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Review

Macromolecular therapeutics

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ABSTRACT

This review covers water-soluble polymer–drug conjugates and macromolecules that possess biological activity without attached low molecular weight drugs. The main design principles of traditional and backbone degradable polymer–drug conjugates as well as the development of a new paradigm in nanomedicines – (low molecular weight) drug-free macromolecular therapeutics are discussed. To address the biological features of cancer, macromolecular therapeutics directed to stem/progenitor cells and the tumor microenvironment are deliberated. Finally, the future perspectives of the field are briefly debated.

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1. Introduction

Macromolecular therapeutics (polymeric nanomedicines, polymer–drug conjugates) are a group of compounds that are characterized by their large molecular weight. There is no clear definition of the term in the literature; some authors use it in the broadest sense, including any macromolecular system with biological activity. In this review, we shall restrict our discussion to water-soluble polymer–drug conjugates and macromolecules that possess biological activity without attached low molecular weight drugs. We shall discuss the main design principles, and the novel approaches that aim to speed up the translation into clinical practice. Finally, we will provide an outlook into the future of this important scientific field.

2. Water-soluble polymers with biological activity

Water-soluble polymers may possess intrinsic biological activity that relates to their structure, molecular weight, charge density, charge distribution, conformation, and stability [1]. Macromolecules such as dextran, poly(*N*-vinylpyrrolidone), and hydroxyethylstarch have been used as blood plasma expanders to restore the blood volume following trauma or shock [2]. Poly(2-vinylpyridine-*N*-oxide) has demonstrated activity against silicosis; its effect has been explained by adsorption of the weakly basic polymer on the weakly acidic surface of silica [1]. Polyelectrolytes stimulate interferon production in cells and living organisms [3,4]. Stereochemistry may have an impact on activity: isotactic poly(acrylic acid) possesses antiviral properties whereas atactic poly(acrylic acid) does not [5].

Water-soluble polymers, PEG [6–13], poly[*N*-(2-hydroxypropyl) methacrylamide] (polyHPMA) [14–18], polyoxazolines [19,20], poly(*N*-vinylpyrrolidone) [21], polyacryloylmorpholine [21], and poly(*N,N*-dimethylacrylamide) [21] have been used to modify proteins and increase their resistance to proteolysis, reduce their antigenicity, and prolong their intravascular half-life [22]. In addition, modification of liposomes, and nanoparticles with semitelechelic (ST) polymers is a widely used method to avoid recognition by the reticuloendothelial system [21,23–26]. These topics are covered in a recent review [27].

3. Water-soluble polymer–drug conjugates

3.1. Historical perspective

The conjugation of drugs to synthetic and natural macromolecules was initiated about sixty years ago — for reviews of the early work see refs. [1,28]. Jatzkewitz used a dipeptide (GL) spacer to attach a drug (mescaline) to polyvinylpyrrolidone in the early fifties [29] and Ushakov's group in Leningrad (now St. Petersburg) synthesized conjugates of poly(*N*-vinylpyrrolidone) with various antibiotics in the sixties and seventies [30–32]. Mathé et al. pioneered conjugation of drugs to immunoglobulins, setting the stage for targeted delivery [33]. DeDuve discovered (Nobel Prize 1974) that many enzymes are localized in the lysosomal compartment of the cell and the lysosomotropism of macromolecules [34], important phenomena for the design of polymer–drug conjugates. Finally, Ringsdorf analyzed the research results of the field and presented a clear concept of the use of polymers as targetable drug carriers [35].

The research on the use of HPMA copolymers as drug carriers commenced in the early 70s in the Kopeček laboratory in Prague. The choice of HPMA for development as a drug carrier was not random. Based on the detailed studies of the relationship between the structure of hydrophilic polymers and their biocompatibility [28,36–45], *N*-substituted methacrylamides were chosen as the target because the α -carbon substitution and the *N*-substituted amide bond ensured hydrolytic stability of the side-chains. We synthesized a series of compounds trying to identify a crystalline monomer for easy purification and reproducible synthesis. The first crystalline *N*-substituted methacrylamide we

succeeded in synthesizing was HPMA, and it was chosen for future development [46,47]. In April of 1974, we filed two patent applications [48,49] which covered the synthesis of *N*-substituted (meth)acrylamides containing oligopeptide sequences and their application as drug (and other biologically active compounds) carriers. The amazing development of this polymer in the scientific community is summarized in Table 1 and Fig. 1. We designed oligopeptide spacers stable in the bloodstream [50] and susceptible to enzymatically catalyzed hydrolysis in the lysosomal compartment [51], demonstrated the targetability of the HPMA copolymer system [52,53], and revealed numerous advantages of polymer–drug conjugates over free drugs as will be described below.

3.2. Design principles

3.2.1. Water-soluble polymer carriers

There are numerous reviews that describe the design of macromolecular therapeutics [1,35,54–60]; thus we shall briefly review the important design principles. The *water-soluble polymer carrier* has to be biocompatible; hence it needs to be either degradable or have a molecular weight below the renal threshold (about 50 kDa for a random coil) to permit elimination from the organism by glomerular filtration. To prevent nonspecific reuptake of the macromolecule after being released into the bloodstream following cell death, its structure should warrant that internalization occurs by fluid-phase pinocytosis. The absence of nonspecific interactions with plasma membranes will minimize the accumulation of the carrier in non-targeted cells thus increasing the biocompatibility of the carrier. In addition, its structure should provide drug attachment/release sites for the incorporation of drugs. Different structures have been used and conjugates based on dextran [61], carboxymethyldextran [62], poly(glutamic acid) [63–65], poly(malic acid) [66,67], polyacetals [68,69], poly(vinyl alcohol) [70, 71], PEG [72–74], poly(L- γ -glutamyl-glutamine) [75], and polyHPMA [76–78] have been successfully evaluated.

3.2.2. Spacers

The drug is bound to the carrier via a spacer that is stable in the bloodstream [50] and interstitial space but enzymatically or chemically cleavable in the lysosomal compartment of the cell. The lysosomal membrane is not permeable to macromolecules [79]. Consequently, the drug needs to be released from the carriers inside lysosomes. One option is to use the pH difference between blood and lysosomes and bind the drug via pH-sensitive bonds [80,81], using hydrazo [82], cis-aconityl [83], or maleic [84] spacers.

The other option is to design spacers that match the specificity of lysosomal enzymes. Based on detailed degradation studies of oligopeptide sequences attached to HPMA copolymers [85,86] with model enzymes [87–91] and lysosomal enzymes [92,93], the sequence GFLG, specific for cathepsin B, was identified [51]; it has been widely used in preclinical [94–96] and clinical settings [76,77]. Another widely used lysosomally degradable sequence is valine–citruline [97,98].

3.2.3. Self-immolative spacers

Elongated spacers, where the enzymatically cleavable bond is separated from the drug by a self-eliminating group, have been designed by several groups [99–101]. Such an approach was used for the design of oral drug delivery systems based on HPMA copolymer–9-aminocamptothecin conjugates [102] and for binding prostaglandin to HPMA copolymer via a cathepsin K sensitive terapeptide (GGPNle) and a self-eliminating 4-aminobenzylalcohol structure [103] (Fig. 2).

3.2.4. Targeting

Optionally, a *targeting moiety* is used that enhances the accumulation of the conjugate in target cells [52,53]. Active targeting of polymer–drug conjugates can be achieved by the incorporation of target cell specific ligands, such as peptides, carbohydrates, lectins,

Table 1
Milestones in HPMA (co)polymer research.

