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Review

Recent advances in pH/enzyme-responsive polysaccharide-small-molecule drug conjugates as nanotherapeutics

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ABSTRACT

Now-a-days, the polysaccharides are extensively employed for the delivery of small-molecule drugs ascribed to their excellent biocompatibility, biodegradability and modifiability. An array of drug molecules is often chemically conjugated with different polysaccharides to augment their bio-performances. As compared to their therapeutic precursors, these conjugates could typically demonstrate an improved intrinsic solubility, stability, bioavailability and pharmacokinetic profiles of the drugs. In current years, various stimuli-responsive particularly pH and enzyme-sensitive linkers or pendants are also exploited to integrate the drug molecules into the polysaccharide backbone. The resulting conjugates could experience a rapid molecular conformational change upon exposure to the microenvironmental pH and enzyme changes of the diseased states, triggering the release of the bioactive cargos at the targeted sites and eventually minimize the systemic side effects. Herein, the recent advances in pH and enzyme -responsive polysaccharide-drug conjugates and their therapeutic benefits are systematically reviewed, following a brief description on the conjugation chemistry of the polysaccharides and drug molecules. The challenges and future perspectives of these conjugates are also precisely discussed.

1. Introduction

Over the decades, the discovery and development of small-molecule drugs have been revolutionised (Hoelder, Clarke, & Workman, 2012). Unfortunately, a vast majority of the marketed small-molecule drugs demonstrate the undesirable physicochemical and biopharmaceutical attributes such as limited solubility, poor pharmacokinetics and biodistribution profiles and unwanted toxicities, which could often reduce their therapeutic outcomes (Hoelder et al., 2012). Currently, with rapidly advancing nanotechnology, a wide variety of nanoplatforms such as liposomes, nanomicelles, polymeric nanoparticles, nanocrystals, nanohybrids *etc.* have been developed and exploited for the drug delivery (Kargozar & Mozafari, 2018). These nanocarriers could improve the biopharmaceutical hurdles of the bioactive agents and exhibit promising therapeutic efficacy. Regrettably, most of these nanoplatforms suffer from the bottlenecks of low targeting efficiency and serious side effects, limiting their clinic translation potentials (Hoelder et al., 2012; Kargozar & Mozafari, 2018).

In recent years, the covalent conjugation of various small-molecule drugs with the polymeric vectors has received an overwhelming interest and created a new prospect in the drug delivery arena (Vogus, Krishnan, & Mitragotri, 2017). Ringsdorf firstly proposed the model of polymer-drug conjugates or polymeric prodrugs in 1975, consisting a biocompatible polymer covalently attached with a drug *via* a biologically responsive linker (Ringsdorf, 1975). Moreover, a targeting moiety or a solubilizer could be grafted into the conjugates to enhance their therapeutic efficiency. Over time, a flurry of reports on the polymer-drug conjugates have appeared (Vogus, Krishnan, et al., 2017). These conjugates could be self-assembled into nano-micellar structures in an aqueous phase and biodegraded under the influence of chemicals or enzymes, releasing the active drug molecules (Li et al., 2013). As compared to the polymeric nanoparticles containing physically encapsulated drugs, these systems display a high drug loading capacity, low premature drug leakage in the systemic circulation and controllable drug release pattern. In addition, these scaffolds could typically improve the physicochemical (solubility and stability against metabolic decomposition) and biological (bioavailability, plasma half-life and biodistribution) characteristics of the drug molecules, while minimizing

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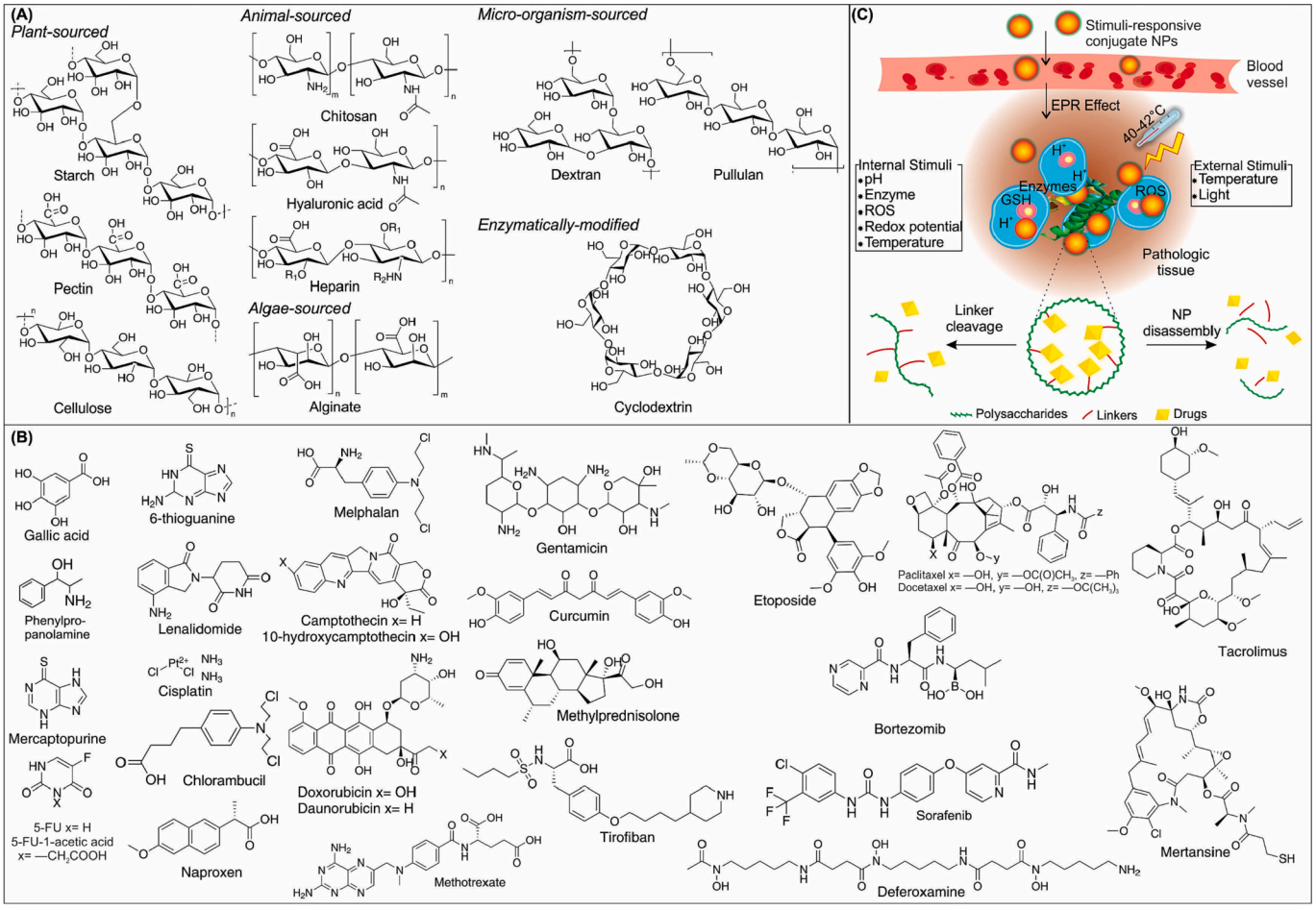

Fig. 1. Chemical structures of various polysaccharides obtained from different sources (A) and small molecule drugs (B) commonly used for fabricating stimuli-responsive conjugates and their schematic drug release mechanisms under different internal and external stimuli (C). Adapted from (Basu, Kunduru, Abtew, & Domb, 2015; Goodarzi, Varshochian, Kamalinia, Atyabi, & Dinarvand, 2013; Wadhwa & Mumper, 2015).

their toxicities (Girase, Patil, & Ige, 2020). A passive site-specific trafficking of these conjugates could be accomplished either through the enhanced permeation and retention (EPR) effects at a tumor sites or *via* electrostatic interactions with the surfaces of the liver and kidney. The conjugates are also coupled with various ligands, which could explicitly recognize and bind to the target receptors on the cells. This could enhance the bio-performances of the scaffolds by active targeting (Zhang et al., 2015).

Numerous formulations based on polymer-drug conjugates are entered into the market and several others are in clinical trials. Among various synthetic polymers, the polyethylene glycol (PEG) based drug conjugates are majorly approved by the US Food and Drug Administration (US-FDA) (Thakor et al., 2020). Despite of various successes, few related products were withdrawn from the market due to their inadequate drug loading capacity and unacceptable toxic residues produced following their biodegradation (Pang, Du, Zhang, Zhai, & Zhai, 2013). To address these shortfalls, the polysaccharides are currently being overexploited to fabricate drug conjugates attributed to their cost effectiveness, abundance in nature, remarkable biocompatibility and biodegradability. Various polysaccharides could also depict the synergistic therapeutic effects with the drug molecules (Deshpande & Jayakannan, 2017). The polysaccharides are carbohydrate polymers derived from algae, plant, microbial and animal origins. These are consisted of monosaccharide residues covalently attached together by α- or β-glycosidic linkages. The polysaccharides are primarily categorized into two subtypes, namely homo-polysaccharides and hetero-polysaccharides depending on their compositions. The homo-polysaccharides are composed of one type of monosaccharide as repeating unit in their chains, while the hetero-polysaccharides contain two or more different types of monosaccharide residues. These polysaccharides possess several reactive functional groups (such as, hydroxyl, amino and carboxyl moieties), which might readily be utilized as active sites for the drug conjugation either directly or *via* linkers (Abbasi, Panda, Arora, Layek, & Bera, 2021). The therapeutic efficacies of these conjugates are significantly influenced by the chemical structure, molecular weight, electric charge, functional groups, polydispersity and branching of the polysaccharides (Li, Ding, Zhuang, Chen, & Chen, 2016; Thummarati, Suksiriworapong, Sakchaisri, & Junyaprasert, 2021).

Preponderance of literatures have also reported several stimuli-responsive polysaccharide-drug conjugates, which could closely relate the surrounding microenvironmental states of the body and synchronize their drug release profiles (Fig. 1) (Hu et al., 2017; Yu, Ran, Shen, Zheng, & Cai, 2020). The distinct characteristics of these systems are their capability to undergo rapid microstructural changes (*viz.*, surface characteristics, shape, solubility, molecular assembly, gel-to-sol transition, *etc.*) in response to the slight changes in the microenvironment. In this context, either external or internal stimuli are utilized (Kargozar & Mozafari, 2018). The external stimuli are generated with the help of various stimuli-producing devices, which could ultimately cause a pulsed drug release from the systems. On the other hand, the drug release rates of the internal stimuli-responsive systems are controlled by the microenvironmental changes caused due to the disease associated altered metabolism (Sui et al., 2020). Among various stimuli, pH and

