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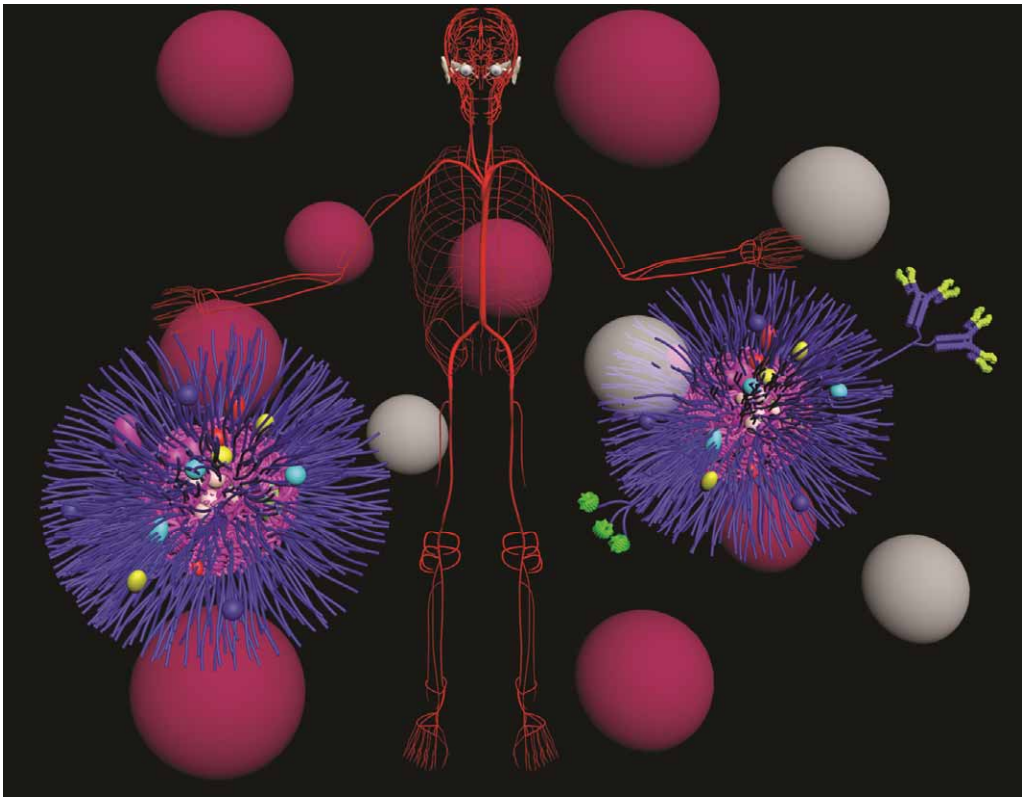

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TUTORIAL REVIEW

Polysaccharides as building blocks for nanotherapeutics†

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The use of polysaccharides as building blocks in the development of nano-sized drug delivery systems is rapidly growing. This can be attributed to the outstanding virtues of polysaccharides such as biocompatibility, biodegradability, low toxicity and low cost. In addition, the variety of physicochemical properties and the ease of chemical modifications enable the preparation of a wide array of nanoparticles. This *tutorial review* describes the properties of common polysaccharides, the main mechanisms for polysaccharide based-nanoparticles preparation, and provides examples from the conceptual design towards pre-clinical and clinical applications.

1. Introduction

Over the past two decades nanoparticles (NPs)-based therapeutics have been introduced for the treatment of cancer, diabetes, allergy, neurodegenerative diseases, infections and inflammation.[1,2] The growing interest in NPs derives from the outstanding advantages they offer, which include protection of the drug from premature degradation, the ability to deliver poorly-water soluble drugs alone or in combination with soluble drugs, controlled drug release mechanisms, improved biodistribution and pharmacokinetics and enhanced intracellular penetration.[2]

The size, geometrical shape and surface characteristics of a NP are crucial for the control of its biodistribution *in vivo*.[1] The small size, which enables NPs to pass through the smallest capillaries, also promotes passive tumor targeting due to the enhanced permeability and retention (EPR) effect of the tumor vasculature (Fig. 1). The passive targeting is achieved by extravasation of NPs through increased permeability of the newly formed tumor blood vessels, caused by rapid and defective angiogenesis.[2] In addition, the impaired tumor lymphatic drainage leads to accumulation of NPs in tumor surroundings in a size dependent manner.[2] Cellular targeting can be achieved by binding a targeting agent (ligand) onto the NPs surface, this will allow the particles to actively bind to specific cells following extravasation (Fig. 1).[2]

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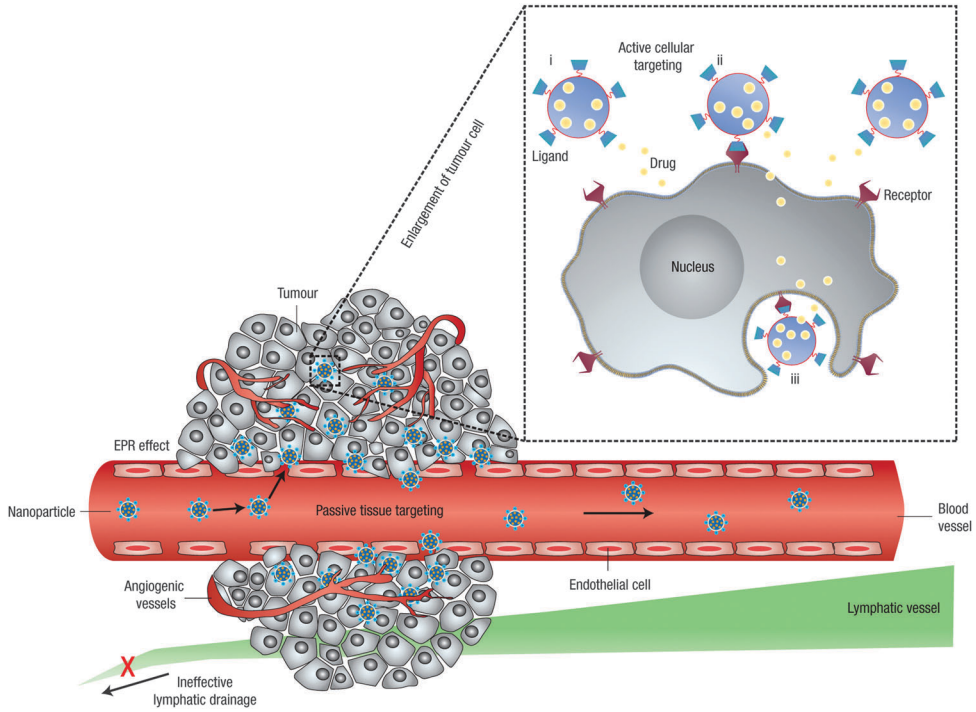

Fig. 1 Passive and active tumor targeting. Passive tissue targeting is achieved by extravasation of nanoparticles through increased permeability of the tumor vasculature and ineffective lymphatic drainage (EPR effect). Active cellular targeting (inset) can be achieved by functionalizing the surface of nanoparticles with ligands that promote cell-specific recognition and binding. The nanoparticles can (i) release their contents in close proximity to the target cells; (ii) attach to the membrane of the cell and act as an extracellular sustained-release drug depot; or (iii) internalize into the cell. Reprinted with permission from ref. 2. Copyright 2007 *Nat. Nanotechnol.*

The effect of spherical, rigid NPs size on circulation time has been studied extensively and size ranges between 100–200 nm have been determined optimal for long circulation.[1] These NPs are large enough to avoid renal clearance and liver uptake and in the appropriate size to evade uptake by the mononuclear phagocyte system (MPS).[1,3] The particle size, shape and the presence of surface ligands also play a crucial role in intracellular delivery since it affects the mechanism of cellular internalization.[1,2] The specific endocytotic mechanism is significant since it determines the intracellular path and microenvironment encountered by the NPs. For example, in clathrin-mediated endocytosis, which is characteristic for specific particle sizes and also for certain ligands, NPs should be capable of endosomal escape in order to avoid the harsh lysosomal environment.[1] The effects of particle geometry on cell internalization and biodistribution are just being revealed and yet a recent literature points out that the particles' geometry is as crucial as its size.[1]

The surface feature of the NP is another major factor in determining the biodistribution. Poly(ethylene glycol) (PEG), which has been initially used to promote long circulation of therapeutic proteins, has been shown to elongate the circulation time of NPs. The PEG coating alters the hydrophobicity of the NP's surface protecting it from protein opsonisation—a process that marks the NP for removal from the circulation by specialized macrophages.[2]

When designing a NP as a therapeutic entity, the mechanism for release of the therapeutic cargo should also be considered. Several activated release methods have been utilized to break the bond between the drug and NP leading to particle degradation, among which are enzymatic cleavage, pH or thermal instability.[1]

As the requirements from NPs as therapeutic carriers are becoming clear so are the requirements from the materials used for their preparation. These materials should be biocompatible and biodegradable, well characterized and easily functionalized.[2] Polysaccharides successfully fulfill all of these requirements and are therefore widely used as the building blocks for the preparation of NPs as drug delivery vehicles.

This *tutorial review* details the properties of polysaccharides used for the preparation of drug delivery vehicles, evaluates the challenges and discusses the opportunities in this exciting field.