Year	Study	Publication
1973	First synthesis	J. Kopeček, H. Bažilová, Poly[N-(2-Hydroxypropyl)methacrylamide]. I. Radical polymerization and copolymerization. <i>Europ. Polym. J.</i> 9 (1973) 7–14.
1974	Characterization of PHPMA solution properties	M. Bohdanecký, H. Bažilová, J. Kopeček, Poly[N-(2-hydroxypropyl) methacrylamide]. II. Hydrodynamic properties of diluted polymer solutions. <i>Europ. Polym. J.</i> 10 (1974) 405–410.
1974	First hydrogels	J. Kopeček, H. Bažilová, Poly[N-(2-Hydroxypropyl)methacrylamide]. III. Crosslinking copolymerization. <i>Europ. Polym. J.</i> 10 (1974) 465–470.
1976	First enzymatic release of ligand from a polymer conjugate in vitro	J. Drobňák, J. Kopeček, J. Labský, P. Rejmanová, J. Exner, V. Saudek, J. Kálal, Enzymatic cleavage of side-chains of synthetic water-soluble polymers. <i>Makromol. Chem.</i> 177 (1976) 2833–2848.
1978	First HPMA modified protein	V. Chytrý, A. Vrána, J. Kopeček, Synthesis and activity of a polymer which contains insulin covalently bound on a copolymer of N-(2-hydroxypropyl)methacrylamide and N-methacryloylglycylglycine 4-nitrophenyl ester. <i>Makromol. Chem.</i> 179 (1978) 329–336.
1979	First HPMA–drug conjugate	B. Obereigner, M. Burešová, A. Vrána, J. Kopeček, Preparation of polymerizable derivatives of N-(4-aminobenzenesulfonyl)-N'-butylurea. <i>J. Polym. Sci. Polym. Symp.</i> 66 (1979) 41–52.
1981	First enzymatic release of ligand from a polymeric substrate by a polymer-modified enzyme	J. Kopeček, P. Rejmanová, V. Chytrý, Polymers containing enzymatically degradable bonds 1. Chymotrypsin catalyzed hydrolysis of p-nitroanilides of phenylalanine and tyrosine attached to side-chains of copolymers of N-(2-hydroxypropyl)methacrylamide. <i>Makromol. Chem.</i> 182 (1981) 799–809.
1981	First enzymatic release of ligand from a polymer conjugate in vivo	J. Kopeček, I. Cífková, P. Rejmanová, J. Strohal, B. Obereigner, K. Ulbrich, Polymers containing enzymatically degradable bonds. 4. Preliminary experiments in vivo. <i>Makromol. Chem.</i> 182 (1981) 2941–2949.
1982	First degradable hydrogels	K. Ulbrich, J. Strohal, J. Kopeček, Polymers containing enzymatically degradable bonds. VI. Hydrophilic gels cleavable by chymotrypsin. <i>Biomaterials</i> 3 (1982) 150–154.
1985	First HPMA–drug–antibody conjugate	B. Řihová, J. Kopeček, Biological properties of targetable poly[N-(2-hydroxypropyl)methacrylamide-antibody conjugates. <i>J. Controlled Release</i> 2 (1985) 289–310.
1994	First combination therapy using polymer-bound drugs	N.L. Krinick, Y. Sun, D. Jonyer, J.D. Spikes, R.C. Straight, J. Kopeček, A polymeric drug delivery system for the simultaneous delivery of drugs activatable by enzymes and/or light. <i>J. Biomat. Sci. Polym. Ed.</i> 5 (1994) 211–222.
1995	First semitelechelic HPMA	S. Kamei, J. Kopeček, Prolonged blood circulation in rats of nanospheres surface-modified with semitelechelic poly[N-(2-hydroxypropyl)methacrylamide]. <i>Pharmaceutical Res.</i> 12 (1995) 663–668.
1999	First clinical trials	P.A. Vasey, S.B. Kaye, R. Morrison, C. Twelves, P. Wilson, R. Duncan, A.H. Thomson, L.S. Murray, T.E. Hilditch, T. Murray, S. Burtles, D. Fraier, E. Frigerio, J. Cassidy, and on behalf of the Cancer Research Campaign Phase I/II Committee, Phase I clinical and pharmacokinetic study of PK1 [N-(2-hydroxypropyl)methacrylamide copolymer doxorubicin]: first member of a new class of chemotherapeutic agents–drug–polymer conjugates. <i>Clin. Cancer Res.</i> 5 (1999) 83–94.
1999	First ATRP polymerization	M. Teodorescu, K. Matyjaszewski, Atom transfer radical polymerization of (meth)acrylamides. <i>Macromolecules</i> 32 (1999) 4826–4831.
1999	First self-assembly into hybrid hydrogels	C. Wang, R.J. Stewart, J. Kopeček, Hybrid hydrogels assembled from synthetic polymers and coiled-coil protein domains. <i>Nature</i> 397 (1999) 417–420.
2005	First RAFT polymerization	C.W. Scales, Y.A. Vasilieva, A.J. Convertine, A.B. Lowe, C.L. McCormick, Direct, controlled synthesis of the nonimmunogenic, hydrophilic polymer, poly[N-(2-hydroxypropyl)methacrylamide] via RAFT in aqueous media. <i>Biomacromolecules</i> 6 (2005) 1846–1850.
2010	First self-assembly of peptide-containing hybrid HPMA copolymers at cell surface	K. Wu, J. Liu, R.N. Johnson, J. Yang, J. Kopeček, Drug-free macromolecular therapeutics: induction of apoptosis by coiled-coil mediated crosslinking of antigens at cell surface. <i>Angew. Chem. Int. Ed.</i> 49 (2010) 1451–1455.
2011	First backbone degradable HPMA copolymer carrier	J. Yang, K. Luo, H. Pan, P. Kopečková, J. Kopeček, Synthesis of biodegradable multiblock copolymers by click coupling of RAFT generated heterotelechelic polyHPMA conjugates. <i>Reactive Functional Polym.</i> 71 (2011) 294–302.
2012	First in vivo self-assembly of peptide-containing hybrid HPMA copolymers resulting in tumor cure	K. Wu, J. Yang, J. Liu, J. Kopeček, Coiled-coil based drug-free macromolecular therapeutics: in vivo efficacy. <i>J. Controlled Release</i> 157 (2012) 126–131.
2013	First combination therapy targeting both stem cells and differentiated cells	Y. Zhou, J. Yang, J. Rhim, J. Kopeček, HPMA copolymer-based combination therapy toxic to both prostate cancer stem/progenitor cells and differentiated cells induces durable anticancer effect. <i>J. Controlled Release</i> 172 (2013) 946–953.
2014	First in vivo self-assembly of oligonucleotide-containing hybrid HPMA copolymers resulting in tumor cure	T.-W. Chu, J. Yang, R. Zhang, M. Sima, J. Kopeček, Cell surface self-assembly of hybrid nanoconjugates via oligonucleotide hybridization induces apoptosis. <i>ACS Nano</i> 8 (2014) 719–730.

antibodies, and antibody fragments. The specific targeting interactions result in biorecognition at the cell surface and enhanced uptake of conjugates by cancer cells through receptor-mediated endocytosis with

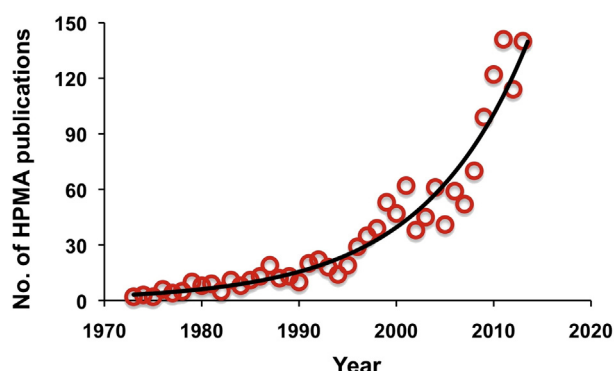


Fig. 1. HPMA publications 1973–present.

concomitant improvement in therapeutic efficacy [59,104]. Attachment of several targeting moieties to one macromolecule provides in a multivalency effect resulting in enhanced avidity of the conjugate [105]. Examples include binding several Fab' antibody fragments [106–108], several saccharide moieties [109,110] or several peptides [111] per HPMA macromolecule. The multivalency effect resulted in enhanced biological activity of the conjugates.

