

Polymer-drug conjugates: current status and future trends

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TABLE OF CONTENTS

1. Abstract
2. From Ringsdorf's model to the clinic: current status of polymer-drug conjugates
 - 2.1. Polymer conjugates carrying orthodox anticancer agents
 - 2.1.1. Polymer-Dox conjugates
 - 2.1.2. Polymer-paclitaxel conjugates
 - 2.1.3. Polymer-camptothecin conjugates
 - 2.1.4. Polymer-platininate conjugates
 - 2.2. Polymer-anticancer drug conjugates designed for active targeting
3. Polymer-drug conjugates: future trends, challenges and opportunities
 - 3.1. Novel polymers and polymer architecture, implications for intracellular targeting
 - 3.2. A novel concept in polymer-drug conjugates: drug combination
 - 3.3. Polymer conjugates carrying novel anticancer agents
 - 3.4. Use of polymer-drug conjugates for the treatments of diseases other than cancer
 - 3.5. The importance of an exhaustive physico-chemical characterization of polymer-drug conjugates
4. Conclusions
5. Acknowledgments
6. References

1. ABSTRACT

Polymer conjugates are nano-sized, multi-component constructs already in the clinic as anticancer compounds, both as single agents or as elements of combinations. They have the potential to improve pharmacological therapy of a variety of solid tumors. Polymer-drug conjugation promotes passive tumor targeting by the enhanced permeability and retention (EPR) effect and allows for lysosomotropic drug delivery following endocytic capture. In the first part of this review, we analyze the promising results arising from clinical trials of polymer-bound chemotherapy. The experience gained on these studies provides the basis for the development of a more sophisticated second-generation of polymer conjugates. However, many challenges still lay ahead providing scope to develop and refine this field. The "technology platform" of polymer therapeutics allows the development of both new and exciting polymeric materials, the incorporation of novel bioactive agents and combinations thereof to address recent advances in drug therapy. The rational design of polymer drug conjugates is expected to realize the true potential of these "nanomedicines".

2. FROM RINGSDORF'S MODEL TO THE CLINIC: CURRENT STATUS OF POLYMER-DRUG CONJUGATES

Synthetic and natural polymers are compounds of great interest in various fields, including biomedical applications. Historically, they have been widely used as excipients in traditional dosage forms (1) as well as materials for prosthesis, valves or contact lenses (2). In more recent years, their applications have been extended to sophisticated drug delivery systems. Examples include biodegradable polymeric implants such as Gliadel® Wafer, a polymeric matrix encapsulating the anticancer agent, carmustine, for the treatment of glioma (3), Zoladex® (4) and Lupron Depot® (5), a polymeric depot containing an inhibitor for luteinizing hormone releasing hormone (LHRH), currently indicated for the treatment of prostate cancer (4-5). Different from these polymeric implants are another class of polymer-based agents known as polymer therapeutics. This term encompasses five types of agents: (i) polymer-drug conjugates, (ii) biologically active polymers, (iii) polymer-protein conjugates, (iv) polymeric micelles where the drug is covalently bound to the polymer carrier, and (v) polymer-DNA complexes (i.e. polyplexes).

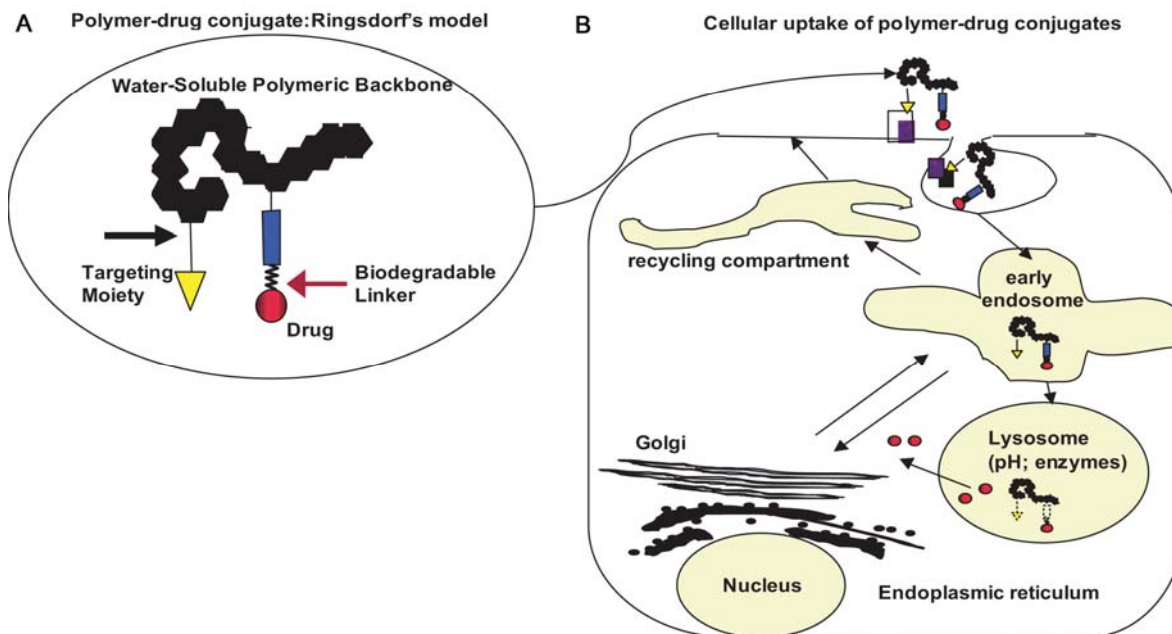


Figure 1. A. Ringsdorf model for polymer-drug conjugates; B. On arrival in the tumor interstitium, the polymer-drug conjugate is internalized by tumor cells through either fluid-phase pinocytosis (in solution) following non-specific membrane binding (due to hydrophobic or charge interactions) or as a result of receptor-mediated uptake due to a ligand–receptor docking.

The aim of this review is to focus only on polymer-drug conjugates. A detailed description of polymer therapeutics can be found elsewhere (6, 7).

