Sweet Fairytale: Carbohydrates as Backbones for Glyconanomedicine

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1. Introduction

Nanoparticle (NP)-based therapeutics have been used for the treatment of cancer, neurodegenerative diseases, infections, allergies, diabetes, and inflammation, both in pre-clinical models and in clinical investigations. In fact, several “nanodrugs” have already been approved by the regulatory agencies.[1–3] The growing interest in NPs for medical applications is attributed to the exceptional advantages they offer, which include protection of the drug from premature degradation, lower therapeutic toxicity, the ability to deliver poorly water soluble drugs, controlled drug release mechanisms, and improved intracellular penetration.[4]

Structural and physicochemical properties, including particle shape, zeta potential, size distribution, and roughness, control the biodistribution of the NPs in vivo.[5] Their small size enables NPs to pass through the smallest capillaries and, thereby, promotes passive tumor targeting, due to the enhanced permeability and retention (EPR) effect of the tumor vasculature.[3,6] The passive targeting is achieved by extravasation of NPs through the fenestrations in the tumor vasculature and ineffective lymphatic drainage.[7] Active cellular targeting is achieved by decorating the surface of NPs with natural or mimetic ligands, such as monoclonal antibodies (mAb) or their fragments.[8,4] As the requirements for clinically applicable NPs are becoming clearer, so are the requirements for the materials used for their preparation. These materials should be biocompatible and, preferably, biodegradable, well characterized, and easily functionalized.[9] Carbohydrates accomplish all of these requirements and are therefore widely used for the preparation of nanoparticles for drug delivery.

2. Carbohydrates

Carbohydrates are highly abundant molecules that are derived from various origins, including algal origin (e.g., alginate and carrageenan), plant origin (e.g., cellulose, pectin, and guar gum), microbial origin (e.g., dextran and xanthan gum), and animal origin (e.g., chitosan [CS], hyaluronan [HA], chondroitin, and heparin).[8] Naturally occurring carbohydrates are diverse in their physiochemical properties, including a great variety of chemical structures (Figure 1), molecular weights (Mw), and ionic nature. This versatility also contributes to a wide range of biological activities. From a pharmaceutical standpoint, carbohydrates possess many favorable characteristics, such as low toxicity, biocompatibility, stability, low cost, hydrophilic nature, and availability of reactive sites for chemical modification.[8,9] Chemical functionalization, using...
mainly the free carboxyl and hydroxyl groups distributed along the carbohydrates’ backbone, have been used to create derivatives with determined/tailored properties.\textsuperscript{[10]}

The solubility, hydrophobicity, and physicochemical and biological characteristics of carbohydrates have been modified. These modifications were achieved using techniques such as oxidation, sulfation, esterification, amidation, and grafting methodologies.\textsuperscript{[10]}

Surface modification of NPs with carbohydrates also has remarkable advantages for generating drug delivery platforms: it endows the NPs with longer circulation time and increased stability. The prolonged circulation time can be attributed to reduction in protein adsorption and opsonization, as well as the hydrophilic coating. In addition, such modification provides active cellular targeting capabilities: pullulan coating has been used for hepatic delivery; alginate and CS coating have been used for mucosal delivery; mannan coating facilitates uptake by macrophages; and HA coated-NPs have been used to target CD44- and CD168-overexpressing cancer cells and inflammatory cells. Surface modification of NPs with carbohydrates can be achieved by adsorption, incorporation, copolymerization, or covalent grafting.\textsuperscript{[11]}

An additional benefit of carbohydrate surface modification of NPs is the “built-in” cryoprotection feature that has been reported for several carbohydrates.\textsuperscript{[12]}

All of these qualities have led to the growing use of carbohydrates in drug delivery systems. The properties of common carbohydrates and their use for the preparation of drug delivery systems are detailed below.

### 3. Carbohydrate NP Preparation: Key Mechanisms

The unique characteristics of carbohydrates have been utilized for the preparation of NPs. Several key mechanisms have been used for this purpose, including cross-linking, self-assembly, and polyelectrolyte-based structures.

#### 3.1 Cross-Linking

Cross-linked NPs have been prepared by using cross-linkers to interconnect polymeric chains and form 3D networks\textsuperscript{[13]} (Figure 2A and B). There are two types of cross-linked NPs, as determined by the cross-linkers used: ionically cross-linked NPs and covalently cross-linked NPs. The nature of cross-linked NPs is mainly determined by the cross-linker density (the molar ratio between the cross-linker and the repeating units of the polymer), which affects properties such as mechanical strength and drug release.\textsuperscript{[13]}

#### 3.1.1 Covalent Cross-Linking

Covalently cross-linked NPs are characterized by a permanent network structure, since the chemical bonds formed are irreversible, unless biodegradable or stimuli responsive materials are employed (Figure 2A).\textsuperscript{[13]}"
teractions that form the 3D network are covalent bonds. Additionally, secondary interactions, such as hydrogen bonds and hydrophobic interactions, are present. The rigid network formed allows absorption of water and bioactive compounds without dissolution of the NP, even upon drastic pH changes.

Common covalent cross-linkers utilized to form such networks with carbohydrates are dialdehydes, such as glutaraldehyde. However, due to toxicity, these cross-linkers are far from optimal for drug delivery systems. Therefore, more suitable alternatives have been tested, such as the natural cross-linker genipin and natural di- and tricarboxylic acids, in combination with the condensation agent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC).