3.2.5. Subcellular targeting

The activity of many drugs depends on their subcellular location; consequently, manipulation of their subcellular fate may result in more effective conjugates. For example, mitochondrial targeting can be achieved by exploiting the negative mitochondrial potential and use of positively charged triphenylphosphonium ions as mitochondrial targeting agents [112]. Steroid hormone receptors (SHRs) have been employed to achieve nuclear targeting. SHSs are known to shuttle between the cytoplasm and nucleus of cells. Once a steroid ligand binds to a receptor such as the glucocorticoid receptor (GR), the ligand–receptor complex actively migrates to the nucleus. This concept was used for

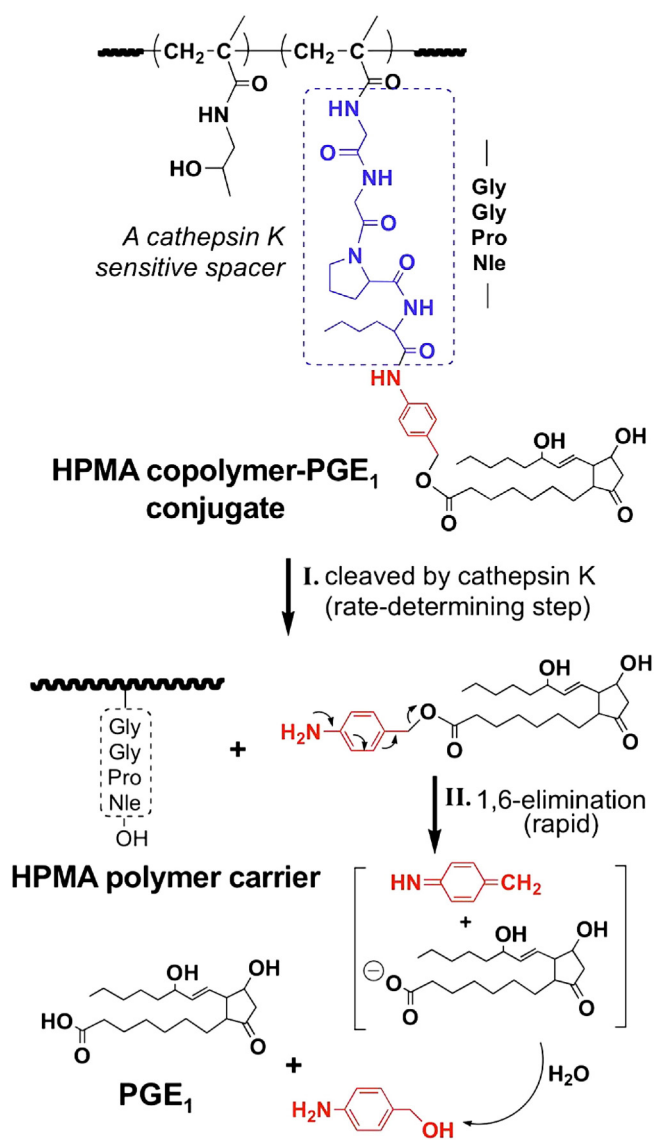


Fig. 2. Example of self-immolative spacer. Scheme of release of unmodified PGE₁ from HPMA copolymer-PGE₁ conjugate by a two-step process — rate controlling enzymatic cleavage followed by fast 1,6-elimination. Adapted from ref. [103].

nuclear transport of DNA [113] or for a cortisol modified photosensitizer bound to HPMA copolymer via a lysosomally degradable GFLG sequence [114].

Structural factors have impact on the biocompatibility and efficiency of a polymer drug carrier as well as on the cellular uptake and subcellular trafficking. General conclusions on the relationship between structure (charge, molecular weight, hydrophobic/hydrophilic balance) and internalization are known [115–120]. However, depending on the type of the cell and detailed structure of the conjugate the subcellular trafficking may vary. The understanding of the endocytic pathways is important in the design of effective conjugates and should be individually evaluated for each design and target.

3.2.6. Gene delivery

Most approaches for gene delivery using water-soluble polymer carriers focus on polyelectrolyte complexes. These will be covered elsewhere in this volume. However, several designs use covalent attachment of DNA/RNA and will be briefly mentioned:

- The dynamic polyconjugate technology for siRNA delivery uses covalently attached RNA and fits the scope of this review. The delivery system is composed of: a) a polymeric carrier that contains amine groups in the side chains. The latter provide attachment/release points for masking poly(ethylene glycol) chains, covalent attachment of siRNA via disulfide bonds (reducible in the cytosol) and optionally a targeting moiety. The PEG chains are attached via pH-sensitive maleic amide bonds [84]. Upon internalization the pH will decrease, the PEG molecule will be released from the conjugate exposing amino groups that will destabilize the endosomal membrane resulting in a reductible environment that will release siRNA into the cytoplasm (Fig. 3) [121].
- Stayton and coworkers used a diblock copolymer that contains a hydrophilic block of HPMA and *N*-(2-(pyridin-2-yl)disulfanyl) ethyl methacrylamide (poly[HPMA-co-PDSMA]) to promote aqueous solubility and permit thiol-disulfide exchange reactions. The second ampholytic block is composed of propylacrylic acid (PAA), dimethylaminoethyl methacrylate (DMAEMA) and butyl methacrylate (BuMA) [122]. This system organizes into micelles. Thiolated siRNA was directly conjugated to the copolymer via thiol exchange reactions with the pyridyl disulfide groups present in micelle corona. Excellent silencing activity was observed in HeLa cells [122].
- Comb-type PEG – siRNA conjugates. A thiol modified siRNA targeting the green fluorescent protein gene was conjugated with reversible addition–fragmentation chain transfer (RAFT) polymerized pyridyl disulfide containing poly(PEG methyl ether acrylate)s. These conjugates demonstrated enhanced serum stability and nuclease resistance when compared with free siRNA [123].

3.2.7. Architecture of conjugates

Polymer architecture has an important impact on the activity of the conjugates. Szóka and Fréchet studied the impact of molecular architecture (hydrodynamic volume, conformation, flexibility, and branching) on the fate of polymers in the organism. They found that molecular architecture has a serious impact on the elimination of the carrier via glomerular filtration, but a much smaller impact on the extravasation of the polymer into the tumor [124,125]. Ulbrich's group studied in detail the relationship between the architecture of HPMA copolymers – linear conjugates, branched conjugates, grafted conjugates, self-assembled micellar conjugates, and grafted dendritic star conjugates – and their activity [126,127]. The linear conjugates with flexible polymer chains were eliminated by glomerular filtration, faster than the highly branched star conjugates with comparable molecular weight. Star conjugates appeared to produce an enhanced number of long-term survivors [128].

Intermolecular and intramolecular aggregation can impact solution properties and activity of macromolecular therapeutics. Multifunctional polymeric drug carriers containing several components, such as targeting modules, drug releasing modules, and endosome disruptive modules, have showed the potential to perform multiple functions within a single structure [55]. Ideally, each component within the delivery system should function independently, without affecting the functionality of the other components. However, the physical and biological properties of multifunctional conjugates can be expected to exert some influence on the other components [103,128]. For example, higher amounts of the hydrophobic drug prostaglandin E₁ bound to polyHPMA macromolecules resulted in a lower rate of drug release [103]. Ding et al. studied the self-association of HPMA copolymers containing an amphipathic CD21-binding heptapeptide (HP = YILHRN) using FRET, light scattering, and SEC [129]. The process of association, largely the result of intra-polymer hydrophobic interactions, resulted in a unimolecular micelle structure. The degree of self-association increased with increased heptapeptide content. The self-association of HPMA copolymer-peptide conjugate was disrupted by the