The original idea of covalently conjugating a low molecular weight drug to a hydrophilic polymeric carrier to increase its therapeutic effect was proposed by Helmut Ringsdorf in 1975 (8). Ringsdorf's model consisted of four different components: a polymeric carrier, a drug, a biodegradable linker and a targeting group (Figure 1a). It was envisaged that covalent conjugation of a low molecular weight drug would alter drug pharmacokinetics at cellular level by restricting its uptake to the endocytic route (Figure 1b). The presence of a targeting moiety would confer specificity against certain cell types (e.g. tumor cells), thus preventing uptake by healthy cells. In addition, the hydrophilic polymeric backbone would also increase drug solubility as conventional chemotherapy agents are often hydrophobic and therefore poorly soluble in biological fluids. These conjugates were designed to obtain selective drug release in the lysosomal compartment ("lysosomotropic delivery") (9). As a consequence, the linker emerged immediately as a key feature. While stable in the blood stream, it needs to be degraded by a biological trigger within the lysosomes (usually low pH or enzymes) due to the accumulation of conjugates in this intracellular compartment following endocytic capture. In the 80s and early 90s a vast number of preclinical studies were conducted to optimize the characteristics of polymeric carriers, of polymer-drug linkers (10–11) and to prove the safety of these novel constructs (12). These studies were primarily the result of collaborative work between Ruth Duncan at Keele University and Jindrich Kopecek at the Institute of Macromolecular Chemistry in Prague, and

resulted in the clinical evaluation of HPMa copolymer-doxorubicin (Dox) (FCE28068, PK1) in 1994, making this conjugate the first synthetic polymer-anticancer drug conjugate to be tested in humans (extensively reviewed in 13).

At the same time, studies carried out by Maeda and colleagues on SMANCS (Zinostatin stimalamer®; Yamanouchi Pharmaceutical Co Ltd., <http://www.yamanouchi.com>), a polymer (styrene-co-maleic anhydride (SMA))-anticancer protein (neocarzinostatin; NCS) conjugate, highlighted for the first time the tendency of macromolecules to passively accumulate in the tumor tissue (14). This effect is now well-described and it is due to two contributing factors, (i) the hyperpermeability of tumor vasculature which allows selective extravasation of macromolecules into the tumor, (ii) poor lymphatic drainage which provides increased retention of macromolecules in the tumor. This effect was described by Maeda who coined the phrase "enhanced permeability and retention effect" (EPR) (15) and it is possibly the most important factor for macromolecular targeting to solid tumors.

Preclinical and clinical studies carried out since the 80s highlighted key features and essential characteristics necessary to rationally design conjugates. In particular, the polymeric backbone needs to be water soluble (to ensure solubility in physiological fluids), non-toxic, non-immunogenic, and have a carrying capacity that is compatible with the drug potency. In addition, it has to be biodegradable or, if non-biodegradable, its size has to be lower than the renal threshold to allow excretion and to prevent accumulation in the body. As different polymers

Table 1. Polymer-drug conjugates that have undergone/are in clinical evaluation

Drug	Conjugate name	Linker type	Clinical Status	Ref
Dox	Oxidized dextran-Dox AD-70, DOX-OXD	Schiff base	Phase I discontinued	21
	HPMA copolymer-Dox PK1; FCE28068	Peptidyl linker	Phase II	22, 23
	HPMA copolymer-Dox-galactosamine PK2; FCE28069	Peptidyl linker	Phase I	58
Paclitaxel	HPMA copolymer- Paclitaxel PNU166945	Ester	Phase I discontinued	24
	PGA-Paclitaxel Xyotax TM	Ester	Phase III	25-30,
Camptothecin	HPMA copolymer-Camptothecin MAG-CPT	Ester	Phase I discontinued	31, 32
	PGA-camptothecin CT-2106	Amide	Phase I/II	33, 34
	PEG-camptothecin Pegamotecan	Ester	Phase II discontinued	35-37
	Carboxymethyldextran-exatecan DE-310	Amide	Phase I	39
	Cyclodextrin-camptothecin IT-101	Amide	Phase I	40-42
Platinates	HPMA copolymer-malonato-platinate AP5280	Malonate	Phase I	44
	HPMA copolymer-DACH-platinate AP5346, ProLindac TM	Aminomalonate	Phase II	46, 47

assume different conformations in solution and present different levels of hydration, the maximum molecular weight (Mw) that still allows renal excretion varies depending on the polymer characteristics. As a rule of thumb, for non-biodegradable polymers, conjugate size should be within 40,000 g/mol. The linker needs to be stable in the blood circulation but ensure drug-release in the tumor following exposure to a precise biological trigger such as an enzyme or variations in pH. It is worth of notice that the degradability of the linker varies depending on the attached drug. For instance, the peptidyl linker glycine-glycine (GG) used in HPMA copolymer-Dox conjugate was non-biodegradable (16). However, the same linker was biodegradable when melphalan (17) was attached to it. The drug has to bear a chemical group that allows conjugation to the polymer. In addition, if the system is designed for lysosomotropic delivery, the drug needs to be stable at low pH, resistant to a harsh enzymatic environment encountered in lysosomes, and be able to diffuse across the lysosomal membrane in order to reach its target. A vast amount of literature has accumulated over the past 30 years and exhaustive reviews have been written on polymer conjugates (18-20).

Although a short historical overview will be given, this review aims to analyze the very latest advances in the field of polymer-drug conjugates, mainly focusing on (i) the development of new polymeric carriers and the importance of polymer-architecture, (ii) the use of polymer-drug conjugates to deliver novel anticancer agents or combinations of anticancer agents, and (iii) the application of the concept of polymer-drug conjugates to diseases other than cancer.

2.1. Polymer conjugates carrying orthodox anticancer agents.

The vast majority of the polymer-drug conjugates synthesized and all conjugates evaluated clinically carry orthodox anticancer agents. In this section we will focus only on those conjugates that have undergone clinical evaluation. These are conjugates containing established chemotherapy agents developed in the 1980s/90s, such as, Dox, paclitaxel, camptothecin and platinates (Table 1).