### 3.1.2 Ionic Cross-Linking

I ionically cross-linked NPs are prepared by the formation of reversible ionic links between charged carbohydrates and oppositely charged ions (e.g., chitosan and tripolyphosphate [TPP]) (Figure 2B). The main interactions inside the network are the ionic bonds that form bridges between the carbohydrate chains, although secondary interactions, such as hydrogen bonds, are also present. This method is considered more biocompatible than covalent cross-linking since no harsh preparation or toxic cross-linkers are used. The cross-linking reaction is mostly influenced by the size of the cross-linker and the global charge of the cross-linker and carbohydrate. I ionically cross-linked NPs are generally pH sensitive, a welcome trait for drug delivery purposes that does not exist for the covalently cross-linked NPs. However, the pH sensitivity can lead to instability of these NPs.

### 3.2 Polyelectrolyte-Based Structures

#### 3.2.1 Polyelectrolyte Complexes (PEC)

Polyelectrolyte complexes (PEC) are formed by direct electrostatic interactions of oppositely charged polyelectrolytes in solution (Figure 2C). PEC represent a biocompatible delivery strategy, since non-toxic covalent cross-linkers are used. These complexes resemble ionic cross-linking, since non-permanent networks are formed that are more sensitive to changes in environmental conditions. However, unlike ionic cross-linking, in which ions react with the polyelectrolyte, in PEC the interaction is between the polyelectrolyte and larger molecules with a broad range of Mw. The formation and stability of PEC is determined mainly by the degree of interaction among the polyelectrolytes. The latter is a function of the charge density and distribution of each of the oppositely charged polyelectrolytes. The chemical environment—the pH of the solution, the ionic strength, the temperature, and the duration and order of mixing—is also crucial. Secondary factors are the Mw of the polyelectrolytes and their flexibility. Ionic cross-linking can reinforce the formed interaction.

#### 3.2.2 Layer by Layer Assembly

Layer by layer (LbL) assembly of polyelectrolyte NPs is a relatively new type of polyelectrolyte-based strategy to generate nano-sized delivery platforms. The LbL technique, which is based on electrostatic interactions, employs alternate adsorption of oppositely charged polyelectrolytes. The structure of the film obtained can be controlled with 1-nm precision, within the range of 5 to 1000 nm, and the molecular composition is well-defined.

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**Figure 1.** Chemical structures of polysaccharides. Amylose is composed of α-(1,4)-D-glucose units. Hyaluronan is composed of alternating β-(1,4)-D-glucuronic acid and β-(1,3)-N-acetyl-D-glucosamine. Chitosan, the N-deacetylated derivative of chitin, is composed of β-(1,4)-N-acetyl-D-glucosamine and D-glucosamine. Alginate is composed of alternating blocks of β-(1,4)-D-mannuronic acid and β-(1,3)-N-acetyl-D-glucosamine acid. Heparin is composed of α- or β-(1,4) linked uronic acid (90% α-L-iduronic acid, 10% β-D-glucuronic acid) and α-D-glucosamine residues. Chondroitin sulfate is composed of alternating β-(1,3)-N-acetyl-D-galactosamine and β-(1,4)-glucuronic acid. Adapted from reference [4].
The advantages of the LbL approach are clear: many therapeutics and biomaterials can be non-covalently incorporated into LbL films under physiological conditions, without compromising their biological properties. In addition, since the technique can be used with multiple components, several factors can be manipulated, among which are the surface chemistry, dimensions of the thin films, and therapeutic moieties used. Thus, a sophisticated nano-sized drug delivery system with a tailored drug release mechanism may be created.

3.3 Self-Assembly

Upon grafting hydrophobic moieties onto a hydrophilic carbohydrate, an amphiphilic copolymer is created. In aqueous solutions, amphiphilic copolymers tend to self-assemble into NPs, in which the inner core is hydrophobic and the shell is hydrophilic. The hydrophilic shell serves as a stabilizing interface between the hydrophobic core and the external aqueous environment (Figure 2D). This self-assembly proceeds via hydrophobic interactions, mainly in order to minimize interfacial free energy. The NPs that form are characterized by prolonged circulation and thermodynamic stability. In addition, since their core is hydrophobic, these particles have been used for the delivery of hydrophobic drugs. Several properties, such as size, surface charge, loading efficiency, stability, and biodistribution, can be altered for particular applications. For example, the size of the NPs can be controlled by adjusting the length of the hydrophobic moiety and the length of the polymer. In addition, the surface charge, which affects particle serum stability and cellular uptake, can be altered by controlling the degree of substitution or the nature of the hydrophobic moiety.

Carbohydrates can be modified with a wide range of hydrophobic moieties, including bile acids (e.g., 5β-cholanic acid, cholic acid, or deoxycholic acid), fatty acids (e.g., palmitoyl acid, stearic acid, or oleic acid), cholesterol, and hydrophobic drugs.