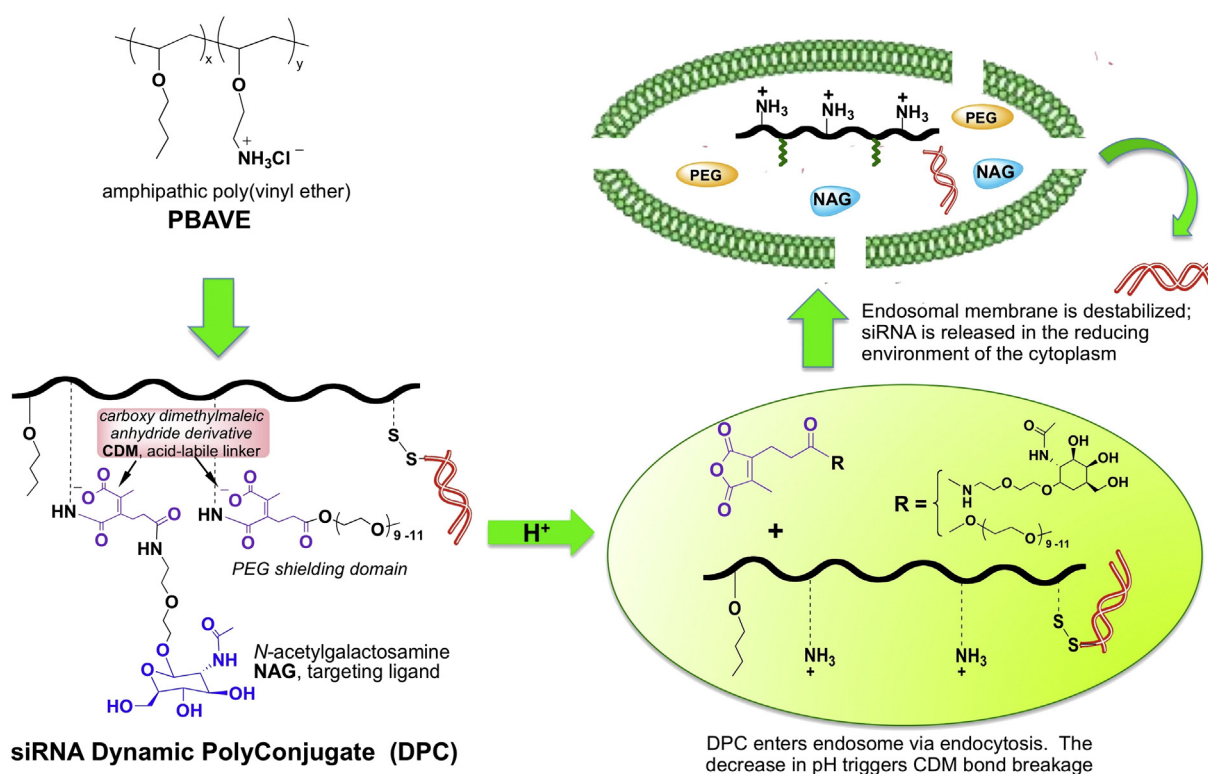


Fig. 3. Design of dynamic conjugates containing siRNA bound via covalent bonds. Scheme of structure of dynamic conjugate, its cellular uptake, release of protecting PEG chains in the endosome and release of siRNA into the cytoplasm following reduction of the disulfide bond. Carboxy dimethylmaleic anhydride (CDM) chemistry was used in the synthesis. Adapted from ref. [121].

incorporation of acrylic acid comonomers into the HPMA copolymer backbone; the ionization of COOH groups along the polymer chains induced a conformational change into an extended conformation. On the other hand the formation of unimolecular micelles (in the absence of ionizable comonomer) resulted in decreased enzyme biorecognizability and accessibility of oligopeptide side-chains (GFLG) by papain [129]. Formation of aggregates of HPMA copolymers by hydrophobic interactions played a major role in blood clearance and body distribution [130]. A better understanding of the relationship between the self-association of polymer conjugates and biological significance is a prerequisite for the rational design of polymeric drug delivery systems.

3.3. Targeted- vs. non-targeted systems

Targeting of polymer–drug conjugates is mostly connected with enhanced biorecognition at the target cell surface mediated by a targeting moiety complementary to a cell surface antigen/receptor. The efficiency of this approach may depend on the type of tumor and structure of the macromolecular therapeutics [27]. For solid tumors the extravasation of long-circulating polymer–drug conjugates via the EPR (enhanced permeability and retention) effect [131,132] might be sufficient to achieve effectiveness. Manipulation of the molecular weight [133,134] or of the architecture [135] may provide a tool to fine-tune the biological properties and efficacy of the conjugates.

For blood tumors, the advantages of targeted conjugates are obvious. Taking into account the limited time a conjugate remains in the bloodstream a biorecognition system can dramatically improve target localization. Examples of treatment of non-Hodgkin lymphoma by targeting to CD20 receptor on B cells will be discussed in Section 4.2.

Targeting may also involve the choice of a drug effective only for a subset of cells. In the section below (Section 3.4) we shall discuss using HPMA copolymer conjugates that target a cell phenotype due to

its mechanism of action. Additional incorporation of targeting moieties specific for the particular phenotype would further improve the efficacy.

3.4. Targeting stem cells

A major challenge for the development of effective anti-cancer macromolecular therapeutics is the heterogeneity of cancer cells. Cancer cells are present in various differentiation statuses, such as cancer stem cells (CSC) and differentiated cells [136,137]. Only the CSCs, with the ability to self-renew and differentiate, have the tumorigenic potential and are able to generate phenotypically heterogeneous tumor cell populations that resemble the original organizations of the parent tumor. The hierarchical CSC theory suggests that the unsuccessful treatment of cancers is largely due to the failure of conventional cytotoxic anti-cancer therapies to eliminate CSCs. Therefore, targeting CSCs in combination with traditional anticancer therapeutics represents a promising strategy to improve cancer patient survival [138,139].

Aiming to improve the outcome of prostate cancer treatments by targeting CSCs, we designed a CSC specific nanomedicine. Cyclopamine, a hedgehog (Hh) pathway inhibitor, was attached to the end of GFLG (glycylphenylalanylleucylglycyl) biodegradable tetrapeptide side chains of HPMA copolymer. To be noted, targeting Hh signaling allows specific interference with malignant CSCs while sparing adult stem cells [140,141]. We evaluated the CSC inhibitory effects of the HPMA copolymer–cyclopamine conjugate in an in vitro prostate cancer epithelial cell model, RC-92a/hTERT cells, with stem cell properties [142]. RC-92a/hTERT cells were chosen since the CD133⁺/integrin $\alpha 2 \beta 1^{\text{hi}}$ /CD44⁺ putative prostate CSCs within the whole cell line could be enriched to 5%, higher than that reported on primary prostate cancer cells or other established prostate cancer cell lines. Following exposure of RC-92a/hTERT cells to HPMA copolymer–cyclopamine conjugate (P-CYP) or HPMA copolymer docetaxel conjugate (P-DTX), we found that P-CYP reduced the percentage of

CD133 + cancer cells while not decreasing the general cell viability, indicating the preferential toxic effect of P-CYP to CD133 + cell subpopulation. In contrast, P-DTX could not reduce the proportion of CD133 + cells, although toxic to the bulk tumor cells [139].

An important end point for evaluating *in vivo* antitumor efficacy is the tumor growth inhibitory effect in long-term. The combination of P-CYP and P-DTX, as well as the P-CYP or P-DTX single treatment all inhibited the PC-3 prostate tumor growth to certain extent compared to saline group, immediately after three weeks of treatment (Fig. 4) [143]. However, after stopping the treatment at day 21 and continuing monitoring for longer periods, tumors in P-DTX group started to regrow faster on average; tumors in P-CYP group continued to grow progressively; strikingly, the combination of P-CYP and P-DTX showed the most persistent tumor growth inhibition, leading to the longest mice survival on average (Fig. 4A) [143].

