Polymer conjugates for focal and targeted delivery of drugs

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Polymer therapeutics is a very promising and rapidly growing area of nanomedicine, which has significantly improved the therapeutic potential of low-molecular-weight drugs and proteins for cancer treatment. Conjugation of toxic drugs to high-molecular-weight carriers can lead to reduction in systemic toxicity, longer retention time in the body, improved biodistribution and therapeutic efficacy, and site-specific passive accumulation thanks to the leaky tumor vasculature. Furthermore, a targeting moiety can be coupled to the polymer–drug conjugate in order to actively and selectively deliver it to the desired tissue and cellular target. This review presents a summary of currently developed polymer therapeutics with detailed focus on their components and supramolecular structure. The use of polymeric nanocarriers for cancer angiogenesis-targeted delivery is illustrated by specific examples. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: polymer conjugates; cancer therapeutics; angiogenesis; targeted delivery; nanomedicine

INTRODUCTION—POLYMER THERAPEUTICS

Almost four decades ago, Helmut Ringsdorf presented his idea regarding the use of polymers as targetable drug carriers.[1] By doing so, he foresaw the basic architecture of polymer–drug conjugates: a polymeric backbone, a drug attached via a hydrolytically—or enzymatically—cleavable spacer, and a targeting group complementary to a receptor/antigen at the target cell. Since then, most of the polymer therapeutics have been designed and developed according to Ringsdorf’s model. Most earlier systems rarely moved forward beyond in vitro testing. However, over the last 25 years, a growing number of polymer therapeutics have been synthesized and characterized. Polyethylene glycol(PEG)-protein conjugates have reached the market, and polymer–drug conjugates and polymeric micelles entered clinical trials mainly as anticancer agents.

Polymer therapeutics, in the sense of nanomedicines bearing low-molecular-weight agents (e.g. drugs and short peptides), proteins, or nucleic acids, possess several advantages: (i) protection of the bound entity from degradation and preservation of its activity during circulation; (ii) increased half-life; (iii) reduction in antigenic activity of the drug or the active entity, leading to a weaker immunological body response; (iv) increase in water solubility of poorly soluble or insoluble drugs; (v) ability to provide passive or active targeting specifically to the site of action; (vi) promotion of cellular uptake and appropriate intracellular trafficking; and (vii) multivalency that adds the possibility to form an advanced complex drug delivery system. All these properties improve the pharmacokinetic and pharmacodynamic profiles of the therapeutic agent, leading to increased efficacy and reduced toxicity, and may enable easier administration and increased patient compliance.[2]

Many of the aforementioned advantages arise from the nanosize of polymer therapeutics. High-molecular-weight therapeutics can only enter the cells via endocytosis, resulting in longer circulation time of the conjugate in the bloodstream compared with the free drug. In contrast, low-molecular-weight therapeutic agents pass rapidly through cell membranes and nonselectively penetrate most tissues. Moreover, the work of Maeda and coworkers[3,4] has established that cancerous tissue is characterized by impaired leaky vasculature and poor lymphatic drainage. They termed this phenomenon as the enhanced permeability and retention (EPR) effect. Macromolecules, e.g. nanosized polymers or other nanocarriers, as opposed to low-molecular-weight compounds, do not extravasate through the capillaries of normal tissues. However, because of the EPR effect, they easily leak through the angiogenic capillaries in the tumors and accumulate within the tumor tissue (Fig. 1).

To avoid rapid renal excretion of macromolecules and to take advantage of the EPR effect, a molecular weight range of 20–200 kDa is commonly used. Particle size range of 30–100 nm was found as an optimum for prolonged circulation, accumulation, and enhanced diffusion within tumor tissue.[5–7] The uptake in most cells is through fluid-phase pinocytosis; yet, a different way of internalization is through receptor-mediated endocytosis, in which macromolecules bind to complementary receptors on the cell surface and enter the cell as receptor-macromolecule complex in clathrin-coated vesicles. Receptor-mediated endocytosis increases the efficiency of internalization of particular macromolecules more than 1000-fold compared with ordinary pinocytosis.[8] A macromolecule that enters the cell through either process finds...
The polymer characteristics, such as molecular weight, polydispersity, architecture, charge, and hydrophilicity, impose the drug solubility, its biodistribution, body excretion, and its interaction with the immune system. The polymeric backbone of the conjugate can be synthetic, natural, or semi-natural. Copolymerization enhances design versatility by allowing systematic variation of the polymer–drug conjugate.

Polymers are used as carriers for therapeutics to deliver the therapeutic agent itself in the interior of the endosome, where the environment is kept acidic (pH 5–6) and many receptors release their bound cargo. From the endosome, the molecules proceed to the digestive lysosome, in which about 40 types of hydrolytic enzymes are active. Many strategies use the acidic pH or the proteolytic enzymes for the release of the therapeutic agent from the polymeric nanocarrier. However, care must be taken that the hostile environment of the lysosome will not hamper the structure and activity of the therapeutic agent.

To design a polymeric nanocarrier, one should pay a careful attention to each one of the different components: the polymer backbone, linker, targeting moiety, if exists, and obviously the therapeutic agent itself. The polymer–drug conjugate should be designed after taking into consideration the (i) stability of the linker between the therapeutic compound and the polymer during circulation and transport; (ii) adequate loading capacity in relation to the potency of the therapeutic agent being carried; and (iii) ability to target the diseased cell or tissue by an active or a passive mechanism. In the next sections, we will elaborate on each of the different components and review the different families of polymeric therapeutics with stimulating examples from preclinical research and clinical trials.

**Polymers**

The choice of a polymeric backbone has great implications on the pharmacokinetics and pharmacodynamics of the conjugated drug. The polymer characteristics, such as molecular weight, polydispersity, architecture, charge, and hydrophilicity, impose the drug solubility, its biodistribution, body excretion, and its interaction with the immune system. The polymeric backbone of the conjugate can be synthetic, natural, or semi-natural. Copolymerization enhances design versatility by allowing systematic variation of the distribution of active units along a polymer chain. Thus, copolymers can be tailor-made to adjust the hydrophilicity or lipophilicity of the entire molecule or of single domains (block systems), or to introduce various functional groups. Properties of the microenvironment in a polymer coil differ drastically from those of the bulk solution and are dictated mainly by monomers composition.

Because the final goal is to use these materials as carriers for pharmaceuticals to be used in humans, some criteria must be met: the polymeric carriers should be biocompatible and nontoxic, and they should avoid interaction with the immune system (unless this is the target), to enable repeated administration. In addition, biodegradable polymers are favored for better clearance from the body. Finally, many groups choose to work with Food and Drug Administration-approved polymers, to facilitate the clinical use of their system. In any way, all polymer therapeutics are considered new chemical entities from the regulatory point of view. The most common polymers used in the field of polymer therapeutics are listed in Table 1. For a suitable conjugation to chemical and biopharmaceutical drugs, many polymers have been proposed as carriers, including N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers, poly (ethyleneimine) (PEI), linear polyamidoamines, polyvinylpyrrolidone, polyglutamic acid (PGA), polyacrylamide, polydiallyldimethylacrylamide, polyvinyl alcohol, chitosan, and dextrin. It is important to note that PEG has a significant contribution specifically in the field of polymer–protein conjugates. PEGylation has been proven to be one of the most straightforward procedures for enhancing the therapeutic and biotechnological potential of peptides and proteins.