3.4 Carbohydrate Drug Conjugates

The concept of a polymer-drug conjugate (Figure 2E) was first introduced in 1975 by Ringsdorf for the delivery of small hydrophobic drugs. The conjugation to a polymer alters the biodistribution and circulation time of the drugs, relative to free drugs, and the conjugated drugs can be selectively delivered and accumulated at the tumor, due to the EPR effect. The benefits of this delivery strategy have led to several phase I/II clinical trials. This promising concept has been used for the preparation of carbohydrate-drug conjugates, especially for the delivery of insoluble anti-cancer drugs, as will be detailed below.

A carbohydrate-drug conjugate consists of three parts: a water soluble polymer, a drug, and a biodegradable spacer connecting the two. Additional components, such as labeling agents and targeting moieties, may also be included. When rationally designing this delivery strategy, special attention should be given to the spacer used. The following principles should be considered for a successful delivery system: first, the spacer should be stable in the bloodstream, in order to increase circulation time, but...
Carbohydrate Backbones for Glyconanomedicine

should be rapidly broken down after cell entry.\textsuperscript{23} In addition, the spacer should release an intact drug molecule without altering its chemical structure.\textsuperscript{23} Furthermore, the carbohydrate itself should be stable in the bloodstream.\textsuperscript{23}

The final size and shape of the carbohydrate-drug conjugate largely depend on the characteristics of the components. For example, conjugation of a hydrophobic drug results in self-assembly into a spherically shaped NP, with the drug physically trapped inside the particle.

4. Carbohydrate Nanoparticles

4.1 Chitosan

Chitosan (CS) is a linear carbohydrate composed of β-(1,4)-linked D-glucosamine and N-acetyl-D-glucosamine (Figure 1). CS is obtained by deacetylation from chitin, a highly abundant carbohydrate, which is the main component of crustaceans’ exoskeleton.\textsuperscript{15}

At physiological pH, CS is the only positively charged carbohydrate and, therefore, can interact with anions, including polyanions and negatively charged carbohydrates, nucleic acids, and proteins.\textsuperscript{14} Among the many advantages of CS are its biocompatibility and low cytotoxicity.\textsuperscript{15}

Indeed, comparison of CS and its derivatives to synthetic cationic polymers, such as polyethylenimine (PEI), reveals significantly lower toxicity.\textsuperscript{24} Interestingly, CS and its derivatives were found to be toxic to several bacteria, fungi, and parasites.\textsuperscript{24} The positive charge of CS also facilitates adherence to mucosal surfaces, which are mostly negatively charged.\textsuperscript{15}

Alongside these advantages, CS also is accompanied by disadvantages, such as its inherent insolubility in aqueous solutions with pH above 6.5.\textsuperscript{25} High degree of deacetylation, low molecular weight, and chemical modification can facilitate water solubility of CS. These factors also affect particle properties, such as size, surface charge, drug entrapment efficiency, and stability.\textsuperscript{15}

4.1.1 Chitosan-Based NPs

The nature of CS and the ease of its chemical modification enable multiple NP preparation schemes, including: covalent cross-linking, ionic cross-linking, polyelectrolyte complexation, and self-assembly.\textsuperscript{15} For example, ionically cross-linked particles can be prepared using polyanions, the most widely used of which is tripolyphosphate (TPP). The wide use of TPP in the preparation of CS NPs is based on a principle reported previously by Bodmeier et al.\textsuperscript{15} The Alonso group later reported the use of these nanoparticles for protein, oligonucleotides and plasmid DNA delivery. The resulting CS/TPP NPs for DNA delivery were in the range of 100-300 nm, depending on the Mw of the CS, and showed high physical stability and encapsulation efficiencies for both plasmid DNA and dsDNA oligomers (20-mers), independent of CS’s Mw.\textsuperscript{27} The efficiency of transfection, however, was highly dependent on the Mw of CS. Only the low Mw CS/TPP NPs mediated strong beta-galactosidase expression in vivo after intratracheal administration.\textsuperscript{27}

Due to its positive charge, polyelectrolyte complexation is one of the most frequently used methods to prepare CS NPs. Recently, PEC of ultrapure CS monomers were used for ocular gene delivery.\textsuperscript{28} The NPs, which were created based on a method developed by Koping-Hoggard et al.,\textsuperscript{29} had an average size of ~100 nm in diameter and a strong positive charge. Upon injection into rat corneas, this formulation demonstrated effective transfection of kidney cell line COS-7 in vitro and 5.4-fold higher luciferase gene expression than with polyethyleneimine-DNA NPs. PEC of CS oligomers (or low Mw CS) and pDNA have several advantages over PEC of high Mw CS with pDNA: high Mw CS form extremely stable PEC with DNA, which delays the release of DNA and, therefore, results in a slow onset of action.\textsuperscript{29} In addition, these PECs are of aggregated shapes, their viscosity at concentrations used for in vivo delivery is very high, and their solubility at a physiological pH is low.