We evaluated prostate CSCs in residual tumors: P-CYP and combination treatments resulted in decreased proportion of CD133 + cells compared to saline group; on the contrary, P-DTX failed to reduce the CD133 + cell population (Fig. 4B). Similarly, the number of prostaspheres formed by tumor cells isolated from P-CYP and combination group was significantly smaller than that isolated from P-DTX treated group (Fig. 4C). In short, P-CYP alone or in combination with P-DTX decreased the CD133 + cancer stem/progenitor cell population; this may contribute to more effective long-term tumor growth inhibition. The achievement of a durable anticancer effect by this combination treatment has been reported as the “top story” in “Prostate Cell News” [144].

Obviously, the efficacy of the system could be further improved by CD133 targeting. CD133 is usually expressed in a confined manner; it is present only in the prostate cancer cell population and in certain tissue stem cells [145,146]. In addition, certain epitopes of CD133 on the surface of CSCs are glycosylated and of unique protein folding pattern, different from CD133 molecules that could also be present on other adult cells [147,148]. Thus it appears that antibodies toward glycosylated CD133 epitopes could be suitable targeting moieties for CSC targeted systems.

3.5. Tumor microenvironment as delivery barrier

The tumor microenvironment cannot be ignored when discussing anti-cancer therapeutics and drug delivery [58]. In the past decade,

numerous studies have indicated the importance of tumor microenvironment in cancer growth, progression, and metastasis [149]. Cancer cells are embedded in unique extracellular matrix (ECM) and are surrounded by various tumor stromal cells. The whole tumor is constantly remodeling through the reciprocal communications between cancer cells and the various tumor microenvironment components by cell–cell interactions, cell–matrix interactions as well as via secreted growth factors and/or cytokines [58]. We shall demonstrate the importance of the microenvironment in pancreatic cancer as an example.

While drugs, such as gemcitabine exhibit efficacy against human pancreatic ductal adenocarcinoma (PDA) cells transplanted into immunodeficient mice [150], they are far less effective in patients. One potential explanation is the dense accumulation of activated fibroblasts (stellate cells), immune cells and extracellular matrix that is characteristic of PDA [151]. The dense stroma creates an elevated interstitial fluid pressure (IFP) that renders the uniform delivery of therapeutics extremely difficult [152–154]. It is important to note that transplantation and xenograft-based animal models do not reproduce this environment. Relevant models in genetically engineered mice have been developed by tissue-specific manipulation of the *Kras* oncogene and key tumor suppressors mutated in human PDA [155–157]. Such models resemble that of human patients; e.g., gemcitabine has no effect on survival.

Provenzano et al. [153] identified hyaluronic acid (HA) as the primary transport barrier and demonstrated that the use of PEG modified hyaluronidase can ablate stromal HA, normalize IFP, and re-expand the vasculature (Fig. 5). Using combination treatment of PEG-hyaluronidase with gemcitabine they doubled the survival time of experimental animals [153]. Interestingly, treatment of genetically engineered mice with Hh pathway antagonist IPI-926 induced transient increase of blood flow and improved gemcitabine delivery [150]. Also, the Hh antagonist cyclopamine was shown to be synergistic with gemcitabine in elimination of cancer stem cells from a direct pancreatic cancer xenograft from patients [158].

Buckway et al. used traditional animal model of pancreatic cancer and demonstrated that tumor targeting could be achieved when co-administering hyaluronidase [159]. However, decisive experiments to prove the suitability of this approach for macromolecular therapeutics will be those using genetically engineered mouse models and

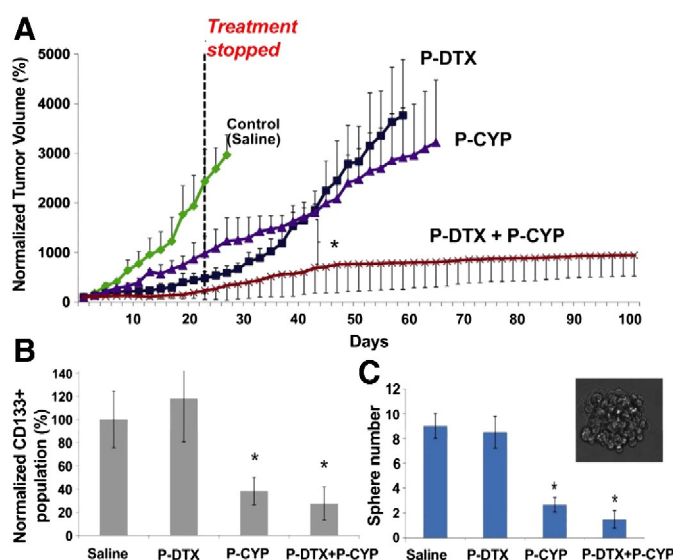


Fig. 4. Treatment of prostate PC-3 tumor bearing nude mice with a combination of two nanomedicines, one targeting cancer stem cells, the other differentiated cells. (A) Tumor growth inhibition by P-CYP (CYP 40 mg/kg, twice per week, 6 doses), P-DTX (DTX 10 mg/kg, single dose), and combination of P-DTX (DTX 10 mg/kg, single dose) and P-CYP (CYP 40 mg/kg, 6 doses) in PC-3 tumor-bearing nude mice. * represents statistically significant difference between single and combination treatment groups ($p < 0.05$); (B) percentage of CD133 + cells in residual tumor cells following *in vivo* treatments; (C) prostasphere forming ability of the residual tumor cells following *in vivo* treatments. *, $p < 0.05$. Adapted with permission from ref. [143].

combination treatment including hyaluronidase and/or hedgehog pathway inhibitors.

The design of suitable ways for macromolecular therapeutics to overcome the transport barrier of the tumor microenvironment is one of the great challenges waiting to be solved.

3.6. Overcoming of multidrug resistance

The appearance of multidrug resistance (MDR) to anticancer drugs is one of major obstacles in cancer chemotherapy [160,161]. Following exposure to anticancer drugs, MDR is induced by overexpression of the MDR1 gene that encodes the transmembrane protein, P-glycoprotein (Pgp). Pgp is an ATP dependent efflux pump that restricts the transport of drugs into the cell interior (Fig. 6A). The mechanism of drug exclusion [162], i.e., partition of the drug into the Pgp drug binding pocket, ATP binding to Pgp resulting in conformational change and release of drug to extracellular space, indicates that nanomedicines that enter cells by endocytosis have a potential to overcome the Pgp type of multidrug resistance. For example, water-soluble polymer–drug conjugates will be internalized in membrane limited organelles and the drug released from lysosomes in the perinuclear region far from the Pgp, securing efficient intracellular concentration [163]. This hypothesis was validated in vivo. We studied the anticancer activities of free DOX and HPMA copolymer-bound DOX [P(GFLG)–DOX] in mouse models of DOX sensitive (A2780) and DOX resistant (A2780/AD) human ovarian carcinoma xenografts [164]. Free DOX was effective only in sensitive tumors, decreasing tumor size about three times, while P(GFLG)–DOX decreased tumor size 28 and 18 times in the sensitive and resistant tumors, respectively (Fig. 6B).

3.7. Combination therapy with macromolecular therapeutics

We have developed a novel concept of using combination therapy with water-soluble polymer-bound drugs. In vivo combination chemotherapy and photodynamic therapy (PDT) studies on two cancer models, Neuro 2A neuroblastoma induced in A/J mice [165] and human ovarian carcinoma heterotransplanted in nude mice [104,166,167], demonstrated that combination therapy produced tumor cures which could not be obtained with either chemotherapy or PDT alone. Other combination systems were quantitatively evaluated by combination index (CI) analysis in A498 renal carcinoma cells [168] and in OVCAR-3 ovarian carcinoma cells [169]. The results demonstrated synergistic effects of HPMA copolymer–drug (SOS thiophene, DOX, and chlorin e_6) conjugate combinations in a wide range of concentrations.

Recently, the regions of synergism for the combination of backbone degradable HPMA copolymer–gemcitabine and HPMA copolymer–paclitaxel conjugates [170] and backbone degradable HPMA copolymer–gemcitabine and HPMA copolymer–platinum conjugates [171] have been identified.

