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Current Status and Trends in Nucleic Acids for Cancer Therapy: A Focus on Polysaccharide-Based Nanomedicines

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The efficacious delivery of therapeutic nucleic acids to cancer still remains an open issue. Through the years, several strategies are developed for the encapsulation of genetic molecules exploiting different materials, such as viral vectors, lipid nanoparticles (LNPs), and polymeric nanoparticles (NPs). Indeed, the rapid approval by regulatory authorities and the wide use of LNPs complexing the mRNA coding for the spark protein for COVID-19 vaccination paved the way for the initiation of several clinical trials exploiting lipid nanoparticles for cancer therapy. Nevertheless, polymers still represent a valuable alternative to lipid-based formulations, due to the low cost and the chemical flexibility that allows for the conjugation of targeting ligands. This review will analyze the status of the ongoing clinical trials for cancer therapy, including vaccination and immunotherapy approaches, exploiting polymeric materials. Among those nanosized carriers, sugar-based backbones are an interesting category. A cyclodextrin-based carrier (CALAA-01) is the first polymeric material to enter a clinical trial complexed with siRNA for cancer therapy, and chitosan is one of the most characterized non-viral vectors able to complex genetic material. Finally, the recent advances in the use of sugar-based polymers (oligo- and polysaccharides) for the complexation of nucleic acids in advanced preclinical stage will be discussed.

1. Introduction

COVID-19 outbreak gave to governments, legislators, and scientists the chance to apply their knowledge derived by more than four decades of work on nucleic acid–based vaccines and medications to combat the pandemic. This opportunity has translated into multiple billions of dollars in revenue for biopharma companies, expanding their RNA-based pipelines. By 2026, the market for vaccines and treatments based only on mRNA is expected to rise to $101.3 billion, representing a compound annual growth rate of 16.8%.[1]

On the wave of more than 2 billion doses of vaccines against SARS-CoV-2 delivered worldwide by BioNTech-Pfizer and Moderna, several formulations applying mRNA technology are currently being developed, such as Moderna's mRNA vaccine against cytomegalovirus that reached phase 3 clinical trial (NCT05085366). The milestones reached by these technologies can be beneficial also for oncology patients. Moderna–Genentech are conducting a phase 2 trial for colorectal cancer (NCT04486378). Lipo-MERIT (NCT02410733), a liposomal RNA (RNA-LPX) vaccine, is under investigation by BioNTech in a dose-escalation phase I trial in patients with advanced melanoma.[2] These are two interesting examples of mRNA vaccines based on lipid nanoparticles (LNPs) for cancer therapy. LNPs and lipoplexes (electrostatic complexes between lipids and nucleic acids) are up to now the most intensively studied and clinically advanced technologies for mRNA delivery.[3] LNPs are widely used for several decades, mainly due to their ease of formulation, low toxicity, and biodegradability of the lipidic components.[4]

Nevertheless, their clinical translation has been limited by the lack of reproducibility, difficult scalability, and low encapsulation efficiency.[5] In addition, some components of LNPs, such as distearoyl phosphatidylcholine and ionizable lipids, are sensitive to temperature and pH.[6]

The delivery of RNA entities has different drawbacks, which can be overcome with a suitable delivery platform. Indeed, they are easily degraded by endonucleases, and they undergo rapid renal excretion, resulting in a short half-life. In addition, the anionic nature of nucleic acids interferes with the interaction with cell membranes and their internalization, making also challenging the endosomal escape.[7] Because of the above-mentioned reasons, it is important to explore different formulation approaches, able to achieve efficient RNA delivery. Typically, the alternatives to LNPs are either viral vectors or polymer-based nanoparticles. Recombinant viral vectors, albeit engineered to minimize the risks of misuse and uncontrolled replication, still suffer from safety concerns.[8]

Polymer-based nanoparticles (NPs) formed by natural or synthetic cationic polymers are a well-established category of drug delivery systems for nucleic acids. Polymeric NPs are generally

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complexed with the genetic material by the electrostatic interactions between the negatively charged phosphate (P) groups of nucleic acids and the positively charged ionizable amine (N) groups of the polymers.[9] The N/P ratio is considered as the ratio between the N and the P groups, which largely impacts on the size, stability, and surface charge of the complex between the polymeric and genetic material (commonly defined as polyplexes).[10]

In this review, we will highlight the recent applications of polymeric NPs for gene delivery in clinical trials for cancer treatments. Among the polymeric materials, we will specifically focus on those exploiting sugar molecules as repetitive units to form a macromolecular backbone, such as oligosaccharides-based polymers and polysaccharides. Sugar-based polymers are of particular interest because they are natural or semi-synthetic, commercially available materials, and many sugar-based compounds are already approved as excipients (e.g., cyclodextrins (CDs),[11] dextran[12]) or drug delivery system (e.g., Sugammadex[13]).

2. Polymer-Based RNA Delivery System in Clinical Trials

Several polymeric backbones for gene delivery have already been under clinical trial investigation.

NU-0129 is a novel precision medicine that consists of gold nanoparticle cores conjugated with short interfering RNA (siRNA) oligonucleotides specific for the glioblastoma (GBM) oncogene Bcl2Like12 (Bcl2L12).[14] GBM is a primary brain tumor, one of the most difficult cancers to treat and it represents a great unmet medical need. The most challenging aspects in developing therapies against GBM are overcoming drug resistance and crossing the blood brain barrier (BBB). Thus, gene therapy appears to be a useful approach to tackle both problems. On one hand, the new druggable molecular target, identified with genomics, can exceed the conventional therapeutic modalities (mainly temozolomide).[15,16] On the other hand, the adequate nanocarrier for the therapeutic nucleotide can allow for overcoming BBB. In this specific formulation, gold nanoparticle cores are decorated with oligoethylene glycol or polyethylene glycol (OEG/PEG) to improve colloidal stability and circulation half-life. A single-arm, open-label phase 0 first-in-human trial (NCT03020017) was conducted on NU-0129 administered intravenously (IV). Following the uptake of NU-0129 by glioma cells, a significant reduction in tumor-associated Bcl2L12 protein expression was recorded. Therefore, NU-0129 is a precision medicine able to accumulate in the brain for the systemic treatment of GBM.