CS-drug conjugates have also been developed, mainly for the delivery of insoluble anti-cancer agents. Recently, an interesting pH responsive CS-drug conjugate has been introduced (Figure 3) for photodynamic therapy.\textsuperscript{30} The system is composed of a glycol CS backbone, a functional group (3-dithyaminopropyl isoithiocyanate, DEAP block), a photosensitizing model drug (chlorine e6 block, C6), and poly(ethyenglycol) (PEG). The system is designed to undergo three-dimensional supramolecular self-assembly (i.e., self-quenched state of photosensitizing drugs) at physiological pH and to destabilize into extended random molecules (i.e., dequenched state for singlet oxygen production) at lower pH, as present in the surrounding tumor, due to protonation of the functional group DEAP. The investigators tested this conformational change by examining particle size and shape, which changed dramatically from 150 nm spherically shaped NPs, at pH 7.4, to 3.4 nm disentangled forms, at pH 6.8. In addition, the zeta potential changed with the pH, confirming the protonation of the DEAP group. The pH-dependent change in photoactivity was confirmed by measuring the amounts of singlet oxygen. In vitro, higher phototoxicity of HeLa cells has been observed at pH 6.8 and 6.4 than at pH 7.4. In vivo, the CS-drug conjugate showed tumor specificity after intravenous administration to nude mice bearing HeLa tumor cells, with a signal lasting for more than 24 hours.
Utilization of the primary amino groups of CS is another way to improve its physicochemical properties. For example, conjugation of hydrophobic moieties, such as deoxycholic acid and cholesterol, to CS allows solvent-induced self-assembly into NPs. This principle was used for preparation of 5b-cholanic acid (HGC)-modified glycol CS NPs for the delivery of the anti-angiogenic peptide RGD. In an in vivo study, the RGD-containing NPs inhibited basic fibroblast growth factor (bFGF)-induced angiogenesis and significantly decreased tumor growth and microvessel density, in comparison to the native RGD peptide. These particles were previously used by the same group for gene delivery and for delivery of the chemotherapeutic agents doxorubicin and paclitaxel.

Other chemical modifications of CS, such as addition of thiol groups and trimethylation, can improve the mucosal and permeation-enhancing properties of CS. Trimethylated CS (TMC) is also characterized by increased solubility at neutral pH.

4.1.2 Chitosan-Coated NPs

The cationic nature of CS is also appealing for the coating of NPs, as it enables them to adhere to mucosal surfaces and to open tight junctions between epithelial cells. For example, CS-coated poly-e-caprolactone (PECL) NPs allowed the bioavailability of the anti-inflammatory drug, indomethacin, in the cornea and aqueous humor, following topical ocular instillation. In addition, CS coating of liposomes (chitosomes) enhanced mucosal adhesion in rat intestine, following oral administration. Chitosome formulations used for the oral delivery of insulin and calcitonin (in separate studies) induced significantly more substantial and prolonged decreases in blood glucose and calcium levels, respectively, relative to uncoated liposomes.

CS coating can be used to replace the cationic polymers and lipids currently used for nucleic acid delivery, thus overcoming the toxicity, which is the major obstacle in using these compounds. CS-coated NPs can interact with nucleic acids, improving the particle loading efficiency and transfection properties.

Structural benefits of coating NPs with CS have also been demonstrated: CS-coated liposomes were more stable than uncoated liposomes in simulated gastric fluids. CS-coated PECL particles also demonstrated enhanced physical stability. In addition, the CS coating facilitated the redispersion of lyophilized PECL nanoparticles.

4.2 Hyaluronan

Hyaluronan (HA) is a linear, high Mw glycosaminoglycan (GAG) composed of alternating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine with β-(1,4) interglycosidic linkages (Figure 1). Hyaluronan holds remarkable hydrodynamic properties, especially viscosity and the ability to retain water. It was previously regarded as important for joint lubrication and structural stability of organs. However, HA was also found to be essential for proper cell growth, embryonic development, healing processes, inflammation, and tumor development. As opposed to other GAGs, HA is not sulfat-
ed, not linked to a protein, and is naturally produced by bacteria as a capsule. Commercially available HA is either produced through bacterial fermentation by Streptococcus species or extracted from rooster combs, umbilical cords, synovial fluids, or vitreous humour.

There are several advantages of HA, which make it suitable for drug delivery: it is water soluble, biodegradable, biocompatible, non-toxic, non-immunogenic, and can be easily chemically modified. In addition, it is the major ligand for CD44 and CD168 (also known as Receptor for Hyaluronan Mediated Motility, RHAMM) and, therefore, is suitable for targeting CD44- and RHAMM-expressing cells. CD44 and CD168 are overexpressed by various tumors—for example, squamous cell carcinoma, ovarian, colon, stomach, glioma, and many types of leukemia and lymphoma—which makes the use of HA as a targeting moiety even more attractive.

4.2.1 HA-Based NPs

HA-based nanocarriers were developed using several approaches, such as HA-drug conjugates—which restore their cytotoxicity upon cell internalization by receptor-mediated endocytosis—PEC with polycations, and ionically cross-linked NPs. Chemically modified HA has also been widely used for the delivery of proteins, peptides, and nucleotides. Chemical modifications assist in prolonging HA half-life. However, beyond a certain level of modification, HA loses the ability to bind its receptors.

HA-based NPs hold a major advantage over other carbohydrate-based NPs, which is the ability to combine both passive targeting, by utilizing the EPR effect in tumors, and active targeting towards the HA receptors overexpressed by the majority of tumors.

