellular uptake, intracellular trafficking, and biological outcomes [18]. Notably, the role of additives extends beyond simple formulation support. The sequence and mode of incorporation (such as pre-mixing with NAs *vs.* post-complexation addition) can generate distinct supramolecular architectures, leading to markedly different TE and cytotoxicity profiles. This sensitivity highlights additives as active formulation components rather than passive excipients. Through non-covalent integration, molecular additives thus provide a powerful and modular means to optimize the balance between TE and cytotoxicity, two frequently competing parameters in non-viral gene delivery.

To appreciate the distinctive merits of this approach, it is instructive to consider alternative strategies that have been pursued to address the same efficiency-cytotoxicity trade-off. PEGylation, the most widely adopted covalent shielding approach, effectively reduces opsonization and extends circulation but introduces the “PEG dilemma”, namely, steric hindrance that attenuates cellular uptake and endosomal escape, as well as anti-PEG antibody-mediated accelerated blood clearance upon repeated administration [19,20]. Covalent ligand conjugation requires multistep synthesis and yields fixed-composition systems that are difficult to reformulate across biological contexts, while *de novo* polymer design demands extensive synthetic effort and iterative screening [21]. Lipid nanoparticles (LNPs), despite clinical validation for mRNA delivery, remain constrained by inherent hepatic tropism, reliance on proprietary ionizable lipids, and cold-chain requirements [22,23]. In contrast, molecular additives offer a fundamentally different paradigm: non-covalent, modular, and operationally simple. Because they are incorporated during complexation via electrostatic self-assembly, additives bypass additional synthetic steps, preserve the chemical identity of both carrier and cargo, and permit facile stoichiometry adjustment to accommodate different vectors, cargoes, and target cell types without re-engineering the core formulation. Moreover, many of the additives surveyed herein, including spermine (SPM), spermidine (SPD), hyaluronic acid (HA), heparin (Hep), and alginic acid (Alg), are commercially available, well-characterized, and in several cases clinically precedented or classified as Generally Recognized as Safe (GRAS), substantially lowering the regulatory barrier relative to novel chemical entities. The timeliness of this analysis is further underscored by the expanding clinical pipeline of non-viral gene therapies, the growing demand for scalable *ex vivo* transfection platforms such as CAR-T cell manufacturing [24], and the increasing recognition that formulation-level optimization, rather than carrier redesign alone, can yield clinically meaningful gains in vector performance.

This review systematically analyzes molecular additives that modulate the performance of non-viral gene delivery systems, providing a mechanistic framework for balancing TE and cytotoxicity. Specifically, we examine three functional categories of additives: those enhancing TE, those mitigating cytotoxicity, and those addressing both challenges simultaneously. By integrating physicochemical, mechanistic, and biological insights, this review offers a unified framework for understanding how molecular additives optimize the efficiency-cytotoxicity balance in non-viral gene delivery.

2. Additives enhancing transfection

The primary limitation in non-viral gene delivery is the inherently low or inconsistent TE observed across different biological systems. This stems largely from multiple physiological barriers that collectively impede successful non-viral NA delivery, including cellular uptake [25], endosomal escape [26], cytosolic trafficking [27], nuclear import [28], and NA release [29]. Among these obstacles, endosomal escape has emerged as a particularly critical bottleneck [26]. Following cell uptake, complexes are typically sequestered within endosomal vesicles, where they encounter a hostile microenvironment characterized by progressively acidic pH (pH 4.5-5.5) and elevated concentrations of hydrolytic enzymes that threaten NA integrity [30]. Failure to escape this degradative compartment invariably results in NA degradation and loss of TE.

To overcome this barrier, one extensively investigated strategy harnesses the “proton sponge effect”. This mechanism is characteristic of polymers bearing protonatable amine groups, exemplified by *b*PEI, which exhibit pKa values spanning the physiological to endo-lysosomal pH range [31–33]. Upon endosomal acidification, extensive polymer protonation triggers active proton influx into the compartment via V-ATPases. To maintain electroneutrality, chloride ions enter concomitantly, accompanied by compensatory water influx driven by osmotic forces. The ensuing ionic imbalance induces a sharp rise in osmotic pressure, culminating in membrane swelling, destabilization, and eventual rupture [26]. Endosomal membrane disruption results in the release of complexes into the cytosol, enabling NA trafficking towards their intracellular targets, the nucleus for DNA and the cytosol itself for RNA [34]. This mechanism extends beyond amines to all compounds bearing protonable functional groups capable of accepting protons, including imidazole and guanidinium moieties [35]. Recent studies have identified additive molecules that can be incorporated into non-viral vector formulations to form ternary complexes with NAs, exploiting this mechanism along with other synergistic processes to enhance TE (Fig. 1). To quantitatively characterize the interactions among the three components in these ternary formulations, we employ a set of stoichiometric ratios for the relative proportions of charged groups within the complex. The widely used N/P ratio represents the molar ratio between protonatable nitrogen groups in the cationic vector (N) and the phosphate groups in the NA (P), thus defining the composition of binary polyplexes. When an anionic additive is incorporated to form the ternary complex, we introduce the A/N ratio, defined as the molar ratio of anionic groups in the polyanion (A) to cationic groups in the polycation (N). We further adopt a unified A/N/P framework that captures the

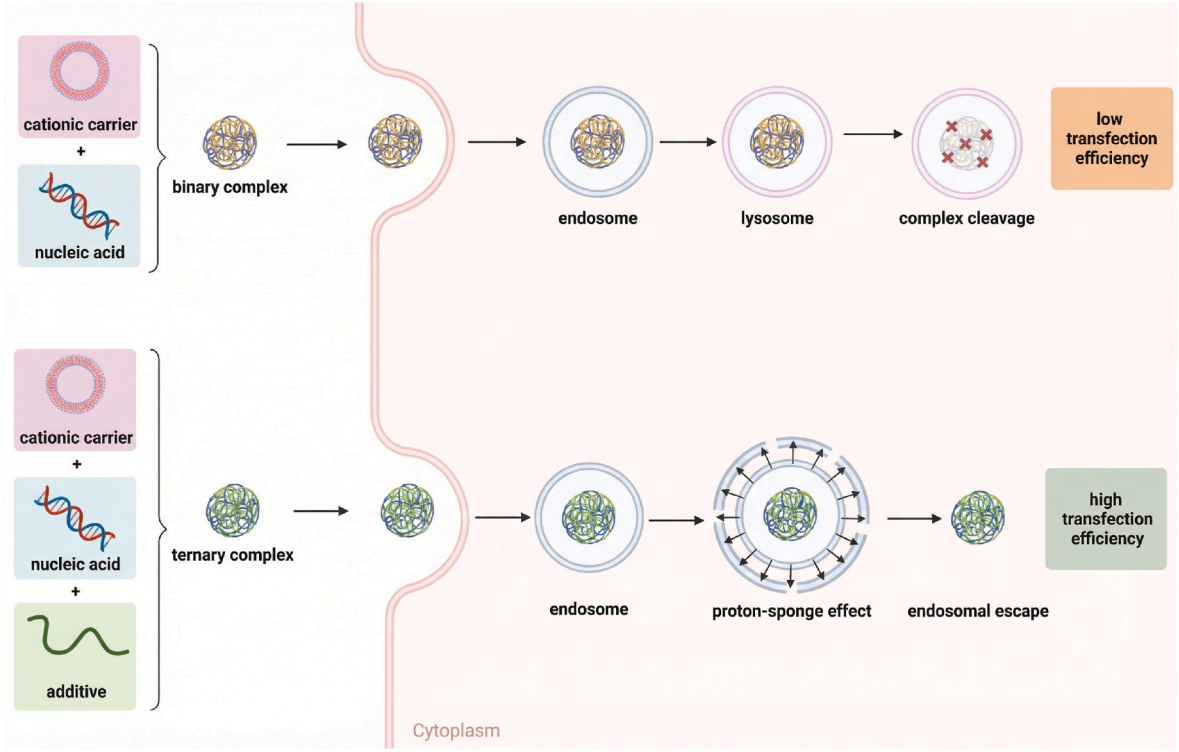

Fig. 1. Schematic comparison of binary and ternary nucleic acid (NA) delivery complexes. A binary complex comprising a cationic carrier and NA enters the cell via endocytosis and is subsequently trafficked to lysosomes, where complex disassembly and cargo degradation result in low transfection efficiency (TE). In contrast, a ternary complex consisting of a cationic carrier, a NA, and an auxiliary molecule, enhances endosomal buffering capacity, promotes the proton-sponge effect, and facilitates endosomal escape into the cytosol, thereby achieving high TE.

