



Design and development of polymer conjugates as anti-angiogenic agents[☆]

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ABSTRACT

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is one of the central key steps in tumor progression and metastasis. Consequently, it became an important target in cancer therapy, making novel angiogenesis inhibitors a new modality of anticancer agents. Although relative to conventional chemotherapy, anti-angiogenic agents display a safer toxicity profile, the vast majority of these agents are low-molecular-weight compounds exhibiting poor pharmacokinetic profile with short half-life in the bloodstream and high overall clearance rate. The “Polymer Therapeutics” field has significantly improved the therapeutic potential of low-molecular-weight drugs and proteins for cancer treatment. Drugs can be conjugated to polymeric carriers that can be either directly conjugated to targeting proteins or peptides or derivatized with adapters conjugated to a targeting moiety. This approach holds a significant promise for the development of new targeted anti-angiogenic therapies as well as for the optimization of existing anti-angiogenic drugs or polypeptides. Here we overview the innovative approach of targeting tumor angiogenesis using polymer therapeutics.

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1. Introduction

The hypothesis that tumor growth is angiogenesis-dependent, and the idea that anti-angiogenic therapy would be an effective strategy to treat human cancer, was first proposed in 1971 by Judah Folkman [1]. Today, the number of anti-angiogenic therapeutic drugs in clinical trials is gradually increasing; yet, the promising potential of these agents alone or in combination with drug delivery systems has not been fully elucidated. With the successful approval of bevacicumb (Avastin), Iressa (Gefitinib), Sorafenib (Bay 43-9006, Nexavar), Sunitinib (SU-11248, Sutent), Tarceva (Erlotinib) and others, inhibition of angiogenesis is becoming the fourth modality of anticancer therapy following chemotherapy, surgery and radiotherapy.

The process of angiogenesis involves many growth factors and their receptors, cytokines, proteases and adhesion molecules [2], thus, multiple targets for therapeutic intervention and targeting opportunities for anti-angiogenic therapy exist. Anti-angiogenic therapy aims either, to prevent the formation of new vessels (cytostatic agents), sequester vascular endothelial growth factor (VEGF) or other angiogenic stimulators, or damage existing vessels (cytotoxic agents). Angiogenesis inhibitors are relatively less toxic than conventional chemotherapy and have a lower risk of drug resistance. Nevertheless, most of the angiogenesis inhibitors are low-molecular-weight-compounds and therefore exhibit poor pharmacokinetic and biodistribution profile. Consequently, relatively small amounts of the drug reach the target site, and therapy is associated with side effects and low efficacy [3]. One of the strategies to target angiogenesis inhibitors is by the use of synthetic polymers as carriers.

The concept of a drug delivery system based on synthetic polymers was first proposed by Helmut Ringsdorf in 1975 [4]. This proposed model consists mainly of five components: macromolecular

polymeric backbone, drug, spacer, targeting group and a solubilizing agent. Macromolecular carriers chosen for the preparation of targeted polymer-drug conjugates should ideally be water-soluble, non-toxic, and non-immunogenic, as well as degraded and/or eliminated from the organism [5,6]. Finally, the macromolecular carrier should exhibit suitable functional groups for attaching the respective drug or spacer. The drug can be conjugated directly or via a degradable or non-degradable linker onto the polymer backbone to allow control of the release rate of the active drug from the conjugate at the target site [7].

A polymeric drug delivery system can be designed for passive or active targeting. Passive targeting refers to the exploitation of the natural (passive) distribution pattern of a drug-carrier *in vivo*. The latter is based upon the phenomenon named the “enhanced permeability and retention (EPR) effect” [8], and attributed to two factors: (I) the disorganized pathology of angiogenic tumor vasculature with its discontinuous endothelium, leading to hyperpermeability to circulating macromolecules, and (II) the lack of effective tumor lymphatic drainage, which leads to subsequent macromolecular accumulation (Fig. 1). The active approach relies upon the selective localization of a ligand at a cell-specific receptor. Targeting tumor vasculature using polymeric drug delivery systems has enormous potential for cancer therapy. A well designed polymeric drug delivery system, whether it is targeting the tumor site passively or actively, improves the therapeutic index of anti-angiogenic agents by increasing the half-life of low-molecular-weight drugs, their water-solubility and their time of exposure to the tumor vasculature (i.e. to the tumor endothelial cells), while reducing their toxicity. Taken together, this new approach may provide a novel strategy to target cancer. This review focuses on the development of drug delivery strategies using polymer therapeutics to target the tumor vasculature.

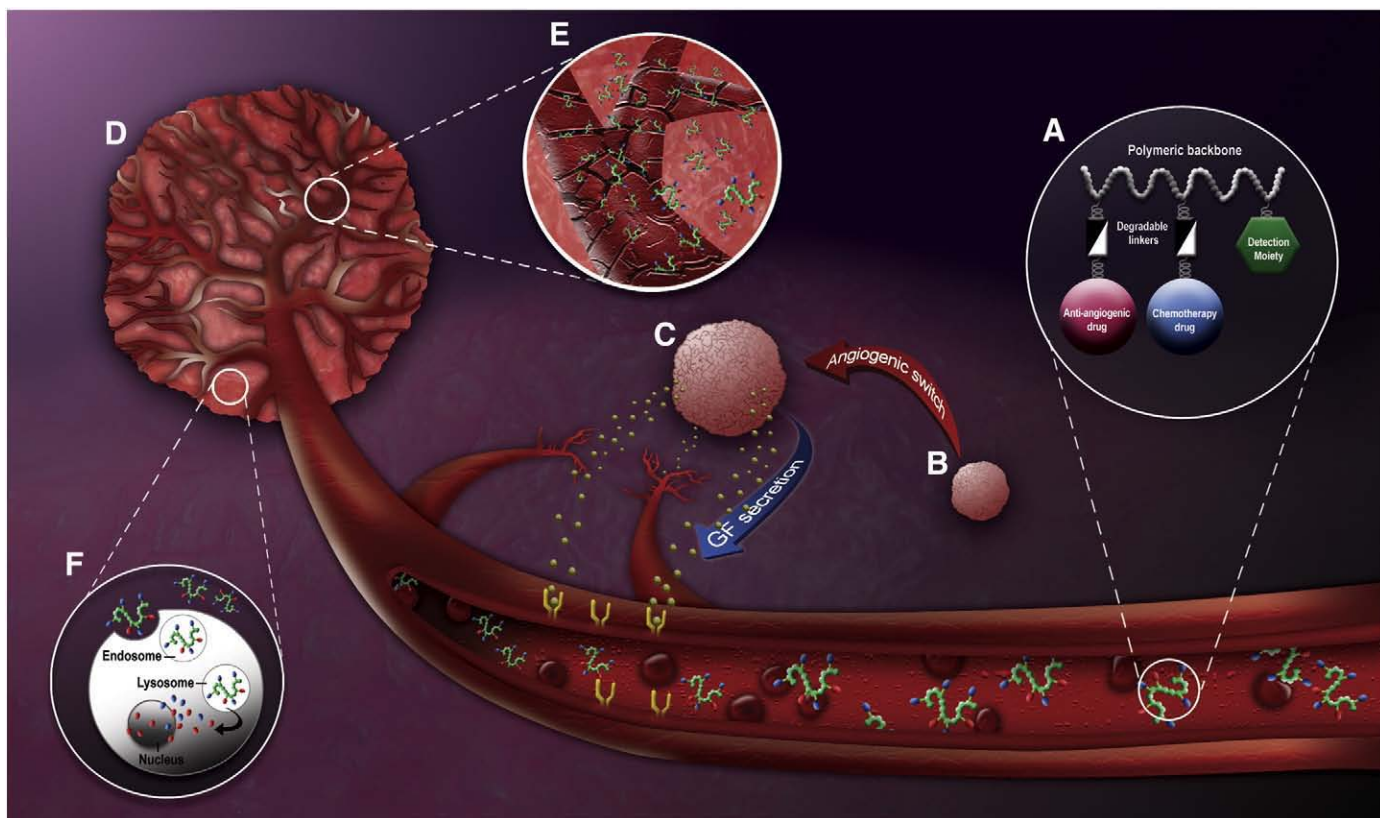


Fig. 1. Targeting tumor vasculature using polymeric conjugates of anti-angiogenic agents. (A) Schematic illustration of a polymeric drug-delivery system consist of (i) polymeric backbone (ii) degradable linkers (iii) anti-angiogenic drug (iv) chemotherapeutic drug and (v) detection moiety. (B) A dormant avascular tumor prior to the angiogenic switch. (C) An angiogenic switch followed by secretion of vascular growth factors (GF) encouraging the recruitment of neovasculature. (D) A well-established mass of a vascularized tumor. (E) The EPR effect allowing extravasation of polymer therapeutics through the hyperpermeable tumor blood vessels and their accumulation at the tumor site. (F) Cellular uptake of the vascular polymeric drug delivery system via endocytosis followed by drug release into the cell cytoplasm.

2. The underlying principles of targeting tumor vasculature

Tumors become clinically detectable only after a tumor mass undergoes continuous expansion [9]. However, expansion of a tumor mass beyond the initial microscopic size of a non-angiogenic tumor is dependent on the recruitment of its own vascular supply, by angiogenesis and/or blood vessel cooption [10,11]. The ability of a tumor to progress from a non-angiogenic to an angiogenic phenotype is central to the progression of cancer and is termed the “angiogenic switch” [12] (Fig. 1). This phenotype is driven by (i) angiogenic balance transition towards the pro-angiogenic state, (ii) increased expression of positive angiogenic regulators (i.e., VEGF, bFGF, TGF- β , and PDGF) by tumor and stroma cells, (iii) decreased expression of negative angiogenic regulators (i.e., thrombospondin-1, endostatin, and angiostatin) by tumor cells and by stroma fibroblasts, and in some tumors (iv) recruitment of bone marrow-derived endothelial precursors [13]. The complex cascade of angiogenesis holds immense potential for therapeutic intervention and targeting opportunities, thus makes the tumor vasculature an attractive target [14]. The prerequisite of a tumor to recruit a functional vasculature in order to expand in mass, led to the development of therapies based on angiogenesis inhibitors to prevent new blood vessels formation (e.g. endostatin, angiostatin and TNP-470) or damage existing blood vessels (e.g. combretastatin and colchicine analogs).

There are two classes of angiogenesis inhibitors – ‘direct’ and ‘indirect’ [15]. Direct angiogenesis inhibitors, such as endostatin, vitaxin, angiostatin and tumstatin, prevent vascular endothelial cells from responding to a spectrum of pro-angiogenic molecules secreted usually by the tumor cells such as VEGF, bFGF, IL-8, platelet-derived growth factor (PDGF) and PD-EGF [16]. Indirect angiogenesis inhibitors such as Iressa and Avastin block the activity of the pro-angiogenic factors e.g. bFGF, TGF- α and VEGF or their receptors on the endothelial cells, thus preventing the stimulation of the endothelial cells [17]. However, tumor cells display genomic instability that can induce acquired drug resistance particularly when using indirect angiogenesis inhibitors [18]. The pro-angiogenic factors blocked by these inhibitors can be compensated by the mutating tumor cells that can produce other pro-angiogenic proteins. In comparison to tumor cells, endothelial cells are considered to be relatively genetically stable [19], therefore direct angiogenesis inhibitors could block endothelial cells from responding to a wide spectrum of pro-angiogenic proteins and appear to be less vulnerable to drug resistance. Nevertheless, Hida et al. demonstrated recently that in contrast to normal endothelial cells, tumor endothelial cells are cytogenetically abnormal and suggested that the tumor microenvironment contributes to these aberrations [20].