HA-drug conjugates utilize several chemical groups on HA: the carboxylate on the glucuronic acid, the N-acetylglucosamine hydroxyl, the reducing end, and the acetyl group, which can be enzymatically removed from the N-acetylglucosamine. HA-drug conjugates are internalized via CD44 receptor-mediated endocytosis, and the drug is released mainly by intracellular enzymatic hydrolysis. In addition to targeting, HA conjugation has been used to increase drug solubility. This quality was used for the delivery of the hydrophobic anti-mitotic chemotherapeutic agent, paclitaxel (PTX). HA-PTX conjugates were shown to increase cellular uptake and cytotoxicity in vitro, in comparison to the free drug. In addition, cellular uptake of the HA-PTX conjugate was shown to be dose-dependent and CD44-specific, as it could be blocked by free HA or by anti-CD44 antibodies, but not by the structurally related carbohydrate, chondroitin sulfates (see Figure 1). In another study, the anti-tumor activity of the HA-PTX conjugate was demonstrated in vivo. This conjugate significantly decreased tumor burden, in comparison to free PTX, in mice bearing abdominal tumors of an ovarian cell line.

As detailed above, PEC have been used to prepare HA-based NPs. HA-CS NPs have demonstrated the ability to transport genes across the ocular mucosa and transport ocular tissue. The NPs were prepared by ionic cross-linking of CS with TPP, which was followed by PEC with HA. Upon topical administration to rabbits, the NPs managed to overcome cellular barriers, were located inside the corneal and conjunctival cells, and achieved significant transfection.

As with other carbohydrates, self-assembly of hydrophobically modified HA has been used for NP preparation. A recent study demonstrated the anti-tumor activity of self-assembled, modified HA-NPs. To this end, poly(γ-benzyl l-glutamate)-modified HA NPs were loaded with DOX. In vivo, the NPs significantly suppressed tumor growth in a breast cancer rat model, in comparison to free DOX, as determined by measuring both tumor volume and burden. In addition, the NPs reduced the cardiotoxicity of DOX.

4.2.2 HA-Coated NPs

The HA capsule of group A streptococci enables them to evade the host immune response and also provides long-term circulation. This feature has been successfully adopted for the delivery of mitomycin C (MMC), using HA-coated liposomes (tHA-LIP) (Figure 5A). HA-LIP were 7- and 70-fold longer circulating than uncoated liposomes and free MMC, respectively, in 3 murine tumor models. The HA on the tHA-LIP was covalently attached to phosphatidylethanolamine in the pre-formed liposomes, using EDC, via the glucuronic carboxylate. The tHA-LIP demonstrated slower MMC efflux and higher encapsulation efficiency than uncoated liposomes (nt-LIP). In addition, since the effect of tHA-LIP is CD44-dependent, these NPs demonstrated significantly higher cytotoxicity in vitro on CD44 overexpressing cells than on other cells and increased drug accumulation in vivo in tumors than in other tissues. The latter resulted in decreased metastasis, inhibition of tumor growth, and prolonged survival. These effects were later demonstrated with doxorubicin-loaded tHA-LIP. This study also compared tHA-LIP to coated stealth (PEG-coated) liposomes. This head-to-head comparison was performed since PEGylation, a common hydrophilic surface modification of drug delivery systems, prevents recognition by the immune system. The tHA-LIP were longer circulating than all tested controls, including uncoated and PEGylated stealth liposomes, in healthy and tumor bearing mice. Furthermore, the HA coating managed to prolong circulation time without activating the complement, which is associated with PEG-coated NPs.

An additional strategy for immune protection and increasing the solubility of highly insoluble single-walled carbon nanotubes (SWCNTs) with a combined phospholipid and HA coating was recently documented. As

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demonstrated for other NPs, the HA coating endowed the SWCNTs with stealth properties, allowing them to evade the immune system. The authors showed reduced immune and mitochondrial toxicity for the coated SWCNTs. In addition, upon systemic administration into healthy mice, the HA coated SWCNTs did not alter the total number of leukocytes nor increased liver enzyme release as opposed to the uncoated SWCNTs.

Another example of selective tumor targeting facilitated by HA coating was demonstrated for lipid-paclitaxel clusters (PTX-GAGs) and MMC-GAGs (Figure 5B). The aqueous insolubility of PTX was utilized in this preparation by mixing it with lipids that self-assembled into nano-sized clusters. The clusters were then covalently coated with HA, via EDC, to facilitate targeting of CD44 and CD168. When tested in vivo, these cluster particles induced tumor arrest in a murine model of colon adenocarcinoma and were significantly more potent than free PTX and Abraxane®, a commercially available PTX formulated in NPs. Similarly, head and neck tumors expressing CD44 were targeted using MMC-GAGs, which showed superior therapeutic outcome compared to free MMC.

Targeting of CD44 was also demonstrated for liposomes decorated with HA oligomers. Unlike the previously described tHA-LIP preparation, the HA oligosaccharides were conjugated to phosphatidylethanolamine by reductive amination prior to liposome preparation. The oligosaccharide-decorated liposomes demonstrated CD44-dependent uptake that could be blocked by both free HA and anti-CD44 antibodies. Liposome uptake was dependent on ligand density. However, as little as 0.1 mol% HA managed to facilitate targeting. In addition, doxorubicin encapsulated in the oligosaccharide-decorated liposomes was significantly more cytotoxic to CD44 overexpressing cells than the free drug.

