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Complete List of Authors:	Duro-Castano, Aroa; Centro de Investigacion principe Felipe, Polymer Therapeutics Lab. Movellan, Julie; Centro de Investigacion principe Felipe, Polymer Therapeutics Lab. Vicent, Maria; Centro de Investigacion Principe Felipe, Polymer Therapeutics Lab.

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A. Duro-Castano,[a] J. Movellan[a]† and M. J. Vicent[a]†

Polymer-drug conjugates represent excellent nanopharmaceutical candidates, as they offer multiple advantages related to their intrinsic characteristics. Many of said characteristics are provided by the covalent bonding between drug and polymer. However, their clinical development has been slow and only one polymer-drug conjugates has reached the market, thus there remains an urgent need for the development of new and smart polymeric systems. Desirable characteristics of these new systems include higher molecular weight and degree of homogeneity, predictable conformations in solution, multivalency, and increased drug loading capacity, amongst others. With these aims in mind, branched polymers are ideal candidates due to their unique rheological, mechanical, and biomedical properties derived from their structure, inaccessible for linear polymers. Within this review, the synthetic strategies developed and the main efforts towards branched polymer implementation as carriers for polymer-drug conjugates will be addressed.

1. Introduction

The nanomedicine field is currently experiencing a notable boom, with 40 products entering into the market in the last ten years and more than 70 currently in cancer clinical trials.[1] This is mainly due to the potential of nanomedicine to greatly contribute towards multiple unsolved pharmaceutical and clinical needs in life-threating diseases.[2] Nanomedicine has gained special attention in recent years in multiple different research areas, with a focus on delivery of drugs or genes, in diagnostics and molecular imaging, as well as in tissue repair and engineering.[3]

Polymer therapeutics can be highlighted as one of the most successful areas contributing to the first generation of nanomedicines with 15 products in routine clinical use. This is exemplified by the polymeric drug glatiramer acetate for multiple sclerosis (Copaxone®, Teva Pharm; $3.7 billion) and the polymer conjugate polyethylene glycol (PEG)-filgrastim for the treatment of neutropenia (Neulasta®, Amgen; $3.6 billion) appearing on the US list of Top 10 selling drugs.[4] Polymer therapeutics encompass a variety of complex multicomponent macromolecular systems, with the presence of a rationally designed covalent bond between a water-soluble polymeric carrier (with or without inherent activity) and the bioactive molecule(s) being the common feature.[5] Polymer therapeutics include polymeric drugs,[6-8] polymer-drug conjugates,[9,10] polymer-protein conjugates,[11-13] polymeric micelles where the drug is attached by covalent bonding,[14-16] and multicomponent polyplexes (polyelectrolyte complexes) that are being developed as non-viral vectors.[17-21] Therefore, they are considered as 'new chemical entities' (NCEs) rather than conventional drug-delivery systems or formulations that simply entrap, solubilize, or control drug release without resorting to chemical conjugation.

Drug conjugation to a polymer not only enhances its aqueous solubility but also changes drug pharmacokinetics (PK) at the whole organism and even subcellular level which therefore may enhance drug therapeutic value. Due to their intrinsic characteristics at the nanoscale (conjugate size, potential for spatially controlled multifunctionality and architecture, and presence of bioresponsive elements) this class of nanopharmaceuticals can be carefully engineered to exhibit unique advantages: (i) increased bioavailability and plasma half-life by means of a higher hydrodynamic volume that will presumably decrease renal clearance; (ii) protection against proteolytic enzymes, or unspecific cellular uptakes; and (iii) modified pharmacokinetics (PK) at the whole body, as well as at cellular and even subcellular, level. Passive targeting based on disease related vasculature abnormalities (the enhanced permeability and retention or EPR effect)[22-26] is also possible, leading to lower systemic toxicity and may even overcome chemoresistant mechanisms (i.e. multiple drug resistance induced by P-glycoprotein 1 overexpression in the plasma membrane) restricting cellular uptake to the endocytic pathway (lysosomotropic intracellular drug delivery).[27] Indeed, polymer-drug conjugates have a greater ability to cross biological barriers and display architecture specific intracellular trafficking allowing for greater control of drug pharmacokinetics (PK) due to the use of bioresponsive chemical conjugation.

Although 16 polymer-drug conjugates are now in advanced clinical trials, progress has been slow due to clinical failures

[a]Centro de Investigación Príncipe Felipe, Polymer Therapeutics Lab., Av. Eduardo Primo Yúfera 3, E-46012 Valencia (Spain)
† Corresponding authors: jmovellan@cipf.es, mjvicent@cipf.es

resulting mostly from poor rational design.[10] This slow progress in some ways parallels that seen for immunoconjugates as both share the challenges of tumor targeting and linker optimization for drug release. Lack of polymer suitability from a clinical viewpoint (safety), poor manufacturing reproducibility, and the lack of validated characterization methods for such complex conjugates and architectures have also limited progress.

Most polymer-drug conjugates use N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers, PEG, or more recently polyglutamic acid (PGA) as carriers.[10] Biopersistent carriers (PEG, HPMA) present disadvantages if chronic parenteral administration and/or high doses are required as there is potential to generate 'lysosomal storage disease' syndrome.[28] Preclinical evidence of intracellular vacuolation[28] and clinically reported hypersensitivity reactions[4] with certain PEG-protein conjugates are raising awareness of the advantages of biodegradable polymers due to their safety benefit alongside the possibility to use higher molecular weight (Mw) carriers allowing for PK optimisation. Higher Mw biodegradable polymeric carriers can maximize EPR-mediated tumor targeting, which is ultimately driven by the circulating plasma concentration of the conjugate.[4] Biodegradable polymers in preclinical or clinical use include: polypeptides,[29-30] dextrins,[31] polysialic acids,[32] polyacetals,[33] and hydroxyethyl starch (HES).[34] Routine clinical use of Copaxone[35] and the promising clinical results with Opaxio® (PGA-paclitaxel (PTX) conjugate)[36] have underlined the high potential of synthetic polypeptides within nanomedicine, in particular polyglutamates (PGA). Biodegradable polymers allow the utilization of higher MW platforms to optimize PK, which is essential for the treatment of diseases that require chronic administration, such as neurological disorders or tissue regeneration.[33, 37]