When comparing the anti-angiogenic therapy versus conventional chemotherapy there are several important key points to keep in mind: (i) The effect of the conventional chemotherapy is directed on all tumor cells whereas the anti-angiogenic therapy affects the endothelial cells of tumor microvessels while each single endothelial cell supplies oxygen and nutrients for 50–100 tumor cells [21,22]. (ii) Anti-angiogenic therapy aims to influence the physiology of endothelial cells which is in direct contact with blood circulation whereas tumor cells are distant in tumor tissue and less accessible to chemotherapy drugs. (iii) Tumor-associated endothelial cells have different functional and phenotypic characteristics [23] therefore, they are easy target for selective therapy as opposed to tumor cells that have few specific tumor cell antigens for selective tumor types [24]. (iv) Traditional cancer therapies make use of chemotherapy at the maximum tolerated dose (MTD) with extended rest periods, usually resulting in significant undesirable toxicities and side effects (e.g. bone marrow suppression, hypersensitivity reactions, anaphylaxis, gastrointestinal disturbances, pulmonary toxicity and major long-term toxicities related to infertility and secondary malignancies). Anti-angiogenic therapy is administered as ‘metronomic

schedule’ (frequent administration of low doses) with relatively limited toxicity and more tolerable side effects [25,26]. Furthermore, it has been established that various conventional cytotoxic agents can function as anti-angiogenic drugs when administered at relatively low doses on a continuous or very frequent ‘metronomic’ schedules [27,28]. In addition, Browder et al. demonstrated that the efficacy of metronomic chemotherapy can be significantly increased when administered in combination with anti-angiogenic drugs [22].

2.1. The advantages of targeting tumor vasculature using polymeric carriers

Although angiogenesis inhibitors (e.g. small molecules, proteins, and antibodies) were designed to target tumor endothelium, most of these agents are delivered systemically and consequently exhibit a non-specific biodistribution and deprived pharmacokinetic properties [29]. The therapeutic index of these agents can be improved by using a polymeric drug delivery system to selectively target the metabolically active endothelium supporting the tumor. Angiogenesis inhibitors such as small molecules differ in their solubility properties in aqueous solutions [30,31]. Accordingly, some of them should be systemically administered in organic and toxic vehicles resulting in limited clinical utility [32]. Furthermore, chemical instability and short half-life of some anti-angiogenic drugs might impair their activity and lower the transient levels of the drug at tumor vasculature [33–35]. Incorporation of such drugs to a delivery vehicle such as soluble polymeric carrier increases their solubility, bioavailability and chemical stability by protecting them from being degraded by the biological environment thus minimizing the systemic toxicity.

Both, direct and indirect anti-angiogenic agents are delivered into the circulation and achieve their therapeutic index by inhibition of proliferation and migration of endothelial cells. Nevertheless, focusing their activity on tumor angiogenesis could potentially enhance the effectiveness of these agents. The use of a macromolecule–drug complex contributes to a longer circulation time and increased accumulation of the biologically active entities at tumor vasculature by exploiting the natural (passive) distribution pattern of a drug-carrier *in vivo*. In addition, a target-specific (active) recognition moiety (e.g. antibodies, oligosaccharides, ligands and peptides) could be attached to the delivery system providing selective localization at the target site [36,37].

A site-specific release of the anti-angiogenic agent is achieved by using a cleavable linker between the active compound and the polymeric backbone. This linker is amenable to cleavage by hydrolysis, lysosomal proteases or acidic conditions leading to release and delivery of the active agent extracellularly or intracellularly depending on the linker features. Another advantage of the polymeric delivery system is the ability to tailor different combinations of anti-angiogenic agents and cytotoxic chemotherapeutic drugs as well as the ability to control their loading percentage on the polymeric backbone. This strategy enables the use of two or more agents that have potentially synergistic inhibitory effect on tumor endothelium or a bi-specific effect on tumor vasculature and tumor epithelium. The concomitant targeted delivery of two agents that act synergistically, allows the administration of lower concentrations of each agent, increasing their combined antitumor efficacy and decreasing their toxicity. An advanced combined drug delivery technique could potentially provide an ideal attack on both tumor and endothelial compartments (see examples in Table 1).

2.2. Passive targeting using the EPR effect

A polymeric vascular drug delivery system should facilitate controlled delivery and release of the anti-angiogenic active agent at target site. The rationale for using polymeric macromolecules as carriers for the delivery of anti-angiogenic and other therapeutic

Table 1
Polymer therapeutics targeted to tumor angiogenesis.

Polymer-drug (linker)	Trade name	Molecular weight (kDa)	Size (nm)	Tumor models/indication	Reference
HPMA					
HPMA–TNP-470 (Gly-Phe-Leu-Gly)	Caplostatin	30		Lewis lung carcinoma (LLC), U87 glioblastoma, A2058 melanoma, PC3 prostate carcinoma, COLO-205 colon carcinoma, Mycen-driven murine neuroblastomas, MG-63 human osteosarcoma.	[56,88,106,168]
HPMA–ALN–TNP-470 (Gly-Gly-Pro-Nle)		80	100		[108]
HPMA–ALN–PTX (Gly-Phe-Leu-Gly)		30	100		[109]
HPMA–RGD4C and HPMA–RGDfK (Gly-Gly)		30		DU145 human prostate carcinoma, LLC (biodistribution assays)	[117–119]
PGA					
PGA–PTX–E-[c(RGDfK) ₂]			30		[169]
PMLA					
β -poly(L-malic acid)-laminin α 4 and β 1-transferrin mAb-PEG-L-valine (disulfide bonds)	Polycefin	550		U87MG and T98G human glioblastoma	[126,127]
PVA					
TNP-470–PVA		220		choroidal neovascularization (rabbits)	[132]
PEG					
mPEG-PLA–TNP-470 (micelles)	Lodamin		8	LLC, B16/F10 murine melanoma	[146]
DSPE–PEG–APRPG, PEG–Lip-SU1498, APRPG–Lip-SU1498			120	C26 NL-17 colon adenocarcinoma	[151]
			178		[154]
			160		
APRPG–LipADM			154	C26 NL-17 colon adenocarcinoma, P388 and P388/ADM leukemia	[153]
PEI-g-PEG–RGD/pCMV-sFlt-1			150	CT-26 colon adenocarcinoma	[157,158]
α v β 3-NP/RAF(–)			Not reported	M21, M21-L human melanoma	[159]
LipCNDAC/APRPG–PEG			100	C26 NL-17 colon adenocarcinoma	[156]
RGD–PEG–PEI (VEGFR2 siRNA nanoplexes)			90–120	N2a mouse neuroblastoma	[160]
PLGA					
PLGA-siVEGF (microspheres)			35,000–45,000	S-180 murine sarcoma cells	[166]
PLGA–PF-4/CTF			40,000	U87MG human glioblastoma	[167]

agents, is based on the biological phenomena observed by Matsumura and Maeda in 1986, known as the EPR effect [8,38]. Different from normal tissues, solid tumors have unique pathophysiological characteristics, such as extensive angiogenesis and hence vascular abnormalities (such as leaky and tortuous blood vessels), and impaired lymphatic drainage [39]. These anatomical and functional abnormalities, allow superior extravasation of macromolecules, nanoparticles and lipidic particles into the tumor tissue (Fig. 2). Once the macromolecules entered the interstitium, they are retained there by lack of intratumoral lymphatic clearance and accumulate at high concentrations [40]. As opposed to macromolecules, low-molecular-weight compounds diffuse rapidly and indiscriminately into normal and tumor tissues through the endothelial cell layer of blood capillaries, therefore causing undesirable systemic side effects followed by quick renal clearance. An important parameter when designing a polymeric drug delivery system is the size of the polymeric carrier which influences the pharmacokinetic profile and the degree of accumulation at the tumor site. Several studies showed a correlation between the half-life in the plasma, the renal clearance, and the accumulation at the tumor site of the respective macromolecule. The normal renal threshold is in the range of 30–50 kDa therefore to achieve an optimal balance between these key elements, polymeric carriers with molecular weights in the range of 20 to 200 kDa are often chosen as the backbone of the drug delivery system [41]. Additional factors that dictate the biodistribution profile of the macromolecule are the charge, conformation, hydrophobicity, and immunogenicity of the polymeric carrier [42].

The EPR effect is affected both by (i) anatomical factors (e.g. extensive angiogenesis and high vascular density, lack of smooth-muscle layer, pericytes, sporadic blood flow, poor lymphatic clearance and slow venous return), and by (ii) permeability-enhancing factors (e.g. VEGF, nitric oxide, prostaglandins, matrix metalloproteinases, tumor necrosis factor and interleukin-2) [43]. Beside these factors, active therapeutic agents affecting the blood vessels can influence the

EPR effect depending on their activity. Compounds that enhance the EPR effect include pro-inflammatory anticancer agents that generate superoxide radical and NO (or activate proMMP to MMP, for example SMANCS, anthracyclins, mitomycin C and nitrosourea) [44–46] as well as drugs that were found to upregulate VEGF such as doxorubicin [47,48]. Enhancement of the EPR effect can result in interstitial hypertension, hypoxia, and acidosis, a state which can interfere with the delivery of low-molecular-weight compounds to solid tumors, render tumor cells resistant to both radiation and several cytotoxic drugs, and induce genetic instability of tumor cells [49]. On the other hand, free and polymer-conjugated angiogenesis inhibitors were shown to have the ability to “normalize” the abnormal blood vessels by balancing the pro- and anti-angiogenic factors [50,51] (e.g. bevacizumab [52], PTK787 [53] and SU6668 [54]) or by decreasing the vascular hyperpermeability through other mechanisms (e.g. TNP-470 [55] and caplostatin [56]), thus reducing the EPR effect. This reversible state can enhance the efficacy of combined anti-angiogenic and chemotherapy or radiotherapy due to improved penetration of cytotoxic chemotherapy drugs and oxygen to the tumor site.