2. Polysaccharides

Polysaccharides are polymers of monosaccharides joined by glycosidic bonds. These highly abundant molecules are 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.* CS, hyaluronan, chondroitin and heparin).[4] Naturally accruing polysaccharides are diverse in their physiochemical properties; there are multiple chemical structures (Fig. 2), the chemical composition greatly varies and so are the molecular weight (Mw) and ionic nature. This versatility also contributes to a wide range of biological activities. From a pharmaceutical standpoint, polysaccharides possess many favorable characteristics such as low toxicity, biocompatibility, stability, low cost, hydrophilic nature and availability of reactive sites for

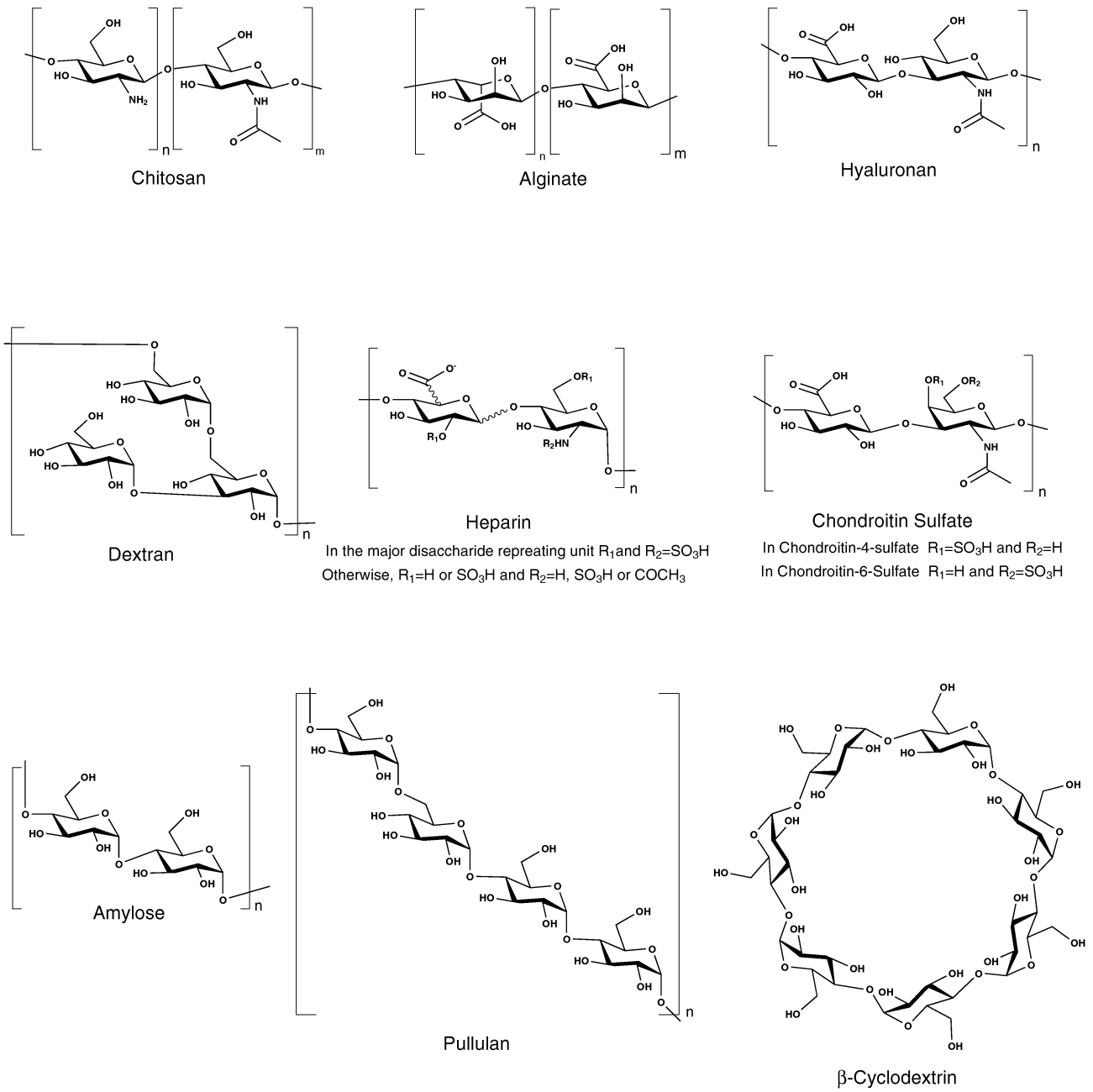

Fig. 2 Chemical structure of polysaccharides. Chitosan, the *N*-deacetylated derivative of chitin is composed of β (1,4)-*N*-acetyl-D-glucosamine and D-glucosamine. Hyaluronan is composed of alternating β-(1,4)-D-glucuronic acid and β-(1,3)-*N*-acetyl-D-glucosamine. Alginate is composed of alternating blocks of β-(1,4)-D-mannuronic acid and α-(1,4)-L-guluronic acid. Chondroitin sulfate is composed of alternating β-(1,3)-*N*-acetyl-D-galactosamine and β-(1,4)-glucuronic acid. Amylose is composed of α-(1,4)-D-glucose units. 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.

chemical modification.[4,5] Chemical functionalization using mainly the free carboxyl and hydroxyl groups distributed along the polysaccharides backbone has been used to create derivatives with determined/tailored properties.[6] The solubility, hydrophobicity, physicochemical and biological characteristics of polysaccharides have been modified. This was performed by using techniques such as oxidation, sulfation, esterification, amidation, or grafting methods.[6]

Another advantage of polysaccharides is bioadhesion, especially for mucosal surfaces, which has been used for targeting specific organs or cells and prolonging the drug residence time. All of these qualities have led to the growing use of polysaccharides in drug delivery systems.

The properties of common polysaccharides used for the preparation of drug delivery systems are detailed below.

2.1 Chitosan

Chitosan (CS) is a linear polysaccharide composed of β-(1,4)-linked D-glucosamine and *N*-acetyl-D-glucosamine (Fig. 2).

CS is obtained by deacetylation from chitin, a highly abundant polysaccharide, which is the main component of the crustaceans exoskeleton.[7] CS based delivery systems have been described for nasal, ocular, oral, parenteral and transdermal drug delivery. Among the many advantages of CS are its low cytotoxicity and biocompatibility.[7] As such, CS is approved for dietary applications in Japan, Italy and Finland and it has been approved by the FDA for use in wound dressings.[8] Interestingly, CS and its derivatives were found to be toxic to several bacteria, fungi and parasites,[8] another welcome trait when aiming for delivery of pharmaceuticals. In addition, CS is positively charged and therefore can interact with negatively charged molecules such as negatively charged polysaccharides, polyanions, nucleic acid and negatively charged proteins.[9] Its positive charge also facilitates adherence to mucosal surfaces, which are mostly negatively charged.[7] In spite of these advantages, CS is inherently insoluble in aqueous solutions above pH 6.5.[10] 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.[7]

Comparison of CS and its derivatives to synthetic cationic polymers such as polyethylenimine (PEI) reveals significantly lower toxicity. However, upon increasing CS charge density in order to improve cell uptake, its toxicity increased[8] as can be expected when taking into consideration the charge related adverse effects of positively charged synthetic lipids and polymers. Nevertheless, this is not necessarily problematic since a significant improvement in cell uptake can be achieved simply by grafting ligands that mediate endocytosis onto CS or any other polysaccharide. CS derivatives that do not increase charge density and all low Mw CS and its derivatives (<10 kDa) are not appreciably toxic.[8] Yet, every newly formed derivative should be examined for toxicity and used in the purest form.[8] This justifies the selection of CS (and its derivatives) that provides health benefits over current formulations as a safe material in drug delivery.[8]

2.2 Alginate

Alginate is a linear anionic polysaccharide composed of alternating blocks of 1,4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues (Fig. 2). The monomer composition of alginate is variable and can consist of homopolymeric blocks and alternating M and G residues.[11] The composition, sequence, and molecular weight determine the physical properties of alginate.[11] Alginates are extracted mainly from brown algae and acetylated forms of alginate can be isolated from the bacteria *Pseudomonas* and *Azotobacter*.[11]

As a polymer used in drug delivery, alginate possesses several attractive properties: it is biocompatible, non-toxic, water soluble and highly mucoadhesive.[11]

2.3 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 linkage[12] (Fig. 2). Hyaluronan holds remarkable hydrodynamic properties especially regarding its viscosity and ability to retain water.[12] It was previously regarded as important for joint lubrication and organ structural stability.[12] However, HA was found to be essential for proper cell growth, embryonic development, healing processes, inflammation, and tumor development.[12,13] As opposed to other GAGs, HA is not sulfated, not linked to a protein[12] and is naturally produced by bacteria as a capsule. Commercially available HA is either produced through bacterial fermentation of *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.[13] 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.[13] CD44 and CD168 are overexpressed by various tumors, for example, squamous cell carcinoma, ovarian, colon, stomach, glioma, and many types of leukemia, lymphoma and multiple myeloma, which make the use of HA as a targeting agent even more attractive.

2.4 Dextran

Dextran is a high Mw polysaccharide composed of α-(1,6)-linked glucan with side chains attached to the 3-positions of the backbone glucose units (Fig. 2). Dextran is obtained from bacterial cultures of lactic acid bacteria such as *Leuconostoc mesenteroides* NRRL B-512.[4] Dextran is water-soluble and also soluble in a wide range of 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.[4] Dextran bears several advantages as a polymer for drug delivery: it is highly water soluble, biocompatible, biodegradable, lacks nonspecific cell binding and resistant to protein adsorption.[5] In addition, dextran is easily functionalized *via* its reactive hydroxyl chemistries.[5]

2.5 Cyclodextrins

Cyclodextrins (CDs) are natural cyclic oligomers of α-(1,4) linked-glucopyranosyl that are produced from starch by enzymatic conversion (Fig. 2). 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.[14,15] 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.[14] 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.[14] The mechanism for this is not completely clear but may be attributed to the fact that CDs have the ability to stabilize biomolecules in physiological media by shielding them from non-specific interactions.[14] This shielding ability may explain the protection against nucleases and interactions with specific proteins that may lead to immune stimulation.[16] In addition, CDs have been shown to have

membrane permeation capabilities.[14] This can attribute to lowering the amount of time the oligonucleotides are found in the circulation and exposed to nucleases.

CDs are biocompatible, do not elicit an immune response, and have low toxicities in animals and humans.[15] Therefore, they are used in pharmaceutical applications for numerous purposes, including improving the bioavailability of drugs.[15] CDs based therapeutics have been reviewed elsewhere.[14,15]

2.6 Arabinogalactan

Arabinogalactan is a long, highly branched natural polysaccharide 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.[17] The unusual water solubility (70% w/w in water), biocompatibility, biodegradability and ease of drug conjugation in an aqueous medium makes arabinogalactan attractive as a potential drug carrier.[17]

2.7 Pullulan

Pullulan is neutral, homopolysaccharide consisting of α–(1,6)-linked maltotriose residues (Fig. 2). It is produced from starch primarily by strains of the fungus *Aureobasidium pullulans*.[18] The unique linkage pattern of pullulan's structure 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 industrially in foods and pharmaceuticals.[18]

2.8 Heparin

Heparin is a linear glycosaminoglycan (GAG) composed of repeating disaccharide units of 1,4-linked uronic acid (D-glucuronic (GlcA) or l-iduronic acid (IdoA)) and D-glucosamine (GlcN) (Fig. 2). The uronic acid usually comprises 90% l-idopyranosyluronic acid (l-iduronic acid, IdoA) and 10% D-glucopyranosyluronic acid (D-glucuronic acid, GlcA). In addition, there are structural variations at the disaccharide level.[19] Due to high content of sulfo and carboxyl groups, heparin has the highest negative charge density of any known biological molecule.[19] The Mw of heparin varies between 5–40 kDa and it is extracted mainly from mucosal tissues of porcine and bovine.[19] Clinically, heparin has been used as an anticoagulant since the 1930s.[19] Heparin is produced exclusively by mast cells (as opposed to the structurally related GAG heparan sulfate). Beyond its anticoagulant activity, heparin has been shown to have antiviral activity, regulate angiogenesis and inhibit complement activation.[20]

3. The main mechanisms of nanoparticle preparation from polysaccharides

3.1 Cross-linking

In crosslinked NPs, the polymeric chains are interconnected by crosslinkers, leading to the formation of a 3D network (Fig. 3A and B).[21] The main factor, which determines the properties of a crosslinked NP such as drug release and mechanical strength, is the cross-linking density, which is determined by the molar ratio between the crosslinker and the polymer repeating units.[21] There are two kinds of crosslinked NPs determined by the nature of the cross-linking agents: covalently crosslinked NPs and ionically crosslinked NPs.