Apart from biodegradability, the development of novel well-defined architectures with higher MW (in order to increase passive targeting provided by the EPR effect), predictable structure and conformation (defined three-dimensional architecture in solution), higher homogeneity, greater drug loading capacity, and increased multivalency are considered crucial research areas in nanomedicine. In this context, the use of branched polymers is emerging in order to achieve the previously described requisites. Branched polymers can be generally, easily prepared in massive scale, and they exhibit special properties when compared to their linear analogues as a result of their different architectures, solution conformations, sizes and shapes as well as greater multivalency. In general, branched systems own unique topological structures and appealing physico-chemical properties. When compared to their linear analogues, they show three-dimensional globular structure, lower solution or melt viscosity, small hydrodynamic radius, improved multifunctionality, enhanced encapsulation capabilities, no or low molecular entanglement and better solubility.[38-39] Furthermore, their high density of functionalities allow to tune their thermal, mechanical, rheological, solution properties (size, conformation, solubility), biocompatibility of the constructs.[40-41] Their high multifunctionality also provides superior stimuli-responsiveness and allows to conjugate a vast amount of bioactive agents, targeting ligands and imaging probes.[42] Moreover, they exhibit controllable supramolecular morphologies and structures and unusual self-assembly behaviors when compared to conventional molecules and linear block copolymers.[43-44] These features can be translated into improved biodistribution and pharmacokinetics profiles, enhanced tumor penetration (derived from their smaller sizes), favored mechanisms to cross biological barriers and cellular trafficking when compared to other systems (classic linear polymer-drug conjugates, multi-block copolymer micelles or core-cross-linked micelles), and overall, different therapeutic output.[45-49] Branched polymers include star, hyperbranched and dendritic-like polymers, dendrimers, graft, brush and comb-like polymers as well as polymer networks (See Fig. 1. for examples of branched polymeric architectures). Dendrimers have been exhaustively reviewed previously,[50-54] while polymer networks are mainly formed from the other polymeric structures by cross-linking strategies. Therefore we will address the other main types of branched polymers, whose relative easy, fast and less cost-effective synthesis, generally via one-pot polymerization, and simpler purification steps are the main advantage over dendrimers.[55-56] Their intrinsic characteristics and potential applications as polymer-drug conjugates will be reviewed herein.

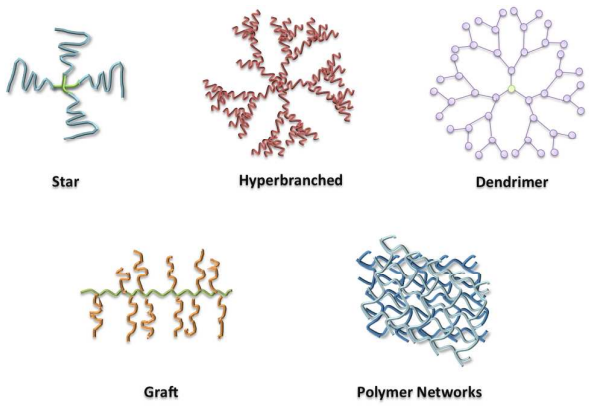

Fig. 1. Some examples of branched architectures

2. Stars polymers

Star polymers are branched polymers consisting of several linear chains attached to a central core, and can be sub-classified depending on the nature of the different branches. If the branches are identical linear chains they are named “Symmetric Stars” and if the branches have different molecular weight or topology they are considered “asymmetric stars”, or “miktoarm stars” if the branches are chemically different. In all cases, these arms can be constituted by one-block or multi-block copolymers.

This special category of polymers has become popular in different research areas (chemistry, physics, biochemistry and

engineering) due to the unique mechanical, rheological, as well as biomedical properties that are unattainable for linear polymers.[46, 57-59] In general, star polymers are characterized by a compact structure, presumably with globular shape, with a large surface area and increased concentrations of functional end groups when compared to polymers of similar MW. Moreover, they offer unique rheological properties which make them ideal platforms for drug delivery[60-62] amongst other biological applications.[63] Furthermore, both multi-arm stars and hyperbranched polymers, display enhanced solubility, lower melt viscosity, and different thermal and physical properties in general in comparison to their corresponding linear structures.[64-65] Viscosity and other properties depend more on arm MW than on the total MW of the star polymer.[66] If compared to dendrimers, star polymers offer the advantages of accelerated and tunable methods of synthesis. Apart from bio-applications, these unique materials are also being considered with growing interest in other areas, including thermoplastics,[67] nanoelectronics,[68] and many other applications. [46, 59] As stated before, star polymers are defined by a smaller size and therefore higher segment density when compared to linear polymers with the same MW. One of the most appealing properties, apart from their rheological characteristics and thermoplastic character, is their self-assembly behavior which can be promoted in solution by the presence of functional moieties along the chain arms (in the case of homopolymers), or by using selective solvents (in the case of star-blocks or miktoarm stars). The micellar structural parameters, such as critical micellar concentration (CMC), aggregation number, core and shell dimensions, overall micelle concentration, as well as the thermodynamics and kinetics of micellization of complex structures, such as star-block copolymers and miktoarm stars, have been poorly investigated when compared to the linear architectures. In general, star structures have higher CMC values and consequently lower aggregation numbers than their linear block copolymers counterparts. He *et al.*,[69] whose study serves as an example of this micellar behavior, synthesized a family of 4-arm star-block copolymers based on polyethylene oxide (PEO) (as inner block forming the core) and poly (methyl methacrylate) (PMMA). They found that the micellar behavior of the polymers was affected by the pH of the aqueous solutions: at high pH values the star-blocks dissolved and adopted an extended conformation, while at low pH and low degrees of neutralization, large spherical micelles formed, presenting lower hydrodynamic radius (Rh) with decreasing degrees of neutralization. In this case, the micellization behavior depended on the balance between the existing interactions, which includes electrostatic (due to the carboxylic groups), hydrophobic, and hydrogen bonding. A second example of arm number influencing micellization character was reported by Strandman *et al.*[70-72] They synthesized two different amphiphilic 4- and 8-arm PMMA-PAA (poly(acrylic acid)) star-block copolymers with PMMA as inner blocks. When studying the 4-arm star polymer, they observed a morphological transition from spherical multimolecular micelles at pH 5 in salt-free aqueous solutions to cylindrical micelles upon the addition of salts that were again transformed into spherical micelles with the increase of the pH up to 12 (swelling of the corona). In contrast, this effect did not occur for the 8-arm star polymer as the higher number of arms resulted in higher repulsion and stretching of the PMMA core, leading only to spherical structures.

Currently, reports suggest that macromolecular architecture is a key parameter for the tuning of micellar behavior and properties, and thus, it must be considered in the design of new materials and their potential biological applications, in particular as drug delivery systems. Although there are many examples of drug encapsulation within these unique architectures due to their inherent nature,[73-76] their description is out of the scope of this review. Only conjugates and polymeric micelles based on covalently bound drugs will be addressed.

2.1. Synthetic approaches

Two major strategies have been widely applied for the synthesis of star polymers: the core-first approach (divergent approach) and the arm-first approach (convergent approach) (Fig. 2).[59]

The core-first approach is based on the use of a multifunctional initiator as a core that initiates the polymerization of several arms simultaneously. Equally reactive initiating sites are crucial to control polymerization and to synthesize homogenous constructs, and this also requires that the initiation step must be always faster than the propagation step. Historically, the major disadvantage of this methodology is the difficulty in the characterization of the polymers obtained, as the arm MW cannot be directly measured. Nevertheless, advances in characterization techniques are progressively solving this problem and this strategy is the most widely used in the synthesis of star polypeptides.[77]

Alternatively, the arm-first approach consists of the reaction of previously synthesized living macromolecular chains with a multifunctional reagent that serves as a core. Two main strategies can be followed in this case; either the use of a multifunctional molecule that will neutralize linear living chains ("multifunctional linking agents") or the strategy can be based on the covalent attachment of telechelic linear polymers to a multifunctional central core ("coupling onto"). The main advantage of the arm first approach is the relative ease of characterization since the living arms can be characterized in a previous step before linkage. However, the main disadvantage of this methodology is the steric hindrance component which could limit the number of arms which can be linked. Moreover, a large excess of living chains is always a requisite, and for this reason, purification-fractionation steps are required to obtain star polymers with high purity.