2.1.1. Polymer-Dox conjugates

Three different conjugates containing Dox were tested clinically, namely dextran-Dox conjugate (AD-70, DOX-OXD), HPMA copolymer-Dox (PK1; FCE28068) and HPMA copolymer-Dox-galactosamine (PK2; FCE28069). The latter will be described in section 2.1.2 as it contains a targeting moiety for active targeting.

Dextran-Dox conjugate (AD-70, DOX-OXD): Dextran-Dox conjugate was the first conjugate to undergo clinical evaluation (21). The conjugate comprised of oxidized dextran (70,000 g/mol) conjugated with Dox, most likely via the formation of a Schiff base. Thirteen patients were enrolled in this phase I clinical trial. Unfortunately, with an MTD of only 40 mg/m², the conjugate resulted more toxic than free Dox (60-80 mg/m²). Toxicity was observed primarily in the liver and was attributed to dextran uptake by the reticuloendothelial system.

HPMA copolymer-Dox conjugate (PK1, FCE28068): N- (2-Hydroxypropyl) methacrylamide (HPMA)-Dox conjugate was the first synthetic polymer drug conjugate to be tested clinically. This experience proved more successful than that of the dextran-Dox conjugate described above (22). The conjugate consisted of an HPMA copolymer containing a tetrapeptidic linker degradable by thiol-dependent lysosomal proteases (glycine-phenylalanine-leucine-glycine (GFLG)). Dox was bound to the carboxy terminus of the glycine via an amide bond. This conjugate had a total drug content of approximately 8.5 wt % and free doxorubicin was less than 2 wt % of the total Dox content. A phase I clinical trial was carried out in 36 patients, and two partial and two minor responses were seen in the study, in non-small cell lung cancer (NSCLC), colorectal cancer, and anthracycline-resistant breast cancer at 80 mg/m² (doxorubicin-equivalent). The MTD dose was approximately 5 times that of free Dox (320 mg/m²). Interestingly, side effects were similar to those observed with anthracyclins, but no congestive cardiac failure (typical of anthracyclins) was seen. These promising results formed the basis to initiate a phase II clinical trial in the three tumor types in which responses had been observed in Phase I trial. The complete results from the Phase II trials have not been published yet, but therapeutic responses were seen in both breast and lung cancer (23, 13).

Polymer-drug conjugates

2.1.2. Polymer-paclitaxel conjugates

Two conjugates containing paclitaxel were evaluated clinically: HPMA copolymer-paclitaxel (PNU166945) and poly-L-glutamic acid (PGA)-paclitaxel (CT-2103, XyotaxTM).

HPMA copolymer-paclitaxel conjugate (PNU166945): As the clinical studies carried out on the HPMA copolymer-Dox conjugate showed no evidence of polymer-related toxicity, another HPMA copolymer conjugate, HPMA copolymer-paclitaxel underwent Phase I clinical evaluation shortly after (24). The polymeric backbone and the peptidyl linker were the same as for the HPMA copolymer-Dox but, in this case, paclitaxel was conjugated to the terminal glycine via an ester bond. Activity was seen in some (1 partial response and 2 stable disease) of the 12 patients enrolled in this study, but unfortunately the trial was discontinued prematurely as parallel preclinical studies showed evidence of neurotoxicity.

PGA-paclitaxel conjugate (CT 2103, XyotaxTM, Paclitaxel-polyglumex (PPX)): Clinical evaluation of paclitaxel poly-L-glutamic acid (PGA) conjugate represents a very exciting story for this field. The biodegradable PGA-paclitaxel conjugate (CT-2103, XyotaxTM, PPX) was firstly designed by Li *et al.* and developed by Cell Therapeutics Inc. (CTI) (25, 26). For this conjugate, paclitaxel is linked to the carrier via an ester bond. This type of linkage had proven unsuccessful for HPMA copolymer camptothecin (CPT) and HPMA paclitaxel, since it led to premature drug release by blood esterases. However, the presence of a different polymeric carrier (PGA as opposed to HPMA copolymer) as well as the high loading (~ 37 wt%) resulted in stabilization of the linker (27). Indeed, it was shown that the main drug release occurred subsequently to polymer degradation by the lysosomal enzyme cathepsin B (28).

This conjugate has been tested clinically for the treatment of a variety of solid tumors. A recent phase III study in patients with NSCLC highlighted a trend of improved survival in women (26, 29). Although statistical significance was not reached, this observation prompted further investigation to understand the reasons for the increased efficacy in women. At the 18th Annual Meeting of European Organization for Research and Treatment of Cancers, National Cancer Institute and American Association for Cancer Research (EORTC-NCI-AACR), new data were presented suggesting that estrogens increase cathepsin B levels (30). Indeed, in January 2007, CTI submitted a new protocol (PGT306) under a Special Protocol Assessment to the Food and Drug Administration Office (FDA) that will focus exclusively on women with normal estrogen levels, the subset where XyotaxTM has demonstrated the greatest survival advantage in the STELLAR trials.

To date, PGA-paclitaxel is the polymer conjugate at the most advanced stage of clinical development, and it is hoped that it will be the first polymer-anticancer conjugate to enter the market. Indeed, Cell Therapeutics

Inc. announced (September 18th, 2006) an exclusive worldwide licensing agreement with Novartis for the development and commercialization of Paclitaxel-polyglumex.

2.1.3. Polymer-camptothecin conjugates

HPMA copolymer-camptothecin (PNU 166148; MAG-CPT): An HPMA copolymer-camptothecin (PNU166148; MAG-CPT) was tested clinically in Phase I studies (31). For this conjugate, HPMA contained Gly-C6-Gly side chains and camptothecin was conjugated to the Gly terminus via an ester bond. Regression of metastasis was seen in one patient although the same patient had progressive disease in other tissues. Overall, no objective antitumor responses were observed (32). The conjugate showed bladder toxicity. This side effect was unexpected but it can be probably attributable to linker instability in the urinary tract (which is acidic) and highlighted once again the importance of linker design.

PGA-camptothecin conjugate (CT-2106): A PGA-camptothecin conjugate (CT-2106) with molecular weight ~ 50,000 g/mol and containing a Gly linker (33-35 wt%) (33) has also entered Phase I/II trials in patients with advanced malignancies. Disease stabilization was seen in 6 of the 24 patients treated and the conjugate was well tolerated (34).