The different types of polymer backbones can be divided into two subgroups according to the feature of biodegradability in the main chain.

**Degradable polymer backbones**

The PGA is synthesized by ring-opening copolymerization of the corresponding N-carboxyanhydrides, initiated by amines or nucleophilic agents. PGA is water-soluble, nontoxic, and biodegradable. Cysteine proteases, particularly cathepsin B, play a key role in the lysosomal degradation of PGA. In addition, PGA has a γ-carboxyl group in each repeating unit of L-glutamic acid that offers multivalent attachment to drugs. Those features make PGA an attractive drug carrier, and indeed, PAG–PTX (OPAXIO™, former names XYTAX and CT-2103) is the most progressed polymer–drug conjugate in the pipeline for market approval.

Several polysaccharides are being used as polymeric carriers. Among natural and semi-synthetic polysaccharides, hyaluronic acid, chitosan, and dextran have been largely investigated for drug bioconjugation. Dextran is a natural polysaccharide containing monomers of the simple sugar glucose. This polyglucose biopolymer is characterized by 1,6 linkages, with hydroxylated cyclohexyl units. Dextran has been particularly popular owing to its clinical approval for use as a plasma expander. Dextran is a water-soluble biopolymer, and it can also be dissolved in some organic solvents. Dextran is biocompatible and biodegradable in the blood and in the gastrointestinal tract. However, it is not degraded in lysosomes. Dextran possesses multiple primary and secondary hydroxyl groups that can be used for binding drugs or proteins directly or via spacers.

Pullulan is another interesting natural, nonionic, and linear homopolysaccharide, formed by repeated units of maltotriose condensed through β-1,6 linkage (β-1,4-linked glucose molecules, polymerized by β-1,6-linkages to the terminal glucose). Because of its excellent biological and physicochemical features, namely biodegradability, low immunogenicity, and polyfunctionality together with its fair solubility in aqueous and few organic solvents, pullulan has become an attractive ingredient for many pharmaceutical applications and chemical manipulations. There are several examples in the literature for the use of pullulan in parenteral drug delivery systems.
of pullulan as a carrier, in which a drug is conjugated to the primary hydroxyl groups via pH-labile bonds or through lysosomal-sensitive peptide spacers.\(^{17,18}\)

**Nondegradable polymer backbone**

The PEG has good water solubility, and it can also be dissolved in many organic solvents. This feature together with its biocompatibility and its ability to abrogate the immunogenicity of many proteins has made it a versatile carrier in polymer therapeutics although it has only one or two attachment points. PEG is commercially available and can be produced as a linear or branched polymer. The functional hydroxyl group at the chain termini can be conjugated with drugs or other functional groups.

The lack of multivalency is one of the main limitations of this polymer. It limits the loading capacity and the potential use of PEG as a carrier. To overcome this limitation, additional reactive groups can be added by reaction of the OH\(^{-}\)/C\(_{0}\) groups with multifunctional compounds, such as glutamic acid dendron.\(^{19}\)

While PEG is mostly common in the field of polymer–protein conjugates, it is also extensively used in the polymer therapeutics field in general, as a drug carrier or as a stabilizing and anti-immunogenic moiety. PEGylation is a common procedure for making liposomes "stealth" nanocarriers.

The HPMA copolymers are one of the most studied platforms for polymer–drug conjugates; it has been studied extensively over the last 30 years.\(^{20}\) Most HPMA copolymer–drug conjugates were developed for the treatment of cancer, with special focus on the site-specific delivery of anticancer drugs. HPMA copolymer is water-soluble, neutral, biocompatible, and nonimmunogenic.

HPMA copolymer conjugated to doxorubicin via a peptidyl linker Gly-Phe-Leu-Gly was the first synthetic polymer-based anticancer conjugate to enter clinical trials in 1994\(^{21}\) (i.e. PK1) and has been the breakthrough that led to the exponential growth of interest in the field of polymer therapeutics. Although the therapeutic approach of PK1 is a promising cancer treatment, the polymer is not biodegradable, so there are issues as to how it is metabolized and cleared from the body. Since then, five other anticancer compounds and few imaging agents conjugated to HPMA copolymer have been evaluated clinically.\(^{22}\)

The HPMA copolymer, PGA, and dextran are most commonly used as a backbone for polymer–drug conjugates.

### Linker

The release of the active agent in its original structure at the target site is a crucial step when using polymers as drug carriers. Thus, the choice of a linker between the active agent and the polymer backbone has a significant role in achieving this aim. The linker should be stable in the bloodstream and the extracellular interstitium in physiological pH and efficiently cleaved at the target tissue. For some medical applications, the drug is biologically inert when bound to the polymer. Therefore, a drug that would otherwise cause side effects can exist in the bloodstream as a nonactive conjugate thus avoiding any harm to the patient. In the case of cancer, one can take advantage of the unique pathophysiology of tumors, such as the acidic, neoplastic supporting microenvironment. Specific proteases are often overexpressed and active in the acidic conditions of the tumor. A drug can be selectively released to its target by hydrolysis at low pH or by lysosomal or tumor-associated overexpressed enzymes. Thus, the linkers in the polymer therapeutics field are

### Table 1. Polymeric backbones

<table>
<thead>
<tr>
<th>Polymer backbone</th>
<th>Monomer</th>
<th>Biodegradability</th>
<th>Immunogenicity</th>
<th>Toxicity</th>
<th>Maximal loading</th>
<th>Clinical status</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG, poly(ethylene glycol)</td>
<td>PGA, poly(glutamic acid)</td>
<td>PEG, poly(lactide-co-glycolide)</td>
<td>HPMA, N2-hydroxymethylacrylamide</td>
<td>PGA, polyethyleneimine</td>
<td>X</td>
<td>Approved</td>
<td>[138]</td>
</tr>
<tr>
<td>Pullulan</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>Approved</td>
<td>[16]</td>
</tr>
<tr>
<td>PEI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[19]</td>
</tr>
<tr>
<td>Dextran</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[20,22]</td>
</tr>
</tbody>
</table>

**Reference**

1. \(^{138}\): Reference number.
2. \(^{20,22}\): Reference number.
3. \(^{142}\): Reference number.
4. \(^{139}\): Reference number.

**Note:**

- PEG, poly(ethylene glycol); HPMA, N-(2-hydroxypropyl)methacrylamide; PGA, poly(glutamic acid); PEI, polyethyleneimine.
- As a plasma expander.
- As a food additive.
either enzymatically cleavable$^{23,24}$ or pH-sensitive.$^{25–27}$ Release of the free drug can occur extracellularly or intracellularly.

Disulfide linkers that are cleaved by reduction have also been proposed as an alternative that has not progressed beyond an experimental stage. Many different classes of enzymes including nucleases, proteases, phosphatases, and lipases are present in the lysosomes. Because water-soluble polymer–drug conjugates enter the cell via endocytosis and then continue to the lysosomes, the presence of an enzymatically cleavable linker between the backbone and the drug enables selective targeting.