Other than these two widely used approaches, the most recent classification takes into account a new synthetic strategy. This approach consists of the reaction of living macroinitiators (MI) (also named macromonomers) with multifunctional molecules acting as cross-linkers giving rise to

star-shaped architectures known as core cross-linked star (CCS) polymers (Fig. 2).[78-79]

The first attempt to synthesize star polymers was made in 1948 by Schaefgen and Flory[80] who, by using the core first approach with multifunctional initiators such as cyclohexanenetetrapropionic or dicyclohexaneneoctacarboxylic acid, polymerized ε-caprolactam to give rise to tetra- and octa-arm star-like polyamides. Morton *et al.*[81] then used the arm first approach for the synthesis of 4-arm star polystyrene by neutralizing polystyryllithium living chains with tetrachlorosilane. From this period onwards numerous efforts have attempted to build novel star-shaped architectures as well as to understand their unique properties from a theoretical and experimental point of view.[82-84] Advances in modern polymer chemistry, especially with the introduction of controlled/living radical polymerizations in the 1990s, made possible the exponential growth of these complex materials. The advances in "click chemistry" represented an important contribution especially when using the arm first approach. However, the synthetic methods for the development of star-related architectures are out of the scope of this review, with more detailed information can be found elsewhere.[46,59]

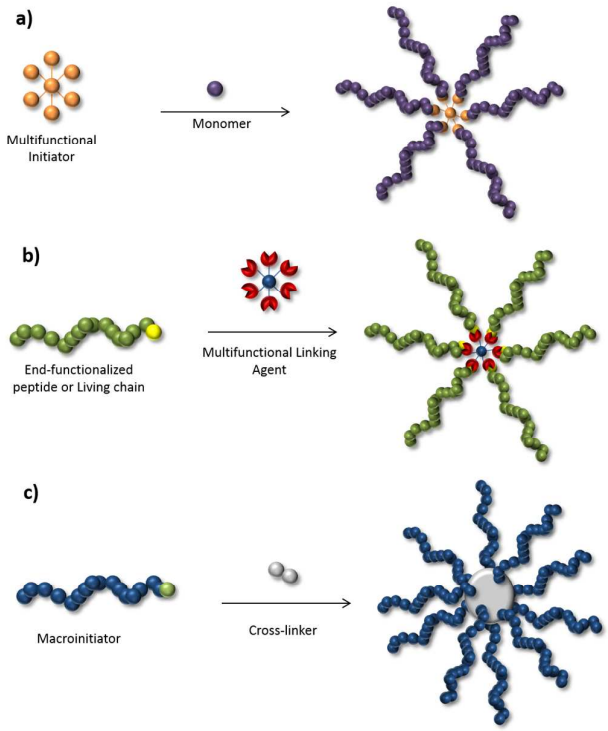

Fig. 2. Strategies for star polymers synthesis. a) Core-first approach. b) Arm-first aproach. c) Core cross-linked stars synthesis.

2.2. Star polymers as drug carriers

Although the star polymers represent promising architectures to be used as polymer-drug conjugates, few examples can be found in the literature and they remain far from clinical application. Most examples are based on the well-known HPMA copolymer, one of the most studied polymer carriers within polymer-drug conjugates.[5] Indeed, PK1 (HPMA copolymer-Doxorubicin (DOX)) was the first synthetic polymer-drug conjugate assessed in clinical trials. In 2000 Wang *et al.*[85] reported the synthesis of star-like HPMA by conjugating semitelechelic HPMA macromolecules to PAMAM dendrimers from generations G2 to G4 as a core. DOX was conjugated to the star-like HPMA copolymer to evaluate its potential as a drug delivery system compared to linear HPMA copolymers in terms of drug release and cytotoxicity against a human ovarian carcinoma cell line (A2780).

Jelínková *et al.*[86] compared two different antibody-targeted HPMA copolymers of GlyPheLeuGly-DOX (star-like vs linear HPMA). The star-like conjugate consisted of 30- to 40-copolymer chains of HPMA bearing DOX linked to the central antibody molecule via an amide bond between the end of each backbone chain and the lysine Ɛ-amino groups of the antibody. Whereas the binding affinity was independent from the polymer architecture, the star-like conjugate exhibited 10-fold higher cytotoxic effect *in vitro* in different cancer cell lines and 6.5-fold higher concentration in blood in the biodistribution studies in mice, as compared to their linear counterparts.

Both types of anti-Thy-1.2 targeted conjugates showed good performance when applied to mice bearing T-cell lymphoma EL4, however the star-like conjugates containing anti-CD71/A or B antibodies performed better than the classic linear ones in colorectal cancer SW620.

Etrych *et al.*[87] then described the synthesis of a family of new biodegradable star polymer-DOX conjugates also based on a macromolecular core formed by PAMAM dendrimers onto which semitelechelic HPMA copolymer DOX conjugates (hydrazone linked) were grafted. They were able to synthesize different MW constructs (from 200 to 1000 g/mol) with relatively low polydispersities (~1.7). The linear HPMA chains were attached to the dendritic core either by stable amide bonds or enzymatically or reductively degradable spacer as shown in Fig. 3. The star conjugates exhibited higher in vivo anti-tumor activities when compared to the free DOX or linear polymer conjugates in a EL4T-cell lymphoma mouse model.[88]

Previously, the same group had developed star-shaped immunoglobulin-containing HPMA-based conjugates[89] with hydrazone-DOX that displayed comparable cytostatic activity as for free DOX-HCl in several cancer cell lines, and significantly higher antitumor activity *in vivo* in mice bearing EL4 T-cell lymphoma than immunoglobulin free conjugates. Another example of the hybrid dendritic-star like polymers is the work of Cao *et al.*[90] who reported the synthesis of a dendrimer-like star polymer based on well-defined poly(L-lactic acid) (PLLA) star polymer with six carboxylic acid-terminated polyester dendrons of 2,2-bis(hydroxymethyl)propionic acid. Amine-functionalized folic acid moieties were effectively conjugated to achieve atargeted drug delivery system, and the constructs were tested in terms of cellular uptake in mouth epidermal

carcinoma (KB) cells (overexpressing folate-receptor). They found that the uptake of folate-conjugated star-like polymers was much higher than the non-targeted ones.

Kowalczuk *et al.*[91] described the synthesis of star-shaped cysplatin nanoconjugates (12-14 nm radii) based on a highly branched poly(styrene) core and poly(tert-butyl acrylate) arms. They were able to achieve a high cysplatin loading (45 wt %) and their *in vitro* evaluation showed a sustained drug release, an endocytic mechanism of uptake, and a lower cytotoxic effect when compared to the free drug.

Very recently, Li *et al.*[92] reported on nanoparticle systems (~15 nm radii) based on star polymers as theranostic vectors bearing aldehyde groups for the covalent conjugation of DOX and activated esters for the 1-(5-amino-3-aza-2-oxypentyl)-4,7,10-tris(tert-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane (DO3A-tBu-NH_2) - a Gadolinium (Gd^{3+}) chelating agent. Amongst other results, they found that the DOX/Gd-conjugated nanoparticles yielded a similar IC_{50} to free DOX for breast cancer cell lines, confirming DOX integrity after nanoparticle conjugation. Moreover, the relaxivity of Gd loaded in star-shaped polymers was found to be 3 times higher than conventional organic non-polymeric Gd/DO3A complexes.