stoichiometric balance of all anionic and cationic moieties within the ternary complex, providing a comprehensive descriptor that enables systematic comparison across formulations. Where molar ratios are not available, we report mass-based ratios (w/w/w), consistent with established literature.

2.1. Spermine (SPM) and spermidine (SPD)

SPM and SPD are naturally occurring quaternary and ternary polyamines, respectively, which play essential roles in numerous biological functions, including cell growth and proliferation. Both molecules are characterized by their cationic nature at physiological pH and their

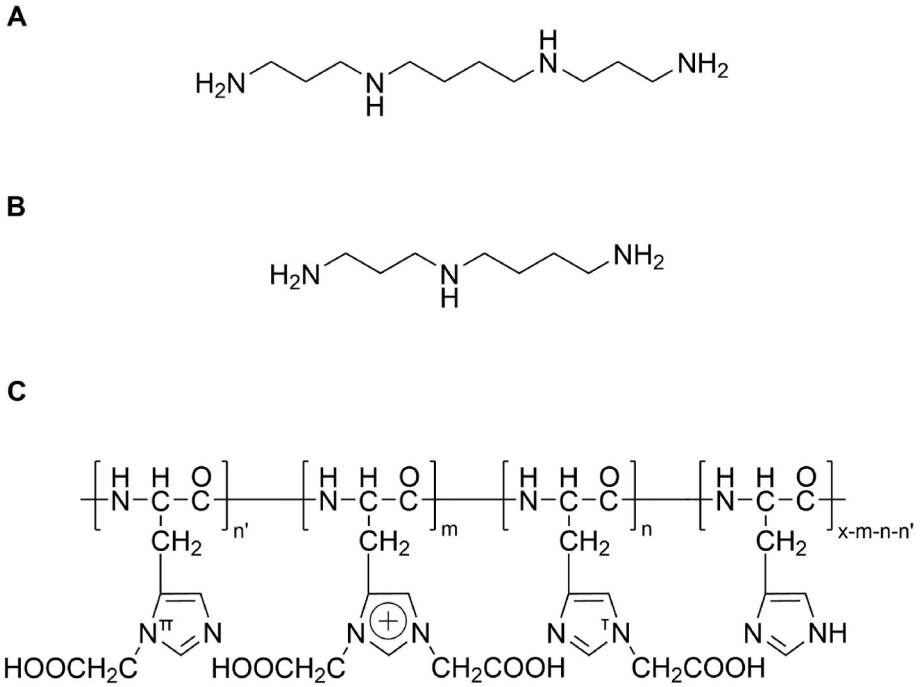

Fig. 2. Chemical structure of A) spermine (SPM), B) spermidine (SPD), and C) carboxymethylated poly-L-histidine (CM-PLH).

capacity to condense NAs under appropriate ionic conditions [36–39]. The two molecules differ structurally: SPM contains two primary and two secondary amines (Fig. 2A), whereas SPD possesses two primary and one secondary amine (Fig. 2B). These functional groups confer a protonable character to both molecules, enabling them to enhance the proton sponge effect and thereby facilitate endosomal escape [36,40]. Consequently, both SPM and SPD represent attractive candidates as additives for enhancing TE of binary complexes. Lv et al. explored this strategy by incorporating SPM as a non-covalent additive into 25 kDa *b*PEI/DNA binary complexes (Table 1). As expected, the resulting ternary complexes exhibited markedly enhanced buffering capacity, as demonstrated by acid–base titration experiments, thereby confirming amplification of the proton sponge effect. This enhancement was further corroborated by confocal laser scanning microscopy, which revealed a more pronounced endosomal escape compared to binary complexes, resulting in increased TE. Although *b*PEI possesses substantial intrinsic buffering capacity, SPM incorporation further augments the proton sponge effect. Notably, ternary complexes containing SPM, formulated at concentrations up to 200 μg/mL preserved cell viability at levels comparable to those observed with *b*PEI/DNA binary complexes, underscoring the potential to enhance efficacy without compromising cell viability [40].

Given the structural and functional similarities between SPM and SPD, comparable transfection-enhancing effects would be expected for both polyamines. Although direct experimental evidence specifically demonstrating SPD-mediated transfection enhancement through the proton sponge mechanism remains limited, recent atomistic molecular dynamics (MD) provide mechanistic support for this hypothesis. These simulations revealed that both SPM and SPD display their protonated amine groups linearly along their aliphatic backbones, resulting in analogous charge distributions and suggesting similar buffering capabilities [44]. Experimental evidence consistent with this interpretation was reported by Apirakaramwong et al., who demonstrated that incorporating either SPM or SPD into chitosan (Chito)/DNA complexes significantly increased TE in HeLa cells compared to binary counterparts (Table 1) [41]. This effect is particularly notable given the limited intrinsic buffering capacity of Chito, suggesting that polyamine addition compensates for this deficiency by reinforcing the proton sponge effect [45]. While the proton sponge effect provides a plausible mechanism for transfection enhancement in systems with limited buffering capacity, such as Chito, it does not fully account for the improvements observed in PEI-based formulations, which already possess substantial intrinsic buffering. This raises a critical question: what additional mechanisms contribute to enhanced transfection efficiency upon polyamine incorporation in buffering-competent binary complexes? MD simulations have provided critical insight into this apparent discrepancy, indicating that the primary contribution of oligoamine incorporation in PEI-based systems lies in enhanced nanoparticle stability rather than additional buffering. Two synergistic mechanisms have been identified: a dehydration effect and hydrogen bond formation between oligoamines and NA. Specifically, oligoamines partially displace hydration water molecules from DNA grooves, thereby establishing stronger and more direct electrostatic interactions with the phosphate backbone. This dehydration-driven process promotes the formation of more compact and stable ternary complexes compared to binary counterparts. Additionally, oligoamines form multiple hydrogen bonds with the DNA backbone, further stabilizing ternary complexes through specific and persistent molecular interactions. The synergistic contribution of these mechanisms likely accounts for the observed increase in TE, as enhanced nanoparticle stability confers improved resistance to lysosomal degradation [40,44].