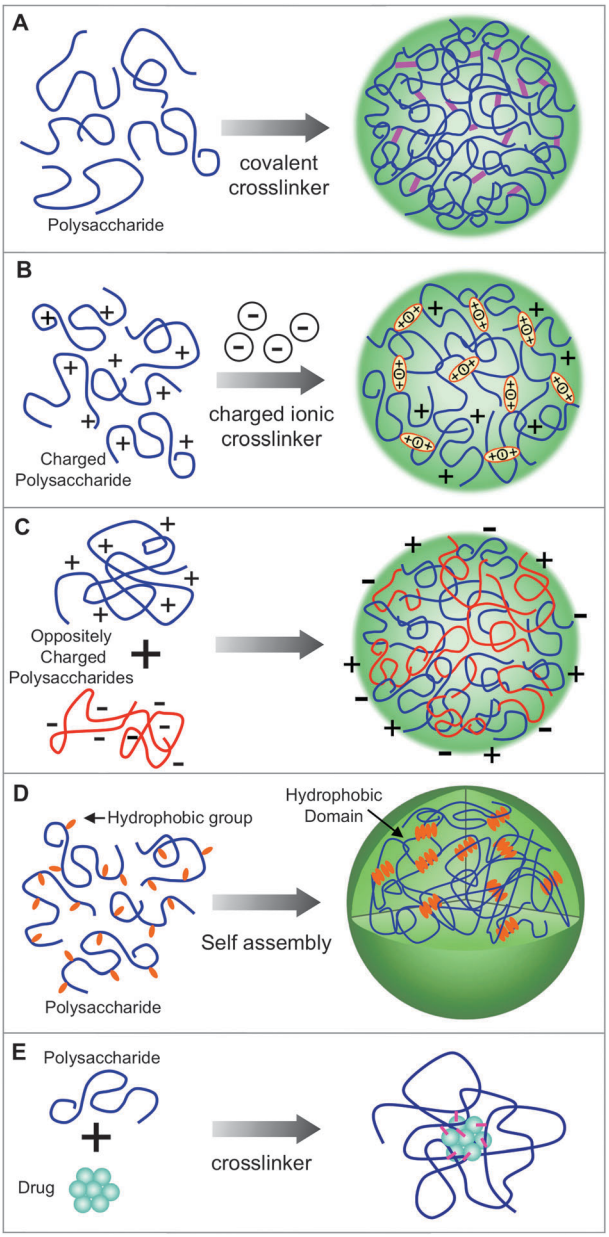

Fig. 3 Common mechanisms for polysaccharide based nanoparticle preparation. (A) Covalent cross-linking. (B) Ionic cross-linking. (C) Polyelectrolyte complexation (PEC). (D) Self-assembly of hydrophobically modified polysaccharides. (E) Polysaccharide–drug conjugate.

3.1.1 Covalent cross-linking. In a covalently cross-linked NP, the network structure is permanent since irreversible chemical links are formed unless biodegradable or stimuli-responsive crosslinkers are employed (Fig. 3A).[21] The rigid network allows absorption of water and bioactive compounds without dissolution of the NP even when the pH drastically changes.[21] A covalently crosslinked NP can contain more than one type of a polysaccharide. The covalent bonds are the main interactions that form the 3D network although secondary

interactions such as hydrogen bonds and hydrophobic interactions also exist.[21] Covalent crosslinkers are molecules with at least two reactive functional groups that allow the formation of bridges between the polymeric chains.[22] The most common covalent crosslinkers used with polysaccharides are dialdehydes such as glutaraldehyde. However, dialdehydes are highly toxic and therefore biocompatible alternatives have been tested. For example, natural di- and tricarboxylic acids have been used for intramolecular cross-linking of CS, which was facilitated by the condensation agent *N*,*N*-(3-dimethylaminopropyl)-*N*-ethyl carbodiimide (EDC).[9] Another alternative is Genipin, a natural biocompatible crosslinker isolated from the fruits of *Gardenia jasminoides* Ellis.[21]

3.1.2 Ionic cross-linking. Ionic cross-linking represents a simple alternative to covalent cross-linking for charged polysaccharides. This method enables the preparation of NPs by the formation of reversible ionic cross-linking and since no harsh preparation or toxic crosslinkers are used these NPs are generally considered biocompatible.[21] Charged polysaccharides can form ionic crosslinked NPs with oppositely charged ions or small ionic molecules (Fig. 3B). For example, the polyanion tripolyphosphate (TPP) has been widely used to crosslink the CS, and divalent cations such as Ca^{2+} have been used to crosslink alginate.[7] The mechanism of NP formation is based on electrostatic interactions between the polysaccharide and oppositely charged ionic crosslinker. The ionic bonds that form bridges between the polysaccharide chains are the main interactions inside the network, although as with covalent cross-linking, additional interactions such as hydrogen bonds are also present.[21] Several factors influence the cross-linking reaction, most crucial are the size of the crosslinker and the global charge of the crosslinker and the polysaccharide.[21] Unlike covalently crosslinked NPs, ionic crosslinked particles are generally pH sensitive, a welcome trait for drug delivery purposes. However, this pH sensitivity can also contribute to instability of the ionic crosslinked network.[21]

3.2 Polyelectrolyte structures

3.2.1 Polyelectrolyte complexes (PEC). Polyelectrolyte complexes (PEC) are formed by direct electrostatic interactions of oppositely charged polyelectrolytes in solution (Fig. 3C). PEC represent another biocompatible option for drug delivery since non-toxic covalent crosslinkers are used. These complexes resemble ionic cross-linking since non-permanent networks are formed that are more sensitive to changes in environmental conditions.[23] However, unlike ionic cross-linking, in which ions or ionic molecules react with the polyelectrolyte, in PEC the interaction is between the polyelectrolyte and larger molecules with broad Mw range.[21] The formation and stability of PEC are determined mainly by the degree of interaction between the polyelectrolytes.[22] The latter is a factor of the charge density and distribution of each of the oppositely charged polyelectrolyte. The chemical environment is also crucial: the pH of the solution, the ionic strength, the temperature, and the duration and mixing order. Secondary factors are the Mw of the polyelectrolytes and their flexibility.[22,24] Ionic cross-linking can reinforce the formed interaction. Positively charged polysaccharides, namely CS, can form PEC with a variety of negatively charged polymers such as the polysaccharides alginate, dextran sulfate, chondroitin sulfate, hyaluronan, carboxymethyl cellulose, carrageenan and heparin. In addition, peptides such as poly-g-glutamic acid, nucleic acids and synthetic polymers could also be used.[21,24]

3.2.2 Layer by layer assembly. Layer by layer (LbL) assembly of polyelectrolyte NPs is a relatively new and therefore less common form of polyelectrolyte-based nano-sized delivery systems. The LbL technique was originally used for generating thin polyelectrolyte layers on solid surfaces and has shown broad biomedical applications in biosensing, regenerative medicine, tissue engineering, and biomimetics research.[25] This technique, which is based on electrostatic interactions, employs alternate adsorption of oppositely charged polyelectrolytes.[26] The obtained film structure can be controlled to 1 nm precision with a range from 5 to 1000 nm and with a definite knowledge of the molecular composition (see also Fig. 9C).[26] The assembly procedure involves the incubation of charged solid support in an oppositely charged polyelectrolyte solution. This results in the adsorption of the first layer, which is subsequently rinsed to remove excess free polyelectrolyte and immersed in a solution containing a second polyelectrolyte to create the second layer. The process is repeated until the desired thickness is obtained.[26] The polyelectrolyte adsorption at each layer is performed to a level of saturation and therefore the terminal charge is alternated after every subsequent layer deposition.[26] The demonstration of multilayer assembly on a three-dimensional support presented this technique for applications in nano-sized delivery systems.[25] The advantages for the LbL approach are clear: many therapeutics and biomaterials can be incorporated into LbL films non-covalently and under physiological conditions, without compromising their biological properties.[25] In addition, since the technique can be used with multiple components, several factors can be manipulated, among them are the surface chemistry, dimensions of the thin films, therapeutic moieties used and, thus a sophisticated nano-sized drug delivery system can be created with a tailored drug release mechanism.[25] Furthermore, LbL NPs can be used for the delivery of multiple active substances with the ability to control the release of each entrapped substance.[25] The possibility to reach sequential drug release from LbL film layers enables the control of the order and timing of multiple drug release.[25]

3.3 Self-assembly

Upon grafting hydrophobic moieties onto a hydrophilic polysaccharide, 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 (Fig. 3D).[27] This self-assembly process is *via* hydrophobic interactions, mainly in order to minimize interfacial free energy.[9,24] The formed NPs are characterized by prolonged circulation and thermodynamic stability.[28] In addition, since the 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 a particular application. For example, the size of the NPs can be controlled by adjusting the

length of the hydrophobic moiety and the length of the polymer.[27] Scaling relations for this purpose have been developed.[27] In addition, the surface charge, which affects particle serum stability and cellular uptake, can be altered by controlling the degree of substitution, the length or nature of the hydrophobic moiety.[24]

Polysaccharides can be modified with a wide range of hydrophobic moieties, among them are bile acids (*e.g.*, 5β-cholanic acid, cholic acid and deoxycholic acid), fatty acids (*e.g.*, palmitoyl acid, stearic acid, oleic acid),[24] cholesterol and hydrophobic drugs.

3.4 Polysaccharide–drug conjugate

The concept of polymer–drug conjugate was first introduced in 1975 by Ringsdorf for the delivery of small hydrophobic drugs.[24] The conjugation to a polymer alters the biodistribution and circulation time of the free drugs, which are capable after conjugation of being selectively delivered and accumulated at the tumor site due to the EPR effect (Fig. 3E). The benefits of this delivery system have led to several phase I/II clinical trials.[24] This promising concept has been used for the preparation of polysaccharide–drug conjugates especially for the delivery of non-soluble anticancer drugs as will be detailed below.