A very promising polymeric nanosized platform has been developed by Sirnaomics, based on a proprietary histidine–lysine co-polymers (HKP).[17] Currently, STP705 and STP707 are under investigation in seven clinical trials for several types of cancer (**Table 1**). Both products are peptide/siRNA nanocomplex-based RNA interference therapeutics. The histidine residues of HKP are mainly responsible for the endosomal escape mechanism, while the lysine primary amino groups are enabling the electrostatic complexation with the negative charges of the phosphate groups of oligonucleotides.[17] The peptide/siRNA nanocomplex STP705 combines two siRNA oligonucleotides, targeting TGF-β1 and COX-2 mRNA. It is formulated with a branched HKP polypeptide and it is currently in phase 2b for squamous cell carcinoma in situ (isSCC) (NCT04293679, NCT04844983), a phase 2 for basal cell carcinoma (NCT04669808), a phase 1/2 for keloid scarring (NCT04844840), a phase 1/2 for hypertrophic scar (NCT05196373, NCT02956317),[18] a phase 1/2 for facial isSCC (NCT05421013), a phase 1 for liver cancer (NCT04676633), and a phase 1 for medical cosmetology treatment in abdominoplasty (NCT05422378).[19]

Similarly, STP707 nanomedicine comprises of two siRNA targeting TGF-β1 and COX-2 mRNA, formulated with a different HKP (HKP+H), is in clinical trial in subjects with advanced, metastatic, or unresectable solid tumors (NCT05037149). Furthermore, STP707 has shown antitumor activity in various solid tumor types[20] and other diseases.[18] Similar to Sirnaomics strategy, other cationic polypeptides mimicking cell-penetrating peptides (CPPs) have been developed. CPPs are short, cationic, hydrophobic, or amphipathic peptides (5–30 residues) that can enter cells with minimal disruption of the membrane.[21] CPPs are endogenous and/or similar to endogenously present proteins. Therefore, they possess appealing properties for drug or gene delivery, such as biodegradability, biocompatibility, and non-immunogenic nature.[22] KK-46 is a polypeptide dendrimer in which the positive charges are bestowed by the arginine and histidine amino acids.[23] This cationic polymer is exploited to complex an anti-SARS-CoV-2 siRNA (siCoV or siRk-12), targeting the nonstructural protein 12 (Nsp 12) of the viral genome. The resulting polyplex called MIR-19, administered as an aerosol for inhalation, has completed the phase 2 clinical trial on participants with symptomatic moderate COVID-19 (NCT05184127).

Another interesting polymer-based delivery system for nucleic acids is the Local Drug EluteR (LODER) developed by Silenseed Ltd. LODER is a biodegradable polymeric matrix in the millimetric scale (FDA approved) that has successfully passed a phase 1/2a clinical trial and it is currently in phase 2 for the therapy of locally advanced pancreatic carcinoma in combination with conventional treatments. Pancreatic ductal adenocarcinoma (PDAC) is the most common pancreatic neoplasm,[24] routinely treated with gemcitabine alone or in combination with nanoparticle albumin-bound (nab-paclitaxel) and FOLFIRINOX (folinic acid, 5-fluorouracil, irinotecan, and oxaliplatin).[25,26] It has been established that KRAS mutation is a hallmark of PDAC tumor growth,[27] and targeting it is a valuable therapeutic approach.[27] LODER encloses the anti-KRASG12D siRNA (siG12D) as a therapeutic moiety, able to target the mutated KRAS.[28]

siG12D-LODER has the dual capacity to protect siRNA from degradation and simultaneously to provide a local prolonged drug release within the tumor over a period of months.[28] The implantation of LODER into pancreatic tumor was carried out using a standard endoscope ultrasound biopsy procedure, which proved to be highly effective and safe. The molecular weight of the copolymer of poly (lactic-co-glycolic) acid (PLGA) forming the matrix is above 50 kDa. Since the matrix must be implanted for a long period, polymers have been selected for their biocompatible and biodegradable properties, mainly to avoid the removal of the empty scaffolds. The polymeric matrix releasing siRNA demonstrated to be safe, avoiding the occurrence of any local or systemic side effects.[29]

Table 1. Clinical trials involving polymeric nanomedicines and therapeutic nucleic acids.

RNA Drug Name	Polymer	Target	Type of RNA	Cancer types	Phase	Current status	Last update	Results	Clinical Trials Identifier	Ref.
STP705 Cotsiranib	Histidine-lysine co-polymers (HKP)	TGF-β1 and COX-2 mRNA	siRNA	Cutaneous squamous cell carcinoma in situ (isSCC)	Phase 2b	Completed	Jun, 2022	78% of patients achieved histological clearance	NCT04293679	[17–19, 36]
						Active, not recruiting	Mar, 2023		NCT04844983	
				Basal cell carcinoma	Phase 2	Active, not recruiting	Mar, 2023	Interim data: 100% complete response (five patients) without any drug-related adverse events.	NCT04669808	
				Keloid scarring	Phase 2	Active, not recruiting	Mar, 2023		NCT04844840	
				Hypertrophic scar	Phase 1/2	Not yet recruiting	Mar, 2023		NCT05196373	
						Completed	Nov, 2021	No results available	NCT02956317	
				Facial isSCC	Phase 1/2	Active, not recruiting	Mar, 2023		NCT05421013	
				Hepatocellular carcinoma liver metastases cholangiocarcinoma	Phase 1	Active, not recruiting	Dec, 2022		NCT04676633	
STP707	Histidine–lysine co-polymers (HKP+H)	TGF-β1 and COX-2 mRNA	siRNA	Solid Tumor	Phase 1	Recruiting	Dec, 2022	Interim data: 20 patients passed the safety requirements.	NCT05037149	[17, 20]
NU-0129	Gold NP decorated with OEG/PEG copolymers	BCL2L12	siRNA	Gliosarcoma recurrent glioblastoma	Early Phase 1	Completed	Aug, 2022	Well-tolerated in glioblastoma patients with no unexpected adverse effects	NCT03020017	[14]
CALAA-01	Cationic cyclodextrin-based polymer (CDP)	Ribonucleotide reductase subunit M2	siRNA	Solid tumor	Phase 1	Terminated	Nov, 2013	Dose limiting toxicity. The adverse effects are attributed to CALAA-01 components rather than siRNA.	NCT00689065	[31, 33]
siG12D-LODER	Copolymer PLGA (LODER)	KRAS	siRNA	Pancreatic ductal adenocarcinoma pancreatic cancer	Phase 2	Recruiting	Jul, 2021		NCT01676259	[30]
						Completed	Apr, 2019	Well-tolerated, safe, and promising potential efficacy	NCT01188785	