A detailed comparison of the efficacy of combination therapy of ovarian carcinoma with 1st and 2nd generation HPMA copolymer – PTX and – GEM conjugates clearly demonstrated the advantage of long circulating 2nd generation conjugates – favorable pharmacokinetics, dramatically enhanced inhibition efficacy on tumor growth, and the absence of adverse effects [172].

3.8. Treatment of non-cancerous diseases

Water-soluble polymer–drug conjugates are suitable for the delivery of drugs to other than cancer disease sites. HPMA copolymers have been used in the treatment of musculoskeletal [173–175], inflammatory [175,176], and infectious [177–180] diseases.

Considerable effort has been devoted to the development of macromolecular therapeutics for the treatment of rheumatoid arthritis. This research was stimulated by the demonstration of profound arthritism of HPMA copolymers in the adjuvant-induced arthritis (AA) rat model [181]. Consequently, the HPMA copolymer–dexamethasone conjugate provided superior and sustained amelioration of AA in rats [182]. Recently, four dexamethasone nanomedicines, namely liposome, core-crosslinked micelle, slow releasing HPMA copolymer–dexamethasone conjugate and fast-releasing HPMA copolymer–dexamethasone conjugate were compared. After a single i.v. injection, the formulations with a slower drug release kinetics (micelle and slow releasing HPMA copolymer–dexamethasone conjugate) maintained longer duration of AA therapeutic activity than those with fast dexamethasone release [183].

4. Novel approaches

New approaches are needed to achieve translation of macromolecular therapeutics into the clinics [184]. Two examples of designs that have a potential to accomplish this task will be discussed below: a) the synthesis of backbone degradable HPMA copolymer carriers that overcome the molecular weight dilemma; and b) drug-free macromolecular therapeutics, a new paradigm in macromolecular therapeutics – a system based on crosslinking of cell surface receptors, which results in apoptosis. This design was inspired by self-assembling biomaterials studied in our laboratory [185,186]. The

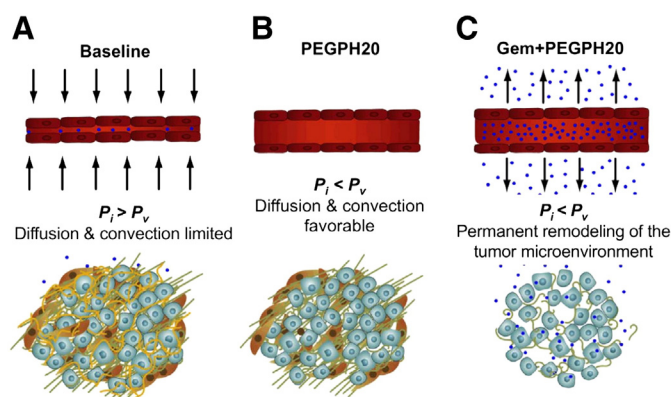


Fig. 5. Altering physicochemicals and remodeling the stroma in pancreatic ductal adenocarcinoma (PDA) to therapeutic advantage. (A) Intratumoral mechanics in PDA impede diffusion and convection of small molecules. (B) Enzymatic degradation of stromal hyaluronic acid decreases interstitial fluid pressure and relieves physical constraints on small molecule perfusion, which can reconstitute in the absence of additional therapy. (C) Combined enzymatic and cytotoxic therapies permanently remodel the tumor microenvironment to favor the delivery and distribution of small molecules. Blue spheres represent chemotherapy molecules, vessels are shown in red, carcinoma cells in light blue, activated pancreatic stromal cells in brown, collagen in green, and hyaluronic acid in yellow. P_i , interstitial fluid pressure; P_v , intravascular fluid pressure. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.) Adapted with permission from ref. [153].

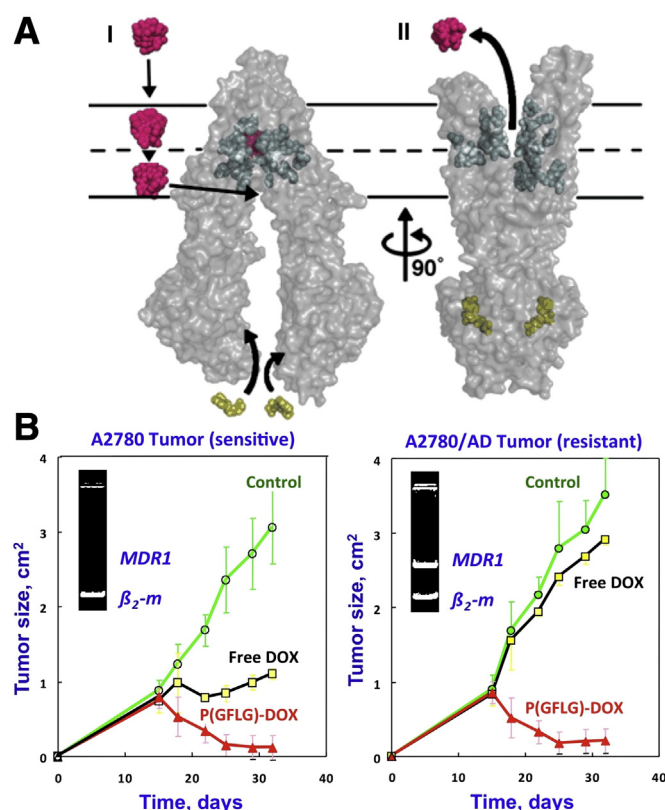


Fig. 6. Multidrug resistance (MDR). (A) Model of substrate transport by P-glycoprotein (Pgp). (B) Efficacy of free doxorubicin (DOX) and HPMA copolymer-bound DOX on the growth of (A) A2780 sensitive, and (B) A2780/AD resistant (expressing the MDR1 gene) human ovarian carcinoma xenografts in mice. Untreated as control. Means \pm SD are shown. (A) Reprinted with permission from ref. [162]. (B) Reprinted with permission from ref. [164].

application of biomaterial design principles on drawing a new strategy of apoptosis induction demonstrates that a bridge between biomaterials and nanomedicines can be created [187].

4.1. Design of backbone degradable long-circulating conjugates

It is well established that high molecular weight (long-circulating) polymer conjugates accumulate efficiently in solid tumor tissue due to the EPR effect [131,132]. To achieve substantial accumulation of the polymer–drug conjugate in solid tumors (due to the EPR effect) a sustained concentration gradient is needed. The concentration depends on the administered dose and the circulation time depends on the molecular weight of the carrier. However, higher molecular weight drug carriers with a nondegradable backbone deposit and accumulate in various organs, impairing biocompatibility.

It was evident a long time ago that polymers with molecular weight above the renal threshold would be advantageous (see p. 193 of ref. [1]). However, previous attempts to design and synthesize long-circulating conjugates produced branched, partially crosslinked copolymers with enzymatically degradable sequences [188]. The synthetic process and the polymer structure were difficult to control; consequently, the process would be difficult to scale-up. Nevertheless, the results proved that a higher molecular weight of polymer carriers transfers into higher accumulation of drugs in the tumor tissue with concomitant enhancement of efficacy [189].

The advances in controlled radical polymerization [190,191] and click chemistry [192–194] offered new vistas for the design and synthesis of long-circulating biocompatible polymer–drug conjugates. To this end we designed new, *second-generation anticancer nanomedicines*

based on high molecular weight HPMA copolymer–drug carriers containing enzymatically degradable bonds in the main chain (polymer backbone) [195–197]. The proposed new design permits tailor-made synthesis of well-defined backbone degradable HPMA copolymers. The synthetic process consists of two main steps: first, the synthesis of a telechelic HPMA copolymer by reversible addition–fragmentation chain transfer (RAFT) polymerization, followed in the second step by chain extension using alkyne–azide [195,196] or thiol–ene [197] click reactions. In addition, we synthesized a new RAFT chain transfer agent (CTA), N^α, N^ϵ -bis(4-cyano-4-(phenylcarbonothioylthio)pentanoyl)glycylphenylalanylleucylglycyl lysine (Peptide2CTA), containing an enzymatically degradable oligopeptide capped at both ends with 4-cyano-4-(phenylcarbonothioylthio)pentanoate [197]. During RAFT polymerization the HPMA monomers incorporate at both dithiobenzoate groups of the Peptide2CTA with identical efficiency. When the final polymer was incubated with papain, a thiol proteinase with similar specificity as lysosomal proteinases, the molecular weight decreased to half of the original value. Thus it is possible to prepare a degradable diblock copolymer with narrow molecular weight distribution in one step, eliminating the chain extension reaction [197].