Polyethylene glycol (PEG)-camptothecin conjugate (Prothecan/ Pegamotecan): Polyethyleneglycol (PEG) (40,000 g/mol) conjugated camptothecin-20-O-glycinate (Prothecan or Pegamotecan), was synthesized and evaluated clinically by Enzon Pharmaceuticals Inc (35-36). PEG has been a very successful polymer for protein conjugation but its limited carrying capacity (unmodified PEG can carry a maximum of 2 drug molecules per chain) constitutes a main drawback for drug conjugation. Indeed, PEG-camptothecin conjugate had only a loading of approximately 1.7 wt% whereas 35 wt% can be obtained with PGA-camptothecin conjugate (see above). PEG-camptothecin conjugate was tested in a phase I clinical trial in 37 patients with advanced solid tumors and lymphomas. A partial response and a minor response were observed and further clinical evaluation is warranted (37). PEG-camptothecin conjugate (pegamotecan) was also evaluated in a phase II trial in patients with advanced and metastatic adenocarcinomas of the stomach and gastroesophageal (GE) junction. Fifteen subjects were enrolled in a first stage and one response allowed for enrolment of 20 additional subjects. Although 4 partial responses and a stabilization of disease were observed (40% and 27%, respectively), Enzon Pharmaceuticals Inc. decided to discontinue further clinical development of Pegamotecan (Press release on February 3, 2005). The company's decision was based on strategic economic analysis and on the analysis of the data coming from Phase IIb trial in patients with gastric or GE cancers, whose disease progressed following prior chemotherapy. Enzon redirected investment to other similar products within its pipeline such as PEG-SN38, a PEG conjugate of 7-ethyl-10-hydroxy-camptothecin (SN-38), a biological active metabolite of irinotecan hydrochloride (CPT-11).

Polymer-drug conjugates

PEG-SN38 (EZN-2208): On November 9th, 2006, Enzon Pharmaceutical Inc. announced new data providing preclinical proof-of-concept in breast, colorectal and pancreatic cancer for PEG-SN38. The data were presented at the 18th EORTC-NCI-AACR annual meeting in Prague (38).

Carboxymethyldextran-exatecan (DX-8951f, an hexacyclic camptothecin) conjugate (DE-310): DE-310 is a macromolecular prodrug of the topoisomerase I inhibitor exatecan mesylate (DX-8951f). The active drug is covalently linked to a biodegradable carboxymethyldextran polyalcohol polymer through a glycyl-glycyl-phenylalanyl-glycyl peptidyl spacer, cleavable by cathepsin B and L. Phase I studies were carried out with DE-310 in 27 patients with advanced solid tumors (39). One patient with metastatic adenocarcinoma achieved complete remission. One partial remission (metastatic pancreatic cancer) and 14 cases of disease stabilization were also observed. This Phase I trial concluded recommending phase II studies at a dose of 7.5 mg/m² given once every 6 weeks, as a prolonged DX-8951 release was achieved.

Cyclodextrin- camptothecin conjugate (IT-101): IT-101 is a conjugate of camptothecin and a linear cyclodextrin-based polymer (CDP). The components of CDP are β -cyclodextrin and PEG. Pharmacokinetics and preclinical studies have demonstrated that this conjugate exhibited prolonged plasma half-life and enhanced distribution to the tumor tissue when compared to CPT alone. Furthermore, IT-101 showed a good tolerability and a potent antitumor activity against a wide range of solid tumors (40, 41). A Phase I safety and pharmacokinetic study of IT-101 in the treatment of advanced solid tumors sponsored by Insert Therapeutics (42), is currently recruiting patients (43). This will be an open-label dose-escalation study of IT-101 administered in patients with solid tumor malignancies. Patients will receive a weekly injection of IT-101 followed by a 1-week rest period.

2.1.4. Polymer-platinate conjugates

HPMA copolymer-malonato-platinate conjugate (AP5280): Following the introduction of the above described HPMA copolymer conjugates into the clinic, another HPMA copolymer conjugate carrying platinum was synthesized and tested clinically (44). This conjugate had a platinum content of 8.5 wt % and was tested in patients with a variety of solid tumors. Although no response was observed, five patients (17%) had stable disease (44).

HPMA copolymer-DACH-platinate conjugate (AP5346, ProLindacTM): ProLindacTM is Access Pharmaceutical's lead oncology drug, which is in Phase II clinical development (45). It is an HPMA conjugate carrying DACH-platinum, the active part of oxaliplatin, linked via a Gly-Phe-Leu-Gly linker, that releases platinum (Pt) much more rapidly in acidic environment. Preclinical studies demonstrated that ProLindac delivered 16-fold more Pt to the tumor than oxaliplatin. This conjugate was studied in more than 10 tumor models and was usually markedly superior to oxaliplatin (46). A 26-patient dose-ranging, open label Phase I study was conducted in Europe.

The recommend dose for Phase II studies was determined as 640 mg Pt/m² to be administered over 1 hour weekly for 3 weeks out every 4 weeks. Partial responses were achieved in 2 patients (melanoma and ovarian carcinoma) and disease stabilizations was seen in patients with a range of other tumors (i.e. melanoma, esophageal cancer and cervix cancer) (47). A Phase IIa clinical study of this conjugate in head and neck cancer patients was initiated in 2006 and it is currently recruiting patients (43).

2.2. Polymer- anticancer drug conjugates designed for active targeting

As described in the previous section, polymer-drug conjugates can passively accumulate in the tumor tissue. However, it has been suggested that the addition of a targeting moiety would increase selectivity by actively targeting the tumor. A number of strategies have been adopted to achieve active targeting, including the use of peptides such as, Melanocyte Stimulating Hormone (MSH) (48), EBV-peptide to promote targeting to lymphocytes (49), RGD4C peptide to promote targeting to endothelial cells ($\alpha_v\beta_3$ integrin receptor) (50), Lutenizing hormone-releasing hormone (LHRH) used to target LHRH receptors (51), antibodies such as antitransferrin receptor antibody (52), or other ligands such as folate (53-57). However, to date only two clinical studies have been conducted on conjugates containing targeting agents. The first one is an HPMA copolymer-Dox-galactosamine (PK2; FCE28069) designed to target the hepatocyte galactose receptor. This conjugate was tested in a Phase I clinical trial in patients with hepatocellular carcinoma (58). The toxicity profile was similar to that seen for anthracyclines but interestingly the MTD was half of that of the non-targeted conjugate although still higher than that of the free drug. It was recently suggested that the different toxicity of the targeted and non-targeted conjugate could be attributed to their different conformations in solution. Indeed, FCE28068 and FCE28069 conformations were recently compared using small-angle neutron scattering (SANS) technique (see paragraph 3.5 for further explanations, (59)).