Navath *et al.*[93] reported the synthesis and biological evaluation of N-acetyl cystein (NAC) conjugated to 6-, and 8- PEG star-shaped polymers via disulfide bonds for applications in neuroinflammation.

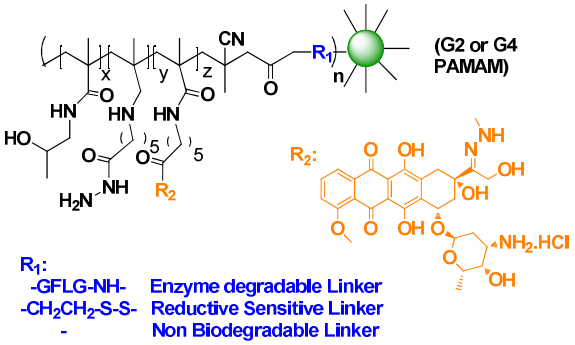

Fig. 3. Star-shaped HPMA copolymers DOX conjugates. Adapted from ref [87].

Conjugates diameter sizes were between 21-28 nm and 34-43 nm for 6-arm an 8-arm polymers respectively. The two synthesized constructs demonstrated a release of NAC of 74% in 2 hours when exposed to glutathione (GSH) at intracellular concentrations (2–10 mM), whereas no release was observed with extracellular concentrations of GSH (2 μM). The conjugates demonstrated a 2-fold increase in antioxidant activity compared to free drug when they were tested by monitoring cytokine release in lipopolysaccharide (LPS) induced inflammatory response in microglial cells looking at ROS (reactive oxygen species), NO (free radical nitrile), anti-inflammatory activity, and GSH depletion.

There are also some examples of star polymer-drug conjugates for antifungal applications. Sedlák *et al.*, in 2008 reported the synthesis of conjugates of β-glucosidase-sensitive star-PEG with the powerful antifungal drug Amphotericin B (AmB) (Fig. 4).[94] Through the use of the linker β-D-glucopyranoside (molecular switch sensitive to β-glucosidases), the release of AmB is ensured to occur only in parasital fungal pathogens that have specific hydrolase β-glucosidases, and not in healthy human tissues where these enzymes are not present. Their preliminary studies demonstrated an efficient targeted delivery at the areas of activity of the pathogens.

The same group has also used the star-shaped PEG platform described before as drug delivery carrier for the antifungal agent nystatin with similar results.[95] Yang *et al.*[96] also reported the synthesis and biological evaluation of PEG-PAMAM star polymer-conjugates of Penicillin V using both biodegradable (ester linkage) and non-biodegradable attachment (amide bond). The authors compared their release profiles but further investigation of this strategy is required in order to characterize these systems for antifungal applications.

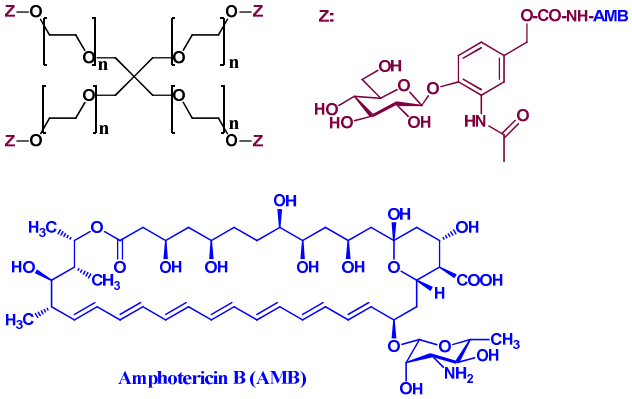

Fig. 4. β-glucosidase-sensitive star-PEG–Amphotericin B (AmB) conjugates. Adapted from [94].

3. Hyperbranched polymers

Hyperbranched polymers (HBPs) are highly branched three-dimensional polymers with a dendritic like architecture and their structural characteristics place them between conventional linear polymers and dendrimers. The main advantage of HPBs over dendrimers is their simpler synthesis, usually involving one-step reactions and purifications by precipitation, implying a great benefit in terms of manufacturing time and costs.

One of the key parameters to take into account in the characterization of these polymers is their degree of branching (DB). DB is 0 for a linear polymer and 1 for a dendrimer, and HBPs are situated between these two with a DB of 0.4 to 0.6. The DB value will consequently have a great influence on the properties of the HBPs, including low molecular entanglement, low melting/solution viscosity, high solubility, host guest interaction capacity, and self-assembly behavior. Notable, all of these properties are tunable through modifying branches, end-groups and DB.

The story of HBP started in the middle of the 20th century with the first theoretical work by Flory.[97] The term hyperbranched polymer appeared later when Kim and Webster[98-100]

synthesized soluble hyperbranched (HB) polyphenylene. Since then, the interest in this category of polymers has grown up constantly and the synthetic approaches as well as their applications have been broadly developed. Some HBPs are already commercially available, such as Boltorn® (HB polyester), Hybrane® (HB polyesteramine), HB PEI (HB polyamine) and HB polyglycerol (HB polyether). HBPs are now used in a wide range of applications,[101-102] with gene/drug delivery, sensors, catalyst, nanoreactors, functional self-assembly, additives, coatings, encapsulations or electron/energy/light-harvesting among the most important.

3.1. Synthetic approaches

The synthetic routes for preparing HBP are usually divided into two main categories both following a bottom-up strategy: the single-monomer methodology (SMM) and the double-monomer methodology (DMM) (see Fig. 5).[101-102]

The SMM technique embraces different approaches including polycondensation of AB_n monomers (n>1), self-condensing vinyl polymerization (SCVP), self-condensing ring-opening polymerization (SCROP), atom transfer radical polymerization (ATRP), and finally proton transfer polymerization (PTP). When using the polycondensation of AB_n method, up to AB_6 monomers can be used leading to highly branched architectures. Side reactions occurring during SCVP can lead to gelation and the polymers obtained by this method often have high polydispersity.

The DMM technique implies the polymerization of two different types of monomers and can be divided into two subcategories. The classic DMM involves the polymerization of A_2 and B_n (n>2) monomers (the "A_2 + B_3" approach) and was first introduced by Jikei and Emrick to prepare soluble HBPs.[103-104] Control of the reaction conditions strongly influences the polymerization results, and the translation of the A_2 + B_3 approach (use of symmetric monomers) to industry is limited by the high cross-linking risk, usually avoided with low monomer concentration, slow monomer addition, and/or by stopping the reaction before the gelation point. An alternative to this strategy is the use of specific monomer pairs with functional groups of different reactivity (asymmetric monomers); the so-called couple-monomer strategy (CMM) first reported by Gao and Yan.[105-109] In the CMM, two types of monomers will preferentially generate an *in situ* AB_n intermediate at the initial stage of the polymerization, minimizing or even eliminating the problem of gelation. This approach can be also considered as the combination of the SMM principle and the multimonomer character of the classic DMM.