The phase 1/2a clinical trial concluded that the combination of siG12D-LODER and chemotherapy was well tolerated by patients, with excellent safety and anticancer efficacy.[30]

CALAA-01 is the first polymer-based siRNA nanomedicine to have reached in-human phase 1a/1b clinical trial for the treatment of patients with solid cancers via the systemic administration of siRNA with a targeted CD-based delivery system.[31]

CALAA-01 is formed by a β-CD-containing polycations in which primary amino groups were modified with imidazole groups which are responsible for endosomal escape. The polymeric system is stabilized by PEG inserted via inclusion complexes into the CD, following conjugation with adamantane (AD). Active targeting properties are conferred by a human transferrin protein (TF), a ligand for transferrin receptor highly upregulated in cancer cells. CALAA-01 was exploited for siRNA against ribonucleotide reductase subunit M2 (RRM2), in which overexpression is related to bad prognosis in cancers.[31]

Initially, CALAA-01 seemed to be a very promising nanomedicine, since it was well tolerated in multi-dosing studies in non-human primates.[32] Nevertheless, in a phase 1b clinical trial, dose-limiting toxicity was observed leading to clinical trial withdrawal. It is postulated that the increased toxicity registered in phase 1b was due to a stability problem of the transferrin-targeting agent over the period of time of the clinical study.[33]

Despite this first unsuccessful trial, the polymeric vectors based on CD units are still extensively studied as promising carriers for siRNA delivery, and the number of studies in literature are on the rise.[34] Furthermore, oligo and polysaccharides-based oligonucleotides delivery systems represent a valuable approach to overcome gene therapy limitations.[35] The excellent biocompatibility, safety profile, biodegradability, and low toxicity of macromolecules based on repeating units of sugars, combined with the ease of chemical modification and the commercial availability of the starting materials, make them perfect candidates for scalable nanomedicines for gene delivery. Although CALAA-01 is the only example of sugar-based nanocarrier for oligonucleotides evaluated in humans, several other candidates can present adequate characteristics for reaching the clinical trial level.

Based on this premise, in the next section, we will describe the most interesting pre-clinical studies concerning sugar-based nanomedicines for cancer therapy.

3. Polymers Based on Sugars for RNA Delivery System

Sugar-based nanocarriers comprise i) oligosaccharides (e.g., CDs[37]), ii) polymers based on repetitive units of oligosaccharides (e.g., nanosponges (NS)[38]), and iii) polysaccharides (e.g., dextran,[39] chitosan,[40] etc.). This category of materials is of great interest for gene delivery due to their unique properties.[41] In view of the good biocompatibility, biodegradability, absence of toxicity, and high availability, saccharides have attracted a lot of attention as novel drug carriers. Natural polysaccharides derived from animals (e.g., chitosan and hyaluronic acid (HA)), plants (e.g., starch, cellulose, CDs, and pectin), algae (e.g., alginate), or bacteria (e.g., dextran). Additionally, the structural diversity of saccharides, which presents different functional groups (i.e., –COOH, $-NH_2$, and –OH), can be derivatized to tailor polysaccharides properties depending on final applications.[42] (**Figure 1**)

Polysaccharides are formed of glycosidic linked monosaccharides, which form carbohydrate linear or branched chains with varying degrees of hydrophilicity. Furthermore, some polysaccharides are able to form a helicoidal structure and they are classified depending on their charge in neutral (e.g., dextran and pullulan (PULL)), cationic (e.g., chitosan), and anionic (e.g., HA).[43] The structure of the most investigated oligo- and polysaccharides are included in **Table 2**.

One interesting property of polysaccharides is that they can bind specific receptors over-expressed on the surface of target cells. For example, HA specifically binds to CD44 receptor overexpressed on several tumor cells, whereasPULL binds with high specificity to asialoglycoprotein receptors (ASGPR) expressed on the surface of hepatocytes. Indeed, the most investigated ligand for ASGPR is the *N*-acetylgalactosamine (GalNAc). Typically, triantennary GalNAc–oligonucleotides conjugates are currently administrated in multiple clinical trials to treat a wide range of liver-based diseases.[50] Recently, a trivalent GalNAc conjugated with an antisense oligonucleotide targeting MyD88 showed promising results in treating hepatocellular carcinoma (HCC).[51]

Besides, cationic polysaccharides, such as chitosan, have been extremely studied for complexing the negatively charged nucleic acids, forming polyplexes. Nonetheless, neutral polysaccharides (e.g., dextran, PULL, etc.) can be derivatized with positively charged groups and become a valuable component to form polyplexes. The positive charge enables the cellular uptake of nucleic acids by the interaction with the negatively charged nuclear membrane. In addition, it can also facilitate the escape of genetic materials from endosomes, preventing their degradation which is a consequence of the endosomal fusion with lysosomes. Indeed, the positive groups give a "proton sponge effect," inducing the entrance into the endosome of chloride and water, causing its swelling and destruction.[42] In addition, some sugar-based macromolecules, such as CDs and derivatives, can also be used for enhancing the solubility, stability, bioavailability, and controlled release of hydrophobic guest molecules allowing for combination therapy of nucleic acids and anticancer drugs.[52] In this review, we present a comprehensive collection of the applications of sugar-based polymers in gene delivery for cancer therapy.