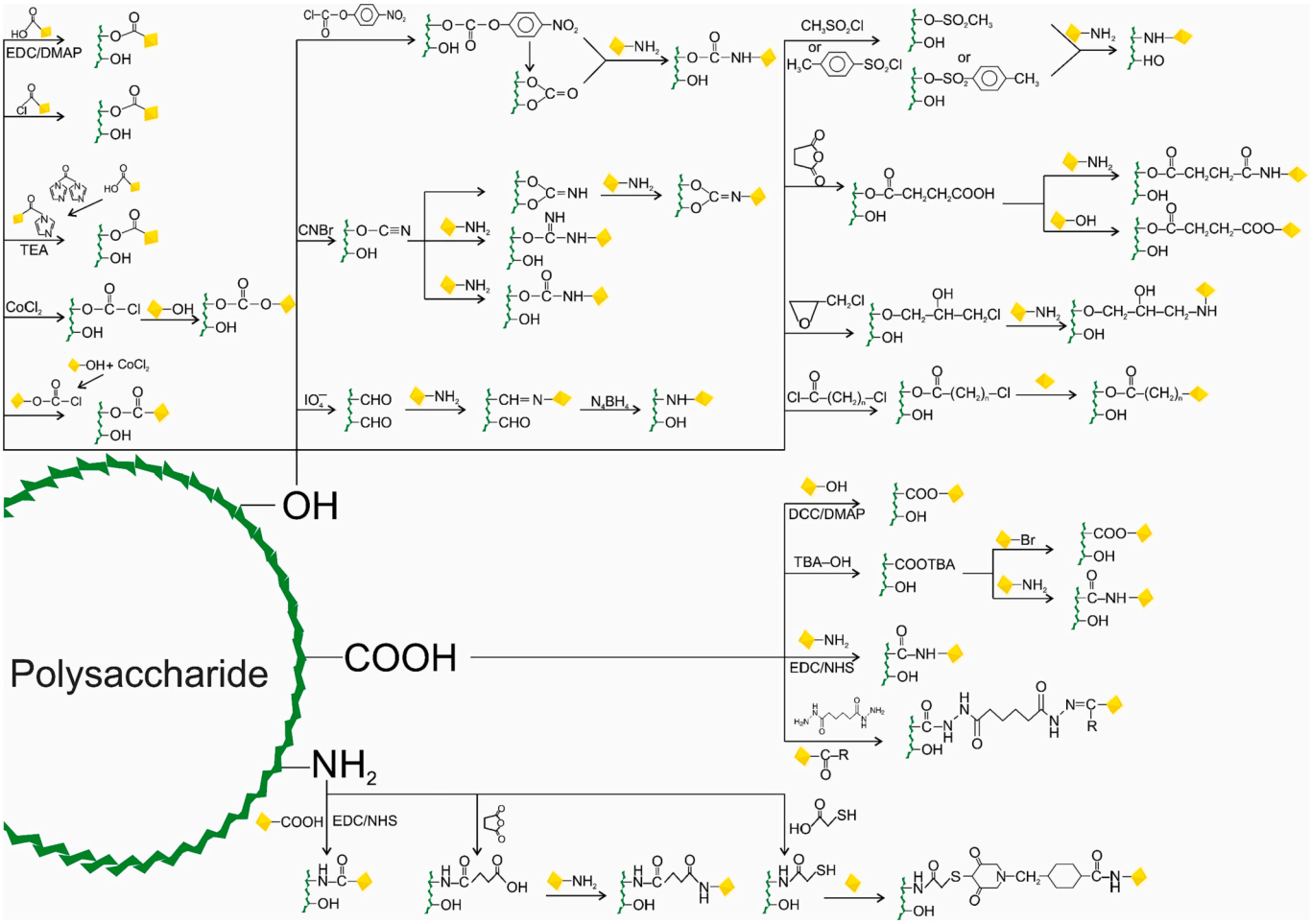

Fig. 2. The polysaccharide-drug coupling chemistry, illustrating conjugations at different groups of polysaccharides. Adapted from (Anwar et al., 2011; De Nicola, Bruni, Traversa, & Ghibelli, 2017; He & Yin, 2017;Qin & Li, 2020; Ravichandran & Jayakrishnan, 2018; Vogus, Krishnan, et al., 2017).

enzyme are considered as the most promising triggers. The changes in the pH values and expression of specific enzymes in tumor or inflammatory regions are widely exploited to achieve targeted accumulation of playlod of various polysaccharide-drug conjugates at the desired biological locations following pH and enzyme-catalyzed reactions (Xu et al., 2015).

This review summarizes the state-of-the-art in pH and enzyme-responsive polysaccharide-drug conjugates and their therapeutic benefits, challenges and future perspectives. Although the bioconjugation of a wide range of therapeutic modalities including neucleotides and macromolecules is extensively reported (Song, Fan, Hu, Cheng, & Xu, 2021), herein, we primarily emphasize on various small-molecule drugs covalently conjugated with the polysaccharides.

2. Polysaccharide-drug conjugation chemistry

The polysaccharides mostly possess the crystalline structure accredited to their strong intramolecular hydrogen bonding networks. Consequently, the polysaccharides display low solubility, which represents a key challenge in their chemical conjugation reactions. The solubility of the polysaccharides is often pH dependent. For instance, the chitosan (CS) dissolves in the acidic medium, while the hyaluronic acid solubilizes in the basic phase (Abbasi et al., 2021). Their solubility also makes the purification process difficult, particularly in the multistep reaction procedures. Over the years, several useful reagents have been designed for the polysaccharide chemistry. Moreover, the recent applications of the microwave assisted conjugation reactions of the polysaccharides could substantially reduce the use of toxic solvents and the reaction time. Typically, these protocols are cleaner, greener and could improve the product yields (Sun, Mu, Mohammed, Dong, & Xu, 2020). The hydroxyl (starch, cellulose, gum arabic, dextran, pullulan, κ-carrageenan, β-cyclodextrin), carboxylic acid (hyaluronic acid, alginate, pectin, heparin, gellan gum) and amino (chitin/chitosan, CS) groups of the polysaccharide molecules are utilized as active sites for the drug conjugation (Abbasi et al., 2021). (Fig. 2).

2.1. Conjugation at polysaccharide hydroxyl groups

The drugs containing carboxyl moieties could directly be conjugated with the hydroxyl groups of the polysaccharides in the presence of carbodiimides as coupling reagents, forming ester linkages (Li, Cai, Qi, & Tang, 2016; Li, Zhao, & Zhao, 2016). It could also be accomplished using the acid chlorides of the drugs in the presence of HC1 acceptors (Sánchez-Chaves & Arranz, 1988). Alternatively, the carboxylic drugs could be activated with the carbonyldiimidazole and the resulting imidazolide intermediates could be coupled with the polysaccharide OH groups in the presence of triethylamine (Sleightholm, Yang, Yu, Xie, & Oupický, 2017). The drugs bearing a hydroxy functional moiety could be conjugated to the polysaccharide OH groups *via* carbonate ester linkages. The activation of the polysaccharide hydroxyl groups with the phosgene is followed by the coupling of the alcoholic drugs. In this context, the chlorocarbonate intermediates of the polysaccharides could produce the intrachain (cyclic) and interchain carbonate esters as side products. To acquire the well-defined polysaccharide carbonate esters,

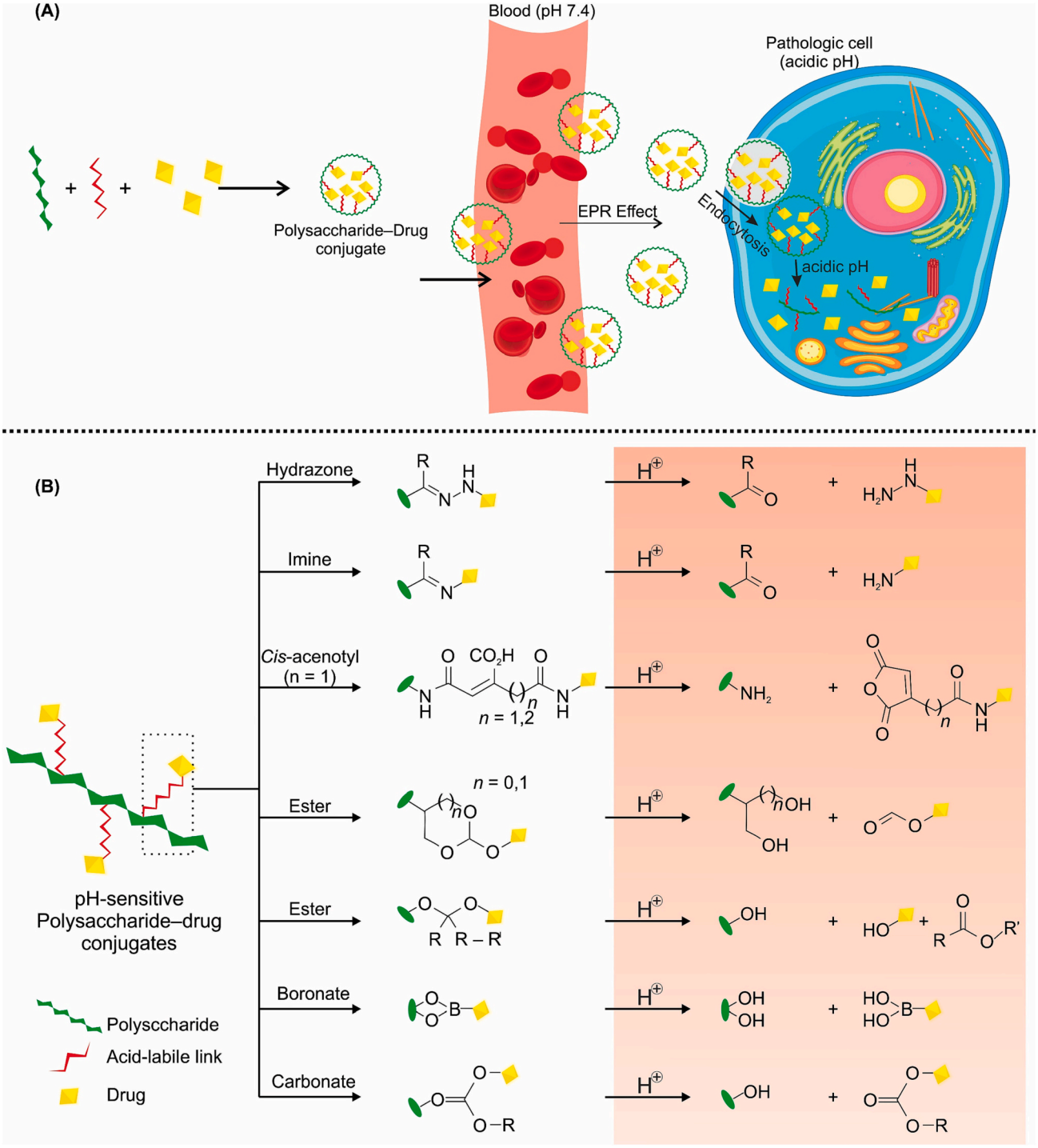

Fig. 3. The schematic mechanisms of pH-responsive drug release from polysaccharide-drug conjugates (A) with various pH-sensitive covalent linkages and their degradation at acidic pH (B). Adapted from (Deirram et al., 2019).

the chlorocarbonate esters of the drugs could initially be synthesized, which are directly coupled with the polysaccharide OH groups (De Nicola et al., 2017). The drugs containing amino moieties could be grafted with the hydroxyl groups of the polysaccharides through the carbamate ester linkages, accomplished *via* several principal routes. Among several methods, the activation of the polysaccharide OH groups with the *p*-nitrophenyl chloroformate is the most popular one. This protocol could lead to the formation of aromatic carbonate ester and less reactive carbonate, which could react with amine drugs to afford carbamate esters (Kothandaraman, Ravichandran, Bories, Loiseau, & Jayakrishnan, 2017). The cyanogen halide activation is also exploited for the covalent attachment of the amine drugs to the polysaccharide backbone. In this reaction, the reactive cyanate esters formed might hydrolyze to yield the inert carbamate or rearrange to produce the intra−/interchain and cyclic imidocarbonate structures. The parallel reactions of these products with the amine drugs could result in the formation of an isourea derivative (Kothandaraman et al., 2017). On the other hands, the dialdehyde polysaccharides obtained after their periodate oxidation could conjugate with the amine drugs, yielding Schiff bases. The subsequent reduction using sodium borohydride could

further stabilize the conjugates (Ravichandran & Jayakrishnan, 2018). The sulfonyl chloride mediated activation of the polysaccharides could result in the production of the corresponding sulfonyl esters, which could further react with drugs containing carboxylic or amino groups (Ehrenfreund-Kleinman, Golenser, & Domb, 2004).