The polysaccharide–drug conjugate consists of three parts: the water-soluble polymer, the drug and the biodegradable spacer connecting the two. Additional components such as labeling agents and targeting moieties may also be included. A special attention as far as rational design of this delivery system 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 to increase circulation time but rapidly broken after cell entry.[29] In addition, the spacer should release an intact drug molecule without altering its chemical structure.[29] Furthermore, the polysaccharide itself should be stable enough in the bloodstream.[29]

The final size and shape of the polysaccharide–drug conjugate greatly depend on the characteristics of the components for example, conjugation of a hydrophobic drug results in self-assembly to a spherically shaped NP with the drug physically trapped inside the particle.

4. Polysaccharide-based nanoparticles

4.1 Chitosan based nanoparticles

Chitosan (CS)'s nature and the ease of chemical modifications enable multiple NP preparation schemes, among them are covalent cross-linking, ionic cross-linking, polyelectrolyte complexation, and self-assembly.[7] Since CS is the only positively charged polysaccharide it has been extensively used to generate polysaccharides particles as drug delivery systems.

Early work describing CS NPs for drug delivery was based on covalently crossing CS with glutaraldehyde.[7] However, since glutaraldehyde is highly toxic, biocompatible alternatives for covalent cross-linking have been developed such as condensation reactions with *N*,*N*-(3-dimethylaminopropyl)-*N*-ethyl carbodiimide (EDC),which was used to facilitate intramolecular cross-linking of CS by natural di- and tricarboxylic acids.[9] This method allows the formation of polycations, polyanions, and polyampholyte NPs.

Since CS is positively charged, ionic cross-linked particles can be prepared using polyanions; among them the most widely used is tripolyphosphate (TPP). The wide use of TPP in the preparation of CS NPs is a result of both being non-toxic and of the ability to modulate particle size, morphological properties and surface charge mainly by controlling the CS to TPP weight ratio.[30] One of the first TPP cross-linked CS NPs for drug delivery were developed by Alonso's group based on a principle reported previously by Bodmeier *et al.*[7] This ionotropic gelation technique involves the addition of an alkaline phase containing TPP into an acidic phase containing CS.[7] The formation of the NPs upon mixing the two phases is immediate and involves inter and intramolecular linkages created between TPP phosphates and CS amino groups.[7] The characteristics of the NPs obtained were shown to be depended on several factors such as the concentrations of CS and TPP, CS's purity and molecular weight.

The group of Alonso later reported the use of these particles 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 both for plasmid DNA and dsDNA oligomers (20-mers), independent of CS's Mw.[31] All obtained NPs showed high physical stability with no detection of DNA release even after incubation with heparin as a competitive anion. The efficiency of transfection, however, was highly depended on the Mw of CS (Fig. 4). Unlike the NPs based on high Mw CS, the low Mw CS/TPP particles gave high gene expression levels in the human embryonic kidney cell line (HEK 293 cells) 2 days post transfection, which was sustained for up to 10 days.[31] In addition, only the low Mw CS/TPP NPs mediated a strong β-galactosidase expression *in vivo* after intratracheal administration.

As discussed earlier, the positive charge of CS enables the formation of polyelectrolyte complexes (PEC) with negatively charged polymers such as negatively charged polysaccharides, nucleic acids, negatively charged peptides and poly(acrylic acid). Polyelectrolyte complexation is one of the most frequently used methods to prepare CS NPs. PEC of CS with γ-poly-(glutamic acid), a natural, non-toxic, and biodegradable negatively charged polymer, have been prepared for oral administration of insulin.[32] These NPs, which were stabilized with TPP and magnesium sulfate, were pH sensitive and had an average size of 218 nm in diameter. The particles were shown to be safe, adhered to mucosal surfaces and induced a significant hypoglycemic action for at least 10 h in diabetic rats when administered orally. The bioavailability of insulin, which was determined from plasma insulin concentration, was about 15%. The same group also reported transdermal delivery of DNA containing CS–γ-poly-(glutamic acid) NPs.[9] These particles showed improved skin penetration and enhanced gene expression in comparison to particles solely comprised of CS and DNA. This can be attributed to a greater density of the γ-poly-(glutamic acid) containing NPs, which contributed to a larger penetration momentum into the skin barrier. Another study describing PEC of CS and DNA was reported by Krishnendu *et al.*,[33] which showed that these PEC can generate immunologic protection in a murine model of peanut allergy. The mice that received NPs containing a dominant peanut allergen gene

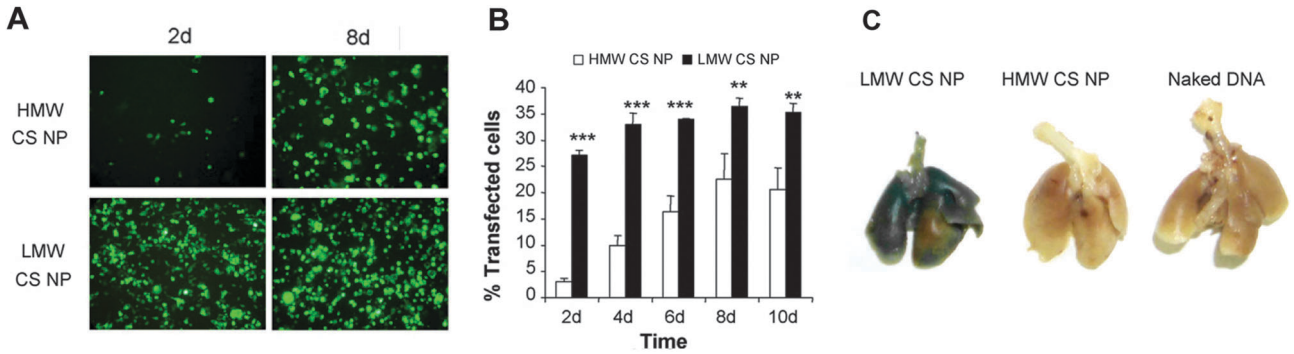

Fig. 4 Ionically crosslinked CS/tripolyphosphate (CS/TPP) nanoparticles for oligonucleotide and plasmid DNA delivery. (A) and (B) Transfection efficiency of LMW CS/TPP nanoparticles. Comparison of GFP transfection efficiency between LMW and HMW CS/TPP nanoparticles at 10 and 20% pDNA loading, respectively, at a dose of 1 μg pDNA. (C) Macroscopic evaluation of β-galactosidase expression in mouse lungs. Lungs were analyzed 72 h after administration of LMW CS/TPP (10% pDNA loading) and HMW CS/TPP nanoparticles (20% pDNA loading) at a dose of 10 μg pCMV-βGal. Naked pCMV-βGal was used as control. Reprinted with permission from ref. 31. Copyright 2009 *Int. J. Pharm.*

produced secretory IgA and serum IgG2a and showed a substantial reduction in allergen-induced anaphylaxis associated with reduced levels of IgE, plasma histamine and vascular leakage. More recently, plasmid–CS PEC were applied in the delivery of basic fibroblast growth factor (FGF-2) and platelet-derived growth factor (PDGF-BB), which regulate cell growth and division.[34] Plasmid–CS PEC containing FGF-2/PDGF-BB genes were injected into BALB/C mice. Several formulations were tested, which differed in the degree of CS deacetylation and Mw. ELISA assays performed on mice sera showed FGF-2 and PDGF-BB expression. In addition, induction of specific antibodies against these proteins has been shown. PEC containing highly deacetylated low Mw CS were found to efficiently induce protein expression with minimal production of neutralizing antibodies, which was also confirmed by histological analyses.

Recently, PEC of ultrapure CS monomers were used for ocular gene delivery.[35] The NPs, which were created based on a method developed by Koping-Hoggard *et al.*,[36] had an average size of ~100 nm in diameter and a strong positive charge. This formulation demonstrated effective transfection of kidney cell line COS-7 *in vitro* and 5.4-fold higher luciferase gene expression than polyethylenimine–DNA NPs had upon injection to rat corneas. The preparation method of PEC of CS and DNA was further improved by P. Artursson *et al.*, who optimized the balance between stability and unpacking of PEC containing CS oligomers of different sizes.[37] PEC of CS oligomers (or low Mw CS) and pDNA have several advantages over PEC of high Mw CS with pDNA: high Mw CS forms extremely stable PEC with DNA, which delays the release of DNA and therefore results in a slow onset of action.[36] In addition, these PEC are of aggregated shapes, their viscosity at concentration used for *in vivo* delivery is very high and their solubility at a physiological pH is low.[36]

CS drug conjugates have also been developed mainly for the delivery of insoluble anti-cancer agents. Very recently, an interesting pH responsive CS–drug conjugate has been introduced (Fig. 5) for photodynamic therapy.[38] The system is composed of a glycol CS backbone, a functional group (3-diethylaminopropyl isothiocyanate, DEAP block), a photosensitizing model drug (chlorine e6 block, Ce6) and PEG. The system is designed to form a 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 tumor surrounding due to protonation of the functional group DEAP. The authors tested this conformational change by examining particle sizes that have changed dramatically from 150 nm spherically shaped NPs at pH 7.4 to 3.4 nm disentangled form at pH 6.8. In addition, the zeta potential also 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 phototoxicities of HeLa cells were 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.

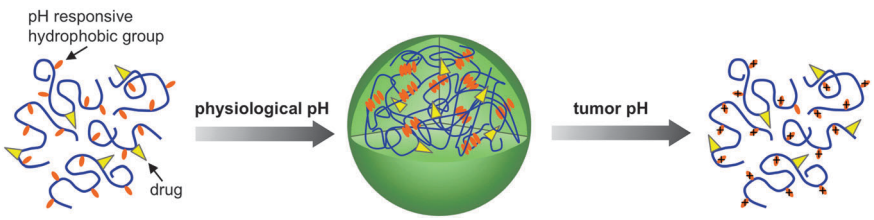

Fig. 5 A pH responsive polysaccharide–drug conjugate. At physiological pH values the polysaccharide–drug conjugate self-assembles into a nanoparticle. When reaching the acidic tumor environment, protonation of the functional group enables disassembly of the nanoparticle and accelerated drug release.