A common example is the oligopeptide spacers, terminated with a drug and susceptible to enzymatically catalyzed hydrolysis in the lysosomes, specifically by cathepsin B or extracellularly by cathepsin K. The cathepsin B- cleavable tetrapeptide, Gly-Phe-Leu-Gly, was extensively used as a pending group on HPMA copolymer for the delivery of several anticancer drugs, because cathepsin B is overexpressed in many tumor and tumor endothelial cells (ECs). This spacer is cleaved intracellularly in the lysosome; therefore, it is used for drug release to the cytosol. Another common enzymatically cleavable tetrapeptide is the Gly-Gly-Pro-Nle spacer, which is cleaved by cathepsin K, an enzyme involved in bone resorption (osteoporosis, osteoarthritis, and bone neoplasms) and overexpressed in osteosarcoma and bone metastases. In contrast to cathepsin B, it is localized and active in the tumor microenvironment. Cathepsin K-cleavable peptides have been used in polymer–drug conjugates to deliver drugs to bone tissues for the treatment of calcified diseases.$^{30,31}$ It should be noted that there are further peptide sequences, which are known to be cleaved by cathepsins B and K and others, such as Phe-Lys or Val-Arg by cathepsin B$^{32}$ and Phe-Arg by cathepsins B and L.$^{33}$

Other proteases, which are overexpressed in tumors and play a critical role in tumor progression, angiogenesis, and metastasis were studied, e.g. the lysosomal protease legumain$^{34,35}$ and the matrix metalloproteinases (MMPs) (i.e. MMP2 and MMP9)$^{36}$ which are active intracellularly and extracellularly, respectively.

Because the enzymes used for prodrug activation are also present in normal cells, activation of an enzymatically cleavable prodrug can be observed in healthy tissues as well. Thus, there is a growing demand for improving tumor uptake through active or passive targeting and to study the overexpression of those enzymes in the individual tumor clinically.

Acid-labile spacers are extensively used in the field of polymer–drug conjugates. There is a significant drop in the pH value from the physiological pH 7.2–7.4 in the blood or extracellular space to pH 4.0–6.5, in the various intracellular compartments.$^{37}$ In addition, the fact that the extracellular pH surrounding the tumor tissue is also slightly lower than that of normal tissue also supports the rationale for the incorporation of these spacers.

Typical examples of acid-sensitive bonds are N-cis-aconityl, hydrazone and carboxylic hydrazone bonds, acetal, imine, and trityl bonds. While N-cis-aconityl and hydrazone linkers are the most studied ones, several prodrugs with acid-labile acetal bonds have also been investigated.$^{25,37}$ Careful attention should be given to the choice of a suitable linker. The chosen chemical bond affects the pH-dependent stability of the prodrug, as well as the molecular weight of the carrier and the attachment site on the carrier.

Another interesting subgroup of acid-labile linkers are pH-sensitive bonds that are incorporated in the backbone of the polymeric carrier. Recently, a number of reports on linear polymers were published in which the monomer units were linked by ketal, acetal, and cis-aconityl bonds.$^{27}$ By doing so, the polymer backbone undergoes a breakdown under acidic conditions after cellular uptake, thus has the benefit of being biodegradable. This strategy still suffers from several disadvantages, such as low molecular weight and absence of functional groups for attaching drugs, and thus requires further development.

Recently, a novel class of polymer backbone in which the drug and the polymer are connected via a noncovalent, biologically inspired binding motif was proposed.$^{38}$ This linker consists of a pair of complementary peptides that are wound around each other in a superhelical fashion to form a tertiary structural motif that is referred to as coiled coil. To form the noncovalent polymer therapeutics, the polymeric carrier functionalized with one peptide is mixed in an aqueous solution with the drug of interest, which is functionalized with the complementary sequence to the peptide. The drug will be intracellularly released when the conjugate will be exposed to the relatively low pH of the endosomal compartment. These innovative coiled coil-based peptide linkers may be useful to form different compound libraries, depending on the pool of carriers and drugs that are created.

**POLYMER–DRUG CONJUGATES**

Nanosized polymer–anticancer drug conjugates are typically composed of three basic components: a water-soluble polymeric carrier, a biodegradable polymer–drug linker, and a bioactive agent. Polymer–drug conjugates progressing through clinical trials as anticancer agents are in fact macromolecular prodrugs.

Most antitumor drugs in clinical use act upon metabolic pathways related to cell growth and high mitotic activity. In addition, many anticancer agents are low-molecular-weight compounds that readily gain access to all cells. These highly nonspecific effects cause serious damage to healthy tissues while treating the tumor, thus leading to dose-limiting side effects and impaired quality of life. Chemotherapeutic treatment is often restricted by dose-limiting systemic toxicity or by the appearance of drug resistance. Moreover, the formulation of poorly soluble drugs may cause additional side effects. Therefore, there is much to gain by conjugating such drugs to a polymeric carrier for antineoplastic therapy. The most common polymer–drug conjugates are camptothecin (CPT), paclitaxel (PTX), doxorubicin (DOX), and platinum.

The first polymer–drug conjugates to enter clinical trials were PK1 and HPMA copolymer–DOX conjugate.$^{21}$ DOX is the most commonly used anthracycline antitumor antibiotic. It has a wide range of antitumor activity and is effective in the treatment of carcinomas of the breast, lung, thyroid, and ovary, and soft tissue sarcomas. However, anthracycline therapy is associated with significant general organ toxicities, especially myelosuppression, mucositis, and accumulating cardiac toxicity. PK2, which is the relative conjugate of PK1, possesses an additional targeting residue—a galactosamine group that is the ligand for asialoglycoprotein. PK2 was designed with the aim of improving the treatment of primary hepatocellular carcinoma and metastatic liver disease. In early Phase I/II clinical trials, both PK1 and PK2 displayed reduced two-fold to five-fold anthracycline toxicity, and the dose-limiting toxicities were typical of the anthracyclines, which included febrile neutropenia and mucositis. Despite high cumulative doses of DOX, no cardiotoxicity was
observed. Furthermore, no signs of immunogenicity or polymer-related toxicity were observed.\textsuperscript{[21,39]} DOX was also conjugated to dextran and tested clinically (Phase II).\textsuperscript{[44]} However, this study was not continued, probably due to the reduced biodegradability of the conjugated dextran.\textsuperscript{[40]}

Paclitaxel is a drug commonly used for the treatment of advanced breast, prostate, and ovarian cancers. It is a very potent cytotoxic drug, although it is hydrophobic and causes side effects such as neutropenia, neuropathies, and hypersensitivity (due to the solubilizing Cremophor EL). PTX was conjugated at high loadings to PGA (paclitaxel poliglumex, OPAXIO™; Cell Therapeutics, Inc) although PTX is conjugated to PGA via an ester bond, the polymer backbone is generally stable in the circulation.\textsuperscript{[41]} OPAXIO™ is currently being evaluated in Phase III clinical trials against nonsmall-cell lung cancer, in ovarian cancer as a single agent, or in combination therapy with carboplatin. A Phase III clinical trial was recently approved for OPAXIO™ as an orphan drug for patients suffering from glioblastoma expressing unmethylated O-6-methylguanine-DNA methyltransferase (MGMT) (http://www.accessdata.fda.gov/scripts/opdlisting/opod/OOPD_Results_2.cfm?Index_Number=377312). Retrospective analysis of clinical data suggests that OPAXIO™’s antitumor activity may be modulated by estrogen levels. Recent in vivo studies indicate that OPAXIO™ metabolism by some cancer cells is enhanced in the presence of estrogen (which correlates with increased levels of cathepsin B), which leads to increased levels of active PTX in tumor tissue and greater antitumor effects.\textsuperscript{[42–45]}