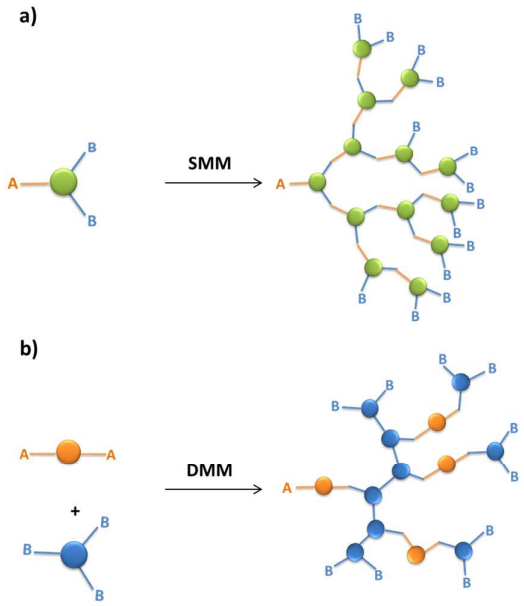

Fig. 5. Two main strategies to reach HBP: a) Single-monomer methodology (SMM), b) Double-monomer methodology (DMM).

3.2. Hyperbranched polymers as drug carriers

As stated above, the applications of HBPs are diverse and some relevant reviews have been already published.[101-102, 110] Herein, we will exclusively focus on HBP-drug conjugates.

In drug delivery applications, HBPs have been taken advantage of for their small molecule encapsulation capacity.[111-113] To the best of our knowledge, the first report of a HBP-drug conjugate was described by Prabaharan *et al.*[114] They prepared an amphiphilic derivative of Boltorn® conjugated with DOX through a pH-sensitive hydrazone linker and actively targeted including folic acid (FA) residues (Fig. 6). The polymer, H40-P(LA-DOX)-b-PEG-OH/FA, was based on the commercial aliphatic dendritic polyester Boltorn® H40 (H40) core, a hydrophobic poly(L-aspartate-DOX) inner arm (DOX loading of 16 wt%), and a hydrophilic PEG and FA-conjugated PEG outer arm (4%). H40-P(LA-DOX)-b-PEG-OH/FA was able to form unimolecular micelles showing a bimodal distribution when analyzed by dynamic light scattering (DLS) (17-36 and 52-76 nm diameter).

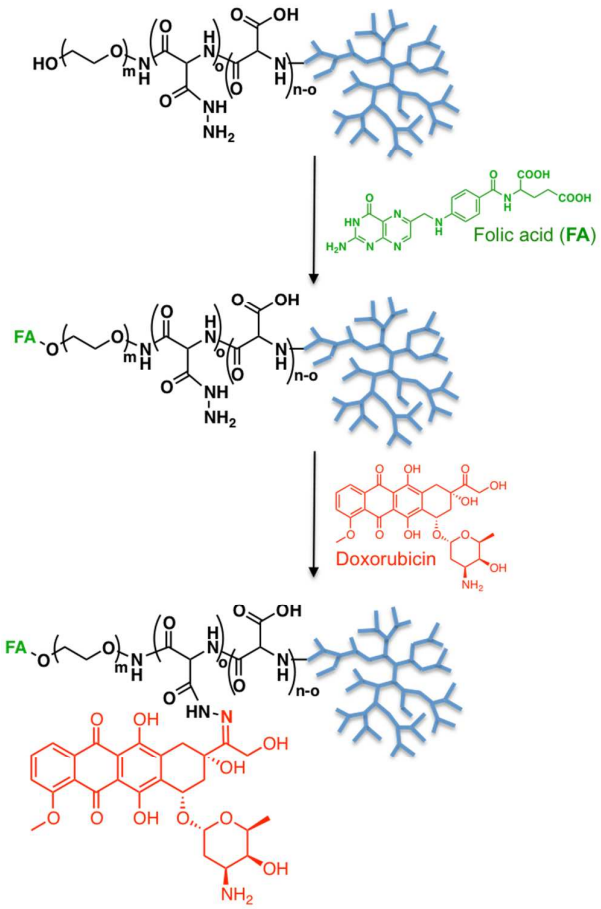

Fig. 6. Amphiphilic derivative of Boltorn® conjugated with DOX through a pH-sensitive hydrazone bond and actively targeted by folic acid. Adapted from ref [114].

A pH dependent release of DOX was obtained through the acid sensitive hydrazone linkage. The cellular uptake of H40-P(LA-DOX)-b-PEG-OH/FA was found to be higher than the one of the control polymer H40-P(LA-DOX)-b-PEG-OH in 4T1 murine breast cancer cell line, resulting in enhanced cytotoxicity against these cells. Combining active agents on the same polymer backbone, such as a drug and a targeting ligand or an imaging moiety, is of great interest to the field of drug delivery. The high number of inner functionalities as well as end-groups that HBPs offer will allow the exploration of this strategy.

Liu *et al.*[115] prepared and evaluated a water-soluble and biocompatible hyperbranched polyphosphate (HPHEEP) (DB 0.48) loaded with chlorambucil, a hydrophobic anticancer agent. The polymer was prepared via SCROP of 2-(2-hydroxyethoxy)ethoxy-2-oxo-1,3-2-dioxaphospholane (HEEP). The authors demonstrated that cells internalized a HPHEEP-rhodamine B conjugate via endocytosis and accumulated in the perinuclear region instead of the nucleus. Covalent binding of chlorambucil to HPHEEP through esterification of the hydroxyl groups led to a 64.2% conjugation ratio of chlorambucil/hydroxyl (approx. 12.8 mol% loading) and particle sizes of between 50-70 nm in diameter. The conjugate was evaluated against MCF-7 human breast cancer cells showing slightly higher IC50 when compared to the free drug. Pang *et al.*[116] reported a second HBP-chlorambucil conjugate, which they generated using a HB poly (amine-ester) series by PTP with a DB between 0.47 and 0.68. Cell viability assays found that these compounds were non-cytotoxic up to 1 mg/mL and their uptake was confirmed by flow cytometry and confocal laser scanning microscopy. The conjugation of chlorambucil (7 wt%) to these HBPs inhibited 50% of MCF-7 growth at a dose of 120 µg/mL.