Chemical modification, usually by utilizing 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.[7] This principle was used for preparation of 5β-cholanic acid (HGC) modified glycol CS NPs for the delivery of the antiangiogenic peptide RGD.[39] The RGD (Arg–Gly–Asp) peptides can specifically target $\alpha_v\beta_3$ integrins on angiogenic endothelial cells and therefore inhibit angiogenesis and tumor growth however, these peptides have short half-live *in vivo* and thus a delivery system is required.[39] The self-assembled polymeric NPs of glycol CS modified with hydrophobic bile acid analogs have hydrophilic shells of glycol CS and hydrophobic cores of bile acid derivatives. The hydrophobically-modified glycol CS was prepared by covalently attaching 5β-cholanic acid through amide formation. The NPs had a diameter of 230 nm and loading efficiency of >85%. The particles demonstrated prolonged and sustained release of drug efflux and inhibition of human umbilical vein endothelial cells (HUVEC) adhesion *in vitro*. In the *in vivo* study, the RGD containing NPs inhibited basic (b)FGF-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 the chemotherapeutic agents doxorubicin and paclitaxel.[9]

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

4.2 Alginate based nanoparticles

The early work reported on alginate-based NPs was focused on the ability of alginate to form a 3D network upon ionic inter- and intramolecular cross-linking with divalent ions.[40] Since then, several preparation mechanisms have been utilized and alginate based NPs have been developed for the delivery of proteins, genes, anti-tubercular and anti-fungal drugs.[41]

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.[42] Alginate encapsulation of anti-tubercular drugs may present a possible solution by improving drug bioavailability and enabling a controlled release profile as has been reported for the anti-tubercular drugs isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol (EMB) in comparison to free drugs.[42] The NPs were prepared by calcium induced gelification followed by polyelectrolyte complexation with CS and were characterized by a size of about 235 nm and high encapsulation efficiencies (70–90%, 80–90%, and 88–95% for INH, RIF, and EMB, respectively). Another advantage of the alginate NPs is the ability to co-encapsulate multiple anti-tubercular drugs. Calcium cross-linked alginate NPs containing econazole and anti-tubercular drugs for the treatment of murine tuberculosis were reported.[42] The encapsulated drugs were detectable above minimum inhibitory concentration for 15 days after administration in lungs, liver and spleen of the treated mice in comparison to 12–24 hours of the free drugs. In addition, the alginate particles 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 in comparison to free drugs.

CS alginate NPs were used for the transmucosal delivery of insulin.[43] The NPs were prepared by ionic cross-linking of CS and TPP, which was followed by complexation with alginate. The obtained NPs size was affected by the overall concentration of the added electrolyte, the mass ratio of alginate and CS, and by the molecular weight of the alginate. Insulin was associated to the CS–TPP–alginate NPs with loading efficiency of 41–52%. The CS–TPP–alginate NPs were administered nasally and exhibited a capacity to enhance systemic absorption of insulin. The CS–TPP–alginate NPs were characterized by a longer hypoglycemic response in comparison to CS–TPP NPs. The duration of the hypoglycemic response also depended on the Mw of alginate.

Recently, surfactant–alginate hybrid NPs have been employed for dual chemo- and photodynamic therapy on a murine drug-resistant tumor model.[44] The obtained NPs had an average size of 70 nm measured by Dynamic Light Scattering (DLS) and contained doxorubicin and methylene blue with encapsulation efficiencies of 78% and 82%, respectively. Following administration to Balb/c mice bearing syngeneic JC tumors (mammary adenocarcinoma), 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 achieved by covalent attachment of cysteine improves the mucoadhesive properties of alginate and provides improved stability of the drug delivery system.[45] Hydrophobically modified alginate derivatives have also been produced for the preparation of self-assembled NPs for the sustained release of vitamin D3.[46]

4.3 Hyaluronan based nanoparticles

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 crosslinked NPs. Chemically modified HA has also been widely used for the delivery of proteins, peptides and nucleotides.[47] Chemical modifications assist in prolonging HA half life however, beyond a certain level of modification, HA loses the ability to bind its receptors.[47]

HA based NPs hold a major advantage over other polysaccharides 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.[13] HA–drug conjugates are internalized *via* CD44 receptor-mediated

endocytosis and the drug is released mainly by intracellular enzymatic hydrolysis.[13] In addition to targeting, HA conjugation has been used to increase drug solubility. This quality was used for the delivery of the hydrophobic antimitotic chemotherapeutic agent, paclitaxel (PTX).[13] HA–PTX conjugates were shown to increase cellular uptake and cytotoxicity *in vitro* in comparison to the free drug.[13] 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 polysaccharide, chondroitin sulfate (see Fig. 2).[13] In another study, the antitumor 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 ovarian cell line.[13]

Recently, HA conjugation was used for the delivery and improved serum stability of Exendin 4.[48] Exendin 4 (exenatide) is a 39 amino acid peptide incretin mimetic that exhibits glucoregulatory activities.[48] Exendin 4 has been shown to induce glucose-dependent enhancement of insulin secretion, glucose-dependent suppression of inappropriately high glucagon secretion, slowing of gastric emptying, reduction of food intake and body weight, and an increase in β-cell mass.[48] However, exendin 4 has a significantly short half-life, which limits its clinical application.[48] Conjugation to vinyl sulfone modified HA resulted in 20-fold increase in serum stability *in vitro* without loss of bioactivity. HA–exendin 4 conjugates lowered glucose levels in type 2 db/db mice and the hypoglycemic effect lasted up to 3 days after injection. In addition, insulin immunohistochemical analysis of islets in db/db mice confirmed the improved insulinotropic activity of the HA–exendin 4 conjugates. HA conjugates have also been suggested for the treatment of acute promyelocytic leukemia (APL).[49] APL patients often relapse due to resistance to the therapy with all-*trans* retinoic acid. Because of the molecular basis of APL alteration and previous success in treating tumors with HA conjugated to the histone deacetylase inhibitor butyric acid,[49] conjugation of HA to both all-*trans* retinoic acid and butyric acid has been tested. *In vitro*, the HA conjugates induced growth arrest and terminal differentiation in retinoic acid sensitive cells and apoptosis in retinoic acid resistant cells. *In vivo*, HA conjugates led to a significant increase in survival time of a retinoic acid sensitive APL murine model in comparison to that induced by a maximum tolerated dose of retinoic acid alone. In addition, in a retinoic acid resistant murine model, the HA conjugates were active in contrast to retinoic acid that was completely ineffective.[49]

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 transfect ocular tissue.[50] The NPs were prepared by ionically cross-linking CS with TPP, which was followed by PEC with HA. The HA-low Mw CS particles led to high expression levels of the transfected alkaline phosphatase in a human corneal epithelium model. Upon topical administration to rabbits, the NPs managed to overcome cellular barriers and were located inside the corneal and conjunctival cells, suggesting that they penetrate the epithelia by a transcellular pathway. In addition, the *in vivo* transfection levels reached were significant.

As with other polysaccharides, self-assembly of hydrophobically-modified HA has been used for NP preparation. In a recent study, HA was modified with the 5β-cholanic acid to form self-assembled NPs that combine both passive tumor targeting based on the EPR effect and a more specific or active targeting exploiting the affinity of HA towards CD44 (Fig. 6A).[51] The obtained NPs size could be controlled in the range of 240–430 nm by varying the degree of substitution of the hydrophobic moiety. *In vitro,* fluorescently labeled Cy5.5–HA-NPs were detected in the cytosol of CD44 overexpressing cells (SCC7) to a much greater extent than cells with low CD44 expression (CV-1) (Fig. 6B). When administered systemically to tumor bearing mice, the particles were shown to selectively accumulate in tumor sites. Smaller HA-NPs were able to reach the tumor site more effectively than larger HA-NPs. In addition, the concentration of the NPs in the tumor site was dramatically reduced when mice were pretreated with free HA (Fig. 6C). This suggests an additional active targeting mechanism, beyond the passive targeting of the EPR effect.

Another recent study demonstrated the antitumor activity of self-assembled modified HA-NPs.[52] To this end, poly-(γ-benzyl L-glutamate) modified HA NPs were loaded with DOX. The NPs presented a sustained drug release pattern at pH 5.5 and 7.4 for up to 10 days. *In vitro*, the NPs were tested on both MCF-7 human breast cancer cells highly expressing CD44 and U87 human glioblastoma cells characterized by low CD44 expression. The accumulation of the NPs in MCF-7 and U87 was consistent with CD44 levels as demonstrated by flow cytometry. Microscopic evaluation revealed that the NPs were present mostly in the cytoplasm of both cell lines. *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.

4.4 Dextran based nanoparticles

Several strategies have been reported for dextran based NPs, among them are dextran–drug conjugates and self-assembly of hydrophobically-modified dextran.[5]

Upon conjugation of poorly water-soluble drugs to dextran *via* activation of the carboxylic groups with *N*,*N*′-carbonyldiimidazole, hydrophobic derivatives that self-assemble into NPs are formed.[53] The self-assembled particles were shown to be stable at pH values ranging between 4 and 11 and with high loading efficiency. The particle size was strongly influenced by the degree of substitution and preparation technique.

Dextrane conjugated with hydroxyethyl methacrylate (HEMA) was used for the preparation of NPs for siRNA delivery using the inverse miniemulsion photopolymerization method.[54] For the preparation of the NPs, HEMA was conjugated to dextran *via* a carbonate ester that is subject to hydrolysis under physiological conditions to enable biodegradation of the NPs. The dextran–HEMA was photopolymerized with cationic methacrylate that enables the NPs to entrap siRNAs based on electrostatic interactions. The obtained particles were characterized by high siRNA loading capacity, no significant cytotoxicity and biodegradability, a trait that was shown to be essential for effective gene silencing. In addition, confocal microscopy analysis revealed endolysosomal localization of the NPs following internalization