Another clinically relevant advantage of the HA-coated NPs is cryoprotection. Cryoprotection provides an outer layer of HA that self-assembled into nano-sized clusters. The clusters were then covalently coated with HA, via EDC, to facilitate targeting of CD44 and CD168. When tested in vivo, these cluster particles induced tumor arrest in a murine model of colon adenocarcinoma and were significantly more potent than free PTX and Abraxane®, a commercially available PTX formulated in NPs. Similarly, head and neck tumors expressing CD44 were targeted using MMC-GAGs, which showed superior therapeutic outcome compared to free MMC.

4.3 Cyclodextrins

Cyclodextrins (CDs) are natural cyclic oligomers of α-(1,4) linked-glucopyranosyl that are produced from starch by enzymatic conversion (Figure 1). There are three main members of the CD family, composed of six, seven, and eight glucose units and known as α-, β-, and γ-CD, respectively. CDs have a hydrophilic exterior and a hydrophobic cavity that enables them to act as hosts to hydrophobic molecules. This unique ability to form inclusion complexes has been utilized by the pharmaceutical industry to improve bioavailability of poorly soluble or biodegradable drugs and to enhance permeability of biological membranes. In addition to stabilization of small drug molecules, CDs have been shown to increase the stability of oligonucleotides against endonucleases and even to modulate undesirable side effects, such as immune stimulation.

CDs are biocompatible, do not elicit an immune response, and have low toxicities in animals and humans. Therefore, they are used in pharmaceutical applications for numerous purposes, including improving the bioavailability of drugs.

4.3.1 Cyclodextrin-Based NPs

Cyclodextrin (CD)-containing polymers have been used in pharmaceutical applications since the 1980’s. In recent years, CD-containing polymers have been used for the formation of NPs designed for controlled or sustained drug release, molecular absorption, tissue engineering, or localized delivery of therapeutic agents. Structurally, CD-containing polymers can be classified according to location of the CD moieties in the polymer network. The CDs can be either located in the polymer backbone or grafted onto a pre-existing polymer. CD-containing polymers are also classified based on their charge, as either non-ionic, cationic, or anionic.

CD-containing polycations (CDPs) have demonstrated unique capabilities for nucleic acid delivery: they were reported to have low in vitro and in vivo toxicities, when compared with other non-CDPs, such as poly-L-lysine and PEI.

Mark E. Davis and co-workers had a major contribution in the area of gene delivery using CDPs. They created a linear CDP and reported the formation of self-assembled nano-sized polyelectrolyte complexes with pDNA with a cell transfection capability compared to PEI and Lipofectamine. Davis’s group further improved this CD-based delivery system to create the first clinically tested targeted NPs for siRNA delivery. This system exploits the CD inclusion capabilities to provide...
the oligonucleotide polycation PEC both steric stabilization and targeting capabilities (Figure 4). The CDP assembles with the siRNA primarily via electrostatic interactions. It condenses siRNA and protects it from nuclease degradation. The CD within the polymer chains that reside on the surface of the NPs are used for assembling PEG, which endows the NP with steric stabilization by preventing aggregation and non-specific interactions with biological components. The PEG is conjugated to an adamantyl group, which forms an inclusion complex with CD. The same principle is applied for the assembly of the targeting ligand transferrin, which was chosen since its receptor is up-regulated on cancer cells. Primary results from phase I clinical trials using siRNA against the M2 subunit of ribonucleotide reductase R2 (RRM2) have recently been published. Following systemic administration to patients with solid cancers, tumor biopsies revealed intracellular and dose-dependent localization of the NPs. In addition, specific reductions of both RRM2 mRNA and protein levels were observed. In a subsequent study, the same NPs were shown to suppress head and neck tumor growth in a murine xenograft model. Very recently, similar CD particles have also been used in a first-in-human phase 1/2a trial for the delivery of camptothecin in patients with advanced solid tumor malignancies.

4.4 Pullulan

Pullulan is a neutral, homopolysaccharide consisting of α-(1,6)-linked maltotriose residues (Figure 1). It is produced from starch, primarily by strains of the fungus Aureobasidium pullulans. Pullulan’s unique linkage pattern contributes to exceptional physiochemical properties, such as adhesiveness, water solubility, and relatively low viscosity upon dissolving in water. Therefore, pullulan and its derivatives have been used in food and in the pharmaceutical industry.

4.4.1 Pullulan-Based NPs

Pullulan has been shown to self-assemble into NPs after modification by hydrophobic molecules, such as cholesterol and stearic acid. In addition, a pullulan-drug conjugate and covalently cross-linked pullulan NPs have been reported. Overall, pullulan-based NPs have been used for the delivery of proteins, anti-cancer drugs, imaging agents, and nucleotides.

The K. Akiyoshi group has been developing self-assembled NPs of cholesterol-modified pullulan for more than a decade. The hydrophobically modified pullulan self-assembles in water into monodisperse and colloidal stable NPs. Akiyoshi and co-workers examined the effect of various modified cholesterol moieties, different Mws of pullulan, and different degrees of substitution of the cholesterol moiety. The self-assembled particles demonstrated the ability to complex various substances, including soluble proteins, such as insulin, mainly by hydrophobic interactions. The complex between the NP and protein in solution was easily formed by simply mixing the two components. The complexation greatly contributed to the thermal stability of the encapsulated proteins (even after heating for 6 h at 90 °C) and protected the proteins from enzymatic degradation, while preserving their bioavailability.