The hydroxyl groups of the polysaccharides could also be modified by introducing the spacers. These procedures allow to introduce a new functional group, which is often more reactive than the OH groups of the polysaccharides. In addition, the spacer groups could reduce the steric hindrance around the bonds conjugating the drug molecules and polysaccharide chains. The succinic or glutaric anhydride have widely been utilized to activate either the hydroxyl groups of polysaccharide chains or the small molecular drugs containing OH moieties prior to their coupling (Deshpande & Jayakannan, 2017). Moreover, the epichlorohydrine could react with the polysaccharides in the presence of bases or zinc tetrafluoroborate as catalysts and produce the 3-chloro-2-hydroxypropyl derivatives, which is used to conjugate the drugs bearing amino groups (Móra & Pató, 1990). Furthermore, the chloroacyl chloride-based modification of the polysaccharides leads to the synthesis of chloroacyl derivatives, which could readily react with the drugs with carboxyl or amino groups (Mihai, Mocanu, & Carpov, 2000; Mocanu, Airinei, & Carpov, 1993; Zhang, Liu, Lan, & Fan, 2008). The amino acids and peptides are also popularly employed as spacers (Cheng, Khin, Jensen, Liu, & Davis, 2003). The degradation of the peptidyl spacers is influenced by their substrate-enzyme specificity, steric crowding and hydrophilicity (Homma et al., 2009).

2.2. Conjugation at polysaccharide carboxylic acid groups

The esterification and amidation with or without linkers are primarily exploited to conjugate drugs with the polysaccharides containing carboxylic acid groups (Lee, Kim, Park, & Lee, 2020; Vogus et al., 2017). The esterification reaction between the carboxylic acid moieties of the polysaccharides and the hydroxyl groups of the drug molecules is carried out in the presence of N,N′-dicyclohexylcarbodiimide (DCC) as a coupling agent and 4-(*N*,*N*′-dimethylamino) pyridine (DMAP) as a catalyst (Sarika, James, Kumar, & Raj, 2016). The tetrabutylammonium (TBA) salt of alginate is also reacted with alkyl halide to afford esters (Yang, Xie, & He, 2011). The 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), a coupling agent, is used to create amide linkages between the carboxylate moieties of the polysaccharides and drug molecules bearing amino groups. In this reaction, the N-hydroxysuccinimide (NHS) is frequently combined with EDC to activate the polysaccharide COOH groups (Liu et al., 2018). The TBA-treated polysaccharides could be grafted with amine containing drugs in presence of triethylamine and 2-chloro-1-methylpyridinium iodide (CMPI), which are used as catalyst and coupling reagent, respectively (Yang et al., 2011). The hydrazination reactions have also been utilized for the efficient drug conjugation to the polysaccharide COOH groups (Schanté, Zuber, Herlin, & Vandamme, 2011).

2.3. Conjugation at polysaccharide amino groups

The conjugation between the amino groups of the polysaccharides and the activated carboxylic moieties of the drug molecules is accomplished through the amide coupling reaction (Anwar et al., 2011). The carboxylic acid groups of the drugs are commonly activated using EDC or EDC/NHS as coupling reagents. The polysaccharide amino groups are also modified with various spacers (*viz.,* succinate linker) or more reactive functional moieties (*i.e.,* sulfhydryl group) to couple with the drug molecules (Anwar et al., 2011; He & Yin, 2017).

3. pH and enzyme–responsive polysaccharide-drug conjugates

3.1. pH-responsive systems

Several scientific reports demonstrated the existence of variable pH gradients across the normal and pathological conditions. An incomplete catabolism of glucose in the cancer cells produces lactic acid even under non-hypoxic conditions, lowering the pH value of the intratumoral region (6.4–6.8) contrary to the normal tissues (7.2–7.4). Similarly, the inflamed synovial joints in the rheumatoid arthritis (RA) and bacterial infection sites are characterized with the acidic microenvironment (Shin et al., 2014). The pH values of early endosomes (5.0–6.5) and late endosomes/lysosomes (4.0–5.0) could also render the acidic intracellular microenvironment (Deirram, Zhang, Kermaniyan, Johnston, & Such, 2019; Li, Ding, et al., 2016). Recently, the pH-responsive systems are potentially exploited for a wide range of drug delivery applications to treat an array of disease states. Various pH-sensitive covalent linkages such as hydrazone, imine, ester, cis-aconityl, carbonate bonds *etc.* are typically incorporated between the drugs and polysaccharides, which could remain stable at the neutral pH, but become labile at the acidic pH. The acidic pH mediated scissoring of these covalent bonds could liberate the attached therapeutic consignments from the polysaccharide backbone and deliver them in the targeted regions (Fig. 3). This could enhance the site-specific actions of the therapeutic entities, consequently alleviating their off-target toxicities.

3.1.1. pH-sensitive linkages

3.1.1.1. Hydrazone bonds. The hydrazone linkages are most popularly employed in the pH-responsive polysaccharide-drug conjugates, which could be attributed to their advantages including ease of synthesis, faster hydrolytic cleavage at the acidic condition and higher stability in the physiological environment (pH 7.4). The hydrazone linkages are commonly produced through the condensation between the hydrazides and ketones or aldehydes. The ketone groups of doxorubicin (DOX) and its structural analogue daunorubicin (DNR) are widely conjugated with the polysaccharides through the hydrazone linkages. The clinical applications of DOX as an anticancer drug are often limited due to its non-specific biodistributions, resulting in serious side effects. To attenuate its severe systemic toxicity, the DOX was conjugated with the hydroxyethyl starch (HES) *via* hydrazone bonds (Zhu, Yao, Chen, & Chen, 2015). The resulting HES–DOX conjugates were self-assembled to yield nanoparticles (NPs) with appropriate size (94–116 nm) and zeta potential ($+8.6 - +10.6$ mV) values suitable for internalization into the cancer cells. The pH sensitivity of the hydrazone linkages ensured negligible drug release under physiological conditions, while demonstrated a controlled DOX release in the intracellular environment. The HES–DOX conjugates could also exhibit a faster drug release profile in the HepG2 cells relative to the corresponding pH-insensitive conjugates. Malfanti and colleagues recently afforded hydrazone linked HA-DOX conjugates, which demonstrated excellent brain diffusion abilities due to their suitable size (< 30 nm) and exhibited an improved therapeutic activity against glioblastoma as compared to other HA-DOX conjugates comprisining amide and adipic hydrazone bonds and free DOX (Malfanti et al., 2022). On the other hands, Mei et al. conjugated the heparin with DOX through acid-sensitive hydrazone linkages (Mei et al., 2017). The conjugates afforded with the feed ratio of low molecular weight heparin (LMWH) and DOX of 4:1 exhibited size of 155.2 nm, polydispersity index (PDI) of 0.198, surface charge of −33.2 mV, drug loading content of 9.23 %, and critical micelle concentration (CMC) value of 3.52×10^{-2}. The acid-labile scaffolds resulted in an enhanced cellular uptake and increased *in vivo* antitumor efficacy than the pure DOX. The conjugates also exhibited an improved safety profile and the hydrophilic LMWH residues depicted an anti-metastatic potential by suppressing the adhesion between the tumor cells and platelets, which could typically be

mediated *via* P-selectin. The affinity of the pullulan towards the asialoglycoprotein receptors of the hepatocytes was exploited for the targeted delivery of DOX to the nuclei of the hepatic carcinoma cells. In this milieu, DOX and pullulan were conjugated through acid-labile hydrazone bonds (Li et al., 2014). The incremental feed ratios of DOX to the amidated pullulan increased the DOX-loading content in the conjugates. Increasing drug-loading content enhanced the dimeters of the spontaneously formed core-shell NPs. About 75 % of DOX was released at pH 5.0 within 2 h, while approximately 15 % of drug was eluted at pH 7.4 after 12 h. After incubation with HepG2, HeLa, and L929 cells, the pullulan–DOX conjugates showed a higher specific internalization in the HepG2 cells. On the contrary, the pure DOX was internalized in the cell nuclei of all the three cell lines. Another study reported an efficient accumulation of pullulan–DOX conjugates at the tumor sites of the nude mice subcutaneous hepatic carcinoma model through an improved EPR effect (Li et al., 2015). Eventually, these conjugates efficaciously inhibited the solid tumor growth and prolonged the survival of the experimental animals. Lu et al. (2009) modified the carboxymethylized pullulan with hydrazine hydrate and subsequently the DOX was covalently attached to the template through pH-sensitive hydrazone bonds. The conjugates self-assembled as spherical NPs having diameter <100 nm and a negative surface charge (−24.6 mV). These released the drug at a faster rate at pH 5.0 (62 % in 24 h) than that at physiological pH (29 % in 24 h). The cytotoxicity of the pullulan–DOX conjugate NPs on 4 T1 mouse breast cancer cells was more effective but less potent than that of free DOX. These conjugates revealed their improved biodistribution and reduced toxicity while maintaining the antitumor efficacy of DOX (Lu, Liang, Fan, Gu, & Zhang, 2010). Another study adapted a similar synthetic approach to accomplish the pullulan–DOX conjugates. In addition to the acid-responsive drug release, the conjugates could also provide an active targeting ability to the hepatic cells owing to the presence of pullulan (Li et al., 2014).

The hydrazone-linked pullulan–DOX conjugates were also utilized to co-deliver another therapeutic agent for achieving the synergistic efficacy. The adipic dihydrazide (ADH) was employed to conjugate DOX onto the pullulan backbone and subsequently, the pyrrolidinedithiocarbamate (PDTC), a chemotherapeutic sensitizer, was encapsulated into the pullulan–DOX conjugate NPs (Li, Sun, Liang, Fan, & Zhang, 2015). These NPs exhibited a diameter of 128.1–179.7 nm with excellent size stability at neutral pH. Moreover, the NPs depicted their active accumulation in the HepG2 cells and conferred a rapid drug release under simulated lysosomal acidic environment. The chemosensitizer-loaded NPs synergistically induced a higher apoptotic potential on the DOX-sensitive HepG2 and DOX-resistant HepG2/ADR cells. These also exerted a greater *in vivo* tumor suppressive effect on both DOX-resistant and DOX-sensitive hepatic cancers as compared to the NPs loaded with single drug and unconjugated DOX. Furthermore, the NPs demonstrated an improved safety profile in terms of the cardiotoxicity. Sui et al. (2017) accomplished the pullulan-DOX conjugates, which were self-assembled to afford NPs encapsulating sorafenib (SFB). These NPs allowed a gradual release of the drugs at acidic pH environment and facilitated their effective cellular internalization, leading to an excellent antiproliferative potential against 4 T1 cells. The animal studies also revealed a significant accumulation of pullulan–DOX/SFB in the tumor sites with improved antitumor effects than the free drugs and pullulan–DOX conjugates. In order to enhance the DOX sensitivity to the triple-negative breast cancer cells, Sui et al. (2020) loaded lapatinib in the hydrazone-linked pullulan-DOX conjugates, which were further surface modified with the thiolated hyaluronic acid (HA). The lapatinib facilitated intratumoral invasion and accumulation of the conjugate NPs, enhanced the antiproliferative efficiency of the DOX and minimized its systemic toxicity with improved anti-recurrence ability. In another study, the anionic carboxymethyl pullulan and cationic polyallylamine (PAA) were exploited to construct DOX conjugated nanocomplexes (NCs), loading plasmid DNA (pDNA) (Vora, Tyagi, Patel, Gupta, & Vavia, 2014). The hydrazone linkages of the pullulan–PAA–DOX/pDNA NCs caused a greater drug release in the acidic condition. The NCs also showed a significantly higher transfection of the DNA in the HEK293 cells relative to the PAA/pDNA complexes.