3.1. Cyclodextrins

CD are cyclic oligosaccharides consisting of α-(1-4)-linked D-glucopyranose units organized in a ring-shaped formation. They are natural, biocompatible, and biodegradable materials derived from the enzymatic degradation of starch.[53] CDs have a toroidal shape, which forms a well-defined truncated cone-shaped lipophilic cavity. Instead, the presence of hydroxyl groups on the outer surface of the molecule gives hydrophilic properties, providing their aqueous solubility. In aqueous solutions, CDs can form "inclusion complexes" in which water molecules in the lipophilic central cavity are replaced by a lipophilic guest molecule with a compatible geometry and polarity. CDs are able to encapsulate a variety of hydrophobic compounds or drugs, changing the physicochemical and biological properties of guest molecules.[54] The native CDs comprise α-cyclodextrin

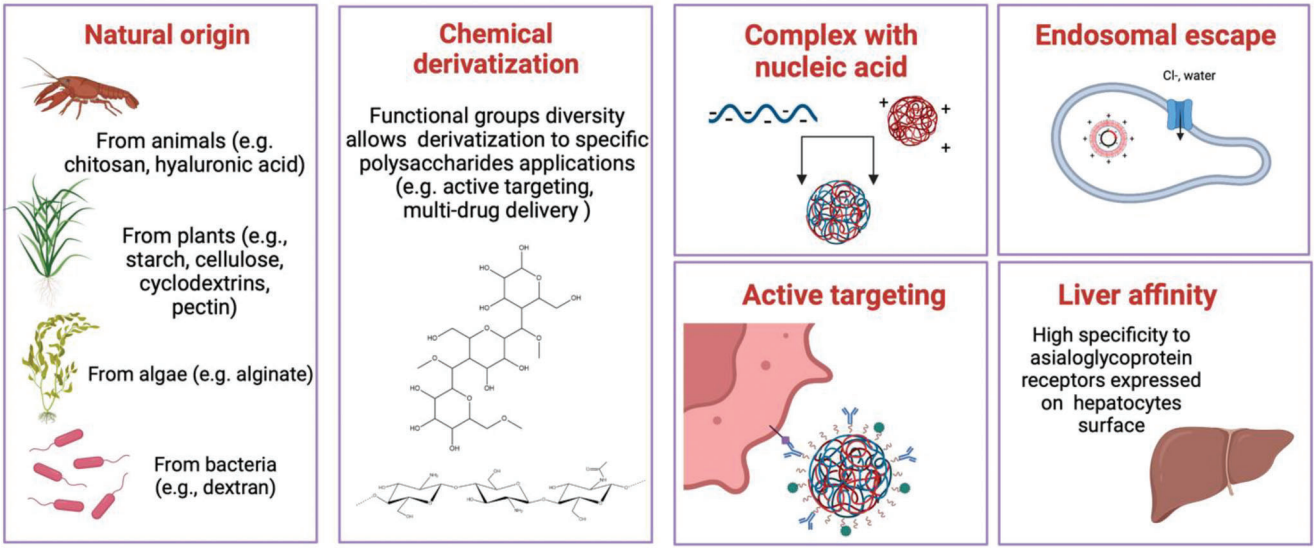


Figure 1. Schematic representation of polysaccharide-based nanocarriers for gene delivery (created with http://BioRender.com).

(αCD), β-cyclodextrin (βCD), and γ-cyclodextrin (γCD), consisting respectively of 6, 7, and 8 glucopyranose units. The presence of hydroxyl groups on CDs backbone allows modifications with different functional groups to improve the technological characteristics of natural CDs.[55] Hydroxypropyl-β-cyclodextrin (HP-β-CD), randomly methylated-β-cyclodextrin (RM-β-CD), and sulfobutylether-β-cyclodextrin (SBE-β-CD) are the most common and used in aqueous pharmaceutical solutions, such as parenteral drug formulations.

Due to their excellent biocompatibility and biodegradability properties, CDs are a promising delivery platform for nucleic acids, especially considering that their hydroxyl groups can be chemically modified to obtain cationic compounds, which can be complexed to negative genetic materials.

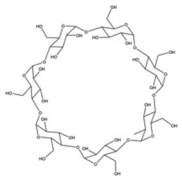
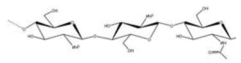
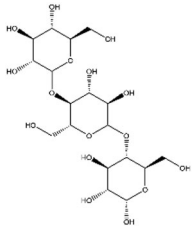
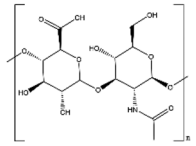
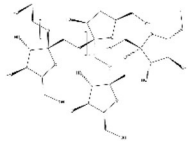
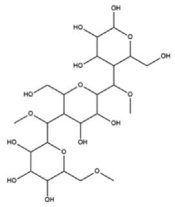
Several applications of CDs in gene delivery have been developed. CDs-based carriers can be used for active targeting by linkage with specific ligands. For example, a CDs-based siRNA delivery vector conjugated with an antibody for active targeting of the IL-3 receptor α-chain, over-expressed on acute myeloid leukemia, was developed for the delivery of siRNA against bromodomain-containing protein 4 (BRD4). In this formulation, the CDs were modified to introduce both cationic, hydrophilic residues and hydrophobic alkylic moieties. First, the oligonucleotide was allowed to form electrostatic interactions with the CDs, while the antibody was grafted through a PEG linker exploiting a ligand post-insertion technique, commonly used in liposomal formulations. The nanomedicine induced the silencing of BRD4 and therefore leukemia apoptosis. Ultimately, a synergistic effect was observed when in combination with the chemotherapeutic agent, cytarabine.[56] Interestingly, the previously described delivery system was modified to reduce the surface charge density by the use of the PEG chains. In a different approach, the surface charge density was reduced by using a mixture of amphiphilic cationic and amphiphilic anionic CDs molecules.[57] Also in this case, the cationic residues were responsible for the electrostatic interaction with the genetic material, while the anionic CDs were able to decrease the charge density for minimizing *in vivo* toxicity and improve the stability in salt- and serum-containing media in order to be more suitable for clinical applications.