2.3. Active targeting using vascular recognition moieties

Normal organ vasculature, as well as tumor vasculature involved in pathological conditions, expresses highly specialized molecular markers [57]. Blood vessels undergoing angiogenesis, whether in regenerating normal tissue or in a tumor, express molecular markers that are not expressed in normal blood vessels [58]. In addition, tumor vasculature during the pre-malignant and fully malignant tumor stages can be distinguished by morphology or biochemistry markers (e.g. expression of integrins, growth factor receptors, proteases and cell surface proteoglycans) [59]. These differences and the molecular diversity of vasculature provide the means by which systemically administered therapies can target tumor blood vessels. Incorporation of a vasculature-targeting moiety in a polymeric drug delivery system

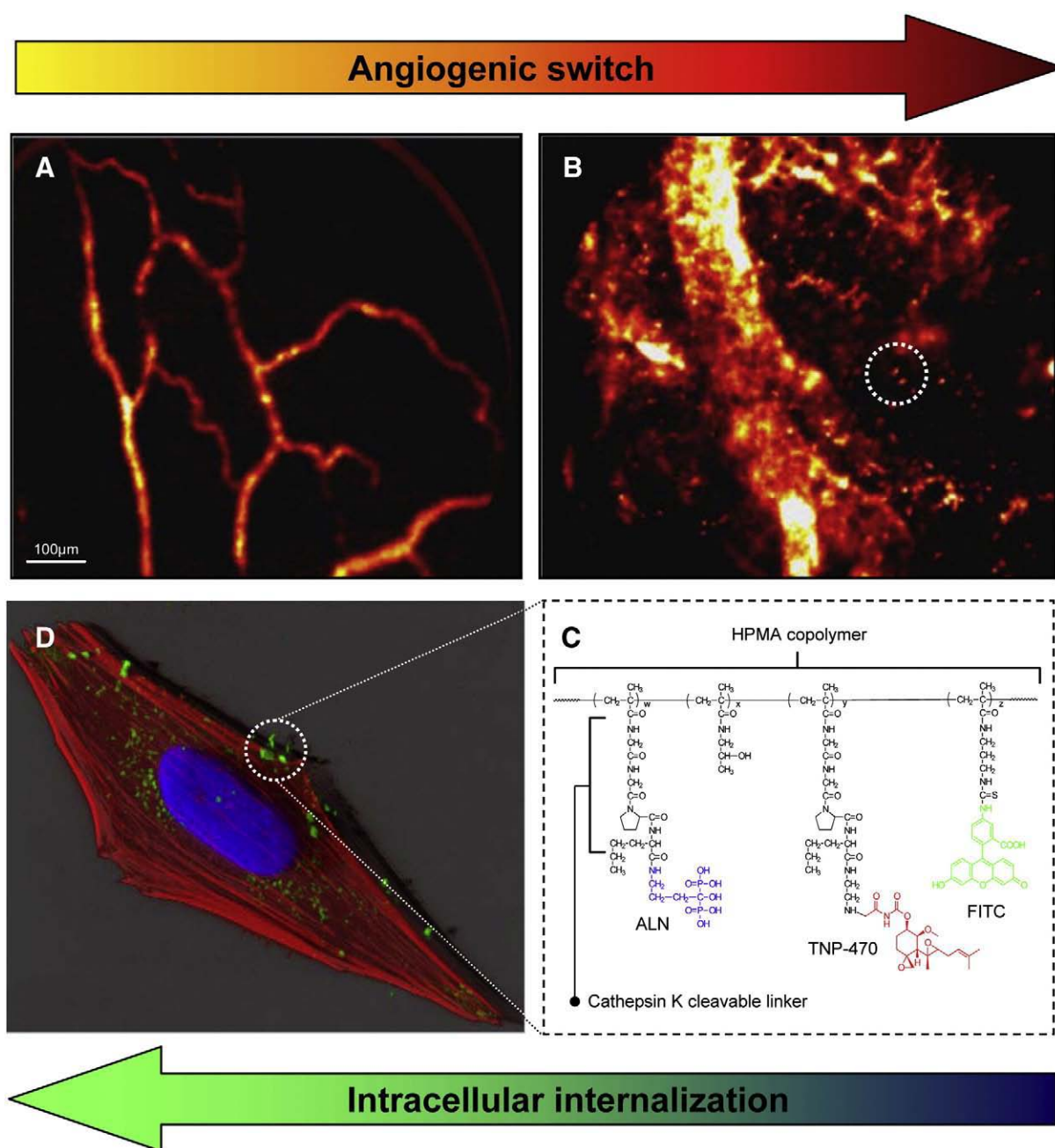


Fig. 2. Fluorescence imaging of vascular polymeric drug conjugate accumulation at normal versus tumor vessels, and its uptake by endothelial cells. (A) Polymeric drug delivery system labeled with fluorescence as imaged in normal healthy tissue blood vessels (B) Extravasation and accumulation of the fluorescence-labeled polymeric drug delivery system at the tumor site due to tumor vessel hyperpermeability and the consequent EPR effect. (C) Chemical structure of the second generation of caplostatin conjugate (HPMA copolymer–ALN–TNP-470). (D) Confocal image of endothelial cellular uptake of a FITC-labeled caplostatin second generation conjugate (green), actin filaments (red) and nucleus stained with DAPI (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in most cases, will lead to a beneficial therapeutic index of the delivered pharmaceutical, that is, a higher efficacy with minimized side effects. One of the leading techniques to isolate peptides that bind to a specific protein is *in vivo* phage display [60,61]. This technology involves the screening of peptide libraries *in vivo*, followed by a selection of the homing peptides that recognize specific tissues such as tumor vasculature [62]. The first tumor-homing peptide described was the Arg-Gly-Asp (RGD) peptide known to selectively bind to $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins [63]. RGD peptides and the higher affinity peptide motif RGD4C have been widely used to deliver cytotoxic compounds such as doxorubicin [64] and proapoptotic peptides [65] selectively to the tumor cell and tumor vasculature. Similar to the molecular markers of blood vessels, angiogenesis markers also include

peptidases/proteases such as aminopeptidase N (CD13) that can be targeted using the homing peptides Asn-hGly-Arg (NGR) [66] as well as angiogenic cell surface receptors such as nucleolin [67] that can be targeted with the F3 peptide (a fragment of the HMG2 protein) [68]. Beside endothelial cells, pericytes that contribute to the tumor angiogenesis were also found to carry specific markers. One such marker is the NG2 proteoglycan, also known as melanoma-associated chondroitin sulphate proteoglycan [69]. NG2 decapeptides have shown to bind both to endothelial cells and to pericytes involved in tumor angiogenesis. Although most of the homing peptides exhibit high specificity to tumor vasculature, phage-displayed peptides isolated for vasculature homing often have the ability to bind to tumor cells as well. This can be the result of the frequent technique where tumor-

bearing animals are used to generate specific homing peptides. Other methods to improve the selectivity of the homing peptides solely to vasculature have been previously described. One interesting example is the isolation of a homing peptide named APRPG that specifically accumulated in angiogenic site by the use of angiogenesis model mice prepared by the dorsal air sac method instead of tumor-bearing mice [70]. The advantage of this method is that the selected phages have the ability to bind only to angiogenic vessels and not to tumor cells. To date, the majority of the vasculature homing peptides were isolated and evaluated for their specificity and binding affinity in mice models. Arap, Pasqualini and their colleagues reported on isolation and synthesis of a prostate homing peptide named SMSIARL that binds specifically to the endothelium of human prostate blood vessels the same way it binds to the mouse prostate vessels [71].

Beside homing peptides, other substances can potentially be utilized as vasculature-targeting moieties. Homing ligands consisting of antibodies or antibody fragments such as recombinant single-chain variable fragments (ScFv) have been immensely investigated and numerous antibodies were generated against vascular targets [72]. However, the vast majority of these antibodies were not used as targeting moieties integrated in a polymeric drug delivery system but as carriers directly conjugated to an active molecule (such as drug, cytokine, toxin, radionuclide or photosensitizer). Several studies have demonstrated that a vascular targeted polymeric system integrated with active homing ligands such as VCAM-1 [73,74], ICAM-1 [75,76] and E-selectin [77] mAbs exhibit avid and selective adhesion to microvasculature in inflammation models. Used as vascular homing moieties, peptide motifs, antibodies and others offer a unique opportunity for selective delivery of high concentrations of anti-angiogenic therapeutic agents to the tumor site. Vascular targeted polymeric systems have been proven to be highly efficacious in various preclinical models of cancer and hold great potential for anti-angiogenic and cancer therapy.

3. N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer

N-(2-Hydroxypropyl)methacrylamide (HPMA) copolymers are hydrophilic, and water-soluble polymers that have been used intensively for therapeutic applications for several decades [37,47,78,79]. Macromolecular therapeutics based on HPMA copolymers are biocompatible, preferentially accumulate in tumors, and possess a higher anticancer efficacy than low-molecular-weight drugs. HPMA copolymer-based macromolecular therapeutics have been shown to be effective against numerous cancer models and are currently being evaluated in clinical trials [3]. HPMA copolymers are uncharged, and have low affinity for cell membranes although many functional groups can be incorporated into the polymer backbone such as charged groups [80,81], targeting moieties and drugs [82]. HPMA copolymers are non-immunogenic [83] and conjugation of antibodies or antibody fragments to HPMA copolymer reduces their immunogenicity [84]. Different anticancer and anti-angiogenic drugs such as doxorubicin [85], paclitaxel [86], cisplatin [87], TNP-470 [56,88] and others have been conjugated to HPMA copolymer. HPMA copolymer–drug conjugates are internalized slowly into most cells by fluid-phase pinocytosis [89,90], but in some cell types, conjugate-bearing hydrophobic drugs interact with the plasma membrane leading to non-specific adsorptive uptake. Once the complexes enter the cells, the linker between the drug and the polymer is cleaved and the drug is liberated slowly. Various cleavable and non-cleavable linkers have been designed for HPMA copolymer drug delivery systems. These include a tetrapeptidyl Gly-Phe-Leu-Gly linker, cleaved by lysosomal thiol-dependent proteases, particularly cathepsin B [91]; a non-degradable linker Gly-Gly; hydrolyzable ester linker [92]; and a Gly-Gly-Pro-Nle linker cleaved by cathepsin K, a protease involved in bone resorption, and overexpressed in bone metastases [93]. The first drug-polymer conjugate to enter clinical trials is the HPMA copolymer–doxorubicin conjugate (PK1) bearing a Gly-Phe-Leu-Gly linker cleaved by lysosomal enzymes of tumor cells [94]. A phase I study revealed a

fivefold higher maximum tolerated dose (MTD) of conjugated doxorubicin relative to standard dose of free doxorubicin with no acute cardiotoxicity. PK2 is an advanced version of PK1 which contains an active targeting moiety galactosamine designed to be taken up by the asialoglycoprotein receptor of liver tumor cells. In clinical trials, PK2 was found to be markedly more active than individual conjugates carrying a single non-targeted drug [95].

The molecular weight of HPMA copolymers can be controlled. HPMA copolymers with molecular weights below the renal threshold (~45 kDa) are rapidly cleared from the blood and are eliminated by glomerular filtration [96]. Increased circulation time can be achieved by synthesizing branched, soluble polymers with high molecular weights [97]. Subsequent renal elimination of the polymers can then be achieved, provided the crosslinks are degradable. The molecular weight and other key elements that dictate the efficacy of HPMA copolymer–drug conjugates such as drug loading percentage and polydispersity can be controlled by using the reversible addition–fragmentation chain transfer (RAFT) polymerization technique [98].