The other targeted conjugate tested clinically is HPMA copolymer-Dox-human immunoglobulin (HuIg) conjugate (60). Preliminary clinical experiments were carried out in 4 patients with this HPMA conjugate. Antitumor activity was seen in some of the patients but conclusions are difficult to draw since this trial was not carried out according to 'Good Clinical Practice' (GCP) guidelines.

3. POLYMER-DRUG CONJUGATES: FUTURE TRENDS, CHALLENGES AND OPPORTUNITIES

3.1. Novel polymers and polymer architecture, implications for intracellular targeting

The development of better polymeric carriers is an ongoing challenge. There is a need to develop higher molecular weight biodegradable polymeric carriers that can maximize EPR-mediated tumor targeting. EPR-mediated tumor targeting is ultimately driven by the plasma concentration of the circulating polymer (61). PEG-polyacetals that show pH-dependent degradation (62), and

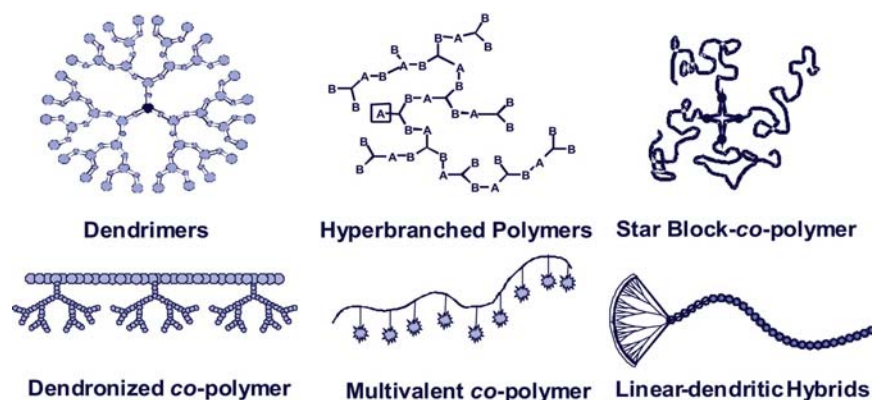


Figure 2. Polymeric architectures being explored as promising carriers for novel Polymer Therapeutics.

dextrins (63) that are degraded by amylase are two possible options that have been suggested. Within this context, we (MV) have recently described a novel system where the drug is incorporated within the polymer main chain. This polymeric drug was designed for pH-triggered activation in the endosomes and lysosomes (64). A ter-polymerization approach was used to incorporate the non-steroidal estrogen diethylstilboestrol (DES) into the main-chain of water-soluble polyacetals using PEG3400 as co-monomer to give a polymer of Mw 43,000 g/mol, and a DES loading 4.7 wt%. The DES-polyacetal displayed greater cytotoxicity than DES against MCF-7 human breast cancer cells and B16F10 murine melanoma cells. Such conjugates can also be prepared with higher molecular weight to maximize their EPR-mediated tumor targeting. Thus, they have considerable potential for further evaluation as a treatment for metastatic prostate cancer.

On the other hand, there is also an urgent need to move away from heterogeneous random coiled polymeric carriers towards better-defined polymer structures. All the conjugates that have been tested clinically so far are linear. However, an increasing amount of new polymeric architectures are being evaluated in preclinical studies. Hyperbranched polymers, star polymers and dendrimers are example of new carriers (Figure 2). Dendrimers and dendronized polymers combine a monodisperse nanoscale geometry with high endgroup density at their surface (i.e. potential for high drug loading), and are thus attractive candidates for immobilizing anticancer drugs, imaging agents, and/or targeting moieties.

Although dendrimer based imaging agents have been evaluated clinically, there is still a need to establish the safety and chemical characteristics of many dendrimer structures currently in pre-clinical development. VivaGel® is another example for a dendrimer-based drug in clinical development by Starpharma as a topical vaginal microbicide for HIV prevention (65). In the case of VivaGel, the dendrimer does not serve as a delivery vehicle but instead is the pharmacological active agent itself. VivaGel® is currently in Phase II clinical trials and expected to be introduced on the market by 2008. Exhaustive review of these architectures is beyond the scope of this review, but detailed information can be found

elsewhere (7, 66-67). Here we have chosen a selected number of studies highlighting the impact of architecture on the biological behavior. In particular we focus on the cellular pharmacokinetics and intracellular trafficking as those properties are crucial in the design of drug delivery systems.

There are relatively few studies where the effect of polymer architecture on endocytosis, cellular internalization and subsequent cellular fate have been investigated systematically. There are a number of studies showing that for instance dextran is internalized, exocytosed and differently trafficked in and out of lysosomes in relation to Mw (68). More detailed studies have been also conducted in Prof Duncan's laboratory with a focus on screening polymers as potential carriers for cytotoxic agents (for example, 69, 70). Uptake studies carried out on endothelial-like ECV304 cells with oregon green labelled-linear and star-shaped PEGs bearing generation 1-4 poly (ester)dendrons showed that the rate of uptake and cellular fate were dependant on conjugate architecture. Internalization was inhibited by incubation at 4 °C suggesting an energy dependent process (i.e. endocytosis). Referring these PEG-dendrons, it was found that the greater molecular weight and branching the lower accumulation inside the cell. This was attributed to an increased rate of exocytosis (71). In a more recent study, Seib *et al.* (72) have compared the uptake of oregon green labeled-PAMAM dendrimers (generation (gen) 2-4), branched and linear PEI by B16F10 murine melanoma cells, using FITC-dextran as a control. All cationic polymers studied were internalized by pinocytosis ('adsorptive' endocytosis), cell-associated fluorescence was maximum for PAMAM gen 4 >> branched PEI > linear PEI > PAMAM gen 3 > PAMAM gen2. These cationic polymers (in particular branched PEI polymer) also showed significant extracellular membrane binding at 4 °C however, they also lacked significant exocytosis when compared with FITC-dextran (89). Superfect-derived dendriplexes incubated with the human endothelial-epithelial hybrid cell line, EA.hy 926, also showed strong binding of the dendriplexes to the plasma membrane (73). Furthermore, preliminary studies with the previously described polycations suggested that these polymers interact with specific membrane component (s), which may