Ye *et al.*[117] reported the synthesis of hyperbranched polyglycerols (HPGs) with hydrophobic cores derivatized with PEG and functionalized with carboxylate groups in order to conjugate and release cisplatin (Fig. 7). They obtained cisplatin loading up to 20 wt% after increasing the number of carboxylate groups on the surface of the HPGs, with conjugates particle size of around 5-10 nm in diameter. The group also observed pH independent drug release and a release rate considerably greater in urine (10% of the dose in 2h), which might represent an advantage for bladder cancer treatment. The HPGs demonstrated good biocompatibility and HPG-cisplatin conjugates effectively inhibited the proliferation of KU-7-luc human bladder cancer cells. In parallel, Xia *et al.*[118] developed a carboxyl-modified HB polyether (Suc-HPMHO) and cisplatin as a pH-responsive complex (Suc-HPMHPO-CDDP). Adjusting the degree of carboxylation of the polymer and therefore its hydrophilic/hydrophobic balance allowed the control of pH-responsive behavior of the polymer. The phase transition was obtained at pH 6.5 for the polymer containing cisplatin and this translated to a faster release of cisplatin at such pH compared to pH 7.4. The complex showed antitumor effect similar to free cisplatin meanwhile the control Suc-HPMHO had low cytotoxicity against COS-7 African green monkey kidney fibroblast cells, and self-assembled into NPs of

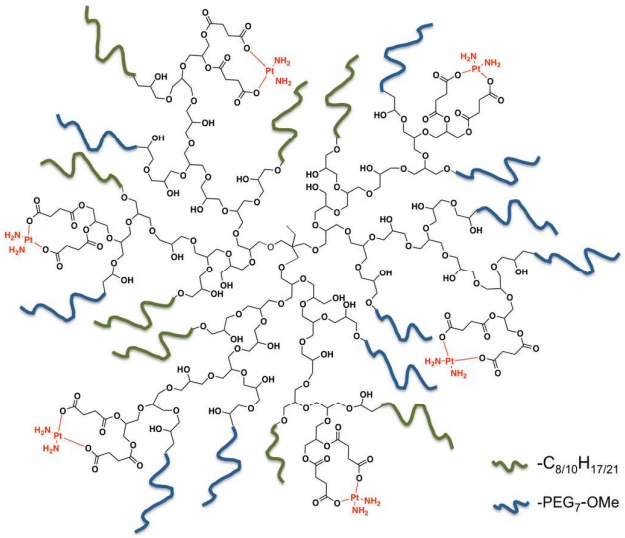

Fig. 7. Representative structure of amphiphilic hyperbranched polyglycerols HPG-C8/10-MePEG-COOH bound to cisplatin.[109]

70-80 nm in diameter in neutral aqueous solution. The authors also found an increased amount of cisplatin with time in cervical cancer HeLa cells at pH 6.5 demonstrating the pH-responsiveness of their polymer-drug conjugate. Subsequently, Lee *et al.*[119] synthesized HB polyglycerols (HBPG)-PEG copolymers aiming to enhance biocompatibility, to increase water solubility, and to improve the clearance of the polymer after drug delivery. DOX was conjugated to the polymer through a pH-labile hydrazone bond (2.1 wt%) and the polymer-drug conjugates spontaneously self-assembled into micelles of an average diameter size of 200 nm (DLS). The constructs showed a pH-responsive DOX release, and the group evaluated conjugate internalization and cytotoxicity in HeLa cells.

Hu *et al.*[120] prepared a family of polyglycerols including ester bonds by oxyanionic initiating hybrid polymerization of glycerol and glycidyl methacrylate in presence of potassium hydride (KH). By adjusting the ratios of KH/glycerol or glycerol/GMA various dHPGs were obtained with DB between 0.43-0.5 and polydispersity (Đ) 1.7-2.2. The polymers were non-toxic up to 10 mg/mL in NIH/3T3 mouse fibroblast cells and quickly degraded due to the cleavage of the ester bonds. 6.7 wt% of methotrexate (MTX) was covalently conjugated through ester linkages at the surface of the dHPGs and the conjugates self-assembled into micelles of ≈ 160 nm. The constructs showed pH dependent release of MTX and an efficient internalization and cytotoxicity against oral adenosquamous cell carcinoma CAL27 cell line and HeLa cells.

There is also one example of photodynamic therapy using HBPs: Li *et al.*[121] used HB poly(ether-ester) and chlorin(e6) as photosensitizer moiety (DB 0.47, Đ 2.84 and total chlorin(e6) loading 4.8 wt%). Particle sizes found were around 50 nm in diameter, and the conjugate was significantly more cytotoxic *in vitro* against CAL-27 cells than the free drug when exposed to 12 J/cm^2 of 660 nm laser light delivered at 100 mW/cm^2.

Sideratou *et al.*[122] reported the conjugation of Gd chelating moieties and FA targeting ligands to Boltorn® H40 HB polyesters. They introduced EDTA (ethylenediaminetetraacetic acid) and DTDA (diethylenetriaminepentaaceticacid) groups on the surface of two different HBPs and covered them with PEG chains (average of 3 PEG/polymer). The authors also added folate moieties to the end of the PEG chains (FA/polymer ratio = 0.92-0.95) aiming to activate targeting through receptor-mediated endocytosis. The r_1 relaxitivities obtained for the Gd^{3+} complexes for BH40-EDTA-PEG-Folate and BH40-DTPA-PEG-Folate were considerably higher than for the Gd-DTPA complex Magnevist® implying a vast improvement. Targeted conjugates demonstrated the expected increased cytotoxicity over the non-targeted conjugate, in FA positive (HeLa) cells, but not in FA negative control cells (adenocarcinoma human alveolar basal epithelial A549 cells).

Finally, it is important to mention that Doxycycline (DXC), a potent inducer of tetracycline transgene systems, has recently been conjugated to a HB polyester (Boltorn® H40) modified with PEG.[123] The final conjugate had 11 wt% DXC loading and formed particles with an average diameter of 77 nm (DLS). The drug release rate was significantly higher at pH 5 than at pH 7.4 and PEG-H40-DXC had a comparable effect on gene expression than free DXC. In this study, the authors found that the PEG-H40-DXC polymer was an effective tool for controlling gene expression in human pluripotent stem cell derivatives.

4. Graft, Comb- and Brush-like polymers

Graft, brush and comb-like polymers belong to the family of segmented polymers and generally consist of a linear polymeric backbone with polymeric branches. A graft polymer is a branched polymer in which the side chains or grafts are different from the main chain. Usually the branches are randomly distributed along the backbone and can be different (copolymers) or similar to the main chain (homopolymers). When the side chains are of the same chemical nature, the polymers are usually called comb-shaped or comb-like copolymers. On the contrary, when the side chains are of a different chemical nature than the backbone, the polymers are called graft or brush depending on their grafting density. Both graft and comb-like polymers are usually defined by a low density of branches and in contrast, brush-like polymers refer to polymers with a high density of grafts. The control of side-chain number and their distribution along the backbone still remains a challenge.[124]

Graft polymers offer the possibility of combining the properties of at least two polymers in a single polymeric structure. One of the characterization parameters of these structures is the grafting yield, which corresponds to the amount of polymer incorporated into the backbone or the amount of polymer grafted depending on the synthetic approach used. These constructs can be easily tuned by controlling the grafting density, length and composition of their backbone and side-chains and thus provide an excellent tool for their application in nanomedicine. In particular, the possibility of using graft polymers as amphiphilic nanocarriers has raised serious interest in the field[125-129] as their micellar properties can be optimized by the appropriate control of the macromolecular polymer structure.

4.1. Synthetic approaches

Graft copolymers are usually prepared in at least two steps, by using three main synthetic approaches: the “grafting through”, the “grafting from”, and finally the “grafting onto” methods.[124,130]

The “grafting through” method first introduced by Schulz and Milkovich[131] consists of the polymerization of macromolecular monomers possessing a polymerizable end-group, and sometimes involves the copolymerization of macro-monomers with low molecular weight monomers. The use of this strategy theoretically allows obtaining completely grafted polymers if each repeating unit contains one side chain, although the complete conversion of macro-monomers is not an easy task. The main drawback of this method is the separation of the graft polymers from the remaining unreacted macro-monomers in the purification steps. The length and MW of the backbone, and thus grafts number, are difficult to determine

except if the side-chains can be separated from the backbone. The principal advantage of this method is the possibility of preparing well-defined polymeric branches that can be previously characterized before the grafting process. The "grafting through" strategy allows using different macro-initiators to incorporate diverse functionalities into the polymer backbone, such as macro-initiators bearing drug molecules, and others carrying a targeting moiety or a solubilizing agent such as PEG, or initiators of different solubility. The polymerization process is usually carried out by radical copolymerization of macro-monomers, ionic polymerization, ring-opening metathesis polymerization, and coordination polymerization.