3.1. HPMA copolymer–TNP-470 conjugate (Caplostatin)

TNP-470 a low-molecular-weight analog of fumagillin, was first shown to be anti-angiogenic in 1990 by Ingber et al. [99]. In clinical trials TNP-470 treatment showed promising antitumor activity when used alone or in combination with conventional chemotherapy [100,101]. However, the efficacy of this drug was significantly limited by neurotoxicity that occurred at the optimal anticancer dose [102,103]. Satchi-Fainaro et al. synthesized and characterized a 30 kDa water-soluble HPMA copolymer–Gly-Phe-Leu-Gly–TNP-470 conjugate [88], named caplostatin (Fig. 3). The tetrapeptide linker (Gly-Phe-Leu-Gly)

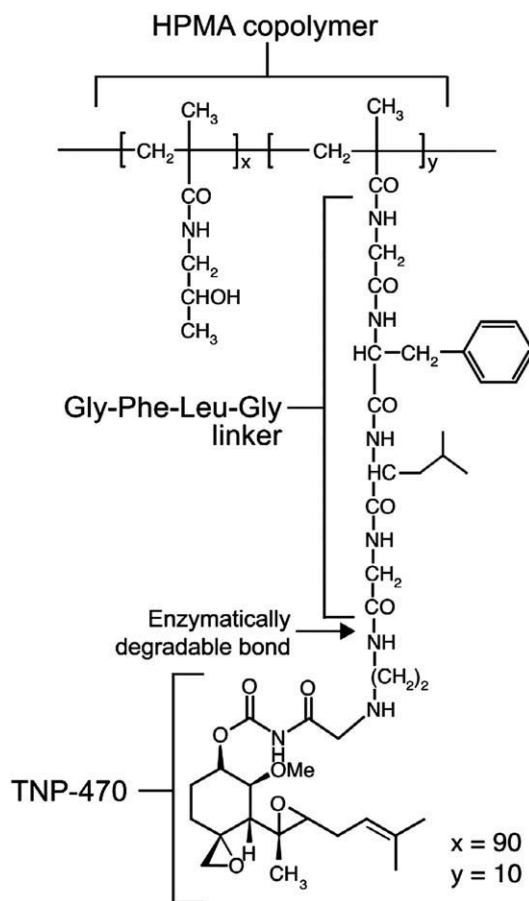


Fig. 3. Chemical structure of HPMA copolymer–TNP-470 conjugate (caplostatin).

that facilitated the conjugation with HPMA copolymer is stable in the circulation [104], and cleavable by the lysosomal thiol-dependent proteases, particularly cathepsin B which is overexpressed in many tumor cells and tumor endothelial cells [79]. Caplostatin is selectively accumulated in the tumor microvasculature due to the passive targeting phenomenon, first described by Matsumura and Maeda, the EPR effect [8,105]. In addition, this conjugate did not cross the blood–brain barrier and did not induce neurotoxicity as did the unconjugated TNP-470. Caplostatin has a broad antitumor spectrum and can be administered over a dose range more than tenfold that of the original TNP-470 without any toxicity. Caplostatin significantly inhibited the tumor growth of Lewis lung carcinoma, U87 human glioblastoma, A2058 human melanoma, PC3 human prostate carcinoma, COLO-205 human colon carcinoma and Mycen-driven murine neuroblastoma in transgenic mice [56,88,106]. In addition to its anti-angiogenic activity, caplostatin is the most potent known inhibitor of vascular permeability [107]. Caplostatin prevented vascular leakage induced by VEGF, bradykinin, histamine, and platelet-activating factor, and prevented pulmonary edema induced by interleukin-2. The mechanism of inhibiting vascular hyperpermeability is partly explained by TNP-470's inhibition of VEGF-induced phosphorylation of the receptor for VEGF (VEGFR-2), calcium influx, and RhoA activation in endothelial cells. Caplostatin represents the most broad-spectrum anticancer agent known, and it is not restricted for targeting a specific endothelial integrin (e.g., $\alpha v\beta 3$ or $\alpha 5\beta 1$) by using RGD motifs, or for targeting bone tumors and metastases.

3.2. HPMA copolymer–alendronate–TNP-470 conjugate

Recently, a second generation of caplostatin was synthesized using an advanced “living polymerization” technique, the reversible addition–fragmentation chain transfer (RAFT). Segal et al. conjugated the aminobisphosphonate alendronate (ALN), and the potent anti-

angiogenic agent TNP-470 with HPMA copolymer through a Gly-Gly-Pro-Nle linker, cleaved by cathepsin K, a cysteine protease overexpressed at resorption sites in bone tissues [108]. In this approach, dual targeting is achieved by passive accumulation due to the EPR effect, thus extravasating from the tumor leaky vessels and not from normal healthy vessels. Active targeting to the calcified tissues is achieved by ALN's affinity to bone mineral. The anti-angiogenic and antitumor potency of HPMA copolymer–ALN–TNP-470 conjugate was evaluated both *in vitro* and *in vivo*. Segal et al. showed that free and conjugated ALN–TNP-470 have synergistic anti-angiogenic and antitumor activity by inhibiting proliferation, migration and capillary-like tube formation of endothelial and human osteosarcoma cells *in vitro*. Evaluation of anti-angiogenic, antitumor activity and body distribution of HPMA copolymer–ALN–TNP-470 conjugate was performed on severe combined immunodeficiency (SCID) male mice inoculated with mCherry-labeled MG-63-Ras human osteosarcoma and by modified Miles permeability assay. The targeted bi-specific conjugate reduced VEGF-induced vascular hyperpermeability by 92% and remarkably inhibited osteosarcoma growth in mice by 96%. This was the first report to describe a new concept of a narrowly-dispersed combined polymer therapeutic designed to target both tumor epithelial and endothelial compartments of bone metastases and calcified neoplasms at a single administration. This new approach of co-delivery of two synergistic drugs may have clinical utility as a potential therapy for angiogenesis-dependent cancers such as osteosarcoma and bone metastases [108] (Fig. 4).

3.3. HPMA copolymer–alendronate–paclitaxel conjugate

Miller et al. developed a new strategy of targeted therapy for the treatment of prostate and breast cancer bone metastases [109]. The strategy rests upon the conjugation of a bone targeting moiety, the aminobisphosphonate alendronate (ALN), and the chemotherapeutic

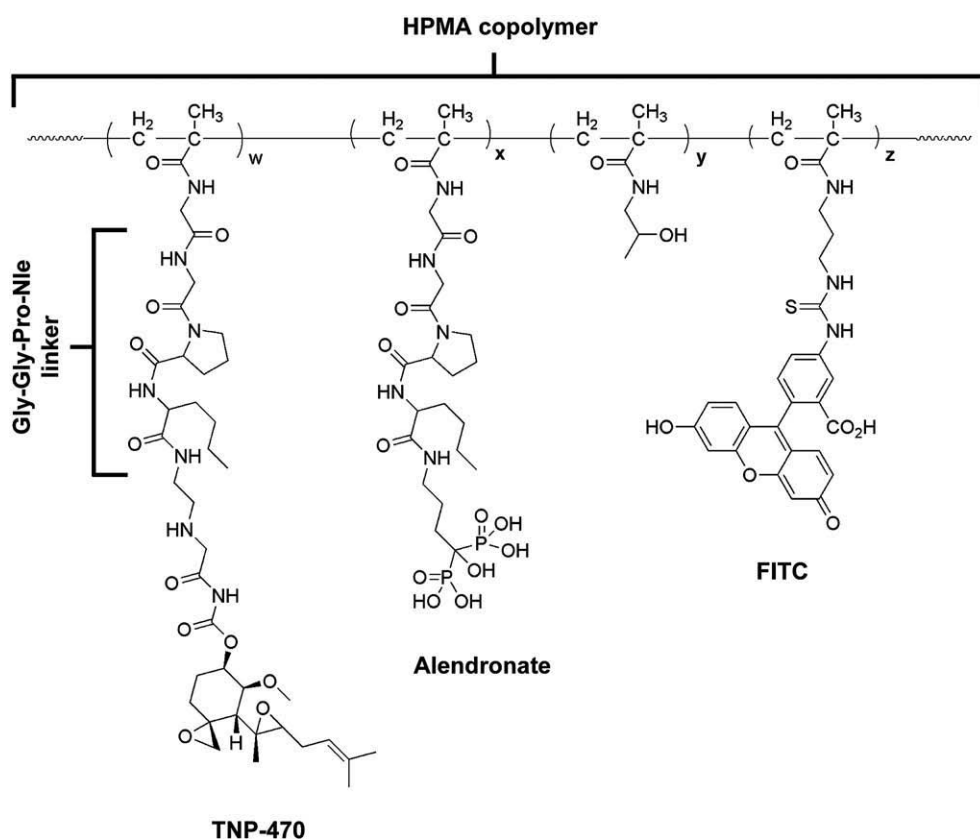


Fig. 4. Chemical structure of second generation targeted caplostatin (HPMA copolymer–ALN–TNP-470).

agent paclitaxel (PTX) to HPMA copolymer (Fig. 5). PTX is commonly used for the treatment of metastatic prostate cancer, however, it is neurotoxic and causes hematological toxicity. Recently, it has been found that ultra low-dose PTX is anti-angiogenic. Taking advantage of the multivalency of polymers, Miller et al. conjugated both drugs with the same polymeric backbone resulting with a nanoconjugate at a size of ~100 nm. PTX was conjugated to HPMA copolymer through the dipeptide phenylalanine-lysine-*p*-aminobenzyl carbonate linker (PTX-FK) (Fig. 5) [109]. This linker was cleaved by the lysosomal enzyme cathepsin B overexpressed in tumor epithelial and endothelial cells and free PTX and ALN were released. HPMA copolymer-PTX-FK-ALN nanoconjugate inhibited the proliferation of prostate carcinoma cells. Furthermore, the conjugate demonstrated anti-angiogenic effect on different steps of the angiogenic cascade such as proliferation, migration and tube formation of endothelial cells. These results warrant its use as a novel bone targeted anti-angiogenic therapy for prostate cancer bone metastases.

3.4. HPMA copolymer-quinic acid conjugates for targeting E-selectin expressing cells

The site-specific expression of selectins (E- and P-selectin) on endothelial cells of blood vessels during inflammatory responses and angiogenesis provides an opportunity to target drugs to the vascular endothelium of diseased tissues. The selectins are known to bind

weakly to the sialylated and fucosylated tetrasaccharide, sialyl Lewis x (sLex) (Kd in the millimolar range). However, the carbohydrate-based molecules rely upon often complex synthesis, which limits their use as targeting ligands. Shamay et al. described an innovative strategy for the selective delivery of HPMA copolymer-conjugates to E- and P-selectin expressing cells with non-carbohydrate analogs of sLex, based on quinic acid (Qa) as targeting ligands (Fig. 6A, B) [110]. These ligands could potentially improve binding affinity through specific, multivalent interactions with selectins. They demonstrated that Qa-based analogs of sLex (Qa-ligands) were able to antagonize adhesion of HL-60 cells to E-selectin. The apparent avidity of the polymer conjugates carrying multiple copies of the Qa-ligands has increased in three orders of magnitude when presented in a multivalent display. The major mechanism of polymer entry into E-selectin expressing cells was endocytosis. The selectin-targetable polymer conjugates provide a foundation that should support targeted delivery of chemotherapeutics and imaging agents to tumor vasculature for therapeutic and diagnostic applications.