Multiblock polyHPMAs with Mw as high as 300 kDa and containing degradable GFLG sequences were obtained by chain extension followed by fractionation using size exclusion chromatography (SEC). The exposure of the multiblock HPMA copolymer to model enzyme papain or lysosomal cathepsin B (pH 6, 37 °C) resulted in complete degradation of GFLG segments and decrease of the molecular weight of the carrier to half (below the renal threshold) [195–197]. These data support our hypothesis and bode well for the success of the proposed design of backbone degradable HPMA copolymers composed of alternating segments of HPMA copolymer, with molecular weight below the renal threshold, and lysosomally degradable GFLG containing oligopeptides (Fig. 7).

The enhanced activity of 2nd generation conjugates when compared to 1st generation conjugates (non-degradable, Mw below the renal threshold) has been proven in vivo. Long-circulating backbone degradable HPMA copolymer conjugates with doxorubicin [198], paclitaxel [199], or gemcitabine (Fig. 8) demonstrated higher efficacy in suppressing the growth of human ovarian carcinoma xenografts in nude mice than 1st generation conjugates. Similarly, bone-targeted long-circulating conjugates containing prostaglandin E_1 had higher accumulation on bone tissue and greater indices of bone formation in an ovariectomized rat osteoporosis model when compared to 1st generation conjugates [200]. Interestingly, in vivo experiments on animal models of ovarian cancer have proven that the diblock structure is sufficient to dramatically enhance efficacy when compared with the first generation conjugates [172,198]. This is of importance for the scale-up of the synthesis and translation into the clinics.

4.2. Drug-free macromolecular therapeutics – a new paradigm in nanomedicines

An exciting new development in the nanomedicine research area is the design of drug-free macromolecular therapeutics. The new paradigm in drug delivery is based on the biorecognition of natural (e.g., peptide or oligonucleotide) motifs at cell surface, formation of heterodimers (e.g., antiparallel coiled-coils or hybridization of complementary oligonucleotides), crosslinking of non-internalizing receptors and initiation of apoptosis [201–203].

4.2.1. Biorecognition in hybrid polymer systems

Hybrid polymer systems are composed from at least two distinct classes of macromolecules, for example, synthetic and biological macromolecules. For instance, conjugation of peptide domains to synthetic polymers produces materials with properties superior to individual components. The peptide domain inserts a level of control over structure resulting from self-assembly at a nanometer scale; the synthetic

part may enhance the biocompatibility of the whole system by reducing the immunogenicity of the natural domain [187].

4.2.2. Drug-free macromolecular therapeutics based on formation of antiparallel coiled-coils

The coiled-coil is one of the basic folding patterns of native proteins. It consists of two or more right-handed α -helices winding together to form (usually) a slightly left-handed super-helix [204,205]. The primary structure of the coiled-coil motif is characterized by a sequence of repeating heptads (motif of seven amino acids) designated as $[a, b, c, d, e, f, g]_n$, in which a and d are usually hydrophobic amino acid residues, while the others are polar. Two helices associate through a hydrophobic interface between a and d , making b, c , and f face outward. Interhelical electrostatic interactions between residues e and g contribute to the stability depending on their detailed structure; α -helices may associate as homodimers, heterodimers in parallel or antiparallel alignments, or form higher order (e.g., tetramer) aggregates [206,207]. Hundreds of native proteins, such as muscle proteins, transcription factors, cytoskeletal proteins, cell and viral surface proteins, tumor suppressors, molecular motors, and many disease- and organ-specific auto-antigens have functional coiled-coil domains [208]. A distinctive feature of coiled-coils is the specific spatial recognition, association, and dissociation of

helices, making it an ideal model for protein biomaterials in which the higher order structures may be predicted based on the primary sequence. Various functional groups may be exactly positioned into the coiled-coil structure, allowing specific intermolecular interactions to occur.

We have designed hybrid systems composed from hydrophilic HPMA copolymer backbone grafted with two pentaheptad antiparallel coiled-coil forming oligopeptide sequences with opposite charge (CCE and CCK [209]) [185]. A mixture of equimolar solutions of P-CCK (P is the HPMA copolymer backbone) and P-CCE self-assemble into hydrogels. This process is mediated by the recognition of CCE and CCK peptide grafts – they fold into antiparallel coiled-coils [185,186]. The excellent biorecognition of the CCE and CCK peptides [185,210] was an inspiration for the design of new nanomedicines; this created a bridge between the design of biomaterials and the design of nanomedicines.

4.2.2.1. Design. CCK and CCE peptides were engaged in the design of a new CD20 + cell apoptosis induction system, called drug-free macromolecular therapeutics [201,202]. CD20 is an ideal target for immunotherapies. It is an integral membrane protein [211] that is expressed from pre-B cells to terminally differentiated plasma cells and is present