Cisplatin is another vastly applied chemotherapeutic drug, used in combination with a wide range of other drugs in treatment of various cancers. Its use is restricted because of severe dose-limiting side effects, such as nephrotoxicity, neurotoxicity, ototoxicity, and myelosuppression, which arise from the indiscriminate uptake of the drug into all rapidly dividing cells and the body’s attempt to excrete the drug through the kidneys. In addition, the limited doses enable the tumor to develop resistance to the treatment. Therefore, the improvement of platinum-based anticancer drugs either by reduced side effects or by overcoming the resistance to this drug is the goal of many studies.\textsuperscript{[50]} Many low-molecular-weight derivatives have been proposed; some have been clinically approved (carboplatin and oxaliplatin). ProLindaç™ is an HMPA copolymer–platinum conjugate that was clinically assessed by Access Pharmaceuticals.\textsuperscript{[47]} The results of Phase II clinical trials with the conjugate revealed excellent tolerability and equivalent efficacy, if not superior to oxaliplatin. Recently, a combination study of ProLindaç™ with PTX has been initiated in the second-line treatment of pretreated advanced ovarian cancer.\textsuperscript{[48]}

Camptothecin is a potent antineoplastic agent with activity against a broad range of cancer types. Unmodified CPT has low solubility, high levels of protein binding, and rapid activation through lactone ring hydrolysis.\textsuperscript{[49]} CPT was recently conjugated to the polymeric backbone Fleximer (poly[1-hydroxymethylenehydroxyethyl formal]), which is also called PHF. This polymeric prodrug derivative of CPT, named XMT-1001, is a hydrophilic, biodegradable 40–70 kDa conjugate. Release of CPT from intravenously administered XMT-1001 involves a dual-phase release mechanism, in which CPT is first released in plasma as the lipophilic prodrugs CPT-SI (a succinimidoglycinate derivative) and CPT-SA (a succinamidoglycinate derivative), which are then hydrolyzed in tissues to release the lactone active form of CPT. The release of prodrugs to the blood is thought to lower bladder toxicity, due to lower levels of active CPT in the urine. XMT-1001 showed enhanced efficacy and safety in animal models and is currently undergoing Phase I clinical trials in cancer patients.\textsuperscript{[49]} Another conjugated form of CPT is CRLX-101 (former name IT-101). CRLX-101 is a conjugate of CPT based on a cyclodextrin polymer. The drug is attached to the polymer at the 20-0H position, which inhibits the ring opening of CPT, so it remains in its active lactone form.\textsuperscript{[50]} CRLX-101 is currently undergoing clinical trials for various types of cancers. So far, evidences of function with low side effects were found.\textsuperscript{[51]}

**NOVEL TARGETED POLYMERIC DRUG DELIVERY SYSTEMS DIRECTED TO TUMOR AND TUMOR ENDOTHELIAL CELLS**

The lack of tumor specificity displayed by drugs for cancer treatments often results in significant toxicity to noncancerous tissues. A well-designed polymeric carrier that can actively target tumor vasculature by ligand-mediated interactions may improve the therapeutic index of chemotherapeutic agents by increasing the time of drug exposure to tumor vasculature and can thus enhance treatment potency.

A diversity of ongoing researches in various fields of drug delivery is being combined to target angiogenesis and cancer; hence, polymers are being conjugated to a variety of entities. One such entity will be a specific targeting moiety to proliferating EC, such as an antibody (to VEGF, endosialin, endothelial cells caveolae proteins, or other), a peptide (i.e. RGD, NRG, or hyaluronan [HA]), or any ligand to the upregulated molecules present on the surface of proliferating EC (Fig. 2). Other entity will include an active moiety that may be an angiogenesis inhibitor, a low-molecular-weight drug, a viral vector/gene expressing an angiogenesis-inhibiting protein or toxins, coagulation factors, vasoactive molecules, or cytotoxic drugs.

![Figure 2](image-url)
This section’s focus is on macromolecules bearing toxic effecter moiety conjugated with a targeting motif directed to the tumor vasculature (Table 2). In well-vascularized tumors with poor vasculature permeability, this strategy might be essential because the tumor ECs are directly exposed to the conjugate in the blood circulation without the need to extravasate from the tumor vasculature into the tumor.

Angiogenesis and vascular-specific markers

Angiogenesis, the generation of new blood vessels from preexisting vasculature,\cite{53} may be considered as an organizing principle in biomedicine\cite{54} and measured as a healthy essential process in embryogenesis, body growth, and wound healing. The expansion of vasculature provides nutrients and oxygen to growing tissues and allows cells movement. Normal blood vessels are lined by a thin monolayer of smooth, tightly joined ECs, which forms a barrier between the circulation and the tissue, controlling the transport of different components from one side to the other. Healthy vessels are organized in stable structures lined with mural cells to form a functional basement membrane.\cite{55}

Angiogenesis is regulated by a number of different growth factors\cite{56} and is dependent on the balance between pro-angiogenic and anti-angiogenic factors. The vascular endothelial growth factor (VEGF) family of proteins is one of the noticeable pro-angiogenic factors.\cite{57} Judah Folkman was the first to propose that tumor growth is angiogenesis-dependent and that anti-angiogenic drugs may serve as a potent and successful anti-cancer therapy.\cite{58} Angiogenesis is a crucial process in tumor growth and progression. This multistep process enrolls (i) activation of earlier quiescent ECs; (ii) degradation of the basement membrane followed by stromal invasion; (iii) increase in proliferation of earlier quiescent ECs; (iv) tube formation and migration of EC accompanied by anastomosis; and (v) tumor vessel anastomosis; and (vi) increase in proliferation of earlier quiescent ECs; (ii) degradation of the basement membrane followed by stromal invasion; (iii) increase in proliferation of earlier quiescent ECs; (iv) tube formation and migration of EC accompanied by anastomosis; and (v) tumor vessel anastomosis.

Ever since angiogenesis inhibitors were recognized as a promising strategy to fight cancer, many potential compounds were identified and isolated, several are currently tested in clinical trials, more than a dozen have been clinically approved, and other clinically approved drugs were found to exert anti-angiogenic effects in addition to their known activity.\cite{54,59}

The objective of targeting toxic agents to the proliferating ECs in the tumor neovasculature, rather than the tumor cells, presents three main advantages: First, directing the drugs to proliferating ECs in the tumor microenvironment can be effective against a variety of angiogenesis-dependent malignancies, because the therapeutic target is not subjected to a tumor type. Second, acquired drug resistance, resulting from genetic and epigenetic mechanisms, often reduces the effectiveness of available drugs.\cite{60–62} Anti-angiogenic therapy targets the ECs of the tumor vasculature, which are considered genetically stable relatively to tumor cells. Therefore, anti-angiogenic therapy possesses the potential to overcome chemotherapy-associated resistance\cite{63} and reduce the incidence of drug resistance,\cite{64,65} especially when direct inhibitors are used. Third, because cancer cells depend upon ECs for survival and growth, damaging the proliferating tumor ECs may amplify the anticancer angiogenic therapeutic effect. This is particularly promising because the elimination of a single EC may act as an initiator of a series of actions, causing a hundred tumor cells to undergo apoptosis.\cite{65}