3.5. HPMA copolymer-RGD4C and HPMA copolymer-RGDfK

The $\alpha v\beta 3$ integrin plays an important role in tumor-induced angiogenesis and tumor metastasis [111]. Peptide-targeting moieties for tumor-vasculature drug delivery have immense therapeutic potential and have been widely investigated [112]. The most well

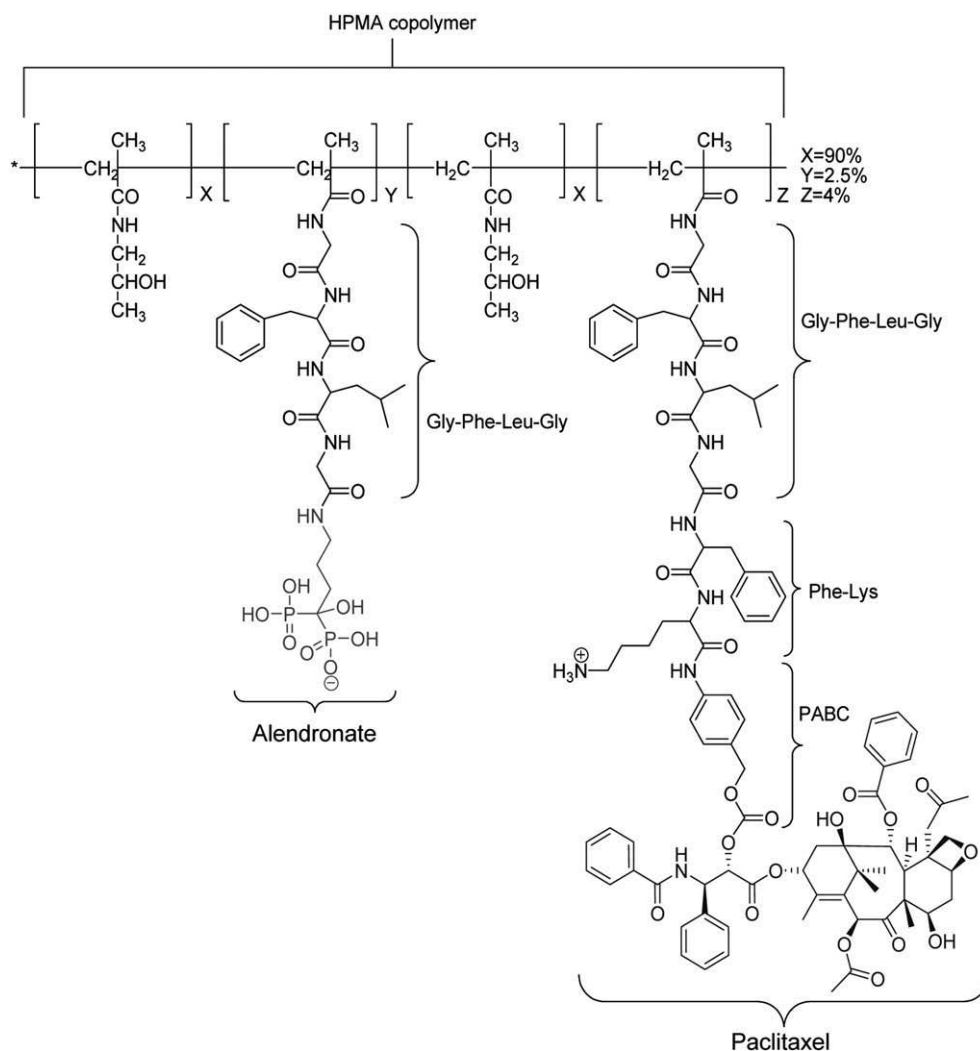


Fig. 5. Chemical structure of HPMA copolymer-ALN-PTX.

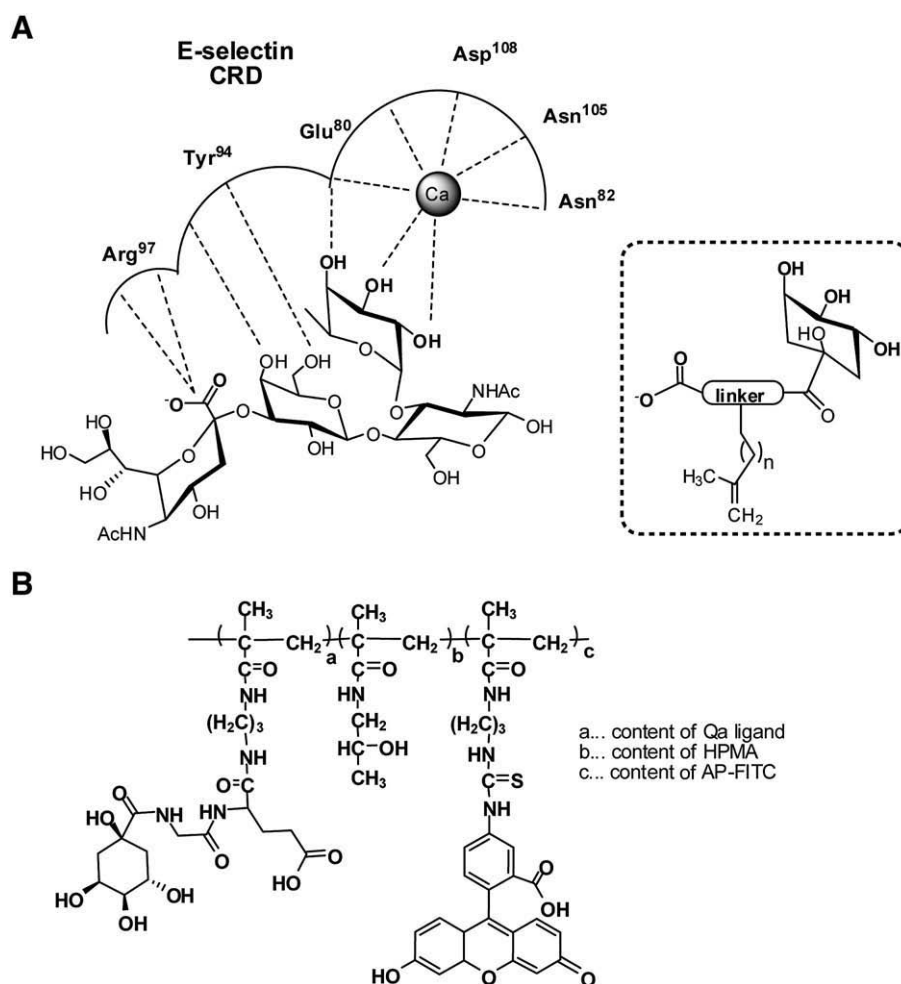


Fig. 6. (A) Schematic description of sLex binding to E-selectin CRD (left), emphasizing the primary inter-molecular interactions. Insert: general structure of the Qa-ligands that are assayed in this study (linker = variable peptide sequence), which show structural analogy to sLex. (B) FITC-labeled HPMA copolymer carrying multiple copies of a Qa ligand.

characterized is the RGD peptide recognizing integrin $\alpha v \beta 3$ which has been effectively used as targeting moiety to deliver drugs to the tumor endothelial compartment [113,114]. Wan et al. designed and characterized an HPMA copolymer–doxorubicin conjugate containing RGD-terminating side-chains, to target tumor endothelial cells [115]. Enhanced uptake by ECV304 cells was demonstrated *in vitro* [115].

Pasqualini and Ruoslahti identified a novel doubly cyclized peptide RGD4C that binds to $\alpha v \beta 3$ with 20–40-folds more avidly than the RGD linear peptides [116]. Mitra et al. reported the synthesis, characterization, *in vivo* imaging and biodistribution of a technetium-99m labeled, water-soluble, HPMA copolymer carrying doubly cyclized Arg-Gly-Asp motifs (HPMA copolymer–RGD4C conjugate) [117]. HPMA copolymer–RGD4C conjugate inhibited $\alpha v \beta 3$ -mediated endothelial cell adhesion and showed high tumor localization. In addition, HPMA copolymer–RGD4C had sustained tumor retention over 72 h and reasonably efficient clearance from the background organs. Comparison study of HPMA–RGD4C with HPMA–RGDfK conjugate showed that HPMA–RGD4C had a significantly higher affinity for $\alpha v \beta 3$ than the monocyclic peptide (RGDfK) [118]. However, on conjugation to the HPMA copolymers both peptide conjugates showed similar activities. This was partially explained because of the multiple numbers of peptides in the conjugates, as polyvalent interactions can be collectively much stronger than corresponding monovalent interactions. Borgman et al. synthesized and characterized HPMA copolymer–RGDfK conjugates for targeted radiotherapy with varying molecular weight and charge content in an attempt to identify a structure that maximizes tumor accumulation while rapidly

clearing other organs [119] (Fig. 7). *In vivo*, HPMA copolymers bearing increased amounts of the CHX-A'-DTPA used as a stable chelating agent for radioactive isotopes resulted in preferential kidney accumulation, causing rapid blood pool clearance and an absence of significant tumor accumulation. The mechanism of kidney accumulation of HPMA copolymer–(RGDfK)–(CHX-A'-DTPA) conjugates requires further investigation. Nevertheless, these studies demonstrate that targeting the $\alpha v \beta 3$ integrin using HPMA copolymer–RGD conjugates could be a promising strategy for selective delivery of radiotherapeutics and anti-angiogenic inhibitors to tumor vasculature and to other tumor sites expressing $\alpha v \beta 3$ integrin.

4. Polyglutamic acid (PGA)–E-[c(RGDfK)₂]-Paclitaxel conjugate

Recently, Eldar et al. designed a PGA–PTX–E-[c(RGDfK)₂] with the hope that a combination of a PGA conjugate containing RGD peptidomimetic motifs with an anti-angiogenic agent might enhance the effects seen for PGA–paclitaxel alone [120]. In situations where a tumor is well vascularized, but vasculature permeability is poor, this strategy might be essential since the tumor endothelial cells are directly exposed to the conjugate in the blood circulation without the need to extravasate from the tumor vasculature into the tumor tissue.