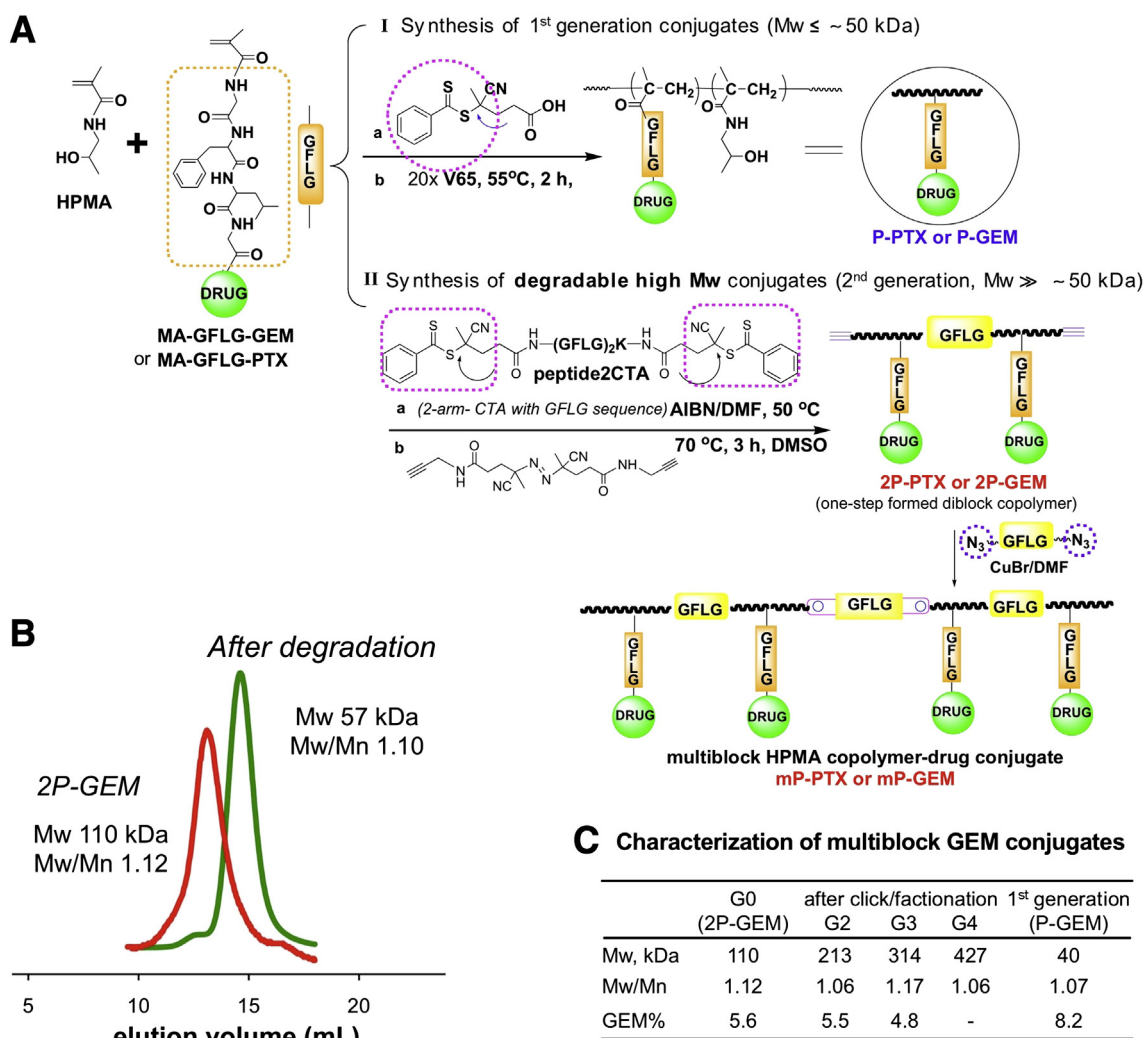


Fig. 7. Design of backbone degradable long-circulating HPMA copolymer–drug conjugates. Two dithiobenzoate chain transfer agents were linked with lysosomal enzyme cleavable peptide GFLGKGLFG resulting in a biodegradable RAFT agent, peptide2CTA. This permits one-step synthesis of diblock copolymers. (A) Post-polymerization click reaction produces multiblock HPMA copolymer–drug conjugates with different chain lengths; (B) the diblock HPMA copolymer–drug conjugates degraded into half of their initial M_w , indicating the potential to employ diblock conjugates with 100 kDa M_w without impairing their biocompatibility (the degradation products are below the renal threshold); (C) characterization of 1st- and 2nd-generation HPMA copolymer–gemcitabine conjugates including molecular weight and drug content [195–197].

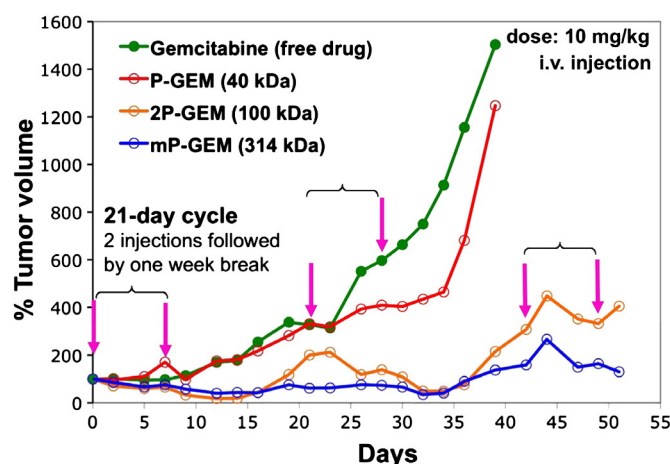


Fig. 8. Comparison of backbone degradable long circulating (second-generation) HPMA copolymer–gemcitabine conjugates (diblock 2P-GEM and multiblock mP-GEM) with low molecular weight (first generation) HPMA copolymer–gemcitabine conjugate (P-GEM): in vivo antitumor activity against A2780 human ovarian xenografts in nude mice. Unpublished data from Kopeček laboratory.

on greater than 90% of B-cell malignancies [212]. CD20 is not shed from the cell surface nor is it present in serum under standard physiological conditions. It is a cell cycle regulatory protein [213] that either controls or functions as a store operated calcium channel. The protein forms dynamic dimers and tetramers [214] constitutively associated in lipid rafts of the cell membrane [215].

Indeed, the biorecognition of CCE/CK peptide motifs at the cellular surface was able to induce apoptosis of CD20 + B cells. Exposure of Raji B cells to a 1F5 anti-CD20 Fab'–CCE conjugate decorated the cell surface with CCE (CD20 is a non-internalizing receptor) through antigen–antibody fragment recognition. Further exposure of the decorated cells to P-CCK (grafted with multiple copies of CCK) resulted in the formation of CCE/CK coiled-coil heterodimers at the cell surface. This second biorecognition induced the crosslinking of CD20 receptors and triggered the apoptosis of Raji B cells in vitro [201] and in a non-Hodgkin lymphoma animal model in vivo [202]. High degrees of apoptosis could be achieved in vitro [201] and long-term survivors produced in Raji B lymphoma mice model [202]. This is a new concept, where the biological activity of drug-free macromolecular therapeutics is based on the biorecognition of peptide motifs.

4.2.3. Drug-free macromolecular therapeutics based on hybridization of morpholino oligonucleotides

After we proved the concept of the new paradigm in nanomedicine by employing a pair of pentaheptad coiled-coil forming peptides, we focused our attention on a hybrid system where the biorecognition is mediated by the hybridization of complementary morpholino oligonucleotides.

4.2.3.1. HPMA copolymer–oligonucleotide hybrids. A recent new design was based on the biorecognition (hybridization) of a pair of morpholino oligonucleotides with complementary sequences [203]. The system is composed of a 1F5 anti-CD20 Fab' antibody fragment, a pair of complementary phosphorodiamidate morpholino oligomers (MORF1 and MORF2 [216]), and a linear polymer (P) of HPMA. We hypothesized that [203]: (1) the exposure of malignant CD20 + B-cells to the anti-CD20 Fab'–MORF1 conjugate (Fab'–MORF1) decorates the cell surfaces with MORF1; and (2) further treatment of decorated B-cells with HPMA copolymer grafted with multiple copies of MORF2 (P–MORF2) results in MORF1–MORF2 hybridization at the cell surface with concomitant CD20 crosslinking, which triggers apoptosis. Indeed, this system produced high levels of Raji B cell apoptosis in vitro and excellent results in vivo (Fig. 9). Treatment of systemically disseminated CD20 + Raji B cell lymphoma in C.B.-17 SCID mice with Fab'–MORF1 and P–MORF2

led to long-term survivors (125 days, Fig. 9C). Eradication of Raji cells after treatment was further confirmed by flow cytometry (Fig. 9D, E), MRI and histology [203]. These results were highlighted in Chemical & Engineering News in January 2014 [217].

The results shown above demonstrate that in the hybridization-mediated system (Fab'–MORF1/P–MORF2), the treatment with equimolar MORF1/MORF2 was sufficient for biorecognition, apoptosis induction, and prevention of lymphoma dissemination. In contrast, for the coiled-coil mediated apoptosis induction a 25× excess of the second conjugate (P–CCK) was needed. In addition, rapid binding kinetics were observed with the oligonucleotide-based system. Other advantages of the MORF oligonucleotides include: (1) specific binding due to a well-defined hydrogen bonding pattern (i.e., base pairing), (2) charge-neutral property that prevents potential off-target effects, and (3) water solubility due to good base-stacking property resulting in favorable pharmacokinetics [203].

The design of drug-free macromolecular therapeutics is a truly novel approach in nanomedicine. It builds on the design of new self-assembling biomaterials [185–187,218,219] and translates the biorecognition principles to nanomedicine [201–203]. The first design of this system was tailored for the non-internalizing CD20 receptor and NHL. CD20 is highly expressed on the surface of malignant and normal B-cells, but not in stem cells or plasma cells. Thus, drug-free macromolecular therapeutics (employing the “B-cell depletion strategy”) can be potentially used to treat B-cell derived *hematological neoplastic diseases and autoimmune diseases*, without non-reversible impact on normal immune function [220]. Other potential disease targets are (in addition to NHL): chronic lymphocytic leukemia (CLL), rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, autoimmune hemolytic anemia, pure red cell aplasia, idiopathic thrombocytopenic purpura, Evans syndrome, vasculitis, bullous skin disorders, type 1 diabetes mellitus, Sjögren's syndrome, Devic's disease, and Graves' ophthalmopathy. All of the above listed diseases have been treated by rituximab anti