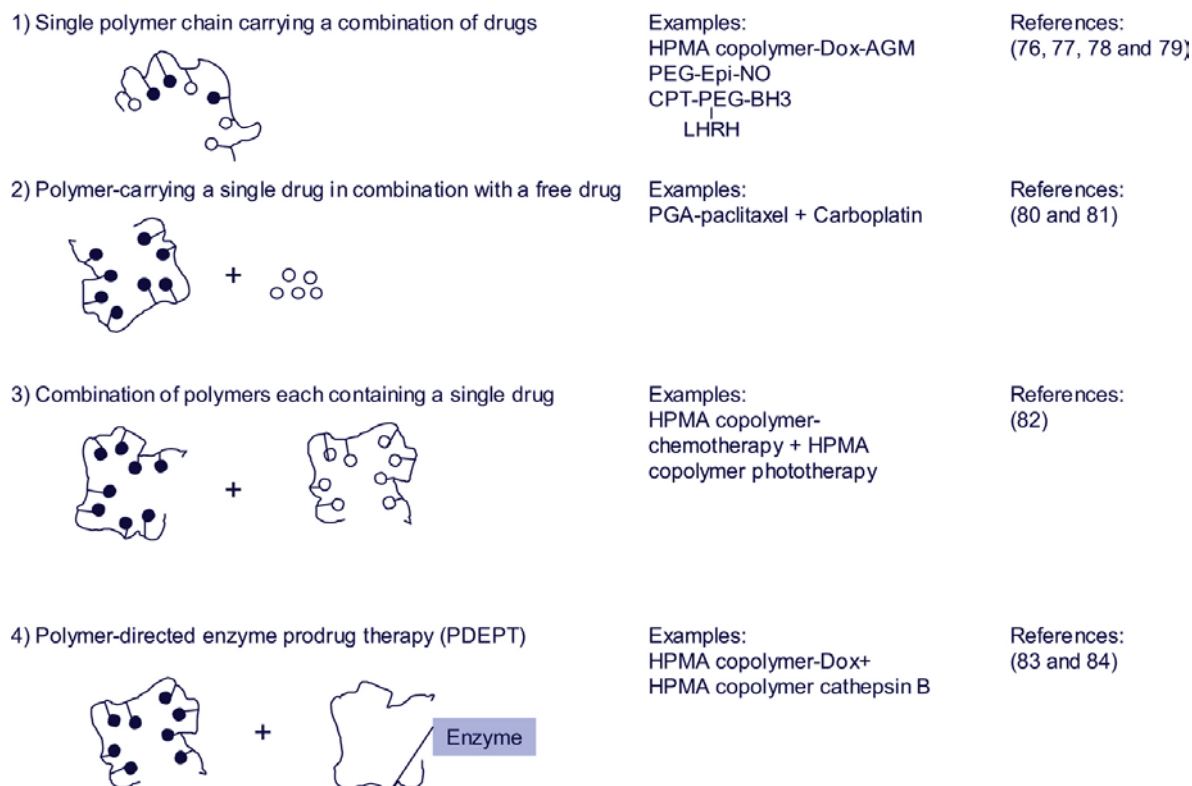


Figure 3. Different possibilities described for drug-combination therapy.

regulate their cellular uptake route. The authors observed that whereas PAMAM gen 4 and the branched PEI were mainly following uptake by cholesterol-dependent pathways, internalization of linear PEI was independent from clathrin and cholesterol (72). Regarding the superfect-derived dendriplexes, it was observed that these systems are at least in part internalized by a cholesterol-dependant pathway (73).

Minko *et al* (74) have also described the relative difference in polymer architectures of dendrimer and linear bis (poly (ethylene glycol)) (PEG) polymer when conjugated to paclitaxel. Both polymers increased aqueous solubility of paclitaxel, however, solubility was higher for dendrimer-paclitaxel when compared to PEG-paclitaxel. On the other hand, conjugation of paclitaxel to both linear PEG polymer and PAMAM gen 4 dendrimer improved its bioavailability as expected. However, the influence of the conjugation on the anticancer activity of paclitaxel was markedly depended on the carrier used. Whereas conjugation to PEG polymer significantly decreased the toxicity of paclitaxel (IC_{50} of PEG-paclitaxel conjugate was more than 25 higher when compared with free drug), PAMAM-paclitaxel conjugate showed a 10-fold increase in cytotoxicity when compared with the free drug (74).

3.2. A novel concept in polymer-drug conjugates: drugs combination

The use of polymer-drug conjugates has been traditionally limited to the delivery of a single therapeutic agent. However, the multivalency of polymeric carriers

allows their use to deliver cocktails of different drugs. This is a remarkable therapeutic opportunity as it is becoming increasingly clear that multi-agent therapy as opposed to single agent therapy, is preferable for diseases such as cancer (75). At present, only few groups have suggested the use of a polymeric carrier for delivery of drug combinations. An HPMA copolymer carrying the aromatase inhibitor aminoglutethimide (AGM) and the chemotherapeutic agent Dox was the first conjugate that combined endocrine therapy and chemotherapy agents on a single polymeric chain (76). This conjugate displayed markedly increased antitumor activity *in vitro* in breast cancer cells, compared to the conjugate carrying only Dox whose activity has been proven clinically (22). A subsequent study investigating the mechanism of action of this combination polymer at a cellular level highlighted that the conjugate conformation in solution and the drug release rates are key parameters for its activity (77) (Figure 3a).