PTX is a potent cytotoxic insoluble drug; however, it is hydrophobic and causes side effects such as neutropenia, neuropathies, and when solubilized in Cremophor EL causes hypersensitivity reactions. PGA–PTX conjugate is currently undergoing phase three clinical trials showing promising results. PGA is a water-soluble, biocompatible,

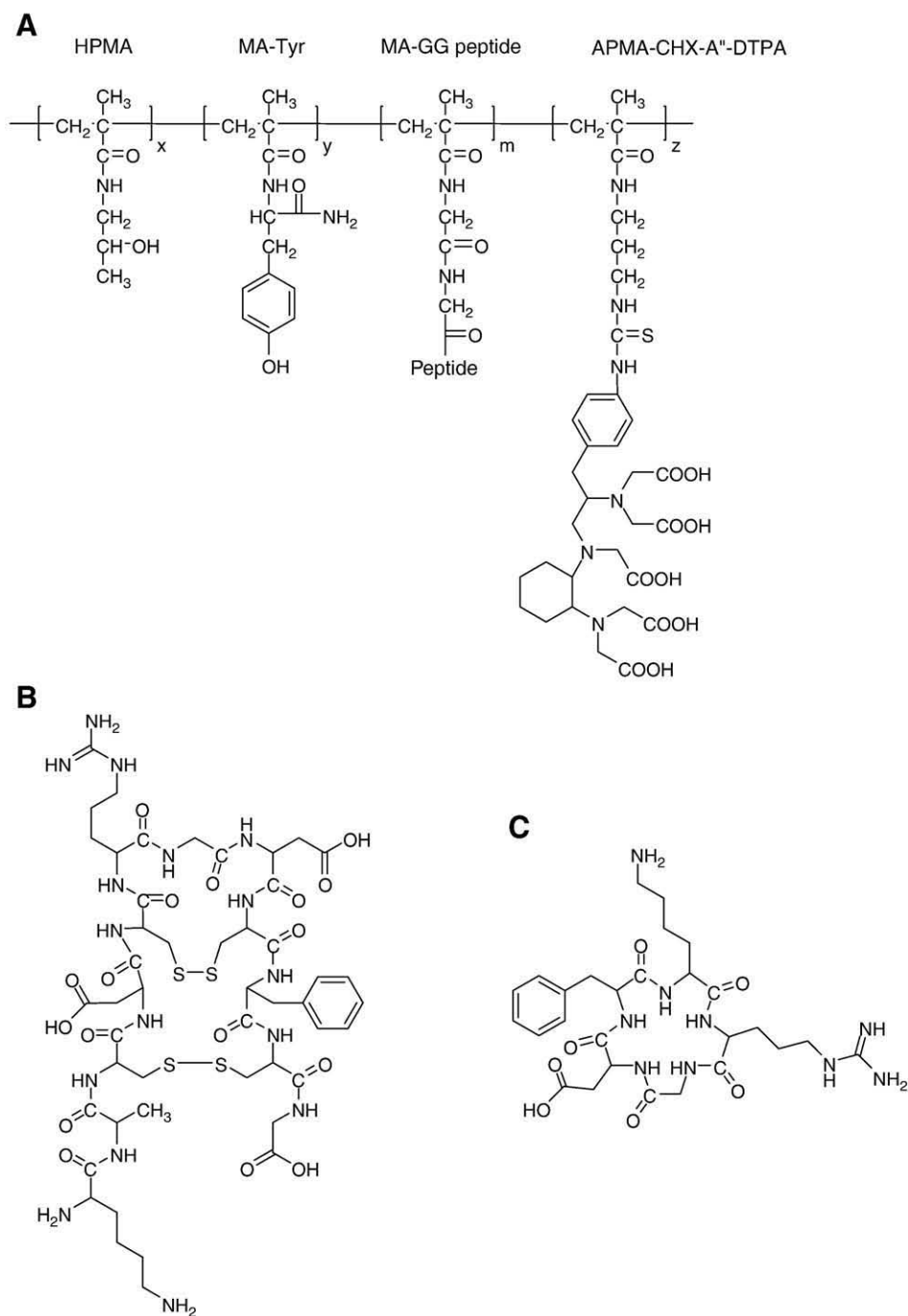


Fig. 7. Chemical structure of a general (A) HPMA copolymer-peptide, where the peptide is (B) RGD4C and (C) c(RGDfk).

non-toxic and biodegradable polymer that accumulates in the tumor bed by the EPR effect when it is used at a nano-scaled size of 10–150 nm. Eldar et al. conjugated PGA with PTX and a targeting moiety, the cyclic RGD peptidomimetic, E-[c(RGDfk)₂], which actively targets the conjugate to proliferating tumor endothelial cells over-expressing $\alpha v\beta 3$ integrin [120]. The resulting PGA-[c(RGDfk)₂]-PTX nanoconjugate was measured at a diameter size of ~30 nm (Fig. 8A). The ester linker between the polymer and the drug is hydrolytically labile and PTX release occurs under lysosomal acidic pH while the PGA itself is degradable by lysosomal enzymes such as cysteine proteases, particularly cathepsin B. PGA-E-[c(RGDfk)₂]-PTX nanoconjugate inhibited the proliferation of endothelial cells, their migration towards VEGF and their formation as capillary-like tubular structures. The adhesion of endothelial cells to fibrinogen-coated wells was inhibited

by PGA-E-[c(RGDfk)₂]-PTX, but not affected by PGA-[c(RADfk)]-PTX control conjugate (Fig. 8B). These results warrant this conjugate as a novel targeted anti-angiogenic anticancer therapy.

5. β -poly(L-malic acid)-PMLA

β -poly(L-malic acid)-PMLA is a non-toxic, non-immunogenic, biogenic polymer purified from the myxomycete *Physarum polycephalum*. PMLA resembles HPMA in carrying abundant carboxyl groups (Fig. 9), but as a polyester of L-malic acid, it is completely biodegraded to carbon dioxide and water. Due to this biodegradability, it is highly suited as a scaffold for tailored nanoconjugate chemistry [121].

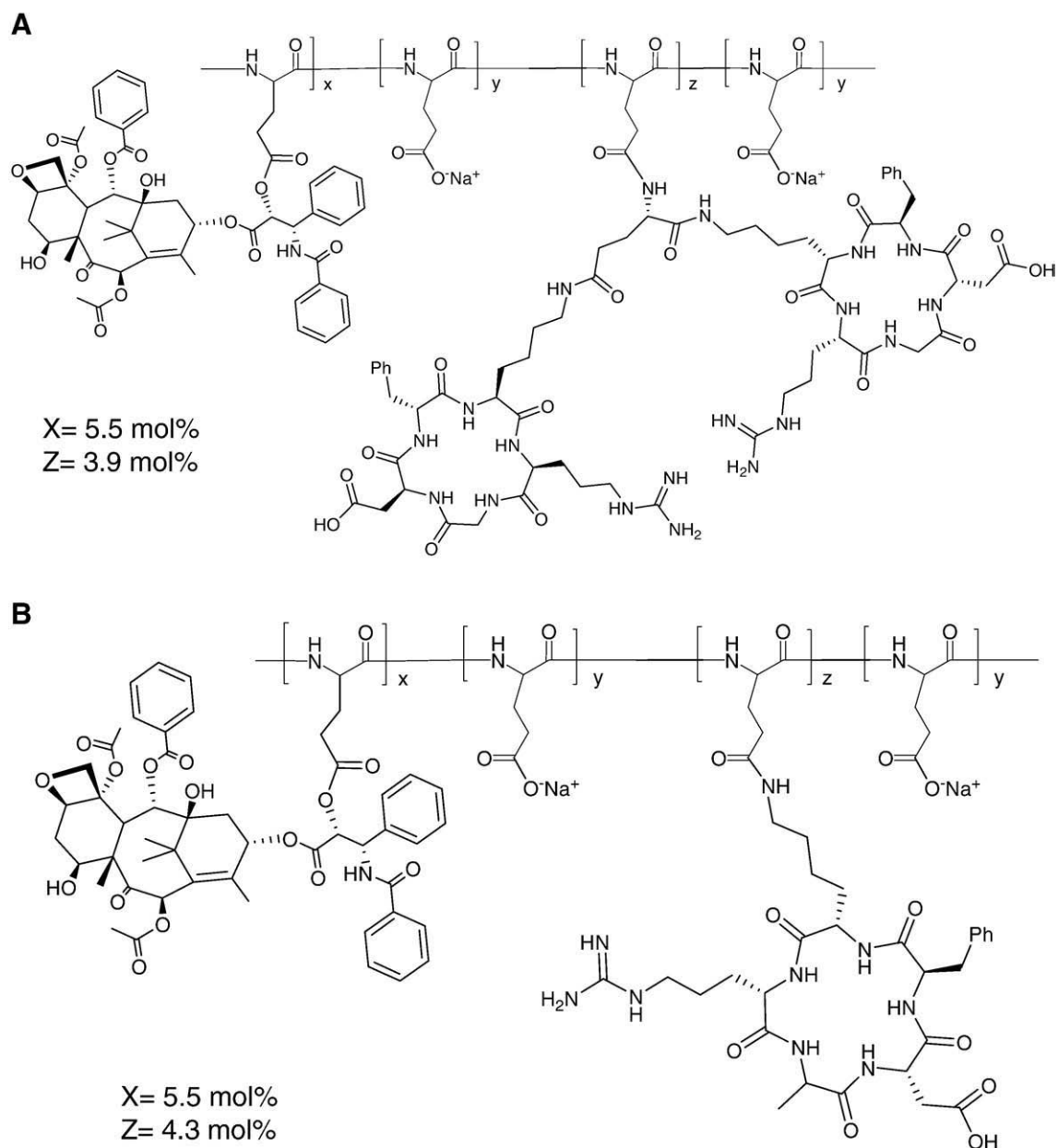
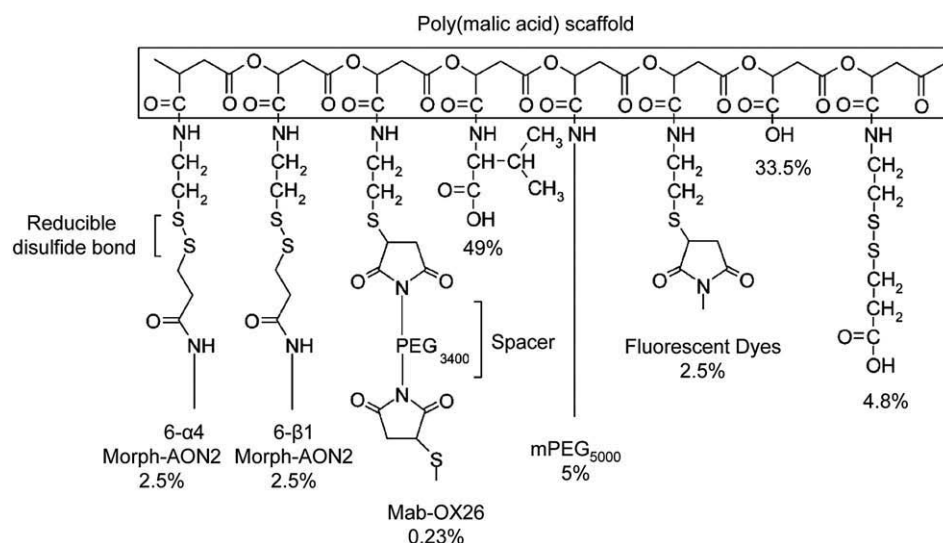


Fig. 8. Chemical structure of (A) PGA-PTX-E-[c(RGDfk)₂] conjugate and (B) PGA-PTX-c(RADfk) conjugate.