Several attempts were also made to conjugate DOX onto the graft *co*-polymers or hydrophobically modified forms of the polysaccharides. For instance, the acid-sensitive hydrazone linkages were exploited to graft DOX onto the dextran backbone copolymerized with poly(ethylene glycol) methyl ether methacrylate (Zhang et al., 2020). The amphiphilic scaffolds were self-assembled to acquire a stable nano-sized bottlebrush-architecture in an aqueous environment. These possessed a densely spaced polymer chains, providing a steric shielding effect on the conjugated drug molecules. Such systems exhibited excellent intratumoral permeability and acceptable circulation stability. These also significantly enhanced the therapeutic potential of the drug with minimal side effects on the normal tissues, which was pertaining to their endo/lysosomal microenvironment (pH 5.0)-responsive drug release behavior. Jin and co-workers attached deoxycholic acid (DCA) to the hydrazine-modified dextran template and subsequently fabricated dextran–DCA–DOX prodrugs (Jin, Guo, Dong, Xie, & Cao, 2017). The conjugates produced nanomicelles under physiological conditions. The optimized nanoscaffolds with smallest particle size (110 nm) and lowest PDI value (0.17) were afforded utilizing the conjugates comprised of dextran of 10 kDa with degree of substitution (DS) of DCA of 9 and DOX loading of 5.5 wt%. These exhibited acid-sensitive drug release patterns, prominent *in vitro* and *in vivo* antitumor activities and superior biosafety profile as compared to the pristine DOX. She et al. (2013) designed the dendronized heparin–DOX conjugates containing pH-labile hydrazone linkages. These conjugates were negatively charged and were self-assembled to accomplish the compact NPs with size of 90 nm. The NPs revealed promising tumor inhibition effects with high anti-angiogenic and apoptosis inducing potentials. In addition, the scaffolds caused minimal toxicities to the healthy organs of either tumor–bearing or healthy mice, evidencing their biosafety profiles. Zhang and co-researchers afforded the methyl glycinate conjugated carboxymethyl chitosan (CMCS) through the amidation reaction (Zhang et al., 2016). Subsequently, the hydrazine hydrate was grafted to the template, attaching DNR *via* hydrazone linkages. The resulting CMCS–DNR conjugates could spontaneously form NPs with core–shell structures in the aqueous phase. The NPs remained stable at pH 7.4 and pH 6.5, but showed a higher sensitivity at pH 5.0. These could facilitate an efficient DNR release in the cancer cells, causing a greater cytotoxic effect on the HeLa cells with two-fold higher IC_{50} value as compared to that of pure DNR.

Several other polysaccharide-anticancer drug conjugates were also fabricated and reported. Thummarati et al. (2021) linked HA and curcumin (Cur) through ester and hydrazone bonds. As compared to the HA–Cur conjugates bearing pH-insensitive ester bonds, the HA–Cur scaffolds containing pH-sensitive hydrazone linkages demonstrated a smaller particle size, lower critical aggregation concentration and greater stability with a higher drug release profile in the acidic microenvironment of the cancer cells. Lai and co-workers afforded an efficient Cur delivery system by conjugating it onto the ADH-modified HA backbone. (Lai, Ding, Ye, Deng, & Cui, 2021) The acid-sensitive hydrazone linkers within the self-assembled HA-ADH–Cur conjugate NPs caused significantly higher drug release rate at pH 5.0 (73 % drug release within 24 h) than that in physiologic pH (22.8 % drug release within 24 h). As compared to the pure Cur, the NPs demonstrated a higher accumulation in the 4 T1 and MCF-7 cells *via* CD44 mediated endocytosis and EPR effects, causing superior therapeutic activity. In another study, the ADH was utilized to create pH-cleavable linkages between HA and ester form of the tacrolimus (TAC) (Dheer et al., 2019). The conjugate significantly increased the aqueous solubility of TAC from 0.008 $mg.mL^{-1}$ to 15.3 $mg.mL^{-1}$ and caused the pharmacokinetic benefits in terms of enhanced C_{max} and half-life and reduced clearance relative to the unconjugated TAC. The conjugate prodrug further showed an augmented cellular uptake, good biocompatibility and a

greater drug release kinetic in the acidic condition (pH 5.4). In an attempt to develop a targeted multimodal anti-glioblastoma therapy, the hydrazone-linked HA-lenalidomide conjugate was synthesized, which was applied as coating onto the Zeolitic imidazolate Frameworks, encapsulating 5-flourouracil (5-FU) and titanocene-loaded lactoferrin (Pandey et al., 2020). The core-shell nanocomposites demonstrated an acid-responsive sustained drug release profiles and superior cytotoxicity against cancer cells as compared to the uncoated systems. Moreover, the nanocomposites were stable in plasma, extracellular fluid and cerebrospinal fluid and exhibited minimal bio-interactions.

3.1.1.2. Imine bonds. The Schiff base, which also refers to imine bonds, is accomplished by the nucleophilic attack of the amines to the aldehyde groups. The acid labile imine bonds are widely being investigated as pH-responsive linkages in the polysaccharide-drug conjugates. The aldehyde groups of the oxidized HES were coupled to the amino moieties of DOX to synthesize HES–DOX conjugates, spontaneously producing micelles in an aqueous phase (Li, Ding, et al., 2016). The micelles with higher drug conjugations demonstrated a smaller diameter, a reduced drug elution in the physiological environment, a faster intracellular drug release and superior antitumor activity. A similar protocol was utilized to conjugate DOX onto the dextran backbone with variable molecular weights of 40 KDa and 50 KDa (Li, Han, Ding, Chen, & Chen, 2017). The prodrug prepared with higher molecular weight dextran bestowed a smaller micellar size, a higher cell internalization efficacy, a faster pH-labile drug release rate in the intertumoral region and more potent tumor suppression effects than that of the conjugates composed of low molecular weight polysaccharide. Feng and co-researchers attached amino groups of DOX to the aldehyde moieties of oxidized dextran (Feng, Li, Han, Zhuang, & Ding, 2017). The imine bonds of the dextran–DOX conjugates remained strenuous in the neutral pH, but were quickly degraded at the lower pH. As compared to the pure DOX, the conjugates displayed an improved cellular uptake, cytotoxicity, antitumor effects on the mouse B16F10 melanoma with attenuated systemic damage. Xu and co-workers also synthesized pH-responsive dextran-DOX conjugates, which presented superior antitumor activity and lower toxicity as compared to the corresponding pH-insensitive derivatives (Xu et al., 2015). The DOX was also conjugated with alginic acid, HA, and dextran through acid-labile imine linkages (Zhang et al., 2019). Among various scaffolds, the dextran–DOX conjugates exhibited a higher drug content and improved stability in the circulation. A further comparison among the scaffolds fabricated with different molecular weight dextran conferred that the dextran-$_{150KDa}$–DOX conjugates released a higher amount of drug in the simulated acidic environment and demonstrated a greater *in vitro* cytotoxicity and improved *in vivo* antitumor efficiency and survival rate as compared to the dextran-$_{6KDa}$–DOX conjugates.

Several micro molecule linkers were also employed to fabricate polysaccharide-drug conjugates bearing imine linkages. The p-carboxybenzaldehyde (p-CBA) was used as linker to conjugate DOX and CMCS (Hu et al., 2017). The conjugates (CMCS–p-CBA–DOX) with imine bonds exhibited a higher stability in the plasma, but decomposed in the acidic microenvironment of the tumors, causing a faster drug release behavior. A more pronounced cytotoxic effect of the conjugates towards SKOV3 cells than that of the pure drug was also evidenced. Moreover, the pH-responsive HA–DOX conjugates were accomplished *via* Schiff base reaction using p-CBA as linker (Hu et al., 2017). The conjugates possessed an enhanced antitumor activity with excellent targeting ability towards HeLa cells. Cao and colleagues synthesized the alkyne-functionalized CMCS employing carbodiimide chemistry. The azide-functionalized DNR was subsequently afforded through the Schiff base reaction between the aldehyde pendants of 4-azidobenzaldehyde and the primary amine group of DNR (Cao et al., 2017). The click reaction between the alkyne-functionalized CMCS and azide-functionalized DNR produced the acid-sensitive amphiphilic prodrug polymer. The conjugates could self-assemble into NPs in an aqueous phase, illustrating narrow size distribution and negative surface charges. The CMCS–DNR NPs demonstrated a superior stability in the physiological environment, controlled drug release profile in the acidic condition, efficient cellular uptake and subsequent translocation into the HeLa cell nuclei, causing a relatively higher cytotoxicity.

3.1.1.3. Ester bonds. The esters bonds, which are produced by condensing hydroxyl and carboxyl groups, are vastly used as pH-responsive pendants of the polysaccharide-drug conjugates. To acquire a targeted therapy of RA, Shin et al. (2014) directly conjugated HA and methotrexate (MTX) through ester linkages cleavable in the mild acidic environment of RA. The conjugates were effectively taken up by the activated macrophages through the HA/CD44 receptor interactions and significantly improved the arthritis indices and paw thickness as compared to the pure MTX. Following systemic administration, the conjugates also significantly reduced the levels of proinflammatory cytokines and pathogenic IgGs. To improve the oral delivery of paclitaxel (PTX), the HA-PTX conjugates were afforded through a similar esterification protocol (Li et al., 2013). In an aqueous environment, the conjugates could self-assemble into NPs with average size of 100 nm. The CS coating onto the HA–PTX NPs was accomplished through electrostatic interactions, which could stabilize the NPs in the stomach pH. This system released the HA–PTX conjugates at the intracellular spaces of the enterocytes following degradation at pH 7.4. Subsequently, the HA–PTX NPs were accumulated in the tumor tissues by the CD44 receptor-mediated internalization. In another study, the PEG was exploited to produce the NCs with HA. The PTX was then grafted to yield ester-linked HA-PTX conjugates, forming nanosized micellar aggregates (~196 nm) in the aqueous solution. The nanomicelles exhibited an acid-labile drug release pattern and pronounced cytotoxicity against HA receptor over-expressing cancer cells (Lee, Lee, & Park, 2008). Li and co-workers afforded HA-podophyllotoxin based pH-sensitive prodrug *via* one-step esterification reaction. HA-mediated targeting facilitated a higher cellular uptake (> 97 %) of the prodrug and its acid sensitive ester bonds caused pH-responsive drug release, leading to an improved tumor inhibition (~ 85 %) with negligible systemic toxicity (Li et al., 2022). On the other hands, the carboxymethyl dextran was directly coupled with the hydroxyl groups of docetaxel (DTX), a structural analogue of PTX, through esterification reaction (Qin, Zhu, Zhang, Zhou, & Wang, 2016). The yielded conjugates demonstrated a faster drug release in the mild acidic environment of the cancer cells as compared to that in the physiological condition, resulting in their superior *in vivo* antitumor efficacy than that of pristine DTX. In another investigation, an ester-linked conjugate of DTX and HA was loaded into nanoliposomes employing thin-film hydration method (Seifu, Nath, & Dutta, 2020). The liposomes exhibited an acid-sensitive drug release profile. In comparison to a commercial formulation, the liposomes showed an enhanced cellular internalization and a higher cytotoxicity against glial tumor cells.