Another group prepared a PEG conjugate containing the combination of epirubicin and nitric oxide (NO) (78). The rationale for this approach is twofold. First, epirubicin and NO have a synergistic effect. In addition NO displays cardioprotective action which was hoped could counterbalance epirubicin-induced cardiotoxicity. *In vitro* studies showed that this conjugate induced apoptosis in Caco-2 cells at higher level than free epirubicin. In addition, the presence of NO on the conjugate conferred protection against epirubicin-mediated cardiotoxicity in adult cardiomyocytes (78). Using this concept of combination therapy, together with the use of targeting

Polymer-drug conjugates

residues, Minko *et al.* have investigated the feasibility of a two tier targeting of camptothecin (CPT)–PEG conjugates to LHRH receptors and cellular antiapoptotic defence using a synthetic analogue of Bcl-2 homology 3 (BH3) domain peptide (79). A branched PEG polymer was used for this study, different loading capacities and component ratios were analyzed to achieve the optimal antitumoral activity *in vivo*. The multicomponent hyperbranched PEG polymer bearing an equimolecular amount of CPT, BH3 and LHRH moieties was almost a hundred times more cytotoxic and displayed enhanced antitumor activity when compared with other synthetic analogues.

An alternative approach is to combine a polymer conjugate carrying a single drug with standard chemotherapy (administered as a free drug). For example, a phase III clinical trials compared PGA-paclitaxel + carboplatin versus paclitaxel + carboplatin (80). The same conjugate has also been tested in combination with radiotherapy in a Phase I trial for esophageal and gastric cancer and four complete clinical responses (33%) were observed in this study (81) (Figure 3b).

In addition, combinations of two conjugates each carrying a single therapeutic agent have also been suggested. For instance, Minko and co-workers tested free CPT, CPT–PEG, CPT–PEG–BH3 or CPT–PEG–LHRH conjugates and the mixture of CPT–PEG–BH3 and CPT–PEG–LHRH conjugates in human ovarian carcinoma cells (52). It was demonstrated that conjugation of CPT to PEG increased its proapoptotic activity and that further enhancement was achieved by using BH3 peptide in a CPT–PEG–BH3 and LHRH peptide in a CPT–PEG–LHRH conjugates and their mixture. Also, Kopecek and colleagues found increased activity *in vivo* following administration of a mixture of an HPMA copolymer-dox (chemotherapy) together with an HPMA copolymer-meso-chlorin e6 monoethylene diamine disodium salt (Mce6) conjugate (photodynamic therapy) (82) (Figure 3c).

Another elegant combination therapy approach is the concept of polymer-directed enzyme prodrug therapy (PDEPT) and polymer-enzyme liposome therapy (PELT) (83, 84). In the case of PDEPT approach, two conjugates are administered sequentially: one carries an anticancer agent (administered first) and the other carries an enzyme capable to degrade the linker between the carrier and the anticancer agent. PELT is a similar approach for liposomes and protein conjugates (Figure 3d).

3.3 Polymer conjugates carrying novel anticancer agents

For years, the use of polymer-drug conjugates has been restricted to established anticancer agents such as doxorubicin, paclitaxel and platinates (discussed in paragraph 2.1.1.). The discovery of new molecular targets for cancer therapy and the subsequent identification of new therapeutic agents prompted the synthesis of polymer conjugates carrying non-orthodox anticancer agents as well as agents that had failed clinical evaluation due to unacceptable toxicity. One such conjugate is the HPMA copolymer-fumagillol (TNP-470), caplostatin. Fumagillol

is a compound that had shown antiangiogenic properties and activity in clinical trials but its development had been stopped due to emergence of neurotoxicity (85). Covalent conjugation of this drug to HPMA copolymers led to the first conjugate carrying an antiangiogenic drug. Preclinical studies showed tumor regression whereas neurotoxicity disappeared due to the inability of the conjugate to overpass the blood-brain barrier (86). Recently, when caplostatin was combined with the monoclonal antibody bevacizumab (Avastin®) eradication of human colon carcinoma in mice was observed (87, 88).

The constant research for targets selectively expressed by cancer cells led to the development of a family of drugs acting on signalling transduction pathways (89). One such compound is the PI3-kinase inhibitor, wortmannin. An HPMA copolymer-11-O-desacetylwortmannin (a derivative of wortmannin) was developed by Kopecek and colleagues. *In vitro* studies showed that this conjugate retained the ability to inhibit type I PI3-kinase activity (90). This is an interesting conjugate, but further studies are needed to evaluate its real therapeutic potential.

Polymer conjugation has also been used for therapeutic agents whose limited solubility undermined their therapeutic application. The synthesis of HPMA copolymer-1,5-diazaanthraquinone conjugates is an example (91). HPMA copolymer conjugated to ellipticine derivatives is another case where polymer-conjugation increased solubility (more than 10-fold) (92).

3.4 Use of polymer-drug conjugates for the treatment of diseases other than cancer

One of the most interesting advances in the field of polymer-drug conjugates was their application to diseases other than cancer. In 2001, the first HPMA copolymer carrying an anti-Leishmania drug was proposed (93). This was an HPMA conjugate of 8-((4-amino-1-methylbutyl)amino)-5-(3,4-dichlorophenoxy)-6-methoxy-4-methylquinoline (NPC1161). A few years thereafter, the same group improved this conjugate with the addition of N-acetylmannosamine to target the mannose receptor of macrophages (94). Studies carried out in RAW 264 murine macrophages showed that the targeted conjugate had an increased uptake as compared to the non-targeted analogue.