Tumor angiogenic blood vessels carry unique molecular markers such as integrin \(\alpha v\beta 3\) and \(\alpha v\beta 5\), aminopeptidase N (APN/CD13), VEGF receptors, MMPs, and P-selectin and E-selectin, some of which are targeted by certain peptides, antibody fragments, or some small molecules\cite{66–69}(Fig. 2). On the basis of the Ringsdorf model,\cite{1} polymeric carriers, which can actively target the tumor vasculature by ligand-mediated interactions, can be used to improve the therapeutic index of chemotherapeutic agents by increasing the time of drug exposure to tumor vasculature and can thus enhance treatment potency.\cite{65}

**RGD-based polymer–drug conjugates**

The tripeptide sequence, Arg-Gly-Asp (RGD), is a fundamental recognition site for cells and proteins. This short but highly conserved sequence was found by Erkki Ruoslahti’s group 25 years ago.\cite{70} To explore the structure–activity relationship between fibronectin and its receptor (later recognized as \(\alpha v\beta 3\) integrin), this group substituted every amino acid in the RGD tetrapeptide with a different one. Using this simple approach, they were able to discover that even the most conservative substitution in the first three amino acids (RGD) abolishes cell attachment activity, whereas the fourth position can undergo changes without altering this function.

As years have passed, this essential recognition epitope was found not only in fibronectin but also in other extracellular matrix (ECM) proteins,\cite{71} playing a central role in cell–ECM, cell–cell, and cell–virus/bacteria interactions.\cite{72,73} The promiscuity of the tripeptide motif and its biological and pharmaceutical importance had led to several therapeutic approaches based on mimicking and blocking this adherence system (Fig. 3).

Given that the RGD sequence is conserved so that any change in it will abrogate its functionality, modification of flanking amino acids, particularly the two subsequent to the aspartic acid, achieves higher affinity and specificity,\cite{74} different conformational features, and better stability. Cyclization is used to improve the binding properties of RGD peptides for a specific subtype of integrin and to improve its stability.\cite{75,76}

The finding that structural restriction within the cyclic RGD backbone can lead to selective integrin-subtype antagonists took the immense research by the group of Kessler a step forward to the discovery of the potent \(\alpha v\beta 3/\alpha v\beta 5\) inhibitor c(RGDf(NMe)\(\gamma\)V), known as cilenitide (Fig. 3(B)).\cite{77,78}

The clinical potential of cilenitide (EMD121974) was promptly recognized and eagerly translated to clinical setting, which was the beginning of an era for the integrin-inhibitor class as anti-angiogenic and anticancer therapies.\cite{79} Presently, cilenitide is tested in several clinical trials as monotherapy or in combination with conventional chemotherapy.\cite{79–81} Despite the reported clinical advances with cilenitide, on 2009, it was reported in two distressing publications that RGD-mimetic agents at well-defined experimental setting may promote, rather than inhibit, angiogenesis. Undoubtedly, the complex effects of integrin antagonists should be further explored and evaluated in properly designed clinical trials.\cite{82,83}

Besides the potential use of integrin antagonists as therapeutic agents per se, these compounds can be successfully applied in...
Table 2. Targeted polymer–drug conjugates

<table>
<thead>
<tr>
<th>Compound</th>
<th>Polymeric Carrier</th>
<th>Effector moiety</th>
<th>Targeting moiety</th>
<th>Target</th>
<th>Example of tumor target</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPMA–AM-GDM–cRGDfK</td>
<td>HPMA</td>
<td>Aminohexylaminogeldanamycin</td>
<td>Cyclic RGDFK</td>
<td>3-integrin</td>
<td>Vasculature endothelial cells and PC-3 (prostate cancer cells) [90,143]</td>
</tr>
<tr>
<td>PGA–PTX–E-[c(RGDfK)₂]</td>
<td>PGA</td>
<td>Paclitaxel</td>
<td>Dimeric cyclic RGDFK</td>
<td>3-integrin</td>
<td>Vasculature endothelial cells [96]</td>
</tr>
<tr>
<td>PEG–DOX–E-[c(RGDfK)₂]</td>
<td>PEG</td>
<td>Doxorubicin</td>
<td>Dimeric cyclic RGDFK</td>
<td>3-integrin</td>
<td>Vasculature endothelial cells and U87 glioblastoma cells [98]</td>
</tr>
<tr>
<td>G3-[PEG-cRGDFK]-[DOX]</td>
<td>G3 poly(lysine)</td>
<td>Doxorubicin siRNA (Luciferase gene)</td>
<td>Cyclic RGDFK</td>
<td>3-integrin</td>
<td>U87 glioblastoma cells [101]</td>
</tr>
<tr>
<td>RGD-4C-PEG-b-P(CL-Hyd-DOX)</td>
<td>PEO-b-PCL micelles</td>
<td>Doxorubicin</td>
<td>Double cyclic RGD-4C</td>
<td>3/5-integrin</td>
<td>Human MDA-435 breast cancer cells * (sensitive and resistant to DOX) [102]</td>
</tr>
<tr>
<td>RGD-4C-PEG-b-P(CL-Ami-DOX)</td>
<td>HPMA</td>
<td>Doxorubicin</td>
<td>Esbp</td>
<td>E-selectins</td>
<td>Immortalized vascular endothelial cells [112]</td>
</tr>
<tr>
<td>HPMA–Esbp–DOX</td>
<td>HPMA</td>
<td>Doxorubicin</td>
<td>Esbp</td>
<td>E-selectins</td>
<td>Human fibrosarcoma HT-1080 cells [121]</td>
</tr>
<tr>
<td>HPMA–(KLAKLAK)₂–NGR</td>
<td>HPMA</td>
<td>NRG</td>
<td>Aminopeptidase N</td>
<td>CD13</td>
<td>Human SK-OV-3 ovarian carcinoma cells [133]</td>
</tr>
<tr>
<td>HPMA-(HA34)-PTX</td>
<td>HPMA</td>
<td>Doxorubicin</td>
<td>Hyaluronic acid</td>
<td>HA receptor (CD44)</td>
<td>B16/F1 melanoma cells [137]</td>
</tr>
<tr>
<td>siRNA/(PEI-SS)-b-HA complex</td>
<td>PEI</td>
<td>siRNA (VEGF gene)</td>
<td>Hyaluronic acid</td>
<td>HA receptor (CD44)</td>
<td>B16/F1 melanoma cells [137]</td>
</tr>
</tbody>
</table>

PEG, poly(ethylene glycol); HPMA, N-(2-hydroxypropyl)methacrylamide; PGA, poly(glutamic acid); PEI, polyethyleneimine.