The succinate spacer linked HA–DTX conjugates with their pH sensitive drug release characteristics in the tumor microenvironment and targeted drug deliveries onto the CD44-overexpressing cells have also been reported (Goodarzi et al., 2014). These conjugates improved the clinical outcomes relative to the precursor drug molecules. The PTX or DTX were also conjugated with the CS employing succinate linker. Following oral administration, these acid-labile conjugates could flawlessly reach into the systemic circulation, causing a superior bioavailability comparative to the parent drugs (Lee et al., 2008; Lee, Kim, Lee, & Jon, 2009). Similarly, an acid-labile succinic acid spacer was employed to conjugate the hydrophobic Cur onto the hydrophilic dextran backbone (Raveendran, Bhuvaneshwar, & Sharma, 2016). The resultant amphiphilic scaffolds facilitated the formation of micelles in an aqueous media and enhanced the solubility and stability of Cur. These conjugate micelles demonstrated a rapid drug release at the acidic pH

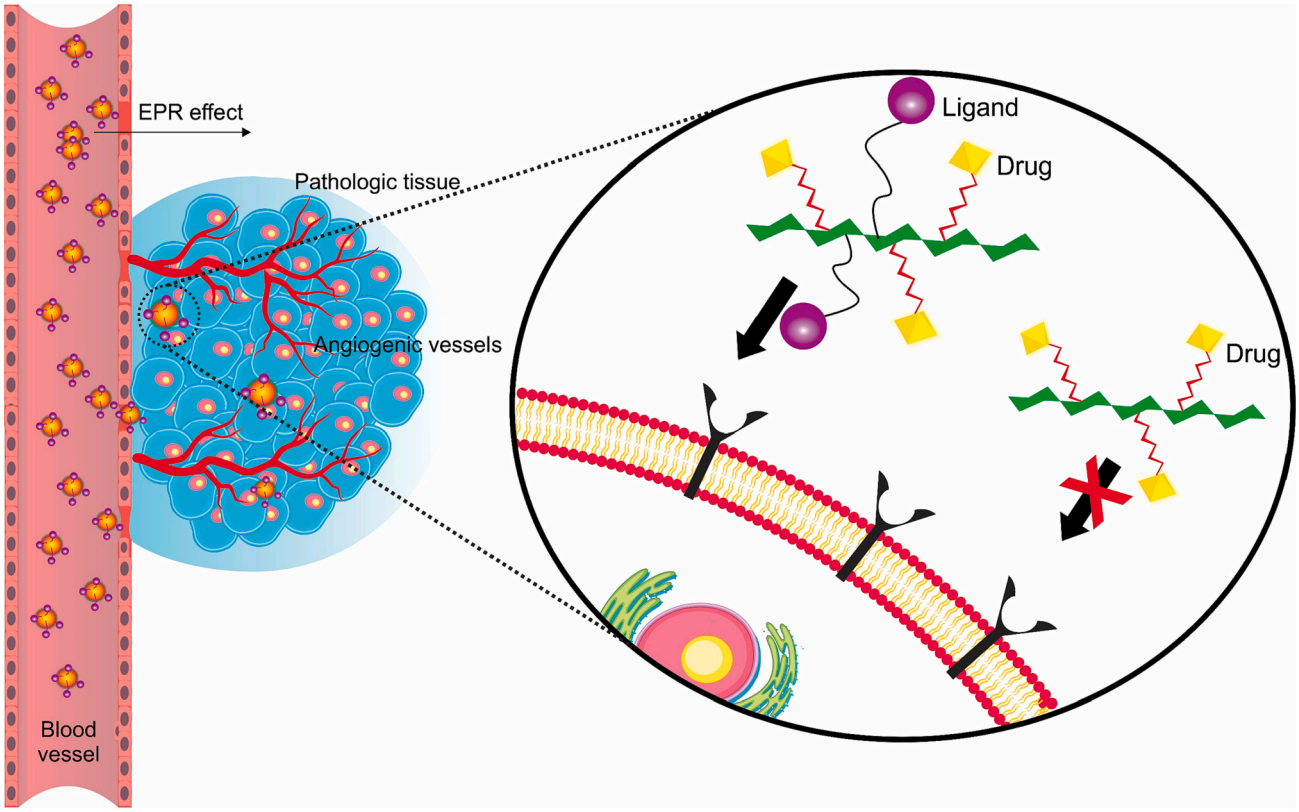

Fig. 4. The schematic presentation of targeting ligand-decorated pH-responsive polysaccharide-drug conjugates. Adapted from (Balasso et al., 2017; Wadhwa & Mumper, 2015).

and exerted profound cytotoxicity on the cancer cells. In another study, Chen and co-researchers reacted glutaric anhydride with Cur to yield Cur–COOH, which was further conjugated with the hydroxyl groups of HES through acid-labile ester linkages (Chen et al., 2020). The resulting conjugates were self-assembled into micellar NPs that protected Cur from the ultraviolet light and heat mediated degradation and significantly improved the drug solubility with excellent colloidal stability in the salt solutions. An improved solubility elicited an obviously enhanced bioavailability, antioxidant and anticancer activities of the conjugated Cur. In a similar manner, Cur–COOH was conjugated with HES *via* ester linkages, which remarkably improved the drug loading efficiency and solubility of Cur. These HES-Cur conjugates were also self-assembled into NPs and conferred obviously higher antiproliferative effects than pristine Cur on HepG2 cells through amplified degree of mitochondrial damage and autophagy (Jiang et al., 2021).

Zhang and colleagues grafted N-(2-aminoethyl)-gluconamide (Glu) onto the HA backbone and the modified HA was then conjugated with bortezomib (BTZ) through the ester bonds (Zhang, Zhang, Yu, Wang, & Liu, 2019). The boronic acid moieties of BTZ could dissociate from the conjugates at lower pH, enabling the scaffolds responsive to the local tumor microenvironment. Interestingly, the boronate-crosslinked HA-Glu–BTZ conjugates demonstrated a 4-fold higher drug release rate in the endosomal pH (5.7) than that in the physiological pH (7.2). As compared to pristine BTZ, the conjugates exerted a more pronounced growth suppression effect on the cancer cells with a lower toxicity towards the healthy cells. Xu and co-researchers employed boron esterification reaction to accomplish an acid-labile dextran–BTZ conjugates (Xu et al., 2015). These scaffolds produced micelles of radius around 50 nm and exhibited efficient internalization into the hepatocarcinoma cells. The intracellular acidic environment could accelerate the release of BTZ from the conjugates. An excellent inhibition efficiency of the dextran–BTZ conjugates towards hypoxic tumor was mediated through the nuclear factor-kappa B (NF-kB) and endoplasmic reticulum stress (ERS)-induced apoptosis signalling pathways.

3.1.1.4. Cis-aconityl bonds. The coupling of various drugs with the polysaccharide *via* pH sensitive cis-aconityl (CA) spacer are widely documented. The COOH groups of stearic acid (SA) was grafted to the amino moieties of chitosan oligosaccharide (CSO) through the EDC-mediated reaction (Hu, Liu, Du, & Yuan, 2009). The CSO-SA and DOX were conjugated further *via* CA bonds. The resulting micelles with drug contents of 3, 6, and 10 % demonstrated sizes of 40.1, 70.7, and 105.8 nm, respectively. In addition, their surface charges were continuously decreased with increasing drug content. The drug release rate of the nanomicelles was increased significantly with decreasing the pH values from 7.2 to 5.0. A faster drug release rate was observed for the conjugates with the lowest drug content. The CSO-SA-CA–DOX micelles were effective in the growth suppression of the multi-drug resistant cells and the reversal of drug resistance. The *in vivo* performances of the conjugate micelles were equivalent to the commercially available DOX injection, but showed an improved maximum tolerated dose. Al-shamkhani reacted daunomycin (DNM) with cis-aconitic anhydride and the resulting product was grafted with the ethylenediamine-modified alginate (Al-Shamkhani & Duncan, 1995). Following the intraperitoneal injection, the alginate-DNM conjugates suppressed the tumor growth with reduced toxicity as compared to the free DNM. In an attempt to co-deliver DOX and PTX, the DOX was modified with cis-aconitic anhydride to accomplish negatively charged CA–DOX. (Ma, Fan, & Li, 2016) Concurrently, the Pluronic F127-CS (F127-CS) conjugates were afforded using succinate as spacer molecules and the resulting scaffolds were further coupled with CA-DOX through the amidation reaction. Eventually, the PTX was entrapped in the lipophilic inner cores of the self-assembled F127-CS–CA–DOX conjugates. The micelles increased their diameter (56.3–403.4 nm) and decreased zeta potential (7.51–0.89 mV) with raising DOX content in the conjugates. An increase in the feed molar ratio of CA–DOX and F127-CS could enhance the DOX content in

micelles, causing an improved PTX loading efficiency. Moreover, the PTX-entrapped F127-CS–CA–DOX micelles presented a pH-triggered release of the drugs and provided a 3.97 and 4.38-fold higher plasma concentrations of PTX and DOX, respectively than those for the solution containing DOX and PTX.

The orientation of the conjugated drug in the hydrophobic cores of the self-assembled NPs could often restrict the release of acidity-cleaved drugs in an intratumoral region, causing a lower cytotoxic potential than that of the free drugs. For instance, the DOX and poly(ethylene imine) (PEI)-grafted dextran were conjugated *via* acid-cleavable CA bonds (Wang, Liu, Shi, Gao, & Gong, 2015). The conjugates were self-assembled into NPs with mean hydrodynamic diameter of 120 nm and zeta potential of +30 mV. Following pH-trigger cleavage, the drugs could physically entrap into the hydrophobic cores of the micelles, resulting in a sustained drug release behavior. Thus, although the PEI–dextran–DOX conjugate NPs efficiently entered into the MCF-7 cells, their cytotoxic potential was lower than that of pure DOX. In another study, the micelles of CSO-CA–camptothecin (CPT) conjugates were afforded, which were sensitive to the acidic pH (5.5) with cumulative drug release of 20 % and 22 % at 8 and 24 h, respectively (Tahvilian, Tajani, Sadrjavadi, & Fattahi, 2016). This indicated that the micellar shells could hinder the release of the detached drugs into the medium.

3.1.1.5. Carbonate bonds. Drugs bearing a hydroxyl functional group could be coupled with the hydroxyl moieties of the polysaccharides through carbonate ester linkages. Nicola and co-researchers accomplished carbonate ester linked dextran-etoposide conjugates for the metronomic therapy. The etoposide was initially activated with carbonyldiimidazole (CDI) and then coupled with dextran *via* hydrolysable carbonate bonds (De Nicola et al., 2017). These were internalized into the cells *via* lysosomes and demonstrated pH-responsive slow drug release pattern and proficient apoptosis inducing potentials. Interestingly, the conjugates mimicked the metronomic therapy by diverting the apoptosis from cytotoxicity to the differentiation.

3.1.2. Targeting ligand-decorated pH-responsive systems

The therapeutic potentials of the pH-responsive polysaccharide-drug conjugates could be often augmented by decorating them with various ligands targeting the overexpressed proteins or receptors on the cells (Fig. 4). The folic acid (FA) has been extensively used as targeting ligand. It could not only enhance the targetability of the acid-responsive dextran-DOX conjugates, but also regulate their architectural morphology. This also provided the merits of superior serum-tolerability (Zhang et al., 2020). Likewise, the folate coupled dextran-DOX conjugates exerted significant antiproliferative property on the DOX-resistant HepG2 cells, pertaining to their ability to deliver larger amount of drug, targetability and pH dependent drug release behavior (Zhang et al., 2015). Another study showed that the *in vivo* antitumor activity of the pH-sensitive trimethyl chitosan (TMC)/PTX conjugates, which were covalently coupled *via* succinate spacers could be enhanced while modified with FA (He & Yin, 2017). The dextran-DTX conjugates and further folate grafting increased the drug solubility by 1200 and 280 folds, respectively (Parhizkar et al., 2017). The cleavage of acid-labile glutarate linkers of dextran–DTX or dextran–DTX-FA conjugates resulted in a faster drug release at pH 5.4 than that in physiological pH. The conjugates presented acceptable hemo-compatibility and higher drug loading content (18–20 mg/100 mg conjugates). The FA coupling to the dextran–DTX conjugates also caused a higher cytotoxicity on the MCF-7 and MDA-MB-231 cells relative to the pure drug.

The FA was also exploited as targeting moieties for several pullulan-drug conjugates. The maleilated pullulan (MP) was chemically conjugated with DOX *via* primary amide bonds (Li, Zhang, et al., 2013). The FA was then anchored on the pendent hydroxyl groups of MP. The resultant FA-decorated MP–DOX NPs were utilized to co-deliver PDTC and chemically and physically loaded DOX through the folate receptor-mediated endocytosis. The drug release rate of the conjugates was significantly higher at acidic pH than at the physiological pH. As compared to the pristine DOX, the FA-MP–DOX/PDTC-DOX NPs demonstrated an enhanced cellular uptake and exerted a lower cytotoxicity but more effective against DOX-sensitive and DOX-resistant ovarian carcinoma cells. Zhang et al. also reported that the FA-decorated MP–DOX NPs possessed the intracellular acid-labile drug release characteristics with higher accumulation in the ovarian carcinoma cells (Zhang et al., 2011). Scomparin and co-workers fabricated the FA coupled and FA free pullulan–DOX conjugates, which presented their particle size of 100 and 150 nm, respectively (Scomparin, Salmaso, Bersani, Satchi-Fainaro, & Caliceti, 2011). These scaffolds exhibited pH-sensitive drug release patterns and could significantly increase the circulation time of DOX in the bloodstream. Regrettably, the FA conjugation could exert a limited impact on the selective cellular uptake of the conjugate NPs.