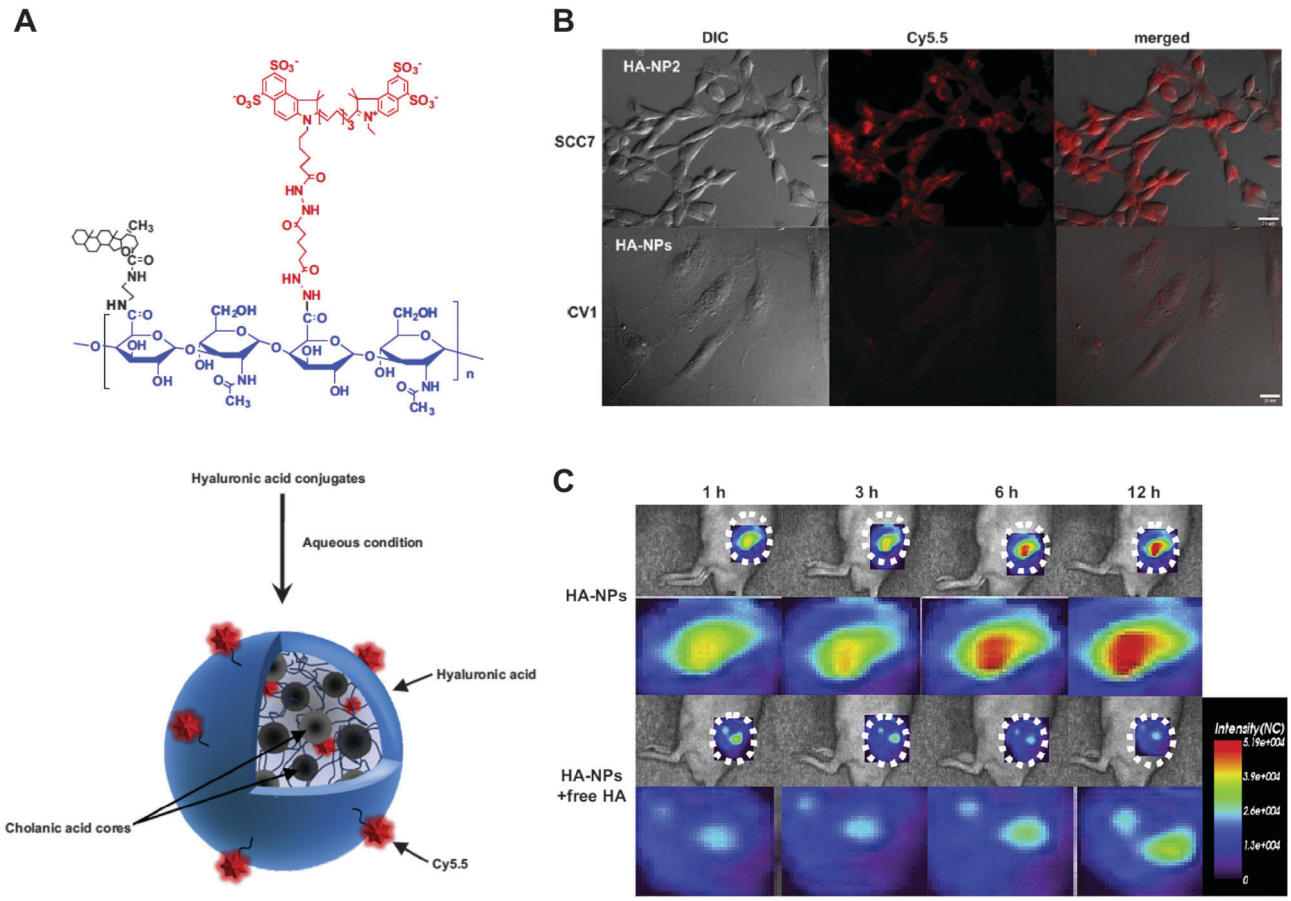

Fig. 6 Self-assembled hyaluronan-based nanoparticles for active tumor targeting. (A) Structure of Cy5.5-labeled hyaluronan nanoparticles in aqueous solution. (B) Confocal images of SCC7 tumor cells and CV-1 cells. The cells were treated with Cy5.5-labeled HA-NPs. (C) *In vivo* accumulation of HA-NPs using fluorescence images. Reprinted with permission from ref. 51. Copyright 2010 *Biomaterials*.

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 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 to transport the viral genome into the cytoplasm following endocytosis.[55] Both methods of endosomal escape significantly improved gene silencing.

Dextran sulfate based NPs have been mostly prepared by PEC, exploiting the anionic nature of dextran sulfate for electrostatic interaction with positively charged polycations (for example, CS and polyethylenimine). Huang *et al.*[56] prepared CS–dextran sulfate PEC for the controlled release of vascular endothelial growth factor (VEGF). VEGF, a growth factor, which stimulates angiogenesis and therefore desired as a therapeutic approach for ischemic conditions, was shown to generate new blood vessels *in vivo*. However, intravenously injected VEGF was not clinically successful and implantable controlled release devices have shown that localized and sustained release of VEGF is required for its favorable action. NPs of ~250 nm were prepared in which the heparin binding domain of VEGF was utilized to bind the polyanion dextran sulfate. The encapsulation efficiency of VEGF was high (85%) and controlled release of active VEGF persisted for more than 10 days. The activity of VEGF was determined by ELISA and by the ability to stimulate endothelial cell proliferation (mitogenic assay). PEC-containing different polycations (polyethylenimine and poly-L-lysine) were also tested, however CS–dextran sulfate complexes were preferred because of their biodegradability, desirable particle size, higher entrapment efficiency, controlled release, and mitogenic activity. In a following study by the same group[57] Repifermin® containing NPs were prepared in the same manner. Repifermin® is a truncated form of fibroblast growth factor-10 that exhibits promise in wound healing applications. The challenge of the delivery lies in the instability of this protein. The resulted 250 nm particles showed high encapsulation efficiency and the release of active Repifermin® was controlled for more than 10 days. In addition, the mitogenic activity of Repifermin® on human umbilical cord vascular endothelial cells was only demonstrated for encapsulated and not free Repifermin®.

Zinc stabilized complexes of dextran sulfate and polyethylenimine (PEI) have been used for the delivery of proteins, DNA and the poorly water-soluble antifungal agent amphotericin B.[58] The preparation method of these NPs was complex coacervation, a method that was previously used for microencapsulation.[58] The effects of preparation conditions

and composition on the physicochemical properties of the particles have been determined.[58] The sizes of the amphotericin B containing particles ranged from 100 to 600 nm, with a zeta potential of 30 mV and drug recovery efficiency of up to 85%. Particle size was shown to be controlled by processing parameters such as the pH of the PEI solutions, the ratio of the two polymers and the concentrations of dextran sulfate and zinc sulfate. The amphotericin B-containing particles displayed no toxicity in tissue culture in contrast to free drug and were almost as efficacious as free drug in killing *Candida albicans*.

4.5 Cyclodextrin based nanoparticles

Cyclodextrin (CD) containing polymers have been used in pharmaceutical applications since the 1980's.[15] 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.[14] 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 preexisting polymer.[14] Another classification of CD-containing polymers relies on the obtained polymer's charge whether it is 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.[15]

The group of Mark E. Davis had a major contribution in the area of gene delivery using CDPs. They have 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.[14] In addition, the relations between the polycation structure and gene delivery capabilities were determined.[14] Among the structural characteristics examined were CDs size, distribution and nature of cationic elements and the polymer size and polydispersity.[14] It was determined that low molecular weight polymers with sufficient distance between the CD units and the amidine cationic centers were optimal, contributing to both high delivery and low cytotoxicity.[14] 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 with both steric stabilization and targeting capabilities (Fig. 7).[14]

Structurally, the CDP assembles with the siRNA primarily *via* electrostatic interactions. It condenses siRNA and protects it from nuclease degradation.[59] The CDs within the polymer chains that reside on the surface of the NPs are used for assembling PEG that endows the NP with stearic stabilization by preventing aggregation and non-specific interactions with biological components. The PEG is conjugated to adamantine, which forms inclusion complexes with CD. The same principle is applied for the assembly of the targeting ligand transferrin, which was chosen since its receptor is upregulated on cancer cells (Fig. 7). Primary results from phase I clinical trials using siRNA against the M2 subunit of ribonucleotide reductase R2

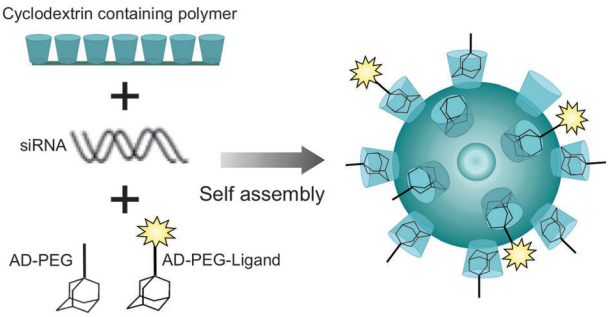

Fig. 7 Cyclodextrin based nanoparticles for systemic siRNA delivery. Schematic illustration of the delivery system. The polyethylene glycol (PEG) molecules are terminated with adamantane (AD) that form inclusion complexes with surface cyclodextrins to decorate the surface of the nanoparticle with PEG for steric stabilization and PEG-TF for targeting. Modified from ref. 59.

(RRM2) have been recently published.[59] Following systemic administration to patients with solid cancers, tumor biopsies revealed intracellular and dose depended localization of the NPs. In addition, specific reductions of both RRM2 mRNA and protein levels were observed. 5′-RLM-RACE analyses have shown that this reduction was mediated by an RNAi mechanism.[59] The paper describing results from the clinical studies did not contain data regarding the effects on human metabolism and immune response. However, following long-term administration to mice, no abnormalities in interleukin-12 and IFN-α, liver and kidney function tests, complete blood counts, or pathology of major organs were observed.[60] The administered NPs used for delivery of *EWS-FLI1* siRNAs, demonstrated a significant and ligand specific inhibition of tumor growth in a murine model of metastatic Ewing's sarcoma (Fig. 8).[60] Only targeted NPs, which contained siRNA against *EWS-FLI1* (a possible transcriptional activator that plays a significant role in the tumorigenesis of Ewing's sarcoma), were able to significantly inhibit tumor growth in comparison to naked siRNA, non-targeted NPs or targeted NPs containing control siRNA (Fig. 8A). The injection of luciferase expressing tumor cells enabled result quantification by measuring the tumor bioluminescent signal (Fig. 8B).