Additionally, the combination between chemotherapeutic agents and nucleic acids can be pursued by the formulation of a CD-based carrier. Indeed, combination therapy is a potential strategy to enhance the efficacy of docetaxel (DTX) against colorectal cancer (CRC). The nuclear factor-κB (NF-κB) signaling pathway is implicated in the development and progression of various cancers. Moreover, it has been shown that NF-κB has a role in resistance to DTX. Therefore, the targeted therapies against NF-κB enhance the antitumor activity of DTX.[58] The amphiphilic, CD-based carrier was decorated with folic acid conjugated with a PEG spacer to specifically target the folate receptor (FR) overexpressed on the surface of CRC cells. The siRNA was complexed with the pending amino groups of the modified CDs, while the hydrophobic DTX was forming inclusion complexes with the CD cavity. The results suggested that the combination of DTX and siRNA significantly inhibited the growth of CRC in mice, without significant adverse effects.[59]

CD-based carriers have also been developed for the delivery of microRNA (miRNA). CDs modified with polyethylenimine (PEI) to introduce a positive nature that allows for complexation with miR-34a were formulated for breast cancer treatment.[60] PEG chains, conjugated through an enzyme cleavable linker, conferred stealth properties to the polyplexes avoiding opsonization and reducing nonspecific interactions.[61] The linker consists of matrix metalloproteinase-2 (MMP2)-cleavable substrate peptides, specifically hydrolyzed by MMP2 overexpressed in cancer cells.[62] The nano-formulation demonstrated excellent stability and antitumor efficacy *in vitro* and *in vivo*.

Recently, a sophisticated nanocarrier with a CD-core has been designed through a facile strategy for the codelivery of gene editing (sgRNA/Cas9, targeting DNA in the nucleus) and gene silencing (antisense, targeting mRNA in the cytoplasm) of the polo-like kinase 1 (PLK1), associated with tumor progression.[63] An azide-modified β-CDs core was used to build a branched DNA structure where seven DNA arms were covalently linked to. The

Table 2. Sugar-based materials exploited for therapeutic nucleic acids delivery.

Name	Structure	Charge	Origin	MW range	References
β-cyclodextrin (CD)		Neutral	Starch	1134.98 Da[44]	[56, 57, 59, 62, 63]
Chitosan		Positive	Chitin crustacean shells	Very low MW (<10 kDa), low MW (from 10 to 80 kDa), high MW (80–300 kDa) and very high MW chitosan (>300 kDa)[45]	[107, 109, 110–112, 114, 116, 118, 119, 120, 122, 123]
Dextran		Neutral	Bacteria	90–500 kDa[46]	[73, 74, 76]
Hyaluronic acid (HA)		Negative	Animals	Up to 20.000 kDa[47]	[122, 123]
Inulin (INU)		Neutral	Plants	3.5-5.5 kDa[48]	[92–95]
Pullulan (PULL)		Neutral	Yeast	50–600 kDa[49]	[86–90]

obtained branched DNA structure was co-assembled with the sgRNA/Cas9/antisense complex. The nanomedicine was decorated with an aptamer for targeting and influenza hemagglutinin peptide for endosomal escape. Both functions were grafted to the CD exploiting host–guest interaction of an adamantine molecule, similarly to the strategy used to introduce TF in CALAA-01 polyplex.[31]

An interesting class of CD-based polymers are nanosponges (NS), consisting of CD units, crosslinked with a crosslinking agent. These macromolecules present excellent swelling properties as well as loading capacities for both hydrophilic and hydrophobic molecules.[55] Over the past decade, different types of NS have been developed based on different types of CDs, and the crosslinkers were tailored for specific applications, ranging from drug delivery and pharmaceutics to agriculture, food production, and cosmetics.[64,65]

Recently, a green synthesis of positively charged water-soluble βCD-based polymers with choline chloride/citric acid using natural deep eutectic solvents has been reported.[66] The resulting polymer's unusual structure enabled it to be cured into a CD-based NS, changing its structure from one that was water-soluble to one that was cross-linked. The presence of quaternary ammonium functionalities, exhibited by the high positively charged zeta potential values, gives these cationic polymers great potential in gene delivery applications.

3.2. Dextran

Dextran is a biocompatible, nontoxic, nonimmunogenic, and water-soluble polysaccharide that has been widely used in drug delivery and gene transfection. Dextran is formed by monomeric

α-D-glucose units, linked by α-(1→6) glycosidic bond, in which the starting end of the chain is α-(1→4) or α-(1→3) glycosidic bond. Dextran is produced by several bacterial strains mostly gram-positive such as leuconostoc and streptococcus strains exposed to sucrose as a carbon source.[67] Several modifications can be performed on the structure such as etherification, esterification, amidation, and oxidation to enhance its properties for an improved delivery system.[68]

As Dextran is a neutral polysaccharide, chemical modification is required to introduce positively charged groups, such as diethylaminoethyl-dextran[69] or aminoethyl methacrylate,[70] eventually responsible for the electrostatic interaction with the negatively charged genetic materials.

A co-delivery carrier based on dextran was designed to enhance the efficiency of the chemotherapeutic drug DTX which clinical application is mainly affected by drug resistance, which has been linked to the sequestration of the drug in autophagolysosomes.[71,72] A tri-drugs platform was formulated using carboxymethyl β-dextran conjugated to protamine sulfate,[73] for the delivery of DTX, the autophagy inhibitor chloroquine (CQ), and siRNA against autophagy-related gene 5 (ATG5 siRNA), which plays a pivotal role in autophagy formation. In this formulation, all the materials are co-assembled via hydrophobic and electrostatic interactions. In particular, the siRNA moieties were complexed to the positive charges of protamine, an arginine-rich nuclear protein, widely exploited for gene delivery. The human breast cancer cell line MDA-MB-231 was used for both studies *in vitro* and to establish a mouse xenograft model. The tridrug-loaded NPs demonstrated excellent anticancer efficacy with good biosafety as a combination therapy against treating triple-negative breast cancer. Moreover, the system led to long-term inhibition of tumor growth.

In another study, a targeted pH-sensitive biocompatible dextran nanocarrier was devised for gene delivery to prostate cancer cells.[74] Prostate-specific membrane antigen (PSMA) is a transmembrane protein, overexpressed on resistant prostate cancer cells, which was targeted through the conjugation of its ligand, urea, to the system.[75] Thus, the novel PSMA-specific acid biodegradable siRNA nanocarrier was developed from a 40 kDa dextran backbone, modified to attach amine groups through acetal bonds, which undergo cleavage in the acidic endosomal microenvironment. Following the cleavage of the acetal bonds, the amine groups are detached from the dextran scaffold and release the siRNA. In this manner, the carrier was designed to remain stable under neutral pH, while releasing the cargo in the cytoplasm. The dextran-based polyplex downregulated simultaneously two selected gene targets, PD-L1 and CD46, which are important for cancer cells in escaping immune surveillance.