The molecular chaperon-like activity of the NPs was demonstrated on proteins, using a system consisting of
the cholesterol-pullulan NPs and β-CD. Capture of heat-denatured, unfolded protein and the release of the refolded form were achieved. In addition, following heating, irreversible protein aggregation was completely prevented, and almost 100% of protein activity was recovered.[14]

The NPs were also tested in the area of cancer vaccine development. Clinical studies demonstrated that subcutaneous injection of NPs carrying the cancer antigen HER2 (CHP-HER2) or NY-ESO-1 (CHP-NY-ESO-1) induced an antigen-specific CD8+ cytotoxic T lymphocyte response and antibody production.[52]

Recently, the cholesteryl-pullulan NPs were used as an antigenic protein delivery system for adjuvant free intranasal vaccines.[52] Intranasal delivery of a non-toxic subunit fragment of Clostridium botulinum type-A neurotoxin, using these NPs, resulted in vigorous induction of botulinum neurotoxin A-neutralizing serum IgG and secretory IgA antibody responses. Furthermore, intranasal immunization of tetanus toxoid with the NPs induced strong tetanus toxoid-specific systemic and mucosal immune responses.

4.5 Arabinogalactan

Arabinogalactan is a long, highly branched natural carbohydrate composed mostly of galactose and arabinose. Arabinogalactan is extracted mainly from the Larix tree and is available at 99.9% purity, with reproducible molecular weight (Mw) and physicochemical properties.[54] The unusual water solubility (70% w/w in water), biocompatibility, biodegradability, and ease of drug conjugation in aqueous media makes arabinogalactan attractive as a potential drug carrier.[54]

4.5.1 Arabinogalactan-Based NPs

Arabinogalactan has been used as a carrier for drug conjugates. Recently, Arabinogalactan-folic acid-drug conjugates for targeted delivery and activated drug release were prepared and characterized.[54] The targeted nanovehicle was formed by conjugation of folic acid and the anti-cancer drug methotrexate to arabinogalactan. The use of folic acid as a targeting ligand derives from the fact that cancer cells overexpress receptors for nutrients in order to maintain their fast-growing metabolism.[3] One of these receptors is the folate receptor, which is overexpressed in malignant cells, including ovary, brain, kidney, breast, colon, and lung cancer cells.[54] Another advantage of using nutrient receptors as receptor targets is that they enable internalization of the nanocarrier via receptor-mediated endocytosis. The activated drug release was achieved by linking methotrexate to arabinogalactan by an endosomally cleavable peptide Gly-Phe-Leu-Gly (GFLG). The nanocarrier displayed significantly greater cytotoxic activity to folate receptor overexpressing cells, in comparison to folate receptor deficient cells.

4.6 Dextran

Dextran is a high Mw carbohydrate composed of α-(1,6)-linked glucan, with side chains attached to the 3-positions of the backbone glucose units (Figure 1). Dextran is obtained from bacterial cultures of lactic acid bacteria such
as *Leuconostoc mesenteroides* NRRRL B-512.\(^8\) Dextran is soluble in water and also in a wide range of other solvents. The presence of dextran degrading enzymes derived from anaerobic Gram negative intestinal bacteria is a motivating factor for the development of dextran-based NPs for colon targeted drug delivery.\(^8\) Dextran bears several advantages as a polymer for drug delivery: it is highly water soluble, biocompatible, biodegradable, lacks nonspecific cell binding, and is resistant to protein adsorption.\(^9\) In addition, dextran is easily functionalized via its reactive hydroxyl groups.\(^9\)

### 4.6.1 Dextran-Based NPs

Several strategies have been reported for preparation of dextran-based NPs, among which are conjugation of dextran with drugs and self-assembly of hydrophobically modified dextran.\(^9\)

Upon conjugation of poorly water soluble drugs to dextran, via activation of the carbohydrate groups with N,N′-carbonyldiimidazole, hydrophobic derivatives that self-assemble into NPs are formed.\(^9\) The self-assembled particles were shown to be stable at pH 4 and 11 and to have a high loading efficiency. Particle size was strongly influenced by the degree of substitution and by the preparation technique.

Dextran conjugated with hydroxyethyl methacrylate (HEMA) was used for the preparation of NPs for siRNA delivery, using the inverse mini-emulsion photo-polymerization method.\(^9\) For the preparation of the NPs, HEMA was conjugated to dextran via a carbonate ester that is subject to hydrolysis under physiological conditions, in order to enable biodegradation of the NPs. The dextran-HEMA was photopolymerized with cationic methacrylate, which enables the NP to entrap siRNAs through electrostatic interactions. The obtained particles were shown to have high siRNA loading capacity and biodegradability, a trait that was shown to be essential for effective gene silencing, and to lack significant cytotoxicity. In addition, confocal microscopy analysis revealed endolysosomal localization of the NPs, following internalization into human hepatoma cell line HuH-7. In order to determine the effect of enhanced endosomal escape on the extent of gene silencing, the authors tested photochemical internalization and the use of an influenza-derived fusogenic peptide. Photochemical internalization is a method in which amphiphilic photosensitizers are utilized to destabilize endosomal vesicles. Fusogenic peptides are peptides of viral origin, which are involved in the fusion between the viral envelope and the endosome and assist in transportation of the viral genome into the cytoplasm following endocytosis.\(^9\) Both methods of endosomal escape significantly improved gene silencing.