The "grafting from" strategy involves the formation of side chains directly on the polymer backbone containing reactive sites that serve as initiation groups (macro-initiator), and is comparable to the core-first approach used for the synthesis of star polymers. The "grafting from" approach was introduced in the late 1950's by Bamford and Smets[132-133] and has been constantly developed since then. The gradual growth of side chains on the polymer backbone represents the main advantage of this method as it decreases the inevitable steric hindrance effect faced when the other methodologies are used. Furthermore, this methodology overcomes the main drawback of purification from the previously described "grafting through" method. However, the characterization of these systems is not simple, as the chains cannot be analyzed separately from the backbone, and obtaining regular side chains remains a challenge. The "grafting from" method is usually carried out using anionic vinyl and ROP, cationic ROP, free radical polymerization, or controlled radical polymerization (particularly ATRP).

Finally, the "grafting onto" approach achieves side chain attachment to the polymer backbone via coupling reactions, and this crucially requires a highly efficient grafting method. The exponential development of *click* chemistry techniques (i.e. copper-catalyzed azide-alkyne Huisgen cycloaddition, activated ester coupling reaction, thiol-ene and thiol-yne reactions, or pyridyl disulfide reaction) has made this approach the most popular strategy. As for the "grafting through" method, the main advantage of this method is the possibility of characterizing the branches before the coupling step. The steric hindrance effect is the major drawback of this approach together with the removal of excess branches that sometimes requires a tedious purification process. "Grafting onto" synthesis via anionic and cationic polymerization, based on coordination polymerization or coupling reactions are the most common. However, this strategy has also been employed using non-covalent interactions between the backbone and the side chains such as hydrogen bonding, host-guest interactions, electrostatic interactions, π-π interactions, and metal-ligand interactions by using the complementary units in the backbone and side chain. This allows reversible and stimuli-responsible structures to be obtained.

4.2. Graft, Comb- and Brush-like polymers as drug carriers

Many examples of drug delivery applications use the possibility of tuning the self-assembly behavior of these polymers by controlling the hydrophilic/hydrophobic balance.[125, 134-136] Herein, we will describe only those examples were the drug is covalently bound to the polymer backbone.

From 1992, Hudecz *et al.*[137] worked on polypeptide-drug conjugates of different side chain architectures with a focus on the influence of the side chain modification on the immunological and pharmacological properties of these conjugates. Their constructs consisted of a general polymer backbone of poly-L-Lysine where different polypeptide branches were grafted from. They first reported the influence of the peptide branches nature on the polymer solution conformation and *in vitro* properties from a series of Daunomycin (DNM) conjugates. A similar study using MTX as anticancer agent was also reported,[138] and subsequently the authors published an *in vivo* study of a polypeptide system of poly[Lys-(Glu$_i$-DL-Ala$_m$)] (EAK) conjugated with DNM.[139] They demonstrated that the conjugate was highly effective in a L210 leukemia model leading to long-term survival whilst the free drug only increased the mean survival. This amphoteric system (EAK) was then studied with MTX and compared with a polycationic polypeptide series for the treatment of *Leishmania donovani* infection.[140] They obtained better results with the polymeric carrier than with the free drug and particularly with one of the polycationic conjugates, poly[Lys(DL-Ala$_m$-Leu$_i$)] (ALK). More recently, Reményi *et al.*[141] carried out a comparative study between two branched polypeptide carriers of different structure, amphoteric poly[Lys(Glu-DL-Ala$_3$] (EAK) and polycationic poly[Lys(Ser-DL-Ala$_3$)] (SAK) and evaluated their anticancer activity when carrying DNM. Cis-acotinyl-daunomycin (cAD) was conjugated to the polymers via amide bonding resulting in comparable drug release (pH and temperature dependent), intracellular distribution and *in vitro* cytotoxic effect from both conjugates. However, the effects on phospholipid bilayers and fluorescence properties were found to be different.

Etrych *et al.*[142] described graft copolymer-DOX conjugates designed for passive tumor targeting. The high-molecular-weight copolymers (MW 90-120 kDa) were composed of shorter polymer chains (MW 20-30 kDa) grafted onto multivalent HPMA containing biodegradable oligopeptide sequences and/or disulfide bridges aiming biodegradability of the polymers. DOX was conjugated to the polymers via pH labile hydrazone bonds and was effectively released at pH 5.5 whilst the conjugates remained stable at pH 7.4. The conjugates sizes were around 20 nm in diameter in all cases, and the polymers exhibited prolonged blood circulation and enhanced tumor accumulation in mice. They also had higher antitumor activity when compared with the free drug and the linear polymer-drug conjugate 38C13 B-cell or EL4 T-cell lymphoma *in vivo* models.

Johnson *et al.*[143] studied drug-loaded bivalent-bottle-brush polymers, synthesizing conjugates via grafting through ring-opening metathesis polymerization (ROMP) procedure of drug-loaded (DOX or/and CPT) PEG based macromonomers. The

drugs were covalently bound through a photocleavable linker (nitrobenzyloxicarbonyl derivatives), and the final brush conjugates pCPT and pDOX carried 8.5 wt% CPT and 12.6 wt% DOX and had sizes between 6-12 nm in diameter. The drugs were released after exposure to 365 nm light with 50 and 64% of DOX and CPT release ten minutes after irradiation respectively. The conjugates showed higher *in vitro* cytotoxicity than the single drugs (up to 30-fold for the combination conjugate) only after irradiation in MCF-7 cells.

Zou *et al.*[144] reported the synthesis of brush polymer-drug conjugates by ring-opening metathesis copolymerization of monomers of exo-norbornene carrying PEG chains or paclitaxel attached through covalent ester bounds (Fig. 8). These polymers displayed a 24 wt% PTX loading, a narrow distribution (Đ = 1.04-1.34) and formed nanostructures from 11.4 nm to 24.6 nm diameter size as measured by DLS. Release of PTX was time- and pH-dependent, with slow release at pH 7.0 and more than 80% of drug release after 24h of incubation at pH 5.5. Although these results were promising, neither *in vitro* nor *in vivo* cytotoxicity studies were reported. Later, the same group described the preparation of a degradable brush polymer-drug conjugate (BPDC) loaded with PTX.[145] The conjugate was synthesized though azide-alkyne cycloaddition of acetylene-functionalized polylactic acid with azide functionalized PEG and PTX. By using this *click* chemistry procedure the authors obtained 23.2 wt% drug loading and the polymer presented nanostructures of 10-30nm in DLS and TEM analysis. This allowed for 50% of the drug to be released after 22h at pH 7 and 37°C, and whilst the polymeric carrier demonstrated no toxicity, the PTX conjugate had greater cytotoxicity in MCF-7 cells than the free PTX.