Finally, NPs for miRNA delivery were developed based on dextran and chitosan, which are attractive polymers because of their biocompatibility and biodegradability. Therefore, a redox-responsive polyelectrolyte complex of thiolated dextran and chitosan with miR-145 was prepared and then decorated with anti-nucleolin aptamer, AS1411 (apt-PEC) for targeted delivery.[76] miR-145 is a tumor suppressive miRNA, abnormally reduced in cancers. Thus, gene delivery of miR-145 can increase its intracellular expression, resulting in a promising therapeutic approach. AS1411 is a DNA aptamer targeting nucleolin, a protein overexpressed in many tumor types.[77] Although AS1411 has been withdrawn from phase 2 clinical trials, the development of nanomaterials based on this aptamer as targeted molecules have been widely investigated.[78] Interestingly, redox-responsive polymers can be prepared with a chemical modification of dextran with thiol groups to form disulfide bonds with the oligonucleotides. Those bonds can be reduced in response to the high level of intracellular glutathione and release the genetic material in the cytoplasm. Chitosan was then complexed to the dextran molecules via electrostatic interaction, to improve colloidal stability.

3.3. Pullulan

Another neutral polysaccharide widely exploited for drug delivery purposes is PULL, a water-soluble carbohydrate extracellularly synthesized by the yeast like fungus *Aureobasidium pullulans*.[79] Structurally, PULL consists of maltotriose and maltotetraose units linked through α-D-(1→6) bonds on its terminal glucose residues, yielding *n* number of α-(1→4) linked trimeric repeating unit.[80] PULL is commercially amply used as a polysaccharide gum. Currently, important commercial applications of PULL include the use as flocculant, blood plasma expander, food additive, adhesive, and dielectric material.[80] It is "generally recognized as safe" by FDA for a wide range of applications, such as an excipient in pharmaceutical tablets, and it is listed in the Japanese Standards as accepted adjuvant for drug delivery.[81] Moreover, PULL has entered clinical trials as vehicle for cancer vaccination.[82]

PULL and its derivatives have been widely investigated for drug and gene delivery due to their promising characteristics. First, pullulans are attractive for their non-toxic, non-carcinogenic, and non-mutagenic properties. Furthermore, PULL can be derivatized by chemical reactions on the nine hydroxyl groups per repeating unit,[83] which can be further hydrophobized, thiolated, PEGylated, or conjugated to cationic substituents.[84] PULL has been exploited for liver targeting because of the deep affinity for the ASGPR in the liver, which possesses biological affinity for sugar residues.[85] For this purpose, PULL was conjugated to PEI to form PULL-PEI/siRNA complexes to safely deliver the oligonucleotide to the liver.[86] In another study, PULL was employed to design an active targeting polyplex loaded with pDNA/siRNA for the specific accumulation in folate receptor overexpressing cancer cells.[87] In this study, the gene vector was synthesized by the conjugation of PEI with PULL and succinic acid as a spacer. Then, the gene vector was targeted with the addition of carboxyl folate to the amino groups of PEI. The resulting gene carrier demonstrated low cytotoxicity and the capacity to specifically deliver gene/siRNA to human cervical carcinoma cells HeLa.

Similar to other polysaccharides, also PULL was developed as a nanocarrier for the co-delivery of the chemotherapeutic agent doxorubicin (DOX) and the short hairpin RNA (shRNA) of beclin1 to enhance the anticancer effect of the drug. Indeed, beclin1 is a pivotal autophage-related gene, which has been observed that induces resistance mechanisms in chemotherapy.[72] Therefore, the efficiency of the drug can be increased by the inhibition of the gene beclin1. An amphiphilic cationic polymer was prepared by linking lipophilic desoxycholic acid (DA), low molecular weight branched PEI, and PULL.[88] The newly synthesized polymer can self-assemble into micelles with a positively charged hydrophilic

shell (PEI) which binds to negatively charged shRNA, while DA provides the hydrophobic core to encapsulate anticancer drugs. Then, folate (FA) was added as ligand for receptor-mediated targeting to the tumor cells. In this study, the amphiphilic functional PULL-based copolymer decorated with FA targeted efficiently the tumor site and suppressed cancer growth more effectively than DOX or shBeclin1 alone.[89] Cationic PULL derivatives are promising carriers also for miRNA delivery. PULL modified with a quaternary ammonium group resulted in the complexation of miRNAs and the polyplexes showed not to be cytotoxic on human umbilical vein endothelial cells (HUVEC).[90] Although deeper investigations are needed, this system could be used as a delivery platform for miRNA therapeutics.

3.4. Inulin-Based Systems

Inulin (INU) is a natural fructan-type polysaccharide, composed of β-D-fructosyl subgroups linked together by (2→1) glycosidic bonds and usually with a (1↔2) bonded α-D-glucosyl end group. Since INU is a biocompatible and biodegradable molecule with a low molecular weight, it has been used for different pharmaceutical applications as a drug carrier and is being recently studied for siRNA delivery. The hydroxyl groups on its structure can be chemically functionalized obtaining derivatives with different potentialities. Among them, positively charged INU derivatives form polyplexes by electrostatic interactions with siRNA to achieve efficient intracellular delivery of the genetic material.[91] For example, INU can be modified with diethylenetriamine (DETA) to introduce positive charges at physiological pH to be exploited for oligonucleotides complexation.[92] The INU-DETA copolymer can be further modified with imidazole (IMI), to obtain a unique pH-responsive polycation (INU-IMI-DETA).[93] Imidazole was added to increase the stability and retention of loaded siRNA and guarantee endosomal escape. This polysaccharide-based cationic system represents a flexible backbone for further chemical modification. PEG was indeed conjugated to confer stealth properties and improve the stability and circulation time of the nanoaggregates. Finally, active targeting properties can be bestowed by the conjugation of ligands, such as the epidermal growth factor (EGF).[94] Surprisingly, the EGF ligand is more effective as targeting agent when PEG chains are conjugated to the system. The polymer without PEG presents an electrostatic interaction between EGF and the siRNA, masking the ability of the EGF to undergo receptor-mediated recognition by cancer cells. The PEG chains instead can mediate the electrostatic interaction between the two molecules, preserving the targeting properties of the ligand.