CS has been also used as a basis for the formation of CDP for gene delivery.[14] Alonso's group has demonstrated that pentasodium polyphosphate-mediated cross-linking of native CS with anionic CDs results in the formation of NPs (100–200 nm in diameter). The obtained NPs were characterized by higher pDNA loading capacity and stabilization in comparison to NPs produced without CDs and presented enhanced cellular uptake and protein expression.[14]

Other CD-based NPs have been developed for the delivery of drug and nucleic acids, among which are CD-based polyrotaxane–pDNA NPs and CD-based dendrimers.[14]

4.6 Arabinogalactan based nanoparticles

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.[17] The targeted nanovehicle was formed by conjugation of folic acid and the anticancer drug methotrexate to arabinogalactan. The use of folic acid as a targeting ligand derives from the fact that cancer

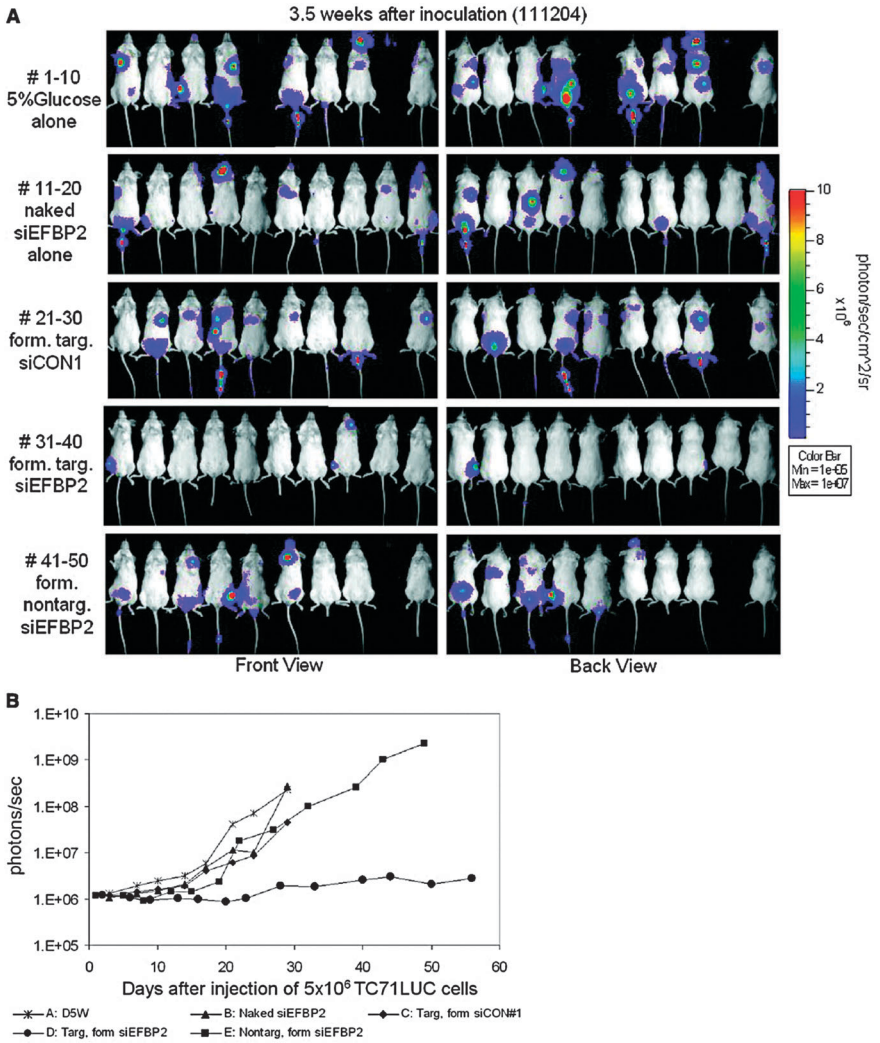

Fig. 8 Tumor growth inhibition in a murine model of metastatic Ewing's sarcoma as a result of sequence specific knockdown of EWS-FLI1 by a targeted cyclodextrin based siRNA delivery. (A) Bioluminescence imaging of NOD/scid mice treated twice weekly with formulated siRNA for 4 weeks. Starting immediately after injection of TC71-LUC cells, mice were treated with formulations containing siRNA targeting EWS-FLI1 (siEFBP2) or a nontargeting control sequence (siCON1). (B) Growth curves for engrafted tumors. The median integrated tumor bioluminescent signal (photons s^{-1}) for each treatment group (n = 8–10) is plotted *versus* time after cell injection. Treatment groups: A, 5% (w/v) glucose only (D5W); B, naked siEFBP2; C, targeted, formulated siCON1; D, targeted, formulated siEFBP2; and E, nontargeted, formulated siEFBP2. Reprinted with permission from ref. 60. Copyright 2005 *Cancer Res*.

cells overexpress receptors for nutrients in order to maintain their fast-growing metabolism.[2] One of these receptors is the folate receptor, which is overexpressed in malignant cells including ovary, brain, kidney, breast, colon, and lung.[17] 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 significant cytotoxic activity to folate receptor overexpressing cells in comparison to folate receptor deficient cells.

4.7 Pullulan based nanoparticles

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

Akiyoshi K. *et al*. have 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 colloidally stable NPs.[61] Akiyoshi and coworkers examined the effect of various modified cholesterol moieties, different Mws of pullulan and different degrees of substitution of the cholesterol moiety.[9] The self-assembled particles demonstrated the ability to complex various substances including soluble proteins such as insulin by mainly hydrophobic interactions.[61,62] The complex between the NP and protein solution was easily formed by simply mixing the two components.[62] The particle size did not change after complexation with the proteins. The complexation with insulin occurred faster than with larger proteins such as α-chymotrypsin or BSA.

These particles showed high colloidal stability with no dissociation of insulin or precipitation. The complexation greatly contributed to the thermal stability of insulin (even after heating for 6 h at 90 °C) and protected insulin from enzymatic degradation. In addition, *in vivo* experiments demonstrated preservation of insulin's bioavailability.[62]

The ability of these NPs to thermally stabilize proteins was further investigated. The molecular chaperon-like activity 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, an irreversible protein aggregation upon heating was completely prevented, recovering almost 100% of protein activity.[9]

Intracellular protein delivery by self-assembled NPs of cationic cholesteryl group-bearing pullulans was also demonstrated.[63] In this study, the cationic derivative of the cholesterol was used instead of cholesterol since it better facilitates cellular uptake. While particle size did not change significantly upon replacing the cholesterol with the cationic cholesteryl group, the zeta potential became positive ($+7.7 \pm 0.1$ mV in comparison to -1.3 ± 0.4). Additionally, the binding constant to the model protein, BSA, significantly grew. The cholesteryl-pullulan NPs demonstrated a more effective internalization of proteins into HeLa cells in comparison to cationic liposomes and a protein transduction domain (PTD)-based carrier. Upon cell internalization, the protein containing NP dissociated and the protein was released.

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.[61]

Recently, the cholesteryl–pullulan NPs were used as an antigenic protein delivery system for adjuvant free intranasal vaccines.[61] Intranasal delivery of a non-toxic subunit fragment of *Clostridium botulinum* type-A neurotoxin using these NPs resulted in antigen adherence to nasal epithelium. The NPs internalized into the nasal epithelium immediately after the intranasal administration and the antigen was later dissociated from the NPs in a controlled manner in the nasal epithelial cells. In addition, the chaperon-like activity reported for other proteins was demonstrated in this system as well. *In vitro*, circular dichroism analysis showed that the secondary structure of the antigen was changed after the incorporation into the NPs, but recovered after it is released. In order to prove induction of antigen specific respiratory immune response, flow cytometric and immunohistochemical analyses were utilized and demonstrated that within 6 h after NPs administration, the antigen released from the nasal epithelium was taken up by $CD11c^+$, dendritic cells. The NPs also induced vigorous 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.

5. Polysaccharide coated nanoparticles

Surface modification of NPs with polysaccharides has remarkable advantages for drug delivery systems: it endows the NP with long-term circulation and increased stability. The prolonged circulation time can be attributed to reduction in protein adsorption and opsonisation. In addition, it provides targeting abilities: pullulan coating has been used for hepatic delivery, alginate and CS coating have been used for mucosal delivery, mannan coating facilitated uptake by macrophages and HA coated NPs have been used to target CD44- and CD168-overexpressing cancer cells. Another advantage is the 'build-in' cryoprotection feature that has been reported for several polysaccharides. Furthermore, polysaccharide coated liposomes demonstrated reduced permeability to water soluble encapsulated materials and protection from degradation by lipases.[64]

Coating NPs with polysaccharides can be achieved by adsorption, incorporation, copolymerization or covalent grafting[65] and has been reported for many polysaccharides, among which are pectin, pullulan, mannan, HA, heparin, CS and dextran.[65]

5.1 Chitosan coated nanoparticles

The cationic nature of CS enables its adherence to mucosal surfaces. In addition, the ability of CS to open tight junctions between epithelial cells has also been demonstrated.[7] These characteristics make CS appealing as a coating agent of nanocarriers designed for mucosal delivery. For example, CS coated poly-ε-caprolactone (PECL) NPs allowed the bioavailability of the anti-inflammatory drug, indomethacin, in the cornea and aqueous humor following topical ocular instillation.[65] In addition, CS coating of liposomes (chitosomes) enhanced mucosal adhesion in rat intestine following oral administration.[7] 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.[7]

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.[65]

Structural benefits of coating NPs with CS have also been demonstrated: CS coated liposomes were more stable in simulated gastric fluids in comparison to uncoated liposomes.[7] CS coated PECL particles also demonstrated enhanced physical stability.[7] In addition, the CS coating also facilitated the redispersion of lyophilized PECL nanoparticles.[7]

5.2 Hyaluronan coated nanoparticles

The HA capsule of group A *streptococci* enables it to escape the host immune response[66] 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) (Fig. 9A).[67] tHA-LIP were 7- and 70-fold longer circulating in comparison to uncoated liposomes and free MMC, respectively, in 3 murine tumor models: BALB/c bearing C-26 solid tumors, C57BL/6 bearing B16F10.9 and D122 lung metastasis.[67] The HA on the tHA-LIP was covalently attached using EDC *via* the glucuronic carboxylate to phosphatidylethanolamine in the preformed liposomes. The tHA-LIP demonstrated slower drug efflux and higher encapsulation efficiency. In addition, since the

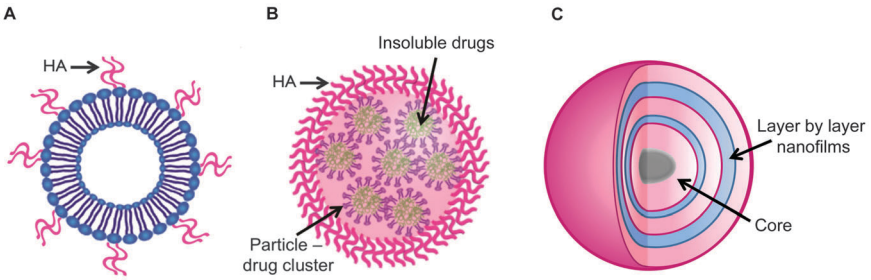

Fig. 9 Hyaluronan coated nanoparticles. (A) A schematic illustration of HA-coated liposome. (B) PTX-GAGs. (C) LbL NP with HA as an outer layer. Adapted from ref. 25.

effect of tHA-LIP is CD44 dependent, these NPs demonstrated significantly higher cytotoxicity *in vitro* on CD44 overexpressing cells and increased drug accumulation in tumors *in vivo*. The latter resulted in decreased metastasis, inhibition of tumor growth and prolonged survival. These effects were later demonstrated with doxorubicin loaded tHA-LIP.[3] This study also compared the tHA-LIP to poly(ethyleneglycol) (PEG) coated liposomes ("stealth" liposomes). This was done since PEGylation, a common hydrophilic surface modification of drug delivery systems, demonstrated prevention of recognition by the immune system. The tHA-LIP were long circulating more than all tested controls including uncoated and PEGylated liposomes in healthy and tumor bearing mice. Furthermore, the HA coating managed to prolong the circulation time without activating the complement, which is associated with PEG coated NPs.[68]

Recently, HA coated lipids–paclitaxel clusters (PTX-GAGs) and MMC-GAGs have been prepared for selective tumor targeting (Fig. 9B).[69,70] 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 *via* EDC with HA to facilitate targeting of CD44. 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 and showed superior therapeutic outcomes compared to free MMC.[70]

Targeting of CD44 was also demonstrated for liposomes decorated with HA oligomers.[71] Unlike the previously described tHA-LIP preparation, the HA oligosaccharides were conjugated by reductive amination to phosphatidylethanolamine 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 depended 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 in comparison to the free drug.