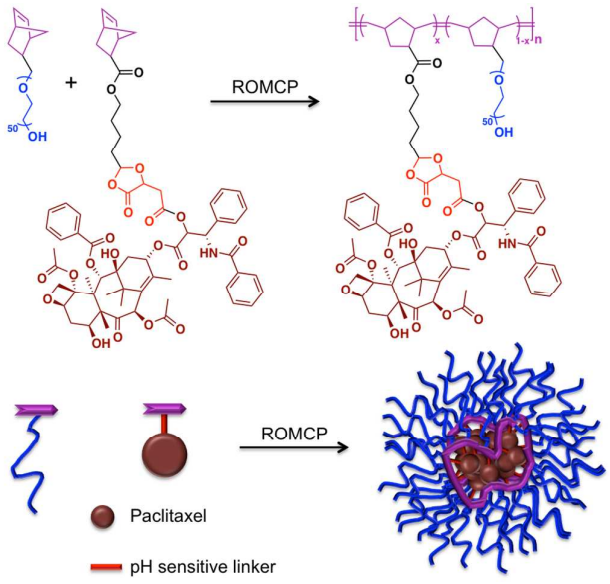

Fig. 8. Preparation of brush polymer paclitaxel conjugate. Adapted from ref [144] with permission from the Royal Society of Chemistry.

Xue *et al.*[146] prepared a cisplatin (CDDP) polymer conjugate from folate-bound PEG-graft-α,β-poly[(N-amino acidyl)-aspartamide] (FA-PEG-g-PAAsp-CDDP) self-assembling into micelles of about 120 nm diameter size according to DLS. The cellular uptake of FA-PEG-g-PAAsp-CDDP was found to be higher than that of the non-targeted micelles on FR-positive KB-cells. *In vivo*, although the anti-tumor activity of the targeted micelles was lower than that of CDDP, polymer conjugate displayed low toxicity against mice indicating their potential use for improved anti-tumor efficacy of CDDP.

Later, Zhang *et al.*[147] reported the development of a biodegradable delivery system for 6-mercaptopurine (6-MP), a drug used against leukemia. They grafted amino-disulfide-PEG and 2-(pyridyldithio)-ethylamine (PDA) on poly(L-succinimide) and the drug was covalently bound to the polymer via thiol-disulfide exchange to give the amphiphilic compound mPEG-SS-NH-graft-PAsp-MP. The spherical micelles of about 160 nm in diameter size were prone to aggregation in the presence of the reductive agent dithiothreitol (DTT). Their *in vitro* evaluation found continued drug release at 40 nM DTT (although far away from mimicking intracellular conditions) and a significant decrease in the cytotoxic effect when comparing with the free drug against HL-60 human leukemia cells. In parallel, Gong *et al.*[148] worked on the design and synthesis of another amphiphilic graft polymer composed of a 6-MP prodrug (PTA) and chitosan (PTA-*g*-CMCS). The polymer assembled into micelles of 104 to 285 nm diameter size (DLS) and the drug was released from the polymeric micelles in presence of glutathione. The authors reported an increase in the growth inhibition of HL-60 human leukemia cells *in vitro* while no cytotoxicity was encountered in a mouse fibroblast cell line.

Some examples of graft-polymer-drug conjugates in the literature are as platforms for the co-delivery of drugs or drug/DNA. Following this strategy, Tai *et al.* (Fig. 9)[149] reported a graft copolymer prepared via polymerization of γ-camptothecin(CPT)-glutamate N-carboxyanhydride on a PEG-based backbone via ROP and obtained loadings up to 25.1 wt% CPT (MB-20). The hydrophobic character of CPT was used to form micelles in which DOX was encapsulated (up to 30 wt%) for dual-drug delivery (MB-20/DOX). The particles of about 50 nm diameter (DLS) were stable over 5 days at 37°C in mice serum and could be internalized via endocytosis.

The *in vivo* study demonstrated that the particles accumulated into the tumor tissues, while both MB-20 and MB-20/DOX had greater activity against the lung cancer xenograft mice model than free drugs.

In parallel, Bao *et al.*[150] prepared a chitosan-graft-polyethyleneimine-candersartan conjugate (CPC) as a targeted drug and gene co-delivery nanovector for cancer therapy. In this study, candersartan (CD) was used as anti-angiogenic drug as well as targeting agent since it can bind to Angiotensin II receptor type 1 (A1TR) which is overexpressed in certain tumor cells. CPC self-assembled with the wild type (wt)-p53 gene to form stable particles of about 150 nm diameter in size, and released the drug and gene efficiently *in vitro*. CPC/wt-p53 complexes had a synergistic effect *in vitro* with a higher inhibitory effect on angiogenesis than mono-delivery and mixed-delivery systems. *In vivo*, the co-delivery system

demonstrated high tumor-targeting and anti-tumor efficacy on nude mice bearing PANC-1 xenografts.

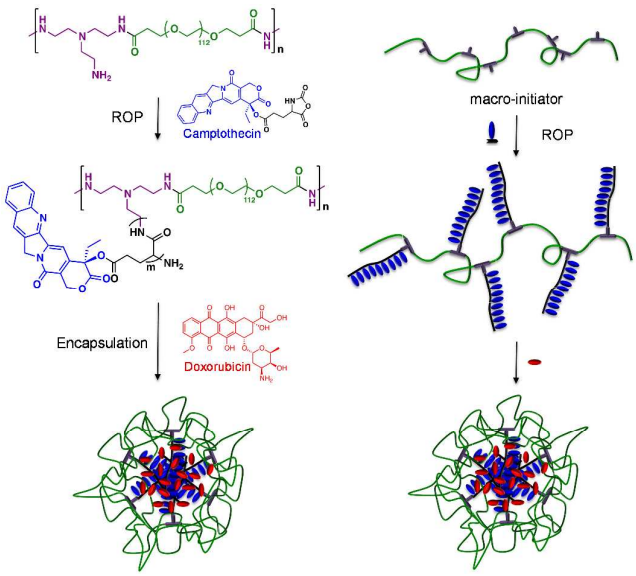

Fig. 9. Graft copolymer synthesis and formation of nanocarrier after DOX encapsulation. Adapted from ref [149].

Conclusions

In this review we have focused our attention on smart branched polymer-drug conjugates and their application in nanomedicine. Covalent conjugation of drugs to polymers has proven to be an excellent strategy to control body distribution (EPR effect), cell trafficking, and drug release kinetics. By optimizing the linking chemistry, scientists have managed to trigger specific drug release kinetics under acidic pH, reductive media, specific enzymatic conditions or light. When compared with their linear and dendritic homologues, star-, hyperbranched- and graft-polymers have demonstrated their competence as suitable drug delivery carriers due to their ease of synthesis, possible high MW, multivalency and, consequently, their different intrinsic properties. The self-assembly behavior observed with these polymers is also an interesting tool as it allows the increase of the particle hydrodynamic diameter which potentially increases their passive targeting of tumors. The progress made on the synthetic and characterization methods, that ultimately lead to low polydispersity and high drug loading capacity among other improvements, will allow their further development towards the desired clinical applications.

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