Dextran sulfate-based NPs have mainly been prepared by PEC, exploiting the anionic nature of dextran sulfate for electrostatic interaction with positively charged poly-

### 4.6.2 Dextran-Coated NPs

Dextran coating of NPs has been shown to promote long term circulation of NPs in vivo, due to reduction in protein adsorption and opsonisation and increasing stability.\(^6\) While the circulation time of uncoated poly (methyl methacrylate) particles was only 3 minutes, the dextran-coated particles eliminated slowly over 48 h. In *in vitro*, heparin- or dextran-coated NPs were also demonstrated to be uptaken more slowly by a macrophagic cell line than were uncoated particles.\(^6\) The steric barrier formed by the dense brush-like arrangement of the attached carbohydrate chains could contribute to the long-circulating properties of the dextran- (or heparin-) coated PMMA NPs.

Recently, an artificial oxygen carrier based on a carbohydrate-decorated NP was reported.\(^6\) The core-shell NPs, developed as red blood cell substitutes, were cov-
ered with a long brush of carbohydrates (heparin, dextran, or dextran sulfate) and demonstrated very low complement activation. The NPs were obtained by using a redox radical polymerization mechanism in an aqueous medium, which was followed by adsorption or coupling of hemoglobin. In addition, the anti-coagulant properties of heparin were preserved upon coating the NPs with hemoglobin. The bound hemoglobin preserved its ability to exchange oxygen.[61]

4.7 Alginate

Alginate is a linear anionic carbohydrate composed of alternating blocks of 1,4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues (Figure 1). The monomer composition of alginate is variable and can consist of homopolymeric blocks or alternating M and G residues.[62] The composition, sequence, and molecular weight determine the physical properties of alginate.[63] Alginites are extracted mainly from brown algae, and acetylated forms of alginate can be isolated from the bacteria Pseudomonas and Azotobacter.[62]

As a polymer used in drug delivery, alginate possesses several attractive properties: it is biocompatible, nontoxic, water soluble, and highly mucoadhesive.[62]

4.7.1 Alginate-Based NPs

The early works reported on alginate-based NPs were focused on the ability of alginate to form 3D networks upon ionic inter- and intramolecular cross-linking with divalent ions.[66] Since then, several preparation mechanisms have been utilized and alginate based NPs have been developed for the delivery of proteins, genes, and anti-tubercular and anti-fungal drugs.[64]

The group of Gopal K. Khuller has presented several papers describing alginate NPs for the treatment of tuberculosis, a disease in which patient non-compliance is one of the main contributors towards treatment failure and multi-drug resistance.[66] Alginate encapsulation of anti-tubercular drugs may present a possible solution, as it has been reported to improve drug bioavailability and enable a controlled release profile of the anti-tubercular drugs isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), and ethambutol (EMB), in comparison to free drugs.[65] The NPs were prepared by calcium-induced gelification, followed by polyelectrolyte complexation with CS, and were about 235 nm in size and characterized by high encapsulation efficiencies. Calcium cross-linked alginate NPs containing econazole and anti-tubercular drugs for the treatment of murine tuberculosis were reported.[65] The NPs significantly increased drug circulation time and managed to reduce bacterial burden in the lungs and spleen of mice infected with Mycobacterium tuberculosis by more than 90% at 15-fold lower dosages than those required of free drugs to obtain the same result.

Recently, surfactant-alginate hybrid NPs have been employed for dual chemotherapeutic and photodynamic therapy on a murine drug-resistant tumor model.[66] Following administration to Balb/c mice bearing syngeneic J774A.1, the dual therapy significantly inhibited tumor growth and improved animal survival. The treatment resulted in enhanced tumor accumulation of both doxorubicin and methylene blue, significant inhibition of tumor cell proliferation, and increased induction of apoptosis.

Chemically modified alginate has also been developed for the preparation of NPs. As with CS, chemical modifications improve the physiochemical characteristics of alginate. For example, thiolated alginate, obtained by covalent attachment of cysteine, improves the mucoadhesive properties of alginate and provides improved stability of the drug delivery system.[67] Hydrophobically modified alginate derivates have also been produced for the preparation of self-assembled NPs for the sustained release of vitamin D3.[68]

4.7.2 Alginate-Coated NPs

Alginate-coated CS NPs were recently developed for the oral delivery of a legumain DNA vaccine.[69] The NPs managed to significantly reduce tumor volume in a murine orthotopic 4T1 breast cancer model and caused an increase in active cytotoxic T lymphocytes.

5. Future outlook

The variety of properties of naturally occurring carbohydrates has been successfully utilized to generate multiple drug delivery systems on the nano-scale. Such properties enable the preparation of nanocarriers for the delivery of proteins, peptides, antibiotics, and nucleic acids, using several administration routes. In addition, promising results from clinical trials using carbohydrate-based nanocarriers have been presented. Further understanding of the mechanisms involved in drug delivery, both at the pathophysiological level (anatomical barriers) and the material and chemical level (chemistry responsible for specificity toward biological targets), coupled with greater knowledge of how the host immune system reacts to different chemicals could result in improved drug delivery systems, tailor-made for a particular application.

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