Beyond the intrinsic physicochemical properties of the individual components, the assembly itself constitutes a critical determinant of the final architecture and performance of ternary gene delivery assemblies. This refers to the specific procedural sequence by which the components, namely NA, cationic polymer, and additive, are combined to form the ternary complex. The order of component addition is not merely a technical detail but rather a fundamental design parameter that governs charge distribution, complex morphology, and ultimately TE [46]. Consistent with this principle, both Lv et al. and Apirakaramwong et al. systematically investigated the impact of assembly sequence on complex structure and function. Using dynamic light scattering measurements, the authors demonstrated that pre-mixing the oligoamine with the cationic polymer before NA addition yielded smaller and more narrowly distributed complex sizes (hydrodynamic diameter, D_H). These findings were further corroborated by electron microscopy analyses, which revealed more compact and uniformly shaped nanoparticles compared to complexes assembled via alternative mixing protocols. Importantly, these structural differences translated into improved biological performance, as complexes formed via polymer–oligoamine pre-mixing consistently exhibited superior TE in comparative studies [40,41]. Collectively, these studies demonstrate that oligoamine incorporation into ternary complexes enhances TE through synergistic mechanisms encompassing improved buffering capacity, stabilized DNA–polymer interactions, and optimized nanoparticle architecture.

2.2. Carboxymethyl poly-L-histidine (CM-PLH)

Poly-L-histidine (PLH) is a cationic polypeptide characterized by protonatable imidazole groups that confer proton sponge activity and promote endosomal escape. However, its application in gene delivery has been hindered by poor aqueous solubility, which compromises colloidal stability, promotes aggregation, and may induce toxicity when incorporated in ternary complexes [42,43,47]. To overcome this limitation, Asayama et al. [47] developed a chemical modification strategy whereby pristine PLH was alkylated with iodoacetic acid, introducing carboxymethyl groups onto the imidazole rings. This modification converted the poorly soluble parent polymer into a hydrophilic derivative (CM-PLH) bearing both cationic and weak anionic functionalities (Fig. 2C). Consequently, CM-PLH exhibited markedly improved aqueous solubility and could be readily incorporated into pre-formed *b*PEI/pDNA polyplexes as a surface-modifying component. This incorporation

Table 1
TE-enhancing additives in ternary complexes, classified depending on the gene delivery vector (GDV), NA, ratios, and complexation order.

Additive	GDV	NA	DNA:GDV:Additive	Complexation Order	TE (fold change)	Viability (fold change)	Ref.
SPM	25 kDa *b*PEI	pDNA	1:1.5:25 (w/w/w)	DNA → (SPM $\overset{?}{\leftrightarrow}$ PEI)	1.4	0.9	[40]
SPM	45 kDa Chito	pDNA	1:4:1 (w/w/w)	DNA → (SPM $\overset{?}{\leftrightarrow}$ Chito)	2.7	0.7	[41]
SPD	45 kDa Chito	pDNA	1:4:512 (w/w/w)	DNA → (SPD $\overset{?}{\leftrightarrow}$ Chito)	2.3	0.9	[41]
SPD	Liposome (DOTAP: cholesterol 3:1 mol)	pDNA	1:3:1 (w/w/w)	(DNA → SPD) $\overset{?}{\leftrightarrow}$ liposome	1.6	1.0	[36]
CM-PLH	Poly(β-amino ester)	pDNA	1:50:10 (w/w/w)	CM-PLH → (PbAE $\overset{?}{\leftrightarrow}$ DNA)	Up to 1.9	Up to 1.3	[42]
CM-PLH	25 kDa *b*PEI	pDNA	1:15:15 (P/N/C)	CM-PLH → (PEI $\overset{?}{\leftrightarrow}$ DNA)	1.5	n.a.	[43]

$\overset{?}{\leftrightarrow}$ stands for "unspecified order of mixing"; → stands for "is added to"; n.a. stands for "not available".

reduced aggregation and enhanced colloidal stability compared to unmodified PLH-based systems (Table 1). Notably, CM-PLH–containing ternary complexes displayed reduced serum-induced aggregation relative to binary counterparts. Furthermore, CM-PLH addition to 25 kDa *b*PEI/DNA complexes conferred pH-responsive behavior, effectively broadening the buffering range from physiological to endosomal pH. Consistent with this enhanced pH sensitivity, CM-PLH exhibited pronounced hemolytic activity under acidic conditions (pH 5.0) while remaining essentially non-hemolytic at physiological pH. Hemolysis assays, which measure erythrocyte membrane rupture and hemoglobin release under acidic conditions [48], have been proposed as a complementary approach to assess membrane-disruptive potential relevant to endosomal escape. Nevertheless, buffering capacity titration and microscopy-based analyses remain the standard methods for direct evaluation of this process [43]. The selective low-pH hemolysis observed for CM-PLH thus suggests superior endosomal disruption capability, correlating with enhanced TE. However, a critical limitation of these studies is the absence of post-transfection viability assessment, which represents a significant gap in evaluating CM-PLH biocompatibility and constrains conclusions regarding its translational potential.

Building on this foundation, Gu et al. [42] developed CM-PLH–based ternary complexes employing hydrolyzable poly(β-amino ester) (PbAE) as the primary cationic vector. These ternary formulations (CM-PLH/PbAE/pDNA) exhibited significantly enhanced TE, coupled with reduced cytotoxicity, compared to PbAE/pDNA binary systems. Critically, the authors provided direct experimental evidence establishing CM-PLH's role in facilitating endosomal escape, thereby confirming a mechanistic link between its pH-dependent membrane-disruptive activity and improved transfection performance.

Collectively, these studies demonstrate that carboxymethylation transforms PLH from a poorly soluble, aggregation-prone polypeptide into a water-soluble derivative with broadened buffering capacity spanning physiological to endosomal pH ranges. This chemical modification not only circumvents the inherent limitations of pristine PLH but also enables its application as a multifunctional additive. By combining extracellular colloidal stability with pH-responsive endosomolytic activity, CM-PLH significantly enhances TE relative to binary complexes while preserving acceptable biocompatibility [41,42].

3. Additives mitigating cytotoxicity

Beyond TE, cytotoxicity represents the other critical parameter determining the practical applicability of any non-viral delivery system. Cytotoxicity refers to the capacity of a substance to damage cells or cause adverse effects within biological systems [49]. A vector may achieve efficient gene expression while simultaneously compromising the viability of target cells, thereby nullifying its therapeutic potential. Therefore, understanding and controlling cytotoxicity is as crucial as optimizing the delivery of the NA itself. Once infused into circulation, complexes are immediately exposed to blood components, including erythrocytes and plasma proteins. Then, complexes adhere to and destabilize erythrocyte membranes, and they can induce the release of hemoglobin. Such membrane disruption triggers oxidative stress through the release of heme, complement activation, potential embolism, and local inflammation [50]. Even when complete lysis does not occur, partial perturbation of the membrane structure can alter erythrocyte deformability and compromise microvascular flow, thereby contributing to secondary tissue damage [51].

Conversely, the rapid adsorption of serum proteins onto complex surfaces leads to protein corona formation, whose consequences depend on the route of administration [52]. Following systemic injection, adsorbed proteins such as complement factors and immunoglobulins expose recognition motifs that trigger opsonization, promoting clearance by macrophages and other mononuclear phagocyte system components and accelerating systemic elimination. In contrast, after local injection, protein corona formation may predominantly induce inflammatory responses through cytokine release. As a result, a substantial fraction of complexes is eliminated before reaching target sites and may elicit local or systemic toxicity associated with inflammatory stress [53]. Besides, multivalent plasma protein binding promotes complex aggregation and agglutination. Large aggregate formation compromises colloidal stability and alters D_H distribution, thereby affecting biodistribution and cellular uptake. Furthermore, aggregation drives sedimentation *in vitro* and may cause microvascular obstructions *in vivo*, further undermining safety and efficacy.