*MDA-MB-435 cells are derived from the M14 melanoma cell line and can no longer be considered a model of breast cancer.
RGD ligand-guided drug delivery polymeric systems. After the discovery of the selective \( \alpha v \beta 3 \) integrin inhibitor and the growing understanding of its importance, it was only natural that new RGD-containing peptides were designed and evaluated. In a pioneering work by Arap and colleagues,\(^{[84]} \) in vivo selection of phage display libraries led to the identification of RGD-4C (Fig. 3(C)). CDCRGDCFC, a small RGD-containing cyclic peptide, was conjugated to the chemotherapeutic agent DOX, giving rise to the covalent construct RGD-4C-DOX. This conjugate showed improved activity and toxicity profile over free DOX in several models, involving tumor cells that did not express integrin \( \alpha v \beta 3 \), suggesting a direct endothelial effect.\(^{[85]} \) Moreover, a number of stable peptide derivatives based on cilengitide had been developed with specific tumor targeting properties.\(^{[86,87]} \)

Multimeric RGD-containing systems were designed to enhance affinity of RGD ligands to their binding site. Embedding multiple copies of the RGD recognition motif on a central scaffold increased integrin affinity and avidity, facilitated their clustering, and induced an active integrin-mediated internalization.\(^{[88,89]} \) One example of this multimeric systems is the stable cyclic RGD-based pentapeptide c(RGDfK) (Fig. 3(D)), an \( \alpha v \beta 3 \)-specific and potent RGD motif, which was conjugated by Borgman et al. through the lysine (K) residue to an HPMA copolymer, giving rise to the covalent construct RGD-4C-DOX. This conjugate showed improved activity and toxicity profile over free DOX in several models, involving tumor cells that did not express integrin \( \alpha v \beta 3 \), suggesting a direct endothelial effect.\(^{[85]} \) Moreover, a number of stable peptide derivatives based on cilengitide had been developed with specific tumor targeting properties.\(^{[86,87]} \)

Further diagnostic studies by Janssen et al.\(^{[86]} \) demonstrated that a dimeric form of c(RGDfK), i.e. E-c(RGDfK)\(_2\), has improved tumor targeting properties over the monomeric form (Fig. 3(E)). Encouraged by these results, Ryppa et al.\(^{[90]} \) coupled the antimicrotubule and anti-angiogenic drug PTX with the E-c(RGDfK)\(_2\). Whereas the in vitro evaluation in this study showed somewhat favorable results toward E-c(RGDfK)\(_2\)-PTX, no antitumor efficacy was demonstrated in the in vivo studies in an OVCAR-3 xenograft model for E-c(RGDfK)\(_2\)-PTX as compared with the moderate efficacy of PTX.

The objective of improving PTX’s delivery and accumulation to the tumor site has been further pursued by designing a PGA–PTX–E-c(RGDfK)\(_2\). Eldar-Boock et al.\(^{[96]} \) conjugated PGA with PTX and E-c(RGDfK)\(_2\) as a targeting moiety, resulting in a ~30 nm diameter size nanoconjugate (Fig. 5). The ester linker between the polymer and the drug is hydrolytically labile, and PTX release occurs under lysosomal acidic pH, whereas the PGA itself is degradable by lysosomal enzymes such as cysteine proteases, particularly cathepsin B. PGA–PTX–E-c(RGDfK)\(_2\) nanoconjugate inhibited ECs proliferation in vitro: their migration toward VEGF, their formation as capillary-like tubular structures, and their adhesion to fibrinogen-coated wells. These results warrant PGA–PTX–E-c(RGDfK)\(_2\) as a novel targeted anti-angiogenic anticancer therapy, yet further in vivo investigation is required to determine its tumor accumulation ability.

In addition to PTX, several reports exist where cyclic RGD-based vehicles were exploited to carry other consignments to tumor vasculature and cells. One such example is DOX that was PEGylated and conjugated to E-c(RGDfK)\(_2\) (Fig. 6). The coupling of DOX to PEG via the (6-maleimidocaproyl)-hydrazone derivative of the drug (DOX–EMCH) and to the bis-cyclic RGD resulted in a 13 kDa PEG–DOX–E-c(RGDfK)\(_2\) conjugate.

**Figure 3.** Chemical structures of various RGD-containing peptide derivatives.

**Figure 4.** Structure of HPMA copolymer–RGDK–AH-GDM conjugate.

**Figure 5.** Chemical structure of PGA–PTX–E-c(RGDfK)\(_2\) conjugate.
The hydrazine linker is relatively stable under neutral pH, yet it is hydrolyzed at the acidic environment of the cellular lysosomes, allowing a rapid intracellular release of DOX.\(^{97}\) Whereas low-molecular-weight DOX entered the cells by diffusion, PEG–DOX–E-[c(RGDfK)]\(_2\) conjugate was designed to internalize into the cells through \(\alpha v/\beta 3\) integrin-mediated endocytosis. PEG–DOX–E-[c(RGDfK)]\(_2\) conjugate exhibited a similar cytotoxicity effect on human umbilical vein ECs and U-87MG glioblastoma cells as free DOX, thus demonstrating that the conjugation of E-[c(RGDfK)]\(_2\) to PEG–DOX did not alter its cytotoxicity. Furthermore, the adhesion of ECs to fibrinogen was inhibited by PEG–DOX–E-[c(RGDfK)]\(_2\) conjugate, whereas the nontargeted control PEG–DOX–c(RADfK) conjugate had no effect. To study the tumor-specific accumulation and preferential biodistribution profile of PEG–E-[c(RGDfK)]\(_2\) in vivo, the conjugate was coupled to a near-infrared cyanine dye (TSCA). Accumulation of PEG–TSCA–E-[c(RGDfK)]\(_2\) in a DA3 mammary tumor inoculated in BALB/c mice was 15-fold and 7-fold higher than PEG–TSCA–c(RADfK) and PEG–TSCA, respectively. Additionally, the biodistribution profile of PEG–TSCA–E-[c(RGDfK)]\(_2\) was tumor-specific, bypassing other selected organs. Although DA3 cells expressed low levels of \(\alpha v/\beta 3\) integrin, the presence of this integrin on tumor ECs was sufficient for the active delivery of PEG–TSCA–E-[c(RGDfK)]\(_2\) to the tumor vasculature. Whereas the conjugation of DOX with PEG facilitates passive tumor tissue accumulation, the addition of the specific targeting moiety, bis-cyclic RGD, to the macromolecule enables the direct delivery of PEG–DOX–E-[c(RGDfK)]\(_2\) to endothelial and tumor cells overexpressing the \(\alpha v/\beta 3\) integrin.\(^{98}\)

Another example is a generation-3 (G3) poly(-lysine) dendrimers with a three-dimensional compact globular morphology. The nanoglobular c(RGDfK)-targeted dendrimeric platform is a co-delivery system for DOX and small interfering RNA (siRNA). siRNA is a short double-stranded RNA, which is regarded as a novel potential therapeutic for the treatment of various diseases by specific gene silencing of the complementary mRNA. However, the efficiency of gene silencing by siRNA is very low because of its extensive degradation by nucleases in the plasma and rapid renal clearance.\(^{99}\) Accordingly, siRNA delivery has emerged as a key issue for the development of siRNA therapeutics.\(^{100}\) The siRNA complexes of the targeted conjugate G3-[PEG-RGD]-[DOX] were readily internalized in U-87MG cells via receptor-mediated endocytosis, followed by intracellular accumulation. Co-delivery of DOX and siRNA resulted in significantly high gene silencing in U-87MG cells due to the combination effects of cytotoxicity and RNA interference (RNAi) activity. It was shown that the compact and three-dimensional nanoglobules are promising carriers for the combined delivery of nucleic acids and chemotherapeutic agents.\(^{101}\)

Multidrug resistance (MDR) is a mechanism by which cancer cells protect themselves from chemotherapy; it is also one of the major obstacles of currently available cancer chemotherapy. The adenosine triphosphate (ATP)-dependent transmembrane transporter P-glycoprotein (P-gp) is responsible for the dominant mechanism that reduces intracellular levels of cytotoxic drugs below lethal thresholds by active pumping of the drug out from the tumor cell. The bypass of P-gp and/or controlled delivery of cytotoxic agents (e.g. DOX) to alternative subcellular sites in cancer cells may enhance the efficacy of the drugs in resistant tumors.