Zhao and co-researchers grafted luteinizing hormone-releasing hormone (LHRH) with the oxidized HES-DOX conjugates *via* acid-sensitive Schiff base bonds for the targeted drug delivery to the prostate cancer cells (Zhao et al., 2017). The HES–DOX conjugates significantly improved the *in vivo* tissue distribution of the pure drug. Interestingly, the HES–DOX/LHRH exerted higher anti-proliferative effects on the RM-1-xenografted mouse model with low systemic toxicities as compared to the HES–DOX and pristine drug. Balasso et al. exploited PreS1 as a targeting agent to enhance the selectivity of the pH-sensitive oxidized pullulan–DOX conjugates to the hepatocellular carcinoma cells through their overexpressed SERPINB3 receptors (Balasso et al., 2017). A two-fold increase in the anticancer activity towards HepG2/SERPINB3 cells was observed for preS1–DOX–pullulan conjugates as compared to the control pullulan–DOX conjugates. In another study, the DOX and BTZ were conjugated onto the oxidized dextran backbone *via* Schiff base and boronic esterification reactions, respectively. The cyclo-(Arg-Gly-Asp-D-Phe-Lys) (c-RGDfK), a targeting ligand, was also grafted to the resulting acid-responsive conjugates (Li et al., 2020). In an aqueous solution, the conjugates formed the micelles with diameter of ~80 nm. These also exhibited a rapid drug release in the acidic conditions, recognized the targeted cancer cells *via* RGD-$\alpha_v\beta_3$ integrin interplay and exerted superior antitumor effects as compared to the non-targeted control prodrug and pristine drugs.

Several bioactive compounds were also employed as targeting agents. Previously, the glycyrrhetinic acid (GCA) was used to target the GCA receptors (GCA-R) highly upregulated in the liver cancer cells. To acquire the hepatoma targeted action of DOX, the micelles of the sulphated hyaluronic acid (sHA)/DOX and HA/GCA conjugates were mixed (Li et al., 2019). The DOX was conjugated onto the sHA backbone *via* acid-labile hydrazone bonds to confer pH-sensitive drug release profiles, whereas the amide bonds coupled HA template and GCA molecules. The mixed micelles possessed spherical shape with nano ranged particle size (~217 nm) and demonstrated the pH-dependant drug release pattern and ability to effectively deliver the drug into the HepG2 cells. Moreover, the conjugate micelles exerted a superior antitumor activity with lower systemic toxicity than that of the pure DOX. Guo and colleagues conjugated the GCA and DOX onto the alginate backbone *via* amide and hydrazone bonds, respectively. The pH-sensitivity of the hydrazone bonds was combined with the liver targeting ability of GCA by mixing these two conjugates (Guo et al., 2013). The alginate-GCA/alginate–DOX NPs demonstrated pH-triggered drug release behavior in the hepatoma cells and caused a higher AUC and half-life in the mice liver than that of the unconjugated DOX. Consequently, the alginate-GCA/alginate-DOX treated animal groups conferred a much higher tumor inhibition rate than that observed in the pure DOX and alginate–DOX treated groups. Interestingly, the mortality rate of the alginate-GCA/alginate–DOX treated mice was significantly lower as compared to the free drug-treated group. In another study, the mitochondria targeted DOX delivery systems were accomplished by affording its conjugates

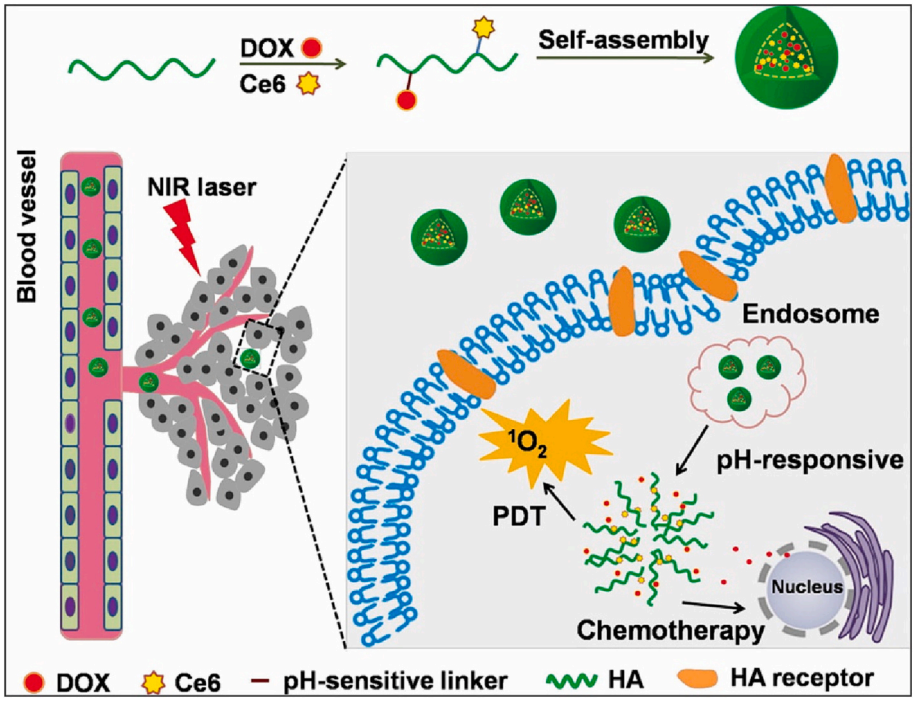

Fig. 5. The chemo-photodynamic therapy through simultaneous delivery of conjugated drug and Ce6 to tumor tissues. Reproduced with permission (Wu et al., 2019). Copyright 2019, Elsevier.

with triphenylphosphonium (DOX-TPP) (Han et al., 2014). HA was further grafted through hydrazone bonds to fabricate acid-responsive HA/DOX-TPP NPs to introduce the specificity of DOX-TPP conjugates towards cancer cells. These NPs released DOX-TPP conjugates in the lysosomes of cancerous cells and caused apoptosis following mitochondrial damage (Liu et al., 2018).

To achieve a target specific delivery of the Cur to the hepatocarcinoma cells, Sarika et al. conjugated pullulan aldehyde (Pul-Ald) with the lactobionic acid ($LANH_2$) *via* Schiff's base reaction. Eventually, the succinic anhydride modified Cur (CurSA) was attached to the pullulan and $LANH_2$–Pul-Ald to accomplish galactosylated and non-galactosylated pullulan–Cur conjugates, respectively (Sarika et al., 2016). Both the conjugates could self-assemble to afford micelles with spherical morphology and demonstrate the ability to increase the stability of Cur in the physiological pH. The galactosylated conjugates ($LANH_2$–Pul-Ald–CurSA) exhibited an enhanced internalization efficiency with a higher cytotoxicity towards the HepG2 cells relative to the non-galactosylated conjugates. To target the macrophages, Coessens and colleagues grafted 6-aminohexyl-α-D-mannopyranoside to the polymeric prodrug of streptomycin (Coessens, Schacht, & Domurado, 1996). The drug was coupled *via* a spacer, glycine hydrazide, onto a polymeric carrier (*i.e.,* poly[N-(hydroxyethyl) -L-glutamine] (PHEG) or dextran), forming hydrazone bonds. The streptomycin release rate was faster in the lysosomal pH as compared to that in physiological pH.

3.1.3. *pH-responsive conjugates for photochemotherapy and bioimaging*

In current years, theranostic nanomedicines for the simultaneous diagnosis, drug delivery and real-time tracking of the chemotherapy efficiency has become an appealing approach for cancer therapy. To date, various theranostic nanoplatforms have been developed by incorporating imaging materials into the pH-responsive polysaccharide-chemotherapeutic drug conjugates. In this context, several photosensitizers are utilized, which could serve as fluorescence imaging agents. When exposed to light irradiation, these could also produce highly cytotoxic ROS and destroy the cancer cells. Such photochemotherapy could efficiently inhibit the growth of the tumor. Apart from photosensitizers, other imaging agents such as carbon dots have also been used. Such turn-on theranostic systems are activatable upon exposure to single stimulus or multiple stimuli of the tumor microenvironment.

To accomplish the chemo-photodynamic therapy, the DOX and photosensitizer chlorin e6 (Ce6) were coupled with HA through pH sensitive hydrazide and amide bonds, respectively (Wu et al., 2019). The HA–DOX–Ce6 conjugates could self-assemble in NPs with average hydrodynamic diameter of 152.8 nm, PDI of 0.105 and uniform spherical morphology. The conjugate NPs dramatically improved the cellular uptake and substantially increased the anticancer activities of the drug. Moreover, the antiproliferative potentials of the conjugate NPs under laser irradiation were significantly higher as compared to free Ce6 or DOX (Fig. 5).

Indocyanine green (ICG) is FDA approved dye and broadly employed as imaging contrast agent for both photothermal and photodynamic therapies of the tumors. It is unstable in the aqueous solutions. In an interesting investigation, specific tumor imaging capability was integrated with photothermal-chemotherapy by conjugating an ICG derivative (*i.e.* ICG-COOH) and PTX onto the HA backbone *via* esterification reaction (Su et al., 2018). The ICG–HA–PTX conjugate easily self-assembled into nanomicelles under ultrasonication. This conjugate acquired significantly lower CMC value (0.0576 mg/mL) than that of HA-ICG (0.1117 mg/mL) and HA-PTX (0.1172 mg/mL). The uniform dimension of the nanomicelles around 130 nm and negative ζ-potential (−26.3 mV) facilitated their EPR-mediated internalization and accumulation into the cytoplasm of the cancerous cells. These conjugates could rapidly release the PTX and ICG intracellularly, exerting synergistic tumor inhibition and powerful apoptotic potentials. Additionally, the conjugates increased the photostability and photothermal effects of ICG-COOH and provided tumor-specific fluorescence.

To fabricate an image-guided drug delivery system, the PEGylated oxidized alginate (PEG–Alg) was initially crosslinked with the amino groups of the fluorescent carbon dots. Subsequently, the DOX was attached onto the crosslinked polymer *via* Schiff base linkages. The conjugate NPs had the size of 27 nm with acceptable DOX content (0.2532 mg/mg of conjugate), demonstrated acid-triggered drug release behavior and exerted an efficient suppression of the cancer cells attributed to the nucleus targeting of the drug (Jia et al., 2016).