Upon reaching the target cell surface, direct interactions with the plasma membrane represent another critical source of cytotoxicity. Membrane-complex contact can induce destabilization, particularly when electrostatic and hydrophobic interactions disrupt local phospholipid organization [54,55]. These interactions may trigger transient pore formation, bilayer thinning, or localized alterations in lipid packing. While transient and localized membrane perturbation is essential for endosomal escape and productive delivery, excessive or prolonged destabilization transitions from functional to cytotoxic. Such perturbations compromise membrane integrity and permeability, enabling uncontrolled ion fluxes, cytoplasmic leakage, and loss of osmotic balance. Furthermore, sustained complex adhesion can interfere with receptor-mediated endocytosis, disrupt cytoskeletal organization, and activate calcium-dependent stress signaling pathways. These cumulative alterations disrupt membrane homeostasis, potentially culminating in cell death [56].

Collectively, these phenomena reveal that cytotoxicity arises from cumulative interactions spanning multiple stages, from the extracellular milieu to the plasma membrane. Notably, despite their mechanistic diversity, these phenomena share a common physicochemical driver: the high cationic surface charge inherent to most gene delivery systems [52]. Cytotoxicity correlates strongly with vector cationic charge density, a property that, while essential for efficient NA complexation and delivery, drives electrostatic attraction to negatively charged biological components, including serum proteins and cellular membranes [49]. This interaction initiates a cascade of adverse processes, including hemolysis, protein corona formation, opsonization, aggregation, and membrane destabilization. The relative contribution of each process depends on the route of administration, and collectively, they account for the observed cytotoxic effects [49–56].

Critically, these biological outcomes are governed not only by surface charge but also by an interplay of physicochemical parameters, including D_H, polydispersity index (PDI), and colloidal stability under physiological conditions. ζ-potential alone provides an incomplete picture; formulations with comparable surface charge can exhibit markedly different biological behavior, depending on their size distribution and aggregation propensity in protein-rich media. Accordingly, the studies discussed below report, where available, D_H and colloidal stability alongside ζ-potential data, although systematic reporting of PDI remains inconsistent across the literature.

A promising strategy to mitigate non-viral vector cytotoxicity involves incorporating anionic additives into binary complexes to partially neutralize or shield surface charge. The following sections examine this approach in detail, exploring how different anionic additives can optimize the efficiency-biocompatibility profile of non-viral gene delivery systems (Fig. 3).

3.1. Poly-γ-glutamic acid (γ-PGA) and polyaspartic acid (PASA)

Both poly-γ-glutamic acid (γ-PGA) and polyaspartic acid (PASA) are anionic polymers bearing pendant carboxylic groups (pKa 4.3–4.5 and 3.8–4.0, respectively) that confer net negative charge at physiological pH (expressed in terms of ζ-potential, approximately −20 to −30 mV) (Fig. 4A, 4B) [57]. Combined with their biodegradability, these properties position them as attractive biocompatible additives for gene delivery. Their anionic character enables electrostatic association with polycation/DNA binary complexes, yielding ternary architectures that

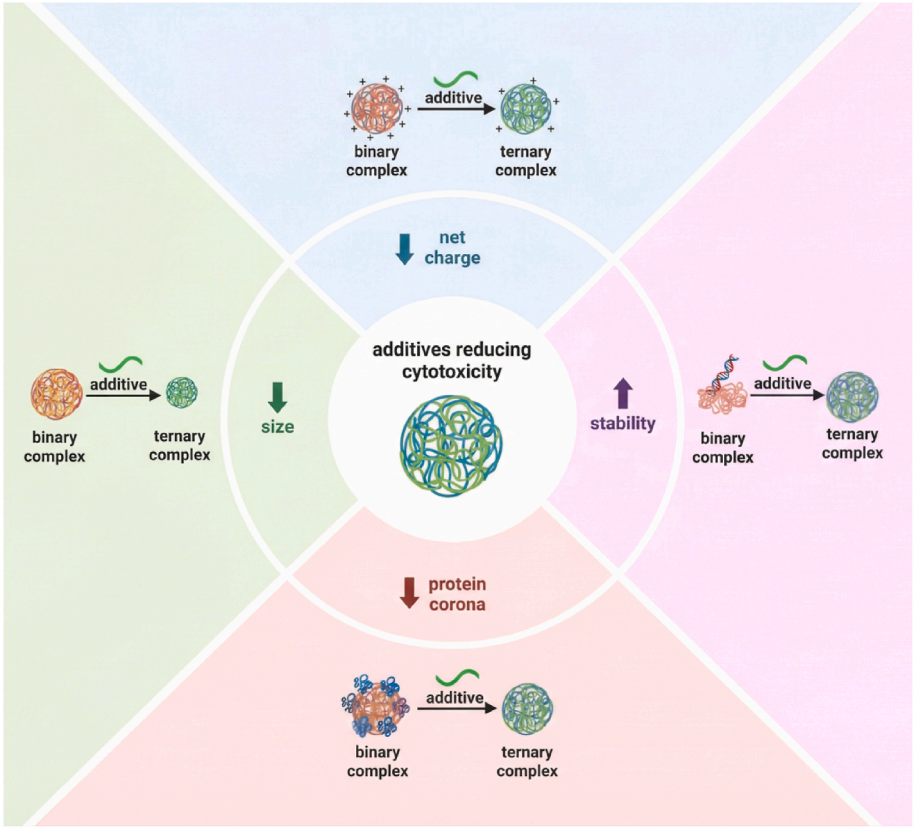

Fig. 3. Schematic representation of the effects of additives on binary complexes. Additive incorporation leads to the formation of ternary complexes with enhanced stability and reduced cytotoxicity through modulation of particle size (D_H), surface charge, protein corona composition, and overall colloidal stability.

attenuate excessive surface charge through anionic shielding, thereby mitigating cytotoxicity.

Multiple studies demonstrate that PASA and γ-PGA exert convergent effects on the physicochemical and biological properties of ternary complexes. When incorporated following binary complex formation, both polymers effectively shield surface charge, reducing ζ-potential from highly cationic (+25 to +45 mV) to near-neutral or slightly anionic (−5 to +5 mV) in PEI [57–59], protamine [60], or other polycation-based systems [61,62]. This charge attenuation diminishes nonspecific plasma membrane binding, inhibits serum protein adsorption that would otherwise drive opsonization and clearance, and prevents agglutination [57,59,61]. Simultaneously, the adsorbed polyanion layer forms a hydrophilic steric barrier at the particle surface that prevents aggregation and inhibits formation of a destabilizing protein corona, thereby maintaining D_H within the 180–300 nm range in physiological media [59,62].

Importantly, when formulated below the critical A/N charge ratio, which is equal to 1 for both PASA and γ-PGA, the polyanion coating preserves DNA condensation and complex stability [57]. Consequently, ternary formulations exhibit markedly reduced cytotoxicity compared to their binary counterparts. However, despite comparable cytocompatibility, the two polyanions differ in their effect on TE: γ-PGA–containing complexes often enhance or maintain TE, whereas PASA incorporation generally compromises it [58,61,62]. Several mechanisms may account for this divergence. First, γ-PGA–coated complexes are internalized via energy-dependent [58] and potentially γ-glutamyl transpeptidase-mediated (GGT) pathways [62] in certain cell lines. Notably, although γ-PGA ternary complexes display reduced overall cellular uptake, they exhibit enhanced perinuclear accumulation, likely due to preferential internalization via caveolae-mediated endocytosis. However, this pathway favors perinuclear trafficking at the expense of endosomal processing, thereby limiting cytosolic release and potentially accounting for the reduced TE observed with polyacid-coated systems [61]. Collectively, polyacid coatings present a characteristic trade-off: they attenuate cytotoxicity through charge shielding and colloidal stabilization but may concurrently impair TE by redirecting intracellular trafficking away from productive release pathways.