The biocompatibility properties of INU were also exploited to formulate INU-coated super-paramagnetic iron oxide nanoparticles (IC-SPIONs).[95] Indeed, SPIONs have been evaluated recently as an effective carrier for drug delivery. However, they require to be coated with a polymeric coating to guarantee the dispersion of SPIONs in water and prevent their agglomeration. An INU-based polycation, prepared by the grafting of ethylenediamine on the polysaccharide backbone allows for the complexation of a siRNA (siGL3) targeting luciferase. The obtained IC-SPIONs resulted to be cytocompatible on tested human colon cancer (HCT116) and normal human bronchial epithelial (16HBE) cells lines, with excellent silencing efficiency in both the absence (70% silencing) and in the presence of an external magnetic field (95% silencing).

Notably, INU is not hydrolyzed by human salivary and intestinal enzymes, due to the β-bonds in its structure. The degradation of the polysaccharide happens only in the distal portion of the digestive system. Therefore, INU is an interesting carrier to deliver drugs to the colon following oral administration.[96]

INU was used as a coating material for a novel site–specific intestine delivery system, the manganese oxide nanocuboids (MNCs) stabilized by arginine for oral delivery of Ephb4 shRNA.[97] The erythropoietin-producing human HCC receptor b4 (Ephb4) is a highly up-regulated oncogene implicated in tumor growth, metastasis, and vascularization regulation in malignant tumors.[98] The MNCs were grafted with arginine to allow the loading of anionic shRNA by electrostatic interactions, then INU specifically consent the accumulation of the MNCs at the target site. The *in vivo* evaluation revealed the knockdown of Ephb4 gene in established Apc mouse models of colorectal cancer.

3.5. Chitosan

Chitosan is a linear polysaccharide composed of D-glucosamine and *N*-acetylglucosamine linked by $\beta(1\rightarrow4)$ glycosidic bonds. It is obtained from partial deacetylation of chitin, presents in crustacean shells. CS is insoluble in physiological water, but it dissolves in acidic solution thanks to protonable amino groups on its backbone. In recent years, chitosan has emerged as an interesting material in biomedical area, such as in drug delivery,[99] gene delivery,[100] and tissue engineering.[101] Chitosan has been widely used in drug delivery because it is a biocompatible, biodegradable, and non-toxic material. Several examples of chitosan in drug delivery have been reviewed and classified according to the delivery route, such as oral drug delivery, ocular drug delivery, nasal drug delivery, pulmonary drug delivery, buccal drug delivery, periodontal drug delivery, dermal and transdermal drug delivery, wound healing, vaginal drug delivery, and vaccine and gene delivery.[102] In gene delivery, chitosan, thanks to its positive nature, has been extremely used for forming stable complexes withnegatively charged genetic materials.[103] The positive groups on chitosan also enable the nuclear uptake of nucleic acids by interaction with the negatively charged nuclear membrane. In addition, chitosan can also facilitate the endosomal escape of genetic materials, due to the proton sponge effect.[104] Chitosan has also a mucoadhesion property, due to the interaction of the cationic D-glucosamine residues with the negatively charged mucin. This feature makes chitosan an excellent delivery carrier for drugs, proteins, and nucleic acids via intranasal administration, exploiting increased residence time in the nasal cavity and therefore the enhanced absorbance of the active molecules.[105] It is evident then the deacetylation degree (DD%) of chitosan, which is the percentage of deacetylated primary amine groups and represents the charge density in acidic conditions,[45] is an important parameter to be considered.

Furthermore, the amine and hydroxyl groups on chitosan backbone can be chemically modified to optimize some of its properties such as the low solubility at physiological conditions, its molecular weight (MW) and to conjugate efficiency enhancers

and targeting ligands. The chitosan molecular weight impacts the polyplexes stability and consequently their biological activity *in vitro*. Chitosan is commercially available at different MW ranging from <10 kDa, up to >300 kDa. Low MW chitosan (from 10 to 80 kDa) and high MW chitosan high MW (80–300 kDa) are the most suitable for the formation of polyplexes, considering their stability and *in vitro* transfection.[45]

Considering chitosan features, such as the naturally occurring positively charged polymer, it has been widely investigated as backbone for polyplexes formulation, often in combination with other cationic molecules, to enhance its transfecting efficiency.[106]

A novel hybrid chitosan-based complex was prepared for systemic delivery of survivin (SVN) siRNA.[107] SVN is an attractive anticancer target as a member of the inhibitor of the apoptosis proteins family, overexpressed in cancer cells to decrease apoptosis.[108] First, SVN-siRNA and protamine were complexed through electrostatic interactions, then a trivalent polyplex was obtained with further complexation with chitosan. Subsequently, lecithin and tripolyphosphate were added to obtain a more stable and efficient hybrid complex. The chitosan-based hybrid complex for systemic delivery of SVN enhanced *in vivo* targetability and anticancer efficacy in a tumor xenograft mouse model of prostate cancer.[107]

In order to solve the limitation of low solubility in physiological pH, chitosan can be chemically modified and grafted with PEG. SVN-siRNA-loaded PEG-CS NPs were prepared and tested for breast cancer therapy decreasing tumor growth in a murine xenograft of 4T1 cells.[109] Interestingly, chitosan can be exploited to stabilize nanobubbles, forming a cationic shell available for the complexation of nucleic acid.[110,111] A different formulation of chitosan-shelled nanobubbles was designed for siRNA encapsulation. In this case, the genetic material is loaded in the internal phase of a water/oil/water emulsion. This type of system has been developed to deliver siRNA against nuclear factor E2-related factor 2 to treat melanoma. The chitosan-shelled in this case enhances the stability of the formulation and provides for interaction with the cellular membrane.[112]

Specific ligands can be added to the CS NPs surface for active cell targeting. Since the expression of integrin $\alpha v\beta 3$ is high in tumors and tumor vasculature, but absent in normal tissues, this receptor is considered an interesting drug target. Integrin $\alpha v\beta 3$ is a receptor for the ligand Arg-Gly-Asp (RGD) peptide. Therefore, a cyclic Arg-Gly-Asp (RGD) peptide-labeled chitosan NP (RGD-CH-NP) was prepared for tumor specific siRNA delivery. RGD was chemically grafted to chitosan by thiolation reaction, and the genetic material was complexed to the polysaccharide via electrostatic interaction. In orthotopic animal models of ovarian cancer RGD-CH-NP loaded with siRNA against FAK (focal adhesion kinase correlated with tumor progression and metastasis)[113] was able to deliver specifically intratumorally.[114]