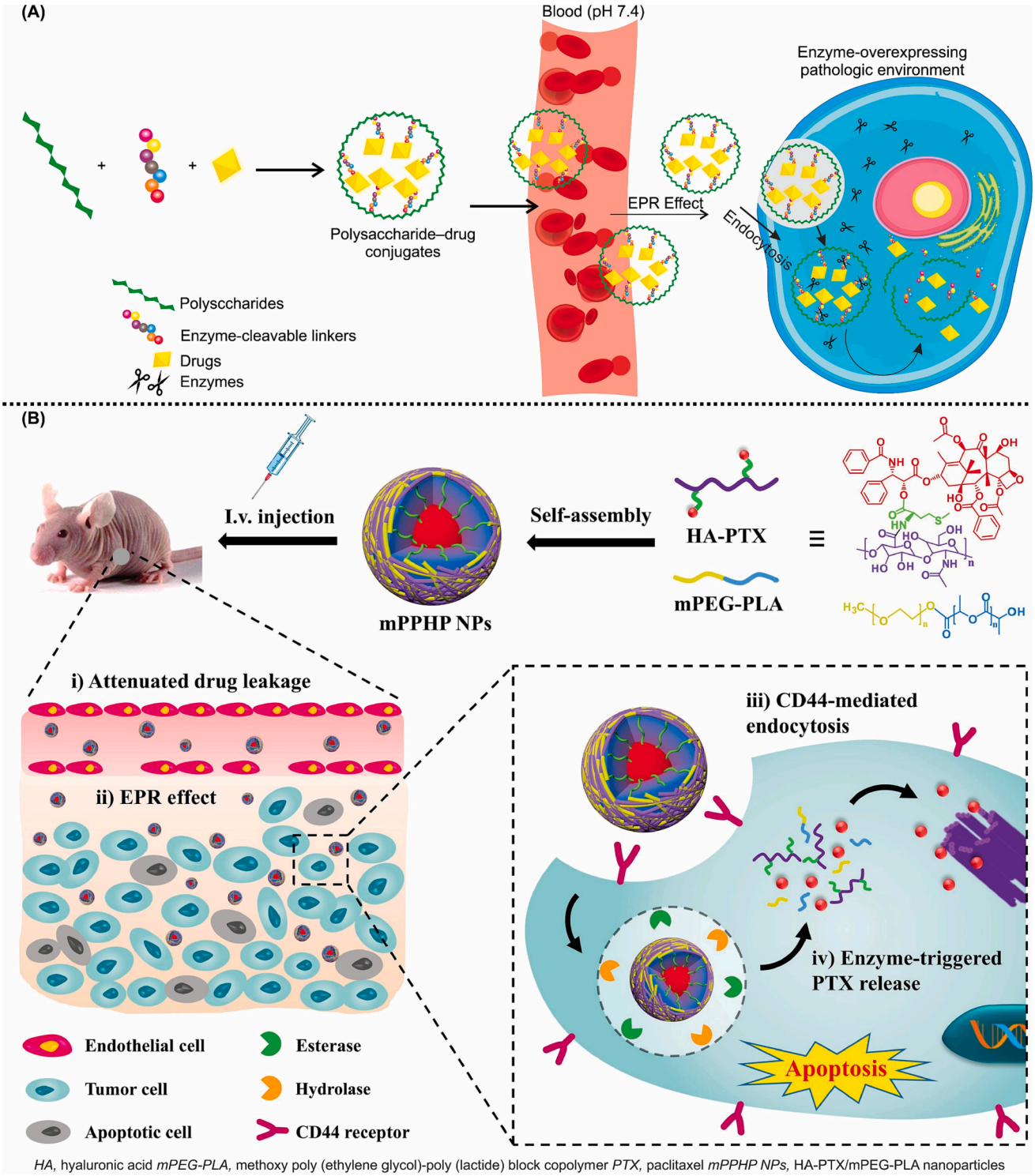

Fig. 6. The schematic illustration of enzyme-stimulated drug release from polysaccharide-drug conjugates in enzyme-overexpressing pathologic environment (A) and preparation, tumor targeted drug delivery, and enzyme-triggered drug release of HA–PTX containing NPs (B). B) Reproduced with permission (Luo et al., 2020). Copyright 2020, Elsevier.

3.2. Enzyme-responsive systems

The enzymes play an imperative role as catalysts in a variety of biochemical reactions. Several enzymes are upregulated in the pathological conditions. These overexpressed enzymes in the disease states have attracted scientists to develop enzyme-degradable drug delivery systems. As compared to other stimuli-sensitive systems, the enzyme-responsive scaffolds experience a rapid reaction rate with high specificity and selectivity. Over the years, various enzyme-sensitive linkers have been exploited in designing polysaccharide–drug conjugates. Once exposed to the specific enzymes, these conjugates are degraded and off-load the conjugated cargoes (Fig. 6A) (Baig et al., 2019; Zhu, Bao, Zhang, Yu, & Lu, 2018).

The esterase enzymes are over-expressed in various cancer cells, which are utilized for the tumor targeted drug delivery. In order to enhance the PTX activity, the self-assembled nanocomposites consisting

enzyme sensitive HA–PTX conjugate and methoxy poly (ethylene glycol)-poly (lactide) block copolymer (mPEG-PLA) were designed (Luo et al., 2020). The HA contributed in the tumor targeting, while the mPEG imparted the hydrophilic attributes to the composites (Fig. 6B). The presence of mPEG could also attenuate the accumulation of scaffolds in the liver. The particle size of the nanocomposites was decreased with increasing degree of substitution of PTX, ascribed to the enhanced hydrophobic interactions among the PTX molecules. The optimized formulations demonstrated the size of 126 nm, PDI of 0.13, zeta potential of −26.7 mV and drug loading of 12.5 %. The esterase-mediated cleavage of the conjugates could rapidly release PTX and selectively exert cytotoxicity against A549 cells. Further, the mPEG-PLA/HA–PTX illustrated an excellent *in vivo* antitumor efficacy with tumor inhibition rate of 75.9 %. Wang and co-researchers fabricated heparin-PTX conjugates by directly anchoring *O*-acetylated heparin and PTX through ester bonds (heparin–PTX1). Alternatively, different amino acids as spacers were exploited to accomplish the conjugates (heparin–PTX2) (Wang, Xin, Liu, & Xiang, 2009). The heparin–PTX2 conjugates progressively liberated a higher amount of drug in presence of esterase at acidic pH (5.0) as compared to the heparin–PTX1 scaffolds. The heparin-PTX conjugates could block the G2/M phase of MCF-7 cells. Moreover, their anticoagulant property was reduced as compared to that of heparin. In another study, a 5-FU derivative *i.e.,* 5-FU-1-acetic acid (5-FUA) was conjugated onto the HES backbone through the esterase sensitive bonds (Luo, Wang, Miao, He, & Tang, 2012). The HES–5-FUA conjugates revealed a higher half-life (121.6 ± 49.9 min) in comparison to 5-FUA (40.1 ± 21.8 min) and 5-FU (17.1 ± 5.4 min). Udo and co-workers conjugated 5-FUA to β-cyclodextrin (β-CD) through amide or ester linkages (Udo et al., 2010). The amide conjugates were not hydrolyzed in presence of enzymes, whereas the ester conjugates were firstly hydrolyzed by the alpha-amylase to small saccharide conjugates, which were consequently hydrolyzed by the carboxylic esterase to liberate 5-FUA. This drug release behavior was comparable to the *in vivo* release in rats. In another investigation, a prodrug of cisplatin (CP) was accomplished by linking it to the pullulan succinate (PulS) *via* esterase-sensitive bonds (Wang et al., 2015). The conjugates promoted the cell apoptosis and arrested the cell cycle, effectively inhibiting the proliferation of HepG2 cells. The PulS–CP conjugates could also accumulate in the tumor tissues and prolong the survival of the tumor-bearing nude mice without causing toxicity to the vital organs.

The human cancer cells also upregulate different protease enzymes such as matrix-metalloproteinases (MMPs) and cathepsin B (CTB). Chau et al. exploited a peptide composed of proline-valine-glycine-leucine-isoleucine-glycine (PVGLIG) residues as MMP-sensitive linker to accomplish dextran–PVGLIG–MTX conjugates (Chau, Padera, Dang, & Langer, 2006). The scaffolds admirably inhibited the growth of MMP-overexpressing tumor cells as compared to the free drug. Additionally, the MMP-sensitive conjugates presented more tolerability than the MMP-insensitive scaffolds with respect to their toxicities in the bone marrow and small intestine. Another study showed that the dextran–PVGLIG–MTX dominantly targeted the tumors by passive targeting and EPR effects (Chau, Dang, Tan, & Langer, 2006). Moreover, the presence of dextran reduced the renal clearance of MTX and subsequently improved its circulation time. In another study, the PVGLIG peptide linkers of dextran–MTX conjugates were shown to be cleaved in the presence of tumor-specific MMP II and MMP IX, releasing the drug selectively in the tumor microenvironment (Chau, Tan, & Langer, 2004). Zhu and co-workers reacted dextran with isocyanate in the presence of dibutyltin dilaurate and the resulting dextran derivative (*viz.,* dextran-maleimide, Dex-Mal) was exploited to synthesize Dex-Mal–CPT conjugates containing valine–citrulline dipeptide as CTB-sensitive linker (Zhu et al., 2018). The conjugates self-assembled into NPs with size ranging from 91.3 to 295 nm and exhibited CTB-sensitive drug release behavior in the acetic acid buffer (pH 5.0). Moreover, the water solubility and antiproliferative activity of the conjugates were comparable with that of irinotecan. Harada et al. reported that the Gly-Gly-Gly linker in the dextran–7-ethyl 10-aminopropyloxy CPT conjugates could exploit lysosomal CTB to release the drug in a slow and prolonged fashion at an optimal pH of approximately 4.0, resulting in an effective exposure of the anticancer drug to the tumor cells (Harada, Sakakibara, Yano, Suzuki, & Okuno, 2000). Cheng and *co*-researchers synthesized PEG-grafted β-CD-based polymer with carboxylate functionality, which was subsequently employed to conjugate CPT through Gly and Gly-Gly-Gly peptide linkages (Cheng et al., 2003). The conjugates enhanced the solubility of CPT and hydrolyzed in a dramatically accelerated rate in either human or mouse plasma at the physiological pH. The rate of hydrolysis of the conjugates was declined with lowering the pH values. In a xenograft mouse model, the conjugates conferred an enhanced efficacy over irinotecan and CPT alone.

Homma and colleagues reported the use of HA as carrier for enzyme-triggered delivery of MTX in the osteoarthritic joints. In this context, the 4,7,10-trioxa-1,13-tridecanediamine and Gly-Phe-Leu-Gly or Asn-Phe-Phe were introduced as linker and lysosomal enzymes-cleavable peptide, respectively (Homma et al., 2009). The HA–peptide– MTX significantly reduced the knee swelling in the rat model, while the marginal effects were evidenced following treatment with HA, free MTX and combination of MTX and HA. Yu and co-researchers fabricated hyaluronidase-responsive HA–gentamicin (Gen) conjugates and were incorporated in the orthopedic implants (Yu et al., 2020). The layer-by-layer assembly technique was exploited to apply multilayer coating of HA–Gen conjugates and CS polyelectrolyte onto the DFO-loaded Titania nanotubes (TNT). The TNT/DFO/HA-Gen demonstrated effective antibacterial and antifouling properties and was suitable for the proliferation, adhesion and osteo/angio-genic differentiation of the mesenchymal stem cells.

3.3. pH and enzyme-responsive systems

Some macromolecular prodrugs were found susceptible to the acidic pH-mediated and enzymatic hydrolysis. The melphalan (MEL) and quantum dots (QDs) were covalently attached onto the HA backbone *via* amidation reaction (Xu, He, et al., 2015). The self-assembled NPs of HA–QDs–MEL conjugates demonstrated diameter of 115 nm, surface charge of 0.75 mV, much faster pH/enzyme responsive drug release behavior under acidic conditions (pH 5.8) and receptor-mediated internalization into human breast cancer cells with significantly superior cytotoxicity than that of pristine MEL. Besides, the toxicity of these NPs on the normal breast cells was lower in comparison to the pure drug. Yu and *co*-researchers fabricated a β-CD polyrotaxane with dialk-poly (propylene glycol) as the axle and β-CD-N_3 as the end-capping group (Yu et al., 2013). The succinate-PTX ester was then attached to the polyrotaxane *via* amidation reaction. The drug release rate of the conjugates was faster at pH 5.0 than that at pH 7.4. The PTX release was further accelerated by the esterase catalysis. The conjugates penetrated deep into the tumors, substantially impeded the tumor growth and prolonged the survival of the tumor-bearing mice, which were superior than a commercial formulation of PTX. Hwang and colleagues conjugated a methylprednisolone (MPS) derivative to a linear β-CD polymer through glycinate ester linkages. The resultant prodrug demonstrated the pH-dependent hydrolysis and enzyme-triggered cleavage, causing the synovial-specific release of the conjugated drug (Hwang, Rodgers, Oliver, & Schluep, 2008). The β-CD–MPS conjugate showed superior efficacy over pristine MPS. In another study, dextran was exploited to fabricate the conjugates of poorly-water soluble drugs like naproxen and ibuprofen (Hornig, Bunjes, & Heinze, 2009). The diameter, size distribution and pH and dextranase dependent hydrolysis of the dextran–drug conjugate NPs were influenced by the degree of the conjugation. Zhu and co-researchers coupled the oxidized cellulose with an amine drug, phenylpropanolamine (PPA), *via* amide bonds using either DCC or EDC as coupling agent. The reaction using DCC was more effective and yielded conjugates containing about 24 % *w*/w of PPA. The conjugates showed a faster drug release rate in the acidic environment and in the rat

Table 1
Various stimuli-responsive polysaccharide/drug conjugates other than pH/enzyme sensitive systems and their therapeutic benifits.