3.2. *Alginic acid (Alg)*

Alginic acid (Alg), also called algin, is a naturally derived, biodegradable polysaccharide composed of β-D-mannuronic (M) and α-L-guluronic (G) acid residues (Fig. 4C). Its abundant carboxylic groups impart net negative charge at physiological pH, rendering it a functional polyanion [63,64]. When incorporated into binary polycation/DNA complexes, Alg functions analogously to synthetic polyacids by partially neutralizing complex surface charge [57]. The resulting polyanion coating substantially reduces ζ-potential, mitigating electrostatic interactions responsible for nonspecific binding to cellular and serum components. Consequently, Alg-coated ternary complexes exhibit diminished serum protein adsorption and protein corona formation, while the electrostatic shielding stabilizes colloidal suspensions and inhibits hemolysis by minimizing disruptive interactions with erythrocyte membranes [63,64].

Alg adopts an *egg-box* supramolecular organization through cooperative interactions between G-block residues and multivalent cations. In ternary complexes, analogous associations may arise via multipoint electrostatic interactions between G residues and protonated polycation amines such as those in PEI [65]. Although not directly visualized in polyplexes, such supramolecular assembly may contribute to enhanced compactness and colloidal stability. Consistent with this hypothesis, Patnaik et al. reported that Alg-containing ternary complexes displayed compact spherical morphology with reduced D_H (~190–200 nm) and

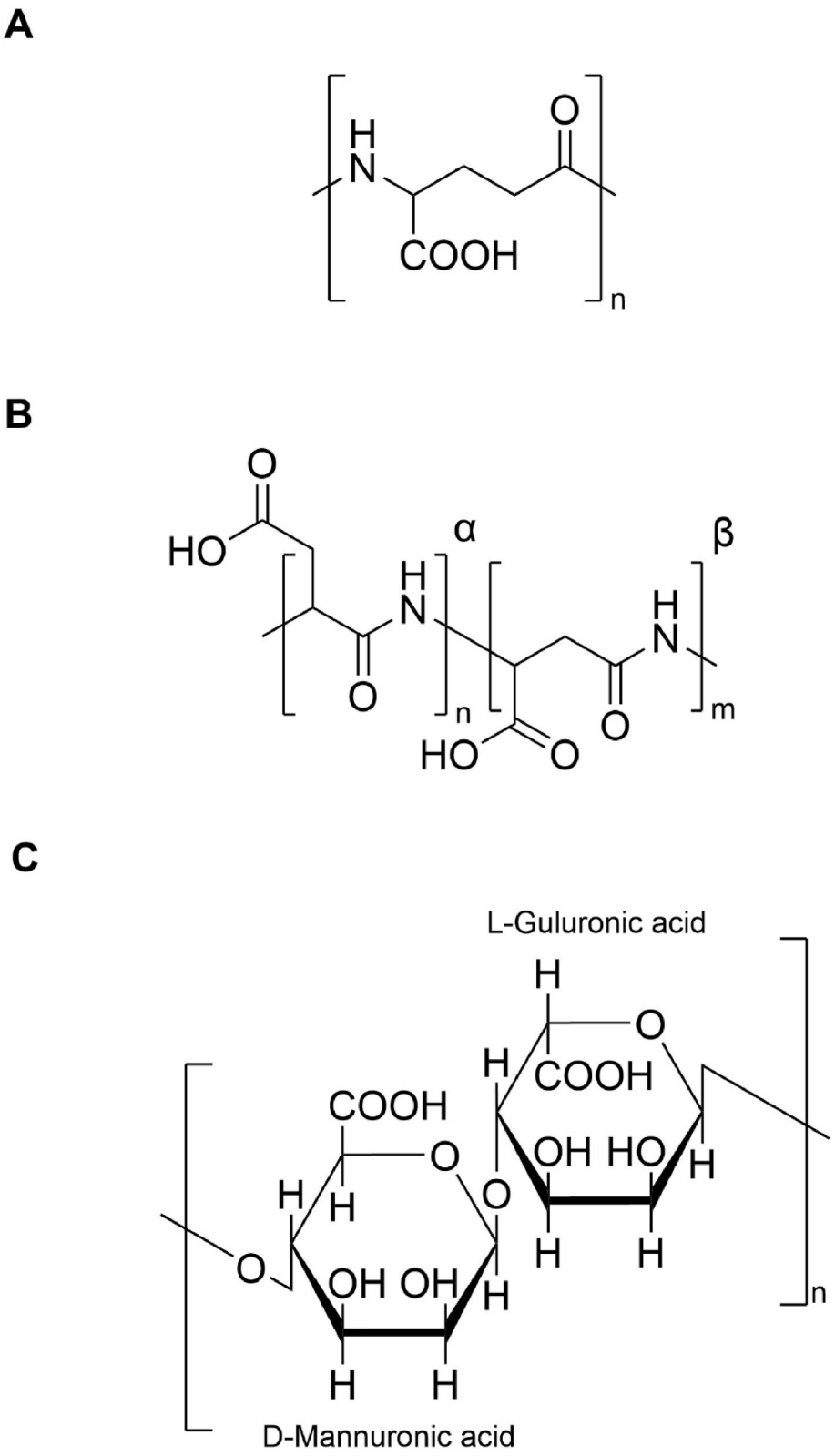

Fig. 4. Chemical structures of A) poly-γ-glutamic acid (γ-PGA); B) polyaspartic acid (PASA): the α and β block consist of aspartic acid residues differing in the position of the amide bond along the backbone (α-*vs*. β-carboxyl group); C) alginic acid (Alg), a polysaccharide composed of D-mannuronic acid and L-guluronic acid residues linked by a O-glycosidic bond.

markedly improved colloidal stability in physiological media. These observations align with earlier findings that Alg incorporation reduces D_H, enhances colloidal stability, and enables controlled payload release while preventing premature NA dissociation [63,64].

This enhanced structural integrity translates into improved biological performance: Alg-coated polyplexes exhibited reduced cytotoxicity compared to binary counterparts in both *in vitro* and *in vivo* settings. Notably, despite comparable cellular uptake, Alg-modified systems

achieved significantly higher TE, indicating that this enhancement derives from improved intracellular processing rather than increased internalization [63]. Endosomal trafficking studies supported this interpretation: while both PEI and PEI–Alg complexes were internalized predominantly via clathrin-independent, energy-dependent endocytosis, PEI–Alg polyplexes exhibited more efficient endosomal release than their binary PEI/DNA counterparts. This was evidenced by greater bafilomycin A1 sensitivity (a V-ATPase inhibitor that prevents endosomal acidification), indicating stronger dependence on proton accumulation for escape. These findings suggest that partial charge neutralization by Alg preserves sufficient buffering capacity to sustain proton sponge-mediated escape while attenuating disruptive membrane interactions, thereby enabling controlled endosomal release and prolonged cytosolic retention [64]. Collectively, Alg-based ternary polyplexes decouple cytotoxicity from TE, effectively circumventing the biocompatibility-performance trade-off inherent to cationic vectors.

A systematic classification of viability-enhancing additives in ternary complexes based on GDV, NA, component ratios, and complexation order is reported in Table 2.