5.1. Polycefin

The basement membrane in the endothelium is likely to contribute to interactions with mural cells and thereby vessel stability, and to the transduction of signals from the lumen of the vessel to the vessel wall [122]. Laminin-8 ($\alpha 4$, $\beta 1$, $\gamma 1$), a vascular basement membrane component, plays an important role in angiogenesis and cell migration and its overexpression in glial tumors, breast cancer and their metastasis suggested that its inhibition could reduce tumor neovascularization [123,124]. Recently, several laminin peptides have been shown to have inhibitory effects on endothelial tube formation in *in vitro* assays [125]. Bong-Seop et al. synthesized a targeted polymeric delivery system based on β -poly(L-malic acid) named Polycefin to target brain tumors [126] (Fig. 9). Polycefin bioconjugate construct of: (i) β -poly(L-malic acid) as the macromolecule carrier, (ii) antisense oligonucleotides targeting Laminin-8 ($\alpha 4$, $\beta 1$), (iii) monoclonal anti-transferrin receptor antibody, (iv) oligonucleotide releasing disulfide units, (v)

L-valine containing, pH-sensitive membrane disrupting unit(s), (vi) protective poly(ethylene glycol) and (vii) a fluorescent detection molecule. Polycefin was found to accumulate in U87MG brain tumor tissue most likely via the antibody-targeted transferrin receptor-mediated endosomal pathway in addition to the EPR effect and inhibited the synthesis of Laminin-8 ($\alpha 4$, $\beta 1$). In addition, Polycefin had no toxic effect on normal and tumor astrocytes in a wide range of concentrations. Manabu et al. reported that Polycefin significantly reduced tumor microvessel density in U87MG human glioblastoma-bearing nude rats causing a reduction in tumor angiogenesis and increased animal survival [127]. Imaging experiments showed significant and specific tumor accumulation of Polycefin in mice bearing U87MG human glioblastoma and MDA-MB 468 human breast carcinoma [128]. This prototype of drug delivery system could potentially be used for specific targeting of several biomarkers simultaneously to reduce tumor neovascularization and treat human gliomas.

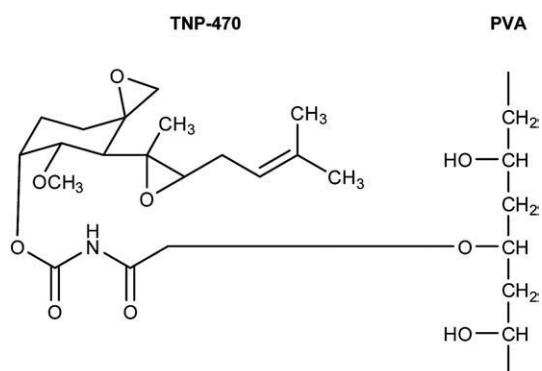


6. Poly(vinyl alcohol)-PVA

Poly(vinyl alcohol) (PVA) is a water-soluble synthetic biodegradable polymer with limited solubility in water and optimal at 87–89% acetate hydrolysis [129]. At more advanced hydrolysis, PVA presents a high tendency to form hydrogen association and easily forming gels. PVA is a polymer of large interest for various pharmaceutical and biomedical applications [130]. Microspheres based on PVA were approved by the Food and Drug Administration (FDA) and other regulatory organizations for embolization. Depending on the type of additives they contain, PVA can be considered as biocompatible and suitable for several biomedical applications [131].

6.1. TNP-470–PVA conjugate

Yasukawa et al. synthesized and evaluated a TNP-470–PVA conjugate for the treatment of choroidal neovascularization (CNV) [132] (Fig. 10). TNP-470 was conjugated to PVA by a dimethylaminopyridine-catalyzed reaction and found to have similar inhibitory effect on human umbilical vascular endothelial cells (HUVEC) growth as free TNP-470 *in vitro*. On the other hand, bovine retinal pigment epithelial cells (BRPECs) were less sensitive to TNP-470–PVA than HUVEC. These findings suggest that TNP-470–PVA preserves the original bioactivity of TNP-470 and that, if this relationship between the two types of cells corresponds to that between choroidal endothelial cells and RPE cells, this conjugate may inhibit the growth of endothelial cells and produce less interference in the proliferation of BRPECs cells. TNP-470–PVA significantly inhibited the progression of CNV induced by subretinal



injection of gelatin microspheres containing β FGF in rabbits. Histologic studies at 4 weeks after treatment demonstrated that the degree of vascular formation and the number of vascular endothelial cells in the subretinal membrane of the eyes treated with TNP-470–PVA were less than those of the control eyes. It was concluded that TNP-470–PVA may enable TNP-470 to be used safely because of prolonged circulation time, passive targeting, and slow release of free TNP-470 and may have potential as a treatment modality for CNV.

7. Poly(ethyleneglycol) (PEG)

Poly(ethyleneglycol) (PEG) is a non-biodegradable polymer synthesized by polymerization of ethylene oxide using methanol or water as initiator to yield methoxy-PEG or diol-PEG, respectively [133]. PEGylation defines the modification of a protein, peptide or non-peptide molecule by the covalent linking of one or more PEG chains. This technique was first recognized for its potential by Davis, Abuchowski et al. in the late 1970s [134,135]. PEGylation is a well-established technology approved by the FDA and used to improve the stability, solubility, bioavailability, and immunological properties of bioactive compounds [136]. PEG is an attractive polymer for conjugation with unique qualities such as high solubility in water and in various organic solvents, lack of toxicity and immunogenicity and flexibility of its chain. PEGylated proteins often lose their biological activity. A typical example is the PEGylated α -interferon Pegasys®, which retains only 7% of the antiviral activity of the native protein, but still shows a greatly improved performance *in vivo* compared with the unmodified enzyme because of improved pharmacokinetics [137]. To overcome this problem improved conjugation techniques have been developed, including site-specific modification following protein mutagenesis [138], the use of the enzyme transglutaminase to PEGylate selectively at glutamine in the protein, and the design of degradable PEG–protein linkages to maximize the return of protein bioactivity [139]. The extended polymer chain provides a hydrodynamic radius that is approximately 5–10 times greater than that of the bioactive compounds of equivalent molecular weight thus preventing rapid renal clearance and prolonging plasma half-life of these compounds. Another advantage of this polymer, important for pharmaceutical applications, is its narrow polydispersity, with Mw/Mn spanning from 1.01 for PEG < 5000 Da and up to 1.1 for PEG as high as 50 kDa [140]. PEG comprises only one or two hydroxyl terminal groups that can be activated, hence have relatively low loading capacity [141]. This causes difficulties particularly when PEGylating non-protein compounds such as low Mw drugs often used in cancer therapy. This issue can be addressed by using branching molecules, such

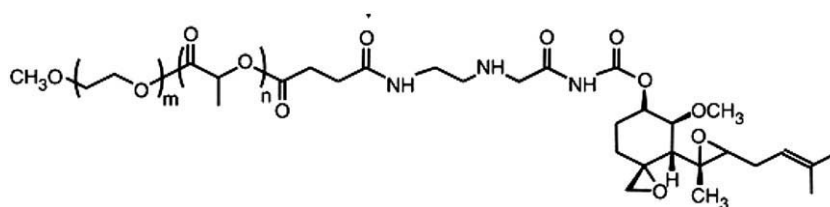


Fig. 11. Chemical structure of mPEG-PLA-TNP-470 (Lodamin).

as bicarboxylic amino acid, to produce forked PEGs with increased loading [142]. Therefore, a small drug PEGylation is mostly used to generate macromolecular prodrugs allowing passive targeting to solid tumors by the EPR effect and slow body clearance [143]. At target site, the drug has to be released to utilize its activity. In recent years, special linkers or bonds between the polymer and the drug have been developed to release the drugs from the conjugate under specific conditions. For example, *N*-cisaconityl acid spacer and hydrazone linker cleaved by acidic pH of endosome or Gly-Phe-Leu-Gly cathepsin B enzyme cleavable linker [144,145]. The PEGylation technique and its applications are becoming more advanced and sophisticated. Among the available polymers for conjugation, PEG is still the most popular option with an established clinical value.

7.1. mPEG-PLA-TNP-470 conjugate (Lodamin)

Benny et al. conjugated the angiogenesis inhibitor TNP-470 to a diblock copolymer, monomethoxy-polyethyleneglycol-poly(lactide) acid (mPEG-PLA) to form nanopolymeric micelles named Lodamin [146] (Fig. 11). This small molecule formulation was designed to be administered orally as an anti-angiogenic therapy. The amphiphilic nature of this polymeric drug enables self-assembly of micelles [147], locating TNP-470 in the core, where it could be protected from the acidic environment of the stomach, thus allowing oral bioavailability. Biodistribution studies showed that Lodamin, administered orally, is effectively absorbed in the intestine and accumulates in tumor tissue. However, a relatively high concentration of Lodamin was also observed in the liver. This was explained by the fact that oral administration directly delivers the drug from the intestine to the liver. The biodegradable PLA polymer hydrolyzes in an aqueous environment and thus allows the slow release of TNP-470 from Lodamin. *In vitro* studies showed a continuous release of TNP-470 from Lodamin over a period of 1 month, with the majority of the drug releasing within the first 4 days (in both gastric and plasma pH conditions). The slow release of TNP-470 in an acidic environment was partially explained by the masking effect of the PEG shell, which delays water penetration to the PLA core and slows the diffusion-mediated release of the drug through this layer. Lodamin inhibited angiogenesis, as shown by inhibition of HUVEC proliferation, by the corneal micropocket assay and in mouse tumor models. Orally delivered Lodamin showed significant antitumor effects compared to free TNP-470 in mouse models of B16/F10 melanoma and Lewis lung carcinoma. In addition, Lodamin prevented liver metastasis of B16/F10 melanoma tumor cells without causing liver toxicity or other side effects and prolonged mouse survival. These results suggest that Lodamin may be a good

candidate for a safe maintenance drug with effective antitumor and antimetastatic properties.