CS nanocarriers can be exploited for the co-delivery of nucleic acids and anticancer drugs to exceed the critical problems related to cytotoxic drugs, such as systemic toxicity and drug resistance mechanisms. As DOX is frequently used for the treatment of several types of cancer, many studies developed nanomedicines for the simultaneous delivery of DOX and nucleic acids. B-cell lymphoma/leukaemia 2 (Bcl-2) protein overexpression in tumor cells can be induced as drug resistance effect given by the administration of DOX.[115] Therefore, a CS nanocarrier for a co-delivery of siRNA against Bcl-2 and DOX for synergistic antitumor efficacy was developed.[116] For this purpose, an amphiphilic chitosan-based polymer was prepared from the positively charged, hydrophilic, *N,N,N*-trimethyl chitosan conjugated to the hydrophobic all-trans retinoic acid (ATRA). ATRA was employed to induce apoptosis in tumor cells and in the meantime to block signaling pathways of tumor stem cells. Finally, lactose acid residues are inserted to facilitate the uptake by HCC cells. The delivery system significantly enhanced *in vitro* and *in vivo* antitumor activity via multiple cooperative effects of the chemotherapeutic drug DOX, the gene silencing by siRNA as well as the nanovector.[116]

Cancer immunotherapy is nowadays considered a valuable therapeutic approach. Dendritic cell vaccination is a promising immunotherapeutic strategy as can offer specificity, low toxicity, and long-term effects due to immunological memory.[117] Chitosan-shelled nanobubbles (NBs) loaded with DNA vaccine were formulated for HER2 + breast cancer therapy, which were functionalized with an antibody to target DCs.[118] These chitosan-shelled NBs resulted in good DNA encapsulation efficiency, high selectivity, and prolonged release. Moreover, DNA-loaded NBs delayed tumor growth *in vivo* after intradermal injection in HER2 + breast cancer mouse model. For cancer vaccine development, tumor cell lysates (TCL) have been recently implemented as tumor antigens. Chitosan nanoparticles with surface mannose moieties for specific DCs targeting were successfully developed.[119] TCL, generated from B16 melanoma cells, was loaded in chitosan-based NPs and Chitosan-shelled NBs vaccine significantly delayed tumor growth in mice.

In the last decade, clustered regularly interspaced short palindromic repeat (CRISPR)-associated Cas nuclease system (CRISPR /Cas9) has become a powerful genome editing technology. However, the development of an efficient CRISPR/Cas9 delivery system is required since the instability of exogenous plasmid limits its applications.[105] CS has been used for delivery systems of CRISPR/Cas9 because of its interesting properties such as biocompatibility, biodegradability, absence of toxicity, and the pH-responsive profile. Paclitaxel (PTX) and sg-VEGFR2/Cas9 plasmid (VC) were co-loaded in CS NPs for cancer therapy, via hydrophobic and electrostatic interactions respectively.[120] CS solubility depends on the pH, thus allowing a sustained and controlled release of PTX. A specific target for the ASGP-overexpressing HCC cells was obtained with the introduction of a β-galactose-carrying lactobionic acid to CS. The nanocomplex inhibited the expression of VEGFR2 protein in HepG2 cells by above 60% and reduced tumor progression by 70% in mice. It has been proved the efficient synergistic therapy of the CRISPR-Cas9 gene-drug co-delivery nanocomplex system.

Another approach that has been widely exploited for the active targeting of nanomedicines is the use of the polysaccharidic ligand HA, as its receptor CD44 is highly expressed in a variety of cancer cells, such as lung, breast, pancreatic, gastric, and colon cancer cells.[121] Nevertheless, nanocarriers based purely on HA are not frequently designed as the negative charge of HA does not facilitate the oligonucleotides loading. In order to give electrostatic interactions with siRNA, the majority of HA-based NPs are chemically modified to include a cationic component, usually a different co-polymer.[42]

For the above-mentioned reasons, HA was conjugated on CS nanoparticles loaded with siRNA against Bcl-2, one of the most important oncogenes involved in cell apoptosis. *In vivo* experiments showed the inhibition of tumor growth through NPs accumulation at the tumor site.[122] In another study, HA was used to form nanocomplexes with CS that already incorporated positively charged DOX and negatively charged miR-34a mimics. miR-34a is a miRNA regulated by the p53 network at the transcriptional level which is commonly downregulated in a variety of cancers. *In vitro* and *in vivo* experiments, the newly synthesized nanocomplexes demonstrated that the co-delivery of DOX and miR-34a synergistically enhanced breast cancer suppression.[123]

4. Summary and Future Perspectives

Carbohydrates-based polymeric delivery systems are largely employed for their advantageous properties like biocompatibility, biodegradability, low toxicity and low environmental impact. The development of polysaccharide-based polymers as efficient carriers in delivering therapeutic substances like drugs, proteins, and genes is still an open challenge. Furthermore, the recent advancements in nanotechnology provided significant knowledge for the enlightened design of novel efficacious formulations for gene delivery and cancer therapy. The potential of nanosized delivery systems became clear, nevertheless, their limitations are still hindering their clinical application. At the preclinical level, saccharides-based systems are widely investigated. On the other hand, this abundance does not reflect in clinical success. The main reason might be ascribed to the difficulty in obtaining compendial material which meets the regulatory requirements (low dispersity index, define composition). The increased availability of novel therapeutic modalities (RNA interference, CRISPR Cas/9, mRNA) with nanotechnological advances might lead to a successful candidate to treat cancer patients.

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Conflict of Interest

The authors declare no conflict of interest.

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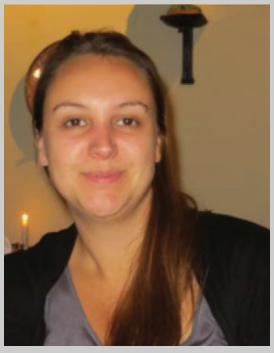

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