4. Double duty glycosaminoglycans (GAGs)

The previous sections examined two complementary strategies: approaches focused on maximizing TE primarily through enhanced proton sponge activity and intracellular NA release, and approaches aimed at mitigating cytotoxicity by reducing surface charge and improving biocompatibility. Glycosaminoglycans (GAGs) represent a third approach that, when incorporated as ternary components, can simultaneously enhance TE and reduce cytotoxicity.

GAGs, also termed mucopolysaccharides, are linear polysaccharides composed of repeating disaccharide units typically comprising a uronic acid and an amino sugar. Their dense array of carboxyl and sulphate groups confers a strong negative charge at physiological pH [66], enabling electrostatic association with cationic carriers [57,67]. When integrated into non-viral complexes, GAGs impart benefits analogous to other polyanions, including reduced non-specific interactions, diminished protein adsorption, prevention of aggregation, and enhanced colloidal stability [68,69]. Critically, GAGs also contain specific recognition motifs that engage strong and selective interactions with cell-surface receptors, enabling receptor-mediated internalization that maintains or enhances TE despite reduced surface charge [70–73]. Among these, CD44, a type I transmembrane glycoprotein involved in cell adhesion, migration, and signaling, serves as the principal receptor for several GAGs, most notably hyaluronic acid (HA) and chondroitin sulphate (CS) [74–76]. CD44 is overexpressed in numerous solid tumors and serves as a marker for cancer stem cell populations characterized by enhanced malignancy and chemoresistance. GAGs-CD44 binding triggers receptor-mediated endocytosis. Therefore, this high-affinity interaction has been extensively exploited in non-viral gene delivery [77,78].

Beyond CD44, additional hyaluronan-binding receptors have been identified, though they remain underexplored in gene delivery applications. These include RHAMM (CD168) and HARE (stabilin-2), which participate in cell motility, endocytosis, and ECM turnover [79–82]. Their tissue- and disease-specific expression patterns offer opportunities for directing GAG-containing complexes toward defined cell populations or pathological microenvironments. Exploiting these alternative receptor pathways may further expand the targeting potentially expanding the targeting capabilities of GAG-based delivery systems.

Rather than supplanting either cationic or anionic strategies, GAG incorporation synergistically integrates their most favorable attributes, balancing electrostatic interactions while introducing biologically guided targeting. This positions GAG-based systems as versatile platforms for developing efficient and well-tolerated non-viral vectors.

4.1. Hyaluronic acid (HA)

Hyaluronic acid (HA) is among the most extensively investigated additives for non-viral gene delivery, owing to its dual function as an anionic shielding agent and a ligand for receptor-mediated targeting [71,72,75,76]. Structurally, HA is a linear glycosaminoglycan composed of repeating D-glucuronic acid and N-acetyl-D-glucosamine disaccharide units, whose carboxyl groups confer net negative charge at physiological pH (Fig. 5A) [73]. When electrostatically assembled with cationic polyplexes, HA effectively neutralizes their positive surface charge, reducing ζ-potential from approximately +30 to +50 mV down to around −40 mV. This surface charge shielding translates into markedly improved colloidal stability, hemocompatibility, and cell viability while maintaining nanometric dimensions (D_H = 70–200 nm, depending on formulation) under physiological conditions [83–85].

Ito and co-workers systematically investigated linear PEI (*l*PEI)-based ternary complexes (DNA/*l*PEI/HA) and demonstrated that optimal gene expression occurs at an A/N ratio of approximately 0.5, corresponding to complete PEI amine protonation and full HA surface coverage, as suggested by Raman spectroscopy (Table 3). At this stoichiometric ratio, HA provides sufficient electrostatic shielding to mask residual cationic sites otherwise responsible for opsonization and aggregation. Raman spectroscopy confirmed the formation of a uniform HA corona. Importantly, HA incorporation does not destabilize the complex but induces moderate relaxation of DNA condensation. This facilitates DNA unpacking following cytosolic release, thereby enhancing accessibility to the transcriptional machinery and improving TE [85].

Table 2
Viability-enhancing additives in ternary complexes, classified depending on the GDV, NA, ratios, and complexation order.

Additive	GDV	NA	DNA:GDV:Additive	Complexation order	TE (fold change)	Viability (fold change)	Ref.
γ-PGA	25 kDa *b*PEI	pDNA	0.4 (A/N)	*b*PEI → (DNA $\overset{?}{\leftrightarrow}$ γ-PGA)	2.4	1.0	[57]
γ-PGA	25 kDa *b*PEI	pDNA	1:8:6 (charge)	(*b*PEI $\overset{?}{\leftrightarrow}$ DNA) $\overset{?}{\leftrightarrow}$ γ-PGA	1.0	4.7	[58]
γ-PGA	25 kDa *b*PEI	pDNA	1:8:6 (charge)	(*b*PEI $\overset{?}{\leftrightarrow}$ DNA) $\overset{?}{\leftrightarrow}$ γ-PGA	1.0	3.0	[59]
γ-PGA	Protamine	pDNA	1:6.4:12 (charge)	(Protamine $\overset{?}{\leftrightarrow}$ DNA) $\overset{?}{\leftrightarrow}$ γ-PGA	1.0	1.6	[60]
γ-PGA	DIP50H50	pDNA	1/5/1 (P/N/C)	PGA → (DIP50H50 $\overset{?}{\leftrightarrow}$ DNA)	0.79	3.0	[61]
γ-PGA	pHDDA-ABOL	siRNA	1:140:2.5 (P/N/C)	γ-PGA → (pHDDA-ABOL $\overset{?}{\leftrightarrow}$ saRNA)	Up to 1.2	Up to 1.5	[62]
PASA	25 kDa *b*PEI	pDNA	1:8:6 (charge)	(*b*PEI $\overset{?}{\leftrightarrow}$ DNA) $\overset{?}{\leftrightarrow}$ PASA	0.5	4.0	[59]
PASA	25 kDa *b*PEI	pDNA	0.1 (A/N)	*b*PEI → (DNA $\overset{?}{\leftrightarrow}$ PASA)	2.7	1.0	[57]
Alg	25 kDa *b*PEI	pDNA	0,1 (A/N)	*b*PEI → (DNA $\overset{?}{\leftrightarrow}$ Alg)	3.0	1.0	[57]
Alg	25 kDa *b*PEI	pDNA	N/P 10; Alg:DNA (w/w) 0.15	(*b*PEI $\overset{?}{\leftrightarrow}$ DNA) $\overset{?}{\leftrightarrow}$ Alg	1.3	1.5	[63]
Alg	750 kDa *b*PEI	siRNA	PEI-Alg 6.26%	(Alg → *b*PEI) $\overset{?}{\leftrightarrow}$ DNA	Up to 1.1	n.a.	[64]

$\overset{?}{\leftrightarrow}$ stands for "unspecified order of mixing"; → stands for "is added to"; n.a. stands for "not available".