Another clinically relevant advantage of the HA coated NPs is cryoprotection.[72] Cryoprotection provides the liposomes with a longer shelf life since it prevents the reversion of lyophilized unilamellar liposomes to multilamellar liposomes upon rehydration. The tested lyophilized tHA-LIP demonstrated the ability to retain the same dimensions, zeta potential, encapsulation efficiencies and half-life of drug release of the original systems upon redispersion. HA cryopreservation is possibly by providing substitute structure-stabilizing H-bonds.

In a recent report, electrostatically assembled LbL NPs for cancer applications with an outer layer of polysaccharides have been presented (Fig. 9C).[25] The authors tested the effect of NPs stabilization on the biodistribution following systemic delivery. The LbL NPs were built on a core template of gold NPs (AuNPs) or quantum dots (QD) and were composed of dextran sulfate (DXS) and poly-L-lysine (PLL) layers with an outer layer of DXS or HA. *In vivo*, NPs showed increased stability when larger number of layers was used. The most outer layer was shown to be of significant importance to both biodistribution and non-specific uptake. An outer layer of HA resulted in a prolonged circulation time and low accumulation in the liver. The authors tested the NPs and nanofilms *in vivo* stability by administering NPs with a QD_{705} core that can be tracked systemically, together with the incorporation of a layer of labeled low Mw PLL. The authors assumed that in the case of *in vivo* instability of the NPs, the incorporated low Mw PLL would be filtered by the kidneys and detected in the bladder shortly after administration. Hence, early detection of high concentrations of the incorporated labeled PLL would indicate *in vivo* instability.

The passive tumor targeting ability *via* the EPR effect was tested post systemic intravenous injection in nude mice bearing subcutaneously induced tumors (Fig. 10A). 24 hours post injection, the HA coated NPs were detected in the tumors (Fig. 10B) and the time dependent NP signal from the tumors was shown (Fig. 10C).

5.3 Heparin and dextran coated nanoparticles

Heparin has been shown to inhibit complement activation at different stages by increasing the activity of protein H.[73] Since the activation of the complement system plays an important role in opsonization and uptake of particles by the mononuclear phagocyte system (MPS), surface modification of NPs with heparin seemed promising.[73] Indeed, coating NPs with heparin significantly promoted long-term circulation of these carriers.[73] The heparin modified poly(methyl methacrylate) (PMMA) NPs significantly elongated the *in vivo* half life.[73] While the circulation time of the uncoated poly (methyl methacrylate) particles was only 3 minutes, the heparin-coated particles circulated for more than 48 h post an initial phase of elimination from the blood with a half-life of 5 h. The heparin coated NPs were

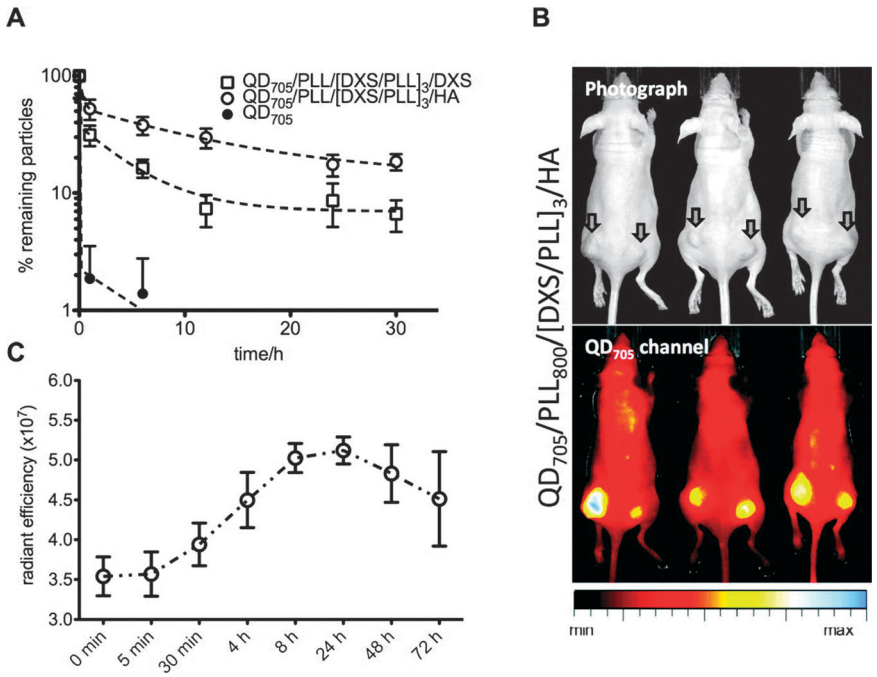

Fig. 10 Blood circulation and tumor targeting of optimized LbL nanoparticles. (A) Blood circulation profiles of QD_{705}, QD_{705}/PLL/(DXS/PLL)$_3$/HA, and QD_{705}/PLL/(DXS/PLL)$_3$/DXS. The longer persistence of QD_{705}/PLL/(DXS/PLL)$_3$/HA in the bloodstream corroborates their superior stability and biodistribution profile. (B) Enhanced permeation and retention (EPR)-based targeting of solid KB tumors induced subcutaneously on both hind flanks using QD_{705}/PLL/(DXS/PLL)$_3$/HA. Image is taken at 24 h time point. (C) Time-dependent accumulation of QD_{705}/PLL/(DXS/PLL)$_3$/HA in KB tumors. Accumulation of the nanoparticles in tumors is transient and typical of EPR dominated targeting. Reprinted with permission from ref. 25. Copyright 2011 *Nano Lett.*

still detectable in the plasma at 72 h. In addition, the dextran-coated NPs were also eliminated very slowly over 48 h. *In vitro,* the heparin or dextran coated particles were also demonstrated to be slower in the uptake by a macrophagic cell line in comparison to uncoated particles.[73] The steric barrier formed by dense brush-like arrangement of the attached polysaccharide chains could contribute to the long-circulating properties of the heparin (or dextran) coated PMMA NPs.

Recently, an artificial oxygen carrier based on a polysaccharide decorated NP was demonstrated.[74] The core–shell NPs, developed as red blood cell substitutes, were covered with a long brush of polysaccharides (heparin, dextran or dextran sulfate) and demonstrated very low complement activation. The NPs were obtained by using a redox radical polymerization mechanism in aqueous medium, which was followed by adsorption or coupling of hemoglobin. In addition, the anticoagulant properties of heparin were preserved upon coating the NPs with heparin. When benzene tetracarboxylic acid (BTCA) was used as a coupling agent for hemoglobin to dextran-coated NPs, the loading capacity showed a 9.3-fold increase over NPs in which BTCA was not used. The modification of NPs by BTCA slightly increased complement activation; however, this activation was reverted by the further addition of hemoglobin. The bound hemoglobin preserved its ability for exchanging oxygen.[74]

6. Summary and discussion

The variety of naturally occurring polysaccharide properties has been successfully utilized to create multiple nano-sized nanomedicines. The advantages of polysaccharides enable the preparation of nanocarriers for the delivery of proteins, peptides, antibiotics and nucleic acids using several administration routes. In addition, preliminary results from the first phase I clinical trial, using polysaccharide-based nanocarriers, have been presented.

Polysaccharides have been shown to posses several favorable traits in comparison to synthetic polymers currently used for drug delivery. Unlike synthetic polymers that can accumulate in the body above a certain Mw that enables renal clearance, polysaccharides have known biodegradation routes in which many of the specific involved enzymes have been identified.[8,12] In addition, for nucleic acid delivery purposes in which a positive charge is required to facilitate electrostatic interactions with the negatively charged nucleic acids, both CS and cyclodextrin containing polymers have been shown to be significantly less toxic than the currently used cationic polymers and lipids.[15] As far as prolonging circulation time, polysaccharides have advantages over the currently used PEG not only related to biodegradation, for example, HA coating of liposomes manage to elongate the circulation time without the adverse complement activation observed for PEGylated liposomes.[68]

Naturally, there are also disadvantages of using polysaccharides for drug delivery purposes, as polysaccharides are natural materials the final product may not be consistent. This may lead to batch variations, an unwelcome feature when aiming for pharmaceuticals. Nevertheless, several reports for polysaccharide production of defined size and content using *in vitro* recombination techniques are available.[75] In addition, the natural nature of polysaccharides can facilitate specific and unwanted reactions with the immune system. For example, it

has been reported that low Mw HA fragments can induce immune stimulation by interactions with Toll like receptors; however, so far no immune stimulation was reported when using NPs containing low Mw HA.[68]

The large variety of polysaccharides especially when also considering all possible modifications gives the advantage of obtaining defined materials for many specific applications but it can also be confusing when trying to choose the appropriate polysaccharide for a specific task. To this end, several issues should be taken into consideration such as the requested drug characteristics, the route of administration, the need for specific targeting and more. For example, several polysaccharide coatings have been shown to prolong the circulation time (*i.e.* hyaluronan, heparin and dextran), which specifies them for systemic applications. Polysaccharides chosen for colon delivery should be mucoadhesive, stable in the rough environment of the stomach and biodegradable by the specific enzymes found in the colon (*i.e.* dextran, amylose and pectin).[4] Upon aiming for specific targeting there are also several options as pullulan can be used to target hepatocytes; alginate and CS are mucoadhesive and can target mucosal surfaces such as vagina, rectal and lungs; mannan can facilitate macrophage targeting, and HA is the primary ligand for CD44 and CD168, and can target many types of cancer cells, stem cells and lymphocytes.

Further understanding of the characteristics of polysaccharides and the mechanisms involved in drug delivery will result in improved drug delivery systems tailor-made for a particular application.

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