Xiong et al.\(^{102}\) developed two polymeric nanocarriers: (i) RGD4C-PEO-b-P(CL-Hyd-DOX)—linked by pH-sensitive hydrazone bond; and (ii) RGD4C-PEO-b-P(CL-Ami-DOX)—linked by a more stable amide bond, in order to increase the therapeutic efficacy of DOX for sensitive and resistant cancers. The delivery systems are based on biodegradable poly(ethylene oxide)-block-poly(3-caprolactone) (PEO-b-PCL) micelles functionalized on the micellar shell (PEO) as well as the micellar core (PCL). Both micellar conjugates were stable in physiological pH 7.2, but at pH 5.0, DOX was released from P(CL-Hyd-DOX) because of the degradation of the acid-labile linker and from P(CL-Ami-DOX) because of micellar core degradation. In both formulations, RGD-4C-containing micelles significantly increased the cellular uptake of DOX in DOX-sensitive (MDA-435/LCC6WT) and DOX-resistant cancer cell lines (a clone expressing a high level of P-gp, MDA-435/LCC6MDR). In MDA-435/LCC6 WT, the best cytotoxic response was achieved using RGD-4C-PEO-b-P(CL-Hyd-DOX), which correlated with the highest cellular uptake and preferential nuclear accumulation of DOX. In MDA-435/LCC6MDR, RGD-4C-PEO-b-P(CL-Ami-DOX) was the most cytotoxic, and this effect correlated with the accumulation of DOX in the mitochondria. Consistent with those in vitro results, RGD-4C-PEO-b-P(CL-Hyd-DOX) and RGD-4C-PEO-b-P(CL-Ami-DOX) nanoformulations were found to be more effective than free DOX in inhibiting the in vivo growth of DOX-sensitive and DOX-resistant tumors, respectively.

**Selectins-targeted polymer–drug conjugates**

Selectins are a family of cell adhesion molecules that bind carbohydrated proteins, and they express on cytokine-activated ECs (E-selectin/CD62E and P-selectin/CD62P), platelets (P-selectin), and leukocytes (L-selectin/CD62L).\(^{103,104}\) E-selectin is a molecular marker associated with tumor vasculature and represents another excellent target for therapeutic agents intended to destroy tumor ECs. It is expressed exclusively by vascular ECs during inflammation and cancer.\(^{105–107}\) and its expression has been associated with tumor angiogenesis and metastasis in a variety of cancers.\(^{108,109}\) E-selectin is a 97-kDa protein that binds the tetrasaccharide sialy Lewis X (sLex) structure. Several E-selectin-mediated targeting strategies were previously exploited for the delivery of macromolecules such as immunoliposomes and nanoparticles into inflamed areas and tumor tissues, using the natural ligand sLex, or its derivatives, as the targeting agents.\(^{110}\)
E-selectin was targeted with a high affinity peptide named E-selectin-binding peptide (Esbp) for the selective delivery of drugs to tumor vascular endothelium. The Esbp moiety contains the DITWDLQWDMK sequence—exclusively binding E-selectin, but not its family members, P-selectin, and L-selectin. Polymers carrying drugs and/or diagnostic agents would benefit from E-selectin-mediated targeting using Esbp because it can be easily attached to a polymeric scaffold. In addition, Esbp binds E-selectin at a low nanomolar range concentration[113] and in a non-competitive manner, compared with sLex (another well-characterized E-selectin-binding peptide).

Shamay et al.[112] demonstrated that copolymers carrying Esbp with an anticancer drug possess strong cytotoxicity against E-selectin-expressing vascular endothelium (Fig. 7). The passive localization associated with the EPR effect was shown to be significantly improved by an active mechanism involving receptor-ligand interactions.[113]

In this study, Esbp-targeted HPMA copolymer was conjugated with DOX via a spacer containing a hydrazide group. Esbp significantly increased the HPMA–DOX conjugate's endocytosis efficiency into immortalized vascular ECs expressing E-selectin upon TNFα (tumor necrosis factor) activation.[114] This was attributed to multivalent interactions between the HPMA–Esbp copolymers and the cell-surface-associated E-selectin. The cellular binding and uptake of the conjugate were consistent with the increased cytotoxicity of P(Esbp)-DOX, as observed by confocal microscopy. This cytotoxicity of P(Esbp)-DOX requires cellular uptake of the copolymer followed by release of the active DOX moieties in endosomal and lysosomal compartments. Esbp peptide was found, for the first time, to be capable of delivering elevated amounts of DOX to E-selectin-expressing vascular endothelium. This is the first successful attempt to implement Esbp-directed chemotherapy to activated ECs using a peptide–polymer–drug conjugate for specific binding to E-selectin.[112]

**Aminopeptidase N-targeted polymer therapeutics**

The APN/anti-CD13 has been identified as one of the important tumor markers overexpressed on the surface of tumor vascular ECs and involved in cancer angiogenesis, invasion, and metastasis.[115,116] APN participates in tumor angiogenesis through regulating filopodia formation and endothelial invasion[117]; furthermore, it is present on tumor neovascularature, thus differentiating it from existing blood vessels.[118] The proteolytic degradation of the extracellular matrix, through the activity of APN and other proteases such as matrix metalloproteinases, contributes to the growth and metastasis of tumors.[119] The NGR motif (Asn-Gly-Arg) has been confirmed to bind exclusively to APN expressed in tumors and not to other types of APN isoforms expressed in normal epithelia and myeloid cells.[118,120]

Adar et al. recently reported the design and synthesis of CD13-targeted HPMA copolymers for the selective delivery of a pro-apoptotic drug into cancer and ECs.[121] The use of the NGR motif facilitates active targeting to the CD13 receptor. A significant benefit of using polymers carrying multivalent display of NGR motifs is that they can act simultaneously on more than one CD13 receptor, to markedly improve the binding affinity.[122,123]

Peptides capable of invading the mitochondria of mammalian cells and triggering apoptosis represent a new therapeutic approach for cancer treatment.[124–126] The mitochondria-disrupting peptide D(KLAKLAK)₂, a 14-amino-acid cationic and α-helical forming peptide, has been shown to induce apoptosis in cancer cells.[127] This D(KLAKLAK)₂ peptide does not penetrate the zwitterionic plasma membranes of eukaryotic cells, but when internalized, it can disrupt the negatively charged mitochondrial membrane[128] resulting in cell death by mitochondrial-dependent apoptosis[124]. The use of this peptide for enhancing apoptotic activity is therefore promising but must involve targeting and internalization strategies. The peptide was attached to the polymer, as described before, through an acid-sensitive hydrazone linkage that is stable in the blood circulation at pH 7.4, but hydrolytically degradable in mildly acidic environment[129] and can be used for the controlled release of the D(KLAKLAK)₂ moieties.