A number of papers have been published regarding the development of zidovudine (AZT)-polymer conjugates and prodrugs for the treatment of HIV. The aim behind many of these has been to produce agents with an equal or higher potency than AZT alone and with an improved toxicological profile. However, only a few studies have investigated the potential of developing controlled release conjugates. One of these studies was described by Vlieghe *et al* (95). In their work, kappa-carrageenan was used as polymer carrier and AZT was linked through an ester bond. *In vitro* studies using MT-4 cells showed that the conjugates displayed increased anti-HIV activity as compared to the free drug. Using sulphated alkyl laminaripentaoside as polymer carrier, Gao *et al* (96) demonstrated that conjugated AZT exhibited considerably

higher anti-HIV activity than the free drug due to synergism with sulphate functionality in the polymer. In addition, the conjugate exhibited a very low to undetectable anticoagulant activity, which is a major side effect of the free drug (sulphate). Finally, within the context of HIV research, it is important to mention the work by Giammona's group, where AZT conjugated to α,β -poly (N-hydroxyethyl)-DL-aspartamide (PHEA) resulted in controlled drug release. Targeting of the conjugate into the brain with an anti-transferrin antibody (OX26) was suggested to promote therapeutic drug levels in the CNS, which cannot be achieved with free drug due to its poor diffusion across the blood-brain barrier (97).

Preliminary studies with polymer-drug conjugates to prevent scar tissue formation have also been described (98). The authors used a polyvalent dendrimer conjugate of glucosamine or glucosamine-6-sulphate. They showed that PAMAM gen 3.5 -glucosamine inhibited synthesis of pro-inflammatory chemokines and cytokines. Also, PAMAM gen 3.5 glucosamine 6-sulfate blocked fibroblast growth factor-2 mediated endothelial cell proliferation and angiogenesis. More importantly, combination therapy with these two conjugates prevented scar tissue formation after glaucoma filtration surgery (98).

Another study generated from our (MV) group, suggested the use of a PGA based conjugate for the delivery of a peptide-based antiapoptotic agent. Peptoid 1, an apoptotic protease activating factor 1 (Apaf-1) inhibitor, was conjugated to PGA (99, 100). We were able to demonstrate that this conjugate enhances the anti-apoptotic activity of the peptide in specific apoptosis cell models and diminishes its non-specific cytotoxicity in a panel of cell lines. This first anti-apoptotic polymeric nanomedicine is showing compelling biological activity in an *in vivo* myocardial infarction model and encourages further investigation.

Although the studies presented in this section are still at an early phase of pre-clinical development, they were included in this chapter as they are exciting examples of how the field of polymer-drug conjugates is expanding beyond cancer therapy to include new therapeutic applications.

3.5 The importance of an exhaustive physico-chemical characterization of polymer-drug conjugates

Characterization of polymer conjugates is generally accomplished using a range of techniques such as chromatography, chemical or enzymatic hydrolysis experiments for determining the amount of bound drug and its release, whereas molecular structure is inferred on the basis of the synthetic route. The strict requirements imposed by regulatory authorities on identity verification of new drugs undergoing clinical evaluation suggest that new methods should be sought for the structural characterization of such conjugates. In recent years, Small Angle Neutron Scattering (SANS) and Nuclear Magnetic Resonance (NMR) appear to be the most suitable analytical tools for this purpose. In NMR as well as SANS experiments, solution conditions, such as temperature, pH, and salt concentration can be adjusted to closely mimic a

physiological environment. Conversely, the solutions may also be modified to mimic extreme non-physiological conditions, for example, for studies of conjugate degradation.

Although few references have been reported, NMR techniques could be considered useful tools to characterize macromolecular structures and their intermolecular interactions with high spatial and temporal resolution. NMR spectroscopy techniques allow the characterization of polymer conjugates. Thus, information such as drug(s) loading, sample heterogeneity and purity, molecular size, aggregation or binding state can be elucidated from the NMR data. The potential of NMR for the characterization of polymer-drug conjugates has been exemplified for several conjugates including PK1, PK2 (101) and the antiapoptotic conjugate poly-L-glutamic acid (PGA)-peptoid (100). Through 1D ^1H and 2D ^1H , ^1H NOESY (Nuclear Overhauser Effect Spectroscopy) and TOSY (Total Correlation Spectroscopy) experiments, the integrity of peptoid 1 and its covalent attachment to PGA were demonstrated (100).

SANS is a technique that only recently has been applied to investigate the behaviour of different HPMA polymer conjugates in solution (60, 76). Two studies were carried out: the first study allowed us to define the radius of gyration (R_g) of HPMA copolymer conjugates containing both Dox and the aromatase inhibitor AGM as a combination therapy (76). The other study was carried out using SANS with two conjugates already in the clinic, PK1 (FCE28068) and PK2 (FCE28069). Despite their similar chemical characteristics, the conjugates displayed a significantly different maximum tolerated dose (MTD) in patients, 320 mg/m² and 160 mg/m², respectively. Therefore, the aim of this study was to use SANS to explore their behavior in solution and establish structure-activity relationships. Clear differences in the scattering behavior for the two conjugates were observed at equivalent concentration. Modeling of the data demonstrated a larger radius of gyration (R_g) (by ~ 2.5 nm) for FCE28069 compared to FCE28068 providing a possible explanation for differences in the MTD (60). This was the first detailed SANS analysis of structurally related polymer-drug conjugates and showed that SANS could be a valuable tool for determining structure activity relationships of this important new class of therapeutics.

Electron paramagnetic resonance spectroscopy allows *in vivo* and *in vitro* degradation studies of polymer-drug conjugates (102). The monitoring of the dynamics in this case provided a rationalization for the drug release kinetics in acidic environments. Finally, it is also important to mention atomic force microscopy (AFM) techniques, which are capable of acquiring a range of physicochemical information on a wide range of bioconjugates (103).

In the future, it will be possible to use information gained from these techniques to assist, guide and control the design and synthesis of optimized second generation polymer conjugates with improved therapeutic value.

4. CONCLUSIONS

The field of polymer-drug conjugates is growing exponentially. From macromolecular prodrugs of established anticancer agents, their application has expanded dramatically in recent years. Delivery of new anticancer agents, combination therapies, treatment of diseases other than cancer, and novel polymer architectures are highly exciting and promising areas. It is hoped that in the next decade some of these new approaches will reach clinical evaluation.

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Abbreviations: Dox: doxorubicin; Pt: platinates, SANS: Small Angle Neutron Scattering; NMR: Nuclear Magnetic Resonance; CNS: central nervous system; Apaf-1: apoptotic protease activating factor 1; EPR: Enhanced Permeability and Retention; NSCLC: non-small cell lung cancer.

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