7.2. PEG-APRPG peptide-modified liposomes: a different type of an anti-angiogenic nanosystem

PEG-modified liposomes have been proven to act as important drug-carrier systems with long-circulating characteristics through avoidance of trapping by the reticuloendothelial system (RES) such as liver and spleen [148,149]. The improved pharmacokinetic profile of such drug-carrier system allows increased tumor accumulation due to size related passive targeting and the EPR effect [8,150]. Maeda et al. designed an anti-angiogenic vasculature-targeting carrier for cytotoxic agents composed of adriamycin-encapsulated liposomes (lipADM), APRPG vascular targeting peptide, PEG and hydrophobic anchor, namely distearoylphosphatidylethanolamine (DSPE) [151] (Fig. 12). DSPE-PEG-APRPG was shown to specifically bind to VEGF-stimulated HUVEC *in vitro*, and to exhibit long-circulating profile and enhanced tumor accumulation *in vivo* [152]. DSPE-PEG-APRPG exhibited enhanced tumor growth inhibition compared to adriamycin-encapsulated liposomes with PEG alone in Colon 26 NL-17 carcinoma-bearing mice, even though, tumor accumulation of both liposomes was similar. Intratumoral distribution studies of fluorescence-labeled APRPG-LipADM in Colon 26 NL-17 carcinoma-bearing mice revealed that APRPG-LipADM was specifically bound to endothelial cells and induced apoptosis in them [153]. Furthermore, APRPG-LipADM was shown to significantly suppress the growth of ADM-resistant P388 tumors in mice. Recently, Katanasaka et al. synthesized APRPG-PEG-modified with liposomal SU1498, an inhibitor of vascular VEGF receptor tyrosine kinase [154]. APRPG-PEG-liposomal SU1498 was shown to inhibit VEGF-stimulated endothelial cell proliferation *in vitro*, significantly decrease tumor microvessel density in mice bearing Colon26 NL-17 carcinoma and prolong the survival time of the mice. An additional role of PEG is to protect hydrophilic moieties on liposomal surface and increase their stability in the serum [155]. Asai et al. used this technique to mask the hydrophilic moiety 5'-*O*-dipalmitoylphosphatidyl CNDAC (DPP-NDAC), a phospholipid derivative of the novel antitumor nucleoside CNDAC, on the liposomal surface with PEG-APRPG conjugate to improve the availability of DPP-NDAC liposomes and to target them to neovasculature [156]. APRPG-EG conjugate reduced the agglutinability of DPP-NDAC liposomes in the presence of serum and raised the blood levels of DPP-NDAC liposomes in colon 26 NL-17 tumor-bearing BALB/c male mice, resulting in enhanced accumulation of them in the tumor. Evaluation of the therapeutic potential of APRPG-

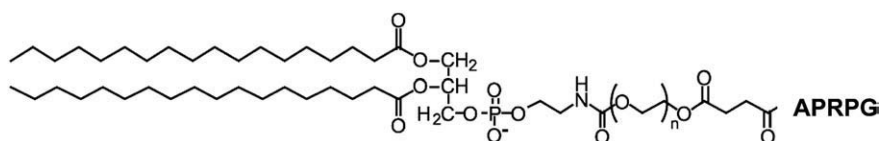


Fig. 12. Chemical structure of modified liposomes with PEG and APRPG-conjugated distearoylphosphatidylethanolamine (DSPE-PEG-APRPG).

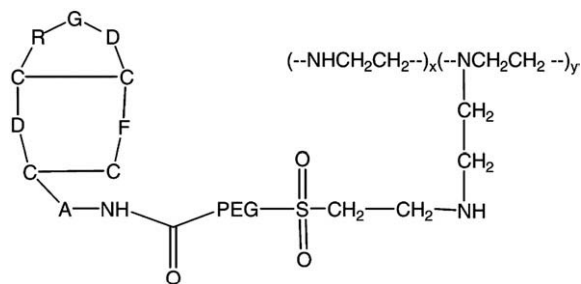


Fig. 13. Chemical structure of PEI-g-PEG-RGD/pCMV-sFlt-1.

PEG-modified DPP-NDAC liposomes revealed superior antitumor activity compared to PEG-modified DPP-NDAC liposomes. The studies mentioned above describe a promising strategy for delivery of liposomes to tumor tissues with enhanced passive targeting through the EPR effect (via PEGylation), as well as active targeting to the tumor angiogenic vasculature (via conjugation of the targeting moiety APRPG).

7.3. Polymeric gene delivery systems to target tumor neovasculature: a combined type of an anti-angiogenic nanosystem

Another interesting strategy to target tumor vasculature is by systemic delivery of an anti-angiogenic gene using PEGylated poly (ethylene imine) (PEI) polymers (Fig. 13). As an example of this approach, a polymeric gene delivery system, PEI-g-PEG-RGD/pCMV-sFlt-1 was developed by incorporating the RGD peptide into the cationic polymer, polyethylenimine (PEI) via an hydrophilic PEG spacer. This delivery system was used to deliver a gene encoding soluble Flt-1 (sFlt-1), a potent and selective inhibitor of VEGF [157]. *In vitro* evaluation found that PEI-g-PEG-RGD/pCMV-sFlt-1 conjugate was able to inhibit the proliferation of endothelial cells by blocking the binding of VEGF to the membrane bound Flt-1 receptor *in vitro*. In addition, PEI-g-PEG-RGD/pCMV-sFlt-1 conjugate exhibited relatively high tumor accumulation *in vivo* and reduced tumor growth of CT-26 colon adenocarcinoma in mice [158]. Another example is a polymerized cationic liposome that has been linked to RGD [$\alpha\beta 3$ -NP/RAF (-)] to selectively deliver a mutant *Raf-1* gene that influences the signaling cascades of two prominent angiogenic growth factors, bFGF and VEGF [159]. Systemic injection of the conjugate into mice resulted in apoptosis of the tumor-associated endothelium, leading to tumor cell apoptosis and regression of established primary and metastatic tumors. The use of multivalent targeting of integrin $\alpha\beta 3$ combined with cationic polymer conjugates to selectively deliver angiogenesis inhibitors can be used as an effective strategy not only for gene

delivery but for siRNA oligonucleotides as well. The major limitations of siRNA therapeutics are deprived blood stability, non-specific immune stimulation and poor intracellular uptake. Schiffelers et al. addressed these issues by constructing a complex of RGD-PEG-PEI (VEGFR-2 siRNA) that combines tissue-targeted selectivity with gene sequence selectivity of siRNA [160] (Fig. 14). Intravenous administration of RGD-PEG-PEI (VEGFR-2 siRNA) complex into N2a neuroblastoma tumor-bearing mice resulted in selective tumor uptake, siRNA sequence-specific inhibition of protein expression within the tumor and inhibition of both tumor angiogenesis and growth rate. Systemic delivery of genes and siRNA oligonucleotides using polymeric complexes could potentially act as an effective strategy to selectively target tumor vasculature.

8. Poly(lactic-co-glycolic acid) (PLGA)

Poly(lactide-co-glycolide) (PLGA) is a biodegradable and biocompatible polymer approved for use in humans by the FDA [161]. PLGA polymeric microspheres have been used as a sustained delivery system for proteins, drugs, and others factors, such as cytokines, hormones, enzymes, and vaccines [162]. PLGA is synthesized by random ring-opening co-polymerization of two different monomers, the cyclic dimers (1,4-dioxane-2,5-diones) of glycolic acid and lactic acid. During polymerization, the monomeric units (of glycolic or lactic acid) are linked together in PLGA by ester linkages, thus yielding linear aliphatic polyester as a product [163]. The polymeric matrix prevents the degradation of the active moiety, and allows control over the release kinetics of the moiety from PLGA. The duration and levels of the active moiety released from the PLGA can be modified by changing the drug:polymer ratio, or polymer molecular weight and composition. The particle surface and the porosity of PLGA can be designed to facilitate passive targeting via the EPR effect or active targeting via ligand binding to specific cell receptors and modification of the drug release profile (e.g., attenuation of burst release) [164]. Moreover, the rate of biodegradation may also be manipulated through polymer modification to achieve half-lives ranging from several hours to several weeks [165]. Taken together, PLGA is an extremely flexible polymer system, which can be adapted to meet the needs of many active moieties to target tumor vasculature.

8.1. PLGA microspheres as an anti-angiogenic therapy

The vast majority of anti-angiogenic polymeric systems are designed for systemic administration through utilization of the unique biodistribution and pharmacokinetics characteristics that were achieved subsequent the conjugation of the anti-angiogenic active moiety to the polymeric backbone. However, locally administered angiogenesis

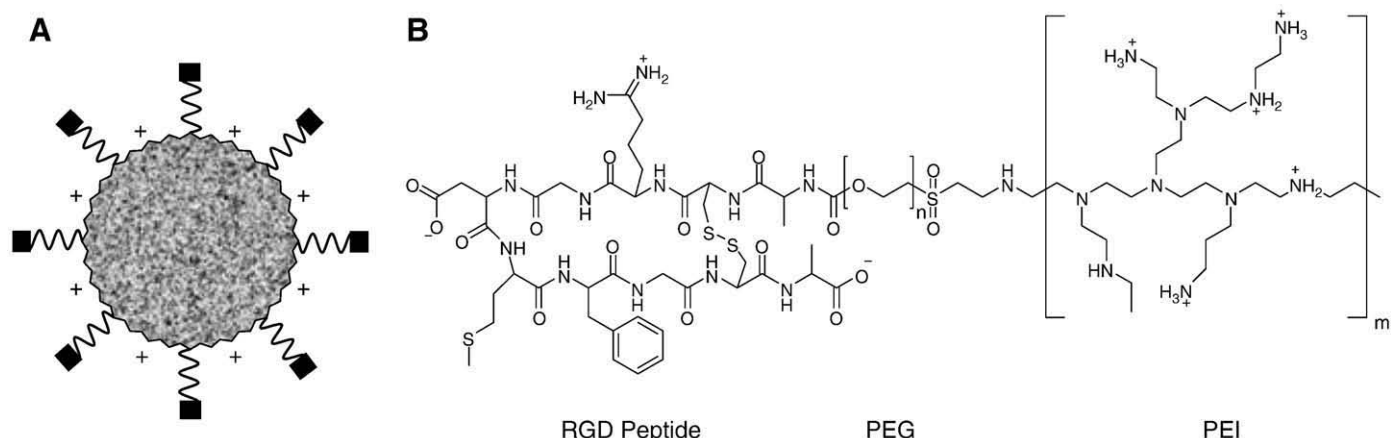


Fig. 14. Chemical structure of self-assembling siRNA nanoplexes RGD-PEG-PEI (VEGFR2 siRNA nanoplexes).

inhibitors can also benefit from the advantages of a polymeric system that will allow long-term sustained release of the active anti-angiogenic moiety. One such example for a different anti-angiogenic nanosystem is PLGA microspheres encapsulating anti-VEGF siRNA for local administration [166]. Release profile showed sustained release of siRNA from microspheres within a month. An intra-tumor injection of PLGA microspheres with encapsulated siRNA suppressed S-180 murine sarcoma tumor growth in mice. Another example for local delivery of angiogenesis inhibitors is PLGA microspheres encapsulating C-terminal fragment of platelet factor 4 (PF-4/CTF), an anti-angiogenic factor [167]. Kinetic and release studies showed that PLGA microspheres maintained their morphological integrity and continued to release biologically active PF-4/CTF for 30 days. In addition, local injection into established intracranial human U87MG glioma tumors in *nu/nu* mice led to a significant inhibition of tumors, decrease in tumor microvessels density and an increase in tumor cell apoptosis. This approach may possess therapeutic advantages for brain tumors including bypassing the blood brain barrier, sustained delivery of the active moiety and localization at tumor site. Nevertheless, this strategy is restricted to local tumors and several issues including the complexity of the administration and the long-term efficacy of this anti-angiogenic therapy require further studies.

9. Conclusions

The neovasculature of solid tumors and growing metastases is an attractive target for targeted polymer therapeutics. There is no barrier between the systemic circulation that transports the macromolecular polymer therapeutics and the target cells. Each target cell, i.e. the tumor endothelial cell, is exposed directly to the systemic supply route and hence the therapeutics. In the last decade, seminal studies in animal models have demonstrated the therapeutic potential of targeted drug delivery to the tumor neovasculature. This review presented a number of novel, potentially exciting therapeutic approaches for further investigation. The challenge for the coming decade will be to address a number of crucial issues in this area described here. Future work may play a role in shaping how we understand and clinically apply successful anti-angiogenic polymer therapeutics in years to come.

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