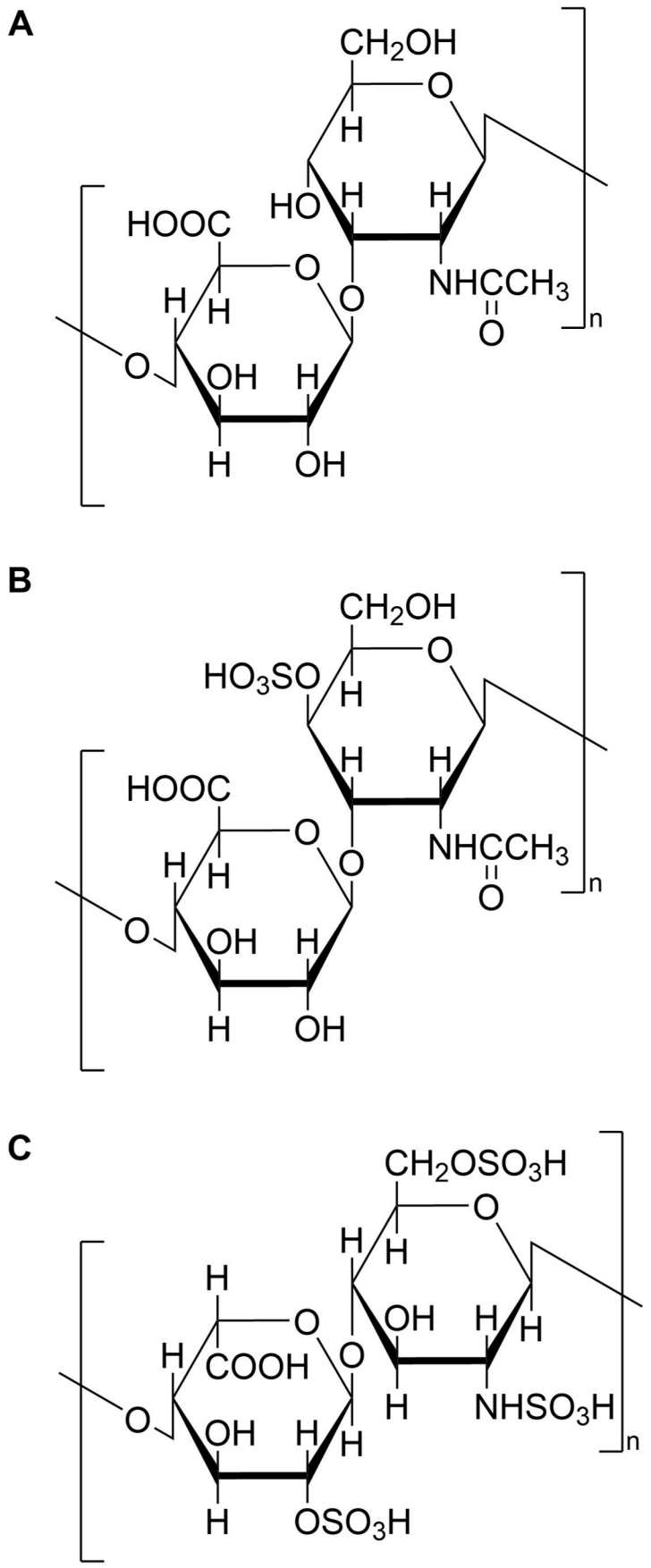

Fig. 5. Chemical structure of A) hyaluronic acid (HA), B) chondroitin sulphate (CS), and C) heparin (Hep).

Table 3
GAGs employed as additives in ternary complexes, classified depending on the GDV, NA, ratios, and complexation order.

Additive	GDV	NA	DNA:GDV:Additive	Complexation order	TE (fold change)	Viability (fold change)	Ref.
Hep	25 kDa *b*PEI	pDNA	0.3 (A/N)	*b*PEI → (DNA $\overset{?}{\leftrightarrow}$ Hep)	2.7	1	[57]
Hep	"Tr4" polycation	pDNA	N/P 20	Hep $\overset{?}{\leftrightarrow}$ Tr4 → DNA	Up to 4.5	n.a.	[70]
HA	25 kDa *b*PEI	pDNA	N/P 10; HA/PEI 7.5 (charge)	(*b*PEI $\overset{?}{\leftrightarrow}$ DNA) $\overset{?}{\leftrightarrow}$ HA	Up to 1.0	1.7	[72]
HA	Hyperbranched polyamido amine	pDNA	1:5:5 (w/w/w)	RHB $\overset{?}{\leftrightarrow}$ DNA $\overset{?}{\leftrightarrow}$ HA	Up to 1.3	1.3	[74]
HA-SS-COOH	25 kDa *b*PEI	pDNA	N/P 10; HA/DNA (w/w) 0.5	HA → (*b*PEI $\overset{?}{\leftrightarrow}$ DNA)	Up to 1.0	Up to 1.0	[71]
HA	25 kDa *l*PEI	pDNA	1:12:12 (charge)	HA → DNA $\overset{?}{\leftrightarrow}$ *l*PEI	1.0	n.a.	[73]
HA	52 kDa CH	pDNA	1:16:0.5 (w/w)	CH $\overset{?}{\leftrightarrow}$ DNA $\overset{?}{\leftrightarrow}$ HA	1.0	1.5	[86]
CS	25 kDa *l*PEI	pDNA	1:12:4 (P/N/C)	*l*PEI → (CS → DNA)	Up to 4.4	Up to 1.3	[69]
CS	25 kDa *b*PEI	pDNA	1:8.5:1.5 (w/w/w)	DNA → (*b*PEI $\overset{?}{\leftrightarrow}$ CS)	Up to 1.2	Up to 3.2	[87]
CS	generation 5 PAMAM	pDNA	1:2.7:8.3 (w/w/w)	CS → (PAMAM $\overset{?}{\leftrightarrow}$ DNA)	1.0	2.3	[67]
CS	25 kDa *b*PEI	pDNA	1:8:6 (charge)	CS → (*b*PEI $\overset{?}{\leftrightarrow}$ DNA)	1.0	4.0	[88]

$\overset{?}{\leftrightarrow}$ stands for "unspecified order of mixing"; → stands for "is added to"; n.a. stands for "not available".

Beyond these physicochemical benefits, HA confers biological selectivity through interactions with specific cell-surface receptors. Among these, CD44 has been validated as the principal mediator of HA-based targeting [72,74–76]. He et al. (Table 3) demonstrated CD44 specificity using reduction-sensitive HA-SS-COOH–shielded *b*PEI/DNA polyplexes: pre-incubation of $CD44^+$(HepG2 and B16F10) cells with free HA (0.1–0.2 % w/v) reduced TE up to 22-fold, whereas $CD44^-$ cells (NIH3T3) showed minimal effect [71].

Two additional hyaluronan receptors merit consideration: HARE and RHAMM. HARE, a multifunctional scavenger receptor predominantly expressed in liver sinusoidal and lymphatic endothelial cells, mediates endocytic clearance of circulating HA and other GAGs through clathrin-dependent internalization and receptor recycling [79]. This mechanism enables HARE-bound ligands to enter endosomal compartments, offering a pathway exploitable for intracellular delivery. RHAMM (CD168), by contrast, functions both at the cell surface and intracellularly, interacting with cytoskeletal and signaling proteins including ERK1/2 and Src [81]. HA binding to RHAMM has been associated with cell migration, cytoskeletal remodeling, and endocytosis of HA-decorated nanoparticles in several delivery models [80]. Collectively, these findings suggest that HARE and RHAMM may serve as auxiliary uptake routes for HA-coated complexes, potentially expanding the range of targetable cell types and tissues. However, whether these receptors retain binding specificity when HA is incorporated into ternary complexes remains uncertain, as electrostatic interactions with cationic components may partially mask receptor-binding domains.