Polysaccharide	Drugs/Agents	Chemistry/linkages	Therapeutic benefits	Reference
Redox-responsive conjugates				
HES	PTX (conjugated) DiR (encapsulated)	Disulfide bonds	• Conjugates caused a synchronized release of loaded DiR and conjugated PTX • These could be applied in chemo-photothermal therapy and photoacoustic imaging	(Li et al., 2019)
	DOX	Disulfide bonds	• Conjugates displayed a prolonged plasma circulation, enhanced tumor accumulation, better antitumor efficacy and reduced toxicity than pristine drug	(Hu et al., 2016)
Thiolated pectin	PTX	Disulfide bonds	• Conjugates had selective *in vitro* and *in vivo* toxicity against colorectal cancer	(Cheewatanakornkool, Niratisai, Dass, & Sriamornsak, 2018)
CS	Dasatinib	Disulfide bonds	• Compared to free drug and non-redox responsive conjugates, redox-sensitive conjugates exhibited better anti-inflammatory effects in mice paw edema model	(Vakilzadeh et al., 2023)
HA	PTX	Disulfide bonds	• Conjugates showed a controlled intracellular drug release behaviour • These had a higher *in vivo* tumor inhibition potential than Taxol and methoxypoly(ethylene glycol)-based conjugates	(Yin et al., 2015)
	TAC	Disulfide bonds	• Conjugates were selectivly uptaken into targeted cells and significantly inhibited IL-2 and IL-1β production by LPS-induced cells	(Singh et al., 2023)
	MTX	Thioketal bonds	• Conjugates had remarkable ROS sensitivity, excellent photostability, high photothermal performance and improved tumor-killing activity	(Yun et al., 2022)
	Hydroxychloroquine	Disulfide bonds	• Conjugates displayed enhanced cellular internalization capacity and 2.23-fold reduced IC_{50} value and inhibited lung metastasis	(He et al., 2022)
Gellan gum	Abietic acid (conjugated) Ribociclib (loaded)	Disulfide bonds	• Gellan gum-SS-abietic acid conjugates conferred higher cellular uptake and cytotoxicity than gellan gum-abietic acid conjugate and free drug	(Shirani, Varshosaz, Rostami, & Mirian, 2022)
Dextran	Diethyldithiocarbamate, a metabolite of disulfiram	Reduction resnitive linkages	• Folate-decorated prodrug exerted anticancer activity superior than disulfiram and folate-free conjugates with minor systemic toxicity in an animal model of colon carcinoma	(Jin et al., 2022)
	PTX	Disulfide bonds	• Conjugates showed a significant cytotoxicity on BT-549 and MCF-7 cells	(Kanwal et al., 2021)
	Pt(IV) (conjugated) DOX (loaded)	Reduction resnitive linkages	• Conjugates released dual drugs under reductive condition, causing an improved therapeutic outcome.	(He et al., 2015)
Light/redox-responsive conjugates				
HES	DOX (conjugated) ICG (crosslinked)	ICG-crosslinked disulfide conjugates of HES and DOX	• Photothermal effects on malignant tumors with dense matrices	(Tang et al., 2019)
pH/redox-responsive conjugates				
HES	DOX	Disulfide and hydrazone bonds	• Conjugates had a high tumor-oriented accumulation and fast intracellular drug release • Dual-responsive conjugates exerted significantly higher tumor-inhibiting efficacy than free DOX and single-responsive conjugates	(Tan, Tian, Liu, Wang, & Wan, 2021)
CS	Cisplatin	Schiff bases, amide bonds and reduction resnitive linkages between cisplatin (IV) and CS	• Conjugates showed a prolonged circulation stability at physiological pH, selective tumor accumulation ability and efficient tumor suppression potential	(Wang et al., 2022)
Thilated HA	DOX	Hydrazone bonds	• These had self-gelation ability and were effective in supressing the growth of human nasopharyngeal carcinoma cells	(Fu et al., 2015)
Oxidized dextran	Pt(IV) and DOX	Esterification with Pt(IV) and Schiff base reaction with DOX	• Prodrug NPs exhibited comparable efficacy to the combination of free drugs in the cervical carcinoma cells • These NPs remarkably reversed the cisplatin resistance in lung carcinoma cells	(Xue et al., 2021)
Redox/enzyme-responsive conjugates				
HES	PTX	Disulfide and α-amylase sensitivite bonds	• Conjugates showed burst drug release under reductive conditions and enhanced cytotoxicity to the tumor cells • These exhibited a longer half-life than commercial product (Toxol)	(Li et al., 2017)
HA	DTX	Disulfide bonds and cathepsin B-responsive tetrapeptide linkages	• Conjugates displayed >99 % tumor inhibition without noticeable toxicity in triple-negative breast tumor-bearing mice model	(Wang et al., 2021)

(*continued on next page*)

Table 1 (*continued*)

Polysaccharide	Drugs/Agents	Chemistry/linkages	Therapeutic benefits	Reference
pH/reduction/enzyme-responsive conjugates				
HES	10-hydroxy camptothecin (10-HCPT)	Ester and disulfide linkages and α-amylase sensitive bonds	• Conjugates significantly improved the physical and chemical properties of 10-HCPT • Prodrug micelles demonstrated better anticancer activity against human liver cancer model	(Li, Zhao, & Zhao, 2020)

liver homogenate, conferring their favorable hydrolysis in these conditions (Zhu, Kumar, & Banker, 2001). In a recent study, Cur conjugated HES based NPs encapsulating dexamethasone (DEX) were developed as a colon-targeted oral formulations for treating inflammatory bowel disease. These NPs exhibited excellent ROS scavenging activity and α-amylase/pH responsive behavior and significantly relieved the ulcerative colitis induced by dextran sulfate (Xu et al., 2022).

4. Challenges and future progresses

As reviewed above, the polysaccharide–drug conjugates with pH and enzyme-responsive characteristics have widely been investigated. Beside pH/enzyme-responsive systems, various other internal and external stimuli sensitive nanotherapeutics based on polysaccharide-small molecule drug conjugates were also developed over the years, which are summarized in Table 1. Although several conjugates are very promising, these are not yet entered into the clinical practices due to the various obstacles (Pang et al., 2013). Most importantly, the structural features (*i.e.,* molecular weight, functional groups, charge, *etc.*) and purity of the polysaccharides are variable from batch to batch and source to source (Roy, Bhattacharyya, Ernsting, May, & Li, 2014). Hence, the fabrication of a reproducible polysaccharide-drug conjugate with uniform attributes is extremely difficult. To eliminate such limitations, the polysaccharides and their drug conjugates could be characterized using small angle neutron scattering, 2D ^{1}H NOESY, TOCSY NMR and pulse-field gradient NMR techniques (Pang et al., 2013). Most of the developed pH and enzyme-responsive polysaccharide-drug conjugates also have sophisticated structures, causing scale-up and industrial production challenges. Thus, the simplification of the conjugate structures is very crucial for their successful clinic translation. Even though the conjugates display excellent repeatability in the *in vitro* conditions, the inter-subject variability could raise concerns with the *in vivo* reproducibility in their efficiency and safety profiles, requiring a long optimization process for these scaffolds. An establishment of a reliable *in vitro* evaluation system, which could effectively predict the *in vivo* behavior, might shorten the optimization process. Furthermore, the polysaccharide-drug conjugates could often show a significant change in biopharmaceutical and pharmacodynamic properties as compared to the native drug molecules. This might require additional FDA approval although the precursor drug is already approved. Therefore, it is crucial to design a clear regulatory agenda to support these conjugates.

Following the drug delivery, the polysaccharide residues of the conjugates could accumulate in the body as these are not readily excreted *via* the kidneys, causing potential toxicity (Goodarzi et al., 2013). Certain pH and enzyme–responsive polysaccharide-drug conjugates could also demonstrate the off-targeted drug release due to the non-specific stimuli, leading to adverse side effects. Moreover, upon administration into the body, the polysaccharide-drug conjugates would experience a series of complex biological barriers such as opsonization, the mononuclear phagocyte system, cellular internalization, endosomal and lysosomal escape, drug efflux pumps *etc.*, significantly limiting their site-specific targeting (Roy et al., 2014). Therefore, it is very crucial to design smart linkers, which could facilitate a higher cellular uptake of the conjugates for site-specific drug delivery and would be sufficiently stable in the transit environment to cross the biological barriers. The linkers should also promote a faster biodegradability of the polysaccharide backbone and eventually these could be eliminated from the body. During designing the smart linkers, a special attention should be paid to the administration routes of the conjugates and heterogeneity, microenvironment and progression of the diseases (Pang et al., 2013).

As the polysaccharides have multiple functional groups, more than one drug with complimentary mode of action and non-overlapping toxicity could be conjugated with their backbone. This could simultaneously deliver two or more drugs, providing synergistic therapeutic effects and overcoming the drug resistance. The multiple components other than the drug molecules could also be conjugated with the polysaccharide. For instance, the polysaccharide could be grafted with PEG to shield the conjugate from the reticuloendothelial system recognition and clearance (Roy et al., 2014). An imaging agent, which would report the therapeutic response or drug delivery in a real time fashion and a targeting ligand, which could increase the cellular bioavailability of the conjugates could also be coupled with the polysaccharides. Unequivocally, the combination therapy could open new therapeutic possibilities, but the complexity of the systems has been tremendously increased. Further, the ratios of the multiple components in the conjugates should be carefully optimized to maximize the therapeutic effects (Pang et al., 2013). The computational modelling of the conjugates could provide the detailed information about their molecular interactions and physicochemical properties and reduce the research time involved to accomplish the complex conjugate systems (Thakor et al., 2020).

5. Conclusions

Due to the speedy advancement in nanotechnology and higher understanding of the distinct physiological variances between the normal and disease tissues, a great deal of research has recently been accomplished to fabricate various pH and enzyme-responsive polysaccharides-drug conjugates as novel therapeutic modalities. These scaffolds could elute the active drugs following scissoring of the conjugated bonds under the influence of specific enzymatic reactions and alterations in pH magnitude of the disease states, providing an opportunity of site-specific drug delivery and reduced adverse effects on the healthy cells. Currently, the pH and enzyme-sensitive conjugates could respond to a combination of two or more stimuli and demonstrate a fine-tuned drug release at the specific sites. Although these systems are appealing for more controlled and precise drug delivery, the translation of polysaccharide–drug conjugates into the clinics is still not so promising owing to many serious obstructions. A better understanding of pH and enzyme -responsive pathways of the polysaccharide-drug conjugates, their structure-efficacy and structure-toxicity relationships could facilitate the development of the optimized conjugate systems with improved therapeutic outcomes, advancing their clinical application prospects. More attention should be paid on their high batch-to-batch reproducibility and industrial scale-up possibility. These might eventually speed up the translation of polysaccharide-drug conjugate based nanotherapeutics from the bench to the clinic. This review article provides a deep insight into the development and challenges of pH and enzyme-responsive polysaccharide-drug conjugates. This would assist the researchers in laying the foundation for further design of such nanomedicines for developing effective therapies to transform human health

in coming years.

Declaration of competing interest

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Data availability

No data was used for the research described in the article.

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