The novel water-soluble HPMA copolymer bearing multivalent display of NGR motifs targets the pro-apoptotic drug to CD13-overexpressing cells. Several NGR sequences of various structural conformations were examined, but the dimeric and cyclic NGR sequences demonstrated superior binding affinity to CD13-overexpressing cells over the linear peptide (Fig. 8). The attachment of the active peptide to a polymeric carrier markedly increased its cytotoxicity and pro-apoptotic activity, relative to free D(KLAKLAK)₂ in CD13(+) cells. Apoptosis was the major mechanism for the induction of cell death following exposure to the polymeric drug. Altogether, this conjugate showed an improved intracellular delivery and cytotoxicity of polymer D(KLAKLAK)₂ conjugate in endothelial and various cancer cells.[121]

**Hyaluronan-based polymer therapeutics**

CD44 is a cell-surface carbohydrate receptor and a component of the ECM.[130] Changes in CD44 expression are associated with a wide variety of tumors and metastasis. The physical and functional properties that are common to CD44 receptors include the high binding affinity to HA (also referred to as hyaluronan or hyaluronate). HA is a linear, negatively charged polysaccharide that is composed of two alternating and repeating units of

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**Figure 7.** Chemical structure of the HPMA–Esbp copolymer containing the terminal hydrazone bonded DOX moiety.

**Figure 8.** Chemical structure of targeted HPMA copolymer bearing (GNRGR)₂ as targeting ligand and D(KLAKLAK)₂ as cytotoxic moiety.
d-glucuronic acid (GlcUA) and N-acetyl-d-glucosamine (GlcNAc) linked together. In contrast to other HA-binding proteins, CD44 binds HA at the cell surface, where multiple, closely arrayed CD44 receptor molecules interact with the highly multivalent repeating disaccharide chain of HA.

The work of Journo et al. presented a correlation between the lengths of low-molecular-weight HA (LMW-HA) oligomers and the binding affinity to the CD44 receptor overexpressed in ovarian SK-OV-3 cells. Despite their relatively simple structure, HA oligomers have wide-ranging and even opposing biological functions depending on the size of the molecule. High-molecular-weight HA (HMW-HA) oligomers pose antiangiogenic, immunosuppressive, and anti-inflammatory properties, whereas smaller molecules exhibit opposite biologic functions. However, HMW-HA oligosaccharides in aqueous solution form very quickly a viscous gel that is not suitable for systemic administration. Therefore, HMW-HA was enzymatically degraded by hyaluronate lyase at different incubation times to acquire a mixture of LMW-HA oligosaccharides. The mixture was separated using a size-exclusion column, and chromatograms of the different sizes of HA oligosaccharides were received according to the different incubation times. Next, LMW-HA oligomers were conjugated to fluorescein isothiocyanate (FITC)-labeled HPMA copolymer and evaluated for their binding ability and intracellular fate in CD44-overexpressing SK-OV-3 cells by means of flow cytometry and confocal microscopy. It was found that polymer conjugates bearing HA oligomers at the size of 10 monosaccharides and above bind most profoundly to CD44-overexpressing cells and internalize to the greatest extent compared with the smaller-size HA-containing conjugates, and thus can be used as a potential drug carrier for CD44-overexpressing ovarian carcinoma cells. Subsequent cytotoxicity experiments were performed with PTX copolymer conjugate bearing HA34, designated as P-(HA34)-PTX (Fig. 9).

P-(HA34)-PTX conjugate exhibited 50 times higher cytotoxicity toward CD44-overexpressing cells relative to the control, nontargeted P-PTX and was less toxic toward cells with low levels of CD44. The cytotoxicity of P-(HA34)-PTX requires cellular uptake of the copolymer followed by the release of active PTX moieties in endosomal and lysosomal compartments. It was therefore concluded that the HA34 is capable of delivering more PTX to CD44-overexpressing cells than to those cells with little or no CD44 expression.

Among other methods, various synthetic polymers such as PEI were investigated for intracellular delivery of siRNA.

Jere et al. reported the conjugation of HA to PEI; PEI-g-HA conjugate reduced the cytotoxicity of PEI and enabled targeted-specific delivery to tissues with various HA receptors. Among several candidates, branched PEI (bPEI, molecular weight = 25 kDa) has been regarded as one of the most effective nonviral vehicles for in vitro gene silencing. It is highly positively charged, hence forming a tight electrostatic interaction with the negatively charged siRNA.

Park et al. synthesized reducible bPEI by crosslinking low-molecular-weight PEI with cystamine bisacrylamide. PEI with a molecular weight of 2000 Da had relatively negligible cytotoxicity and low siRNA delivery efficiency. The crosslinked PEI containing disulfide bonds (PEI-SS) showed comparable siRNA delivery capability to high-molecular-weight bPEI with remarkably reduced cytotoxicity. The amine groups of PEI-SS were further conjugated to the carboxyl groups of HA in the form of block copolymer by reductive amidation, resulting in (PEI-SS)-b-HA conjugate complexed with siRNA. Tumor angiogenesis was reported to be suppressed efficiently by downregulating the gene expression of VEGF. Moreover, there were many reports on the therapeutic application of VEGF siRNA for the treatment of cancer.

The cytotoxicity of (PEI-SS)-b-HA appeared to be negligible, and the effective cellular uptake of siRNA/(PEI-SS)-b-HA complex by HA-receptor-mediated endocytosis was confirmed by flow cytometry and confocal microscopy analyses. In addition, siRNA/(PEI-SS)-b-HA complex showed a 50–80% gene silencing efficiency in vitro. HA in the outer surface of siRNA/(PEI-SS)-b-HA complex contributed not only to effective cellular uptake by HA-receptor-mediated endocytosis but also to enhanced serum stability, alleviating the nonspecific binding of siRNA to serum proteins. (PEI-SS)-b-HA conjugate was developed as a target-specific and nontoxic delivery system for siRNA therapeutics and was proved successful when VEGF siRNA/(PEI-SS)-b-HA complex dramatically retarded tumor growth in a mouse model.

**SUMMARY**

Conjugation of small molecules to macromolecular carriers, such as polymers, holds great therapeutic potential, particularly in the field of cancer treatment. Focal tumor tissue accumulation of nanoscale polymer–drug conjugates is induced because of the EPR effect. This passive accumulation can be further augmented by the active targeting of polymer–drug conjugates to a specific cell marker known to be overexpressed in the tumor bed or in ECs lining the tumor vasculature.

For the past three decades, many polymer-based drug delivery systems were designed and developed, several entered clinical trials, and most did not develop beyond preclinical trials. One of the main reasons is the complexity of these systems that makes them very difficult to be characterized. Hence, careful rational design of polymer–drug conjugates is required. The choice of a polymeric backbone, degradable or not, will influence physicochemical factors and pharmacokinetic parameters of the system. Biodistribution and biocompatibility will determine the potential of the new system to enter the clinic.

Conjugation of a small therapeutic molecule with a polymer via different linkers allows trigger-dependent release of the drug at the site of interest. The growing understanding of tumor tissue and its stroma together with the advances in the field of organic chemistry allow further development of smart drug release mechanisms in a controlled and well-defined way.
Further improvement in the biodistribution of polymer–drug conjugates can be achieved by active targeting of those to specific, tumor-associated, cellular markers. Tumor vasculature is a highly attractive niche due to its immediate exposure to the administered system, its relatively stable genetics, and vast expression of unique extracellular receptors. Furthermore, receptor-mediated cellular penetration may bypass tumor cell resistance resulting in better therapeutic effect.

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POLYMER CONJUGATES FOR FOCAL AND TARGETED DELIVERY OF DRUGS