The order of complexation also warrants consideration. In early work, Ito et al. employed a pre-addition strategy wherein HA was mixed with DNA before *l*PEI complexation, yielding finely dispersed nanoparticles ($D_H \approx 100$ nm) with superior TE both *in vitro* and *in vivo*, suggesting that HA-DNA pre-association promotes homogeneous condensation and uniform surface coating [83]. However, subsequent studies, including later works from the same group, adopted post-complexation coating, whereby HA was added after PEI/DNA complex formation [84]. This method more closely resembles typical ternary self-assembly processes and allows better control of the outer anionic layer, improving colloidal stability and reproducibility. Despite differing assembly sequences, both strategies yield comparable outcomes: negatively charged, HA-coated nanocomplexes ($D_H \approx$ 70–200 nm) combining reduced cytotoxicity with enhanced receptor-mediated uptake.

In summary, HA incorporation confers dual benefits to ternary complexes: favorable modulation of physicochemical properties and CD44-mediated cellular targeting, establishing HA as one of the most versatile polyanionic additives for non-viral gene delivery.

4.2. Heparin (Hep) and chondroitin sulphate (CS)

Across multiple studies, heparin (Hep) and chondroitin sulphate (CS) have been employed in electrostatically assembled ternary complexes [67–70]. Both additives reduce surface charge to near-neutral values, thereby minimizing nonspecific interactions with cellular membranes and serum proteins. Their incorporation prevents aggregation and protein corona formation while maintaining nanometric particle dimensions and colloidal stability in physiological media [69,70]. Consistent with other polyanionic additives, these effects translate into reduced hemolysis and improved cell viability compared to unmodified polyplexes.

CS (Fig. 5B) displays receptor specificity for CD44, a property well established for the free polysaccharide and exploited for targeted delivery. Although this specificity has not been directly confirmed for CS-containing ternary complexes, CD44 recognition is likely at least partially retained. Supporting this hypothesis, $CD44^+$ cells exhibit higher TE with CS-coated complexes than $CD44^-$ cells [88].

Although direct evidence for specific CS–HARE interaction remains lacking, potential involvement of the HARE receptor (stabilin-2) has also been proposed. This hypothesis is based on two observations: first, HARE is a known receptor for hyaluronan, a structurally related GAG; second, several studies have demonstrated that CS-containing complexes are internalized through clathrin-mediated endocytosis, a pathway frequently associated with HARE activity [67,88].

The internalization mechanism, however, appears to be cell-type dependent. While Kurosaki et al. [88] and Imamura and co-workers [67] observed clathrin-dependent endocytosis for CS-based polyplexes, Hagiwara et al. [68] reported macropinocytosis predominantly in COS-7 cells, which lack both HARE and CD44 expression. The shift likely represents a compensatory, non-specific entry mechanism engaged when receptor-mediated pathways are unavailable, rather than an intrinsic property of the polyplexes themselves.

Owing to its high sulfation degree (Fig. 5C), Hep exhibits a strong negative charge density that promotes tight electrostatic interactions with cationic carriers. This reduces surface charge and relaxes polyplex structure, facilitating NA release and enhancing TE compared to binary complexes. However, excessive Hep can over-neutralize complexes, causing premature NA dissociation either extracellularly or during endosomal trafficking [57]. Therefore, careful optimization of the A/N ratio is essential to balance NA condensation with controlled release.

Given Hep's known specificity for HARE, receptor-mediated uptake via this pathway represents a potential internalization mechanism for Hep-coated complexes. Harris et al. demonstrated that HARE specifically recognizes Hep and mediates its endocytosis via clathrin-coated vesicles, coupled to intracellular signaling and receptor recycling [79].

Although this pathway was characterized in the context of Hep clearance rather than NA delivery, it provides a mechanistic foundation for receptor-specific internalization. Nevertheless, Boyle et al. [70] reported that Hep-based ternary complexes are internalized through macropinocytosis, suggesting that the uptake route may vary with nanoparticle D_H, formulation composition, or cell type.

In summary, both Hep and CS function as biologically active polyanions that not only modulate surface charge and improve cytocompatibility but also engage receptor-mediated and alternative endocytic pathways. Their integration into polyplex architecture thus represents an evolution from purely physicochemical optimization toward a more biologically informed approach to non-viral gene delivery.

5. Conclusions and future perspectives

The development of efficient and biocompatible non-viral gene delivery systems requires addressing two fundamental challenges: maximizing TE while minimizing cytotoxicity. Herein, we have systematically examined how ternary complex formulations offer versatile solutions through strategic incorporation of molecular additives. Of note, ternary complex design enables flexible modulation of the balance between TE and cytotoxicity, with additive selection and assembly protocol critically determining overall performance.

The additives can be classified into three complementary categories. TE-enhancing additives, including oligoamines (SPM, SPD) and modified polypeptides (CM-PLH), amplify TE through enhanced buffering capacity, improved proton sponge effect, and stabilization via dehydration and hydrogen bonding. Cytotoxicity-reducing additives, comprising polyacids (γ-PGA, PASA) and polysaccharides (Alg), alleviate cytotoxicity by partially neutralizing surface charge, thereby mitigating hemolysis, protein corona formation, and aggregation while preserving colloidal stability. Double-duty GAGs (HA, Hep, CS) represent the most versatile class, simultaneously enhancing both parameters through charge-shielding and receptor-mediated endocytosis via CD44, and potentially HARE and RHAMM.

While the studies reviewed herein demonstrate the potential of molecular additives to modulate the efficiency-cytotoxicity balance of non-viral gene delivery systems, several gaps remain that need to be addressed to advance these formulations toward translational applications.

First, the vast majority of the evidence base relies on pDNA as model cargo. As the field increasingly shifts toward mRNA-based therapeutics, CRISPR-Cas9 ribonucleoproteins, and short regulatory RNAs (siRNA, ASOs), the compatibility of additive-based ternary formulations with these structurally and physicochemically distinct cargoes warrants systematic investigation. Differences in molecular weight (M_W), topology, and charge density between pDNA and RNA species may substantially alter additive-cargo-vector interactions and, consequently, optimal stoichiometric ratios and assembly pathways.

Second, nearly all studies examined here were conducted *in vitro* using two-dimensional monolayer cultures. Validation in more physiologically relevant models, including three-dimensional spheroids, organ-on-chip systems, and *in vivo* biodistribution studies, will be essential to confirm that the improvements in TE and cytocompatibility observed at the bench translate to biologically meaningful outcomes.

Third, the current literature has explored individual additives in isolation. Combinatorial strategies employing two or more additives simultaneously, for instance a TE-enhancing oligoamine paired with a cytocompatibility-improving polyanion, remain unexplored. Such multicomponent formulations could enable independent and concurrent optimization of complementary performance parameters.

Fourth, the growing availability of high-throughput and computational screening tools, including machine learning-assisted formulation design, offers an opportunity to accelerate the identification of optimal additive-vector-cargo combinations. Systematic mapping of the formulation space through design-of-experiment (DoE) approaches would complement the largely empirical optimization reported to date.

Finally, future studies should adopt a comprehensive physicochemical characterization framework encompassing D_H, PDI, ζ-potential, and colloidal stability in biologically relevant media as a minimal reporting standard. Many of the studies reviewed herein provide only partial datasets, making cross-study comparison and formulation ranking difficult. Standardizing complexation protocols, including mixing order, incubation time, and buffer composition, will also be essential to ensure batch-to-batch reproducibility, a prerequisite for any translational application, for instance in *ex vivo* settings such as CAR-T cell manufacturing, where polyanion-based additives may enable high TE at clinical scale, provided that formulation robustness can be demonstrated under process-relevant conditions.

CRediT authorship contribution statement

G. Protopapa: Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **F. Fruzzetti:** Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **I.M. Coizet:** Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **G. Dadomo:** Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **N. Bono:** Conceptualization, Formal analysis, Investigation, Writing – review & editing. **G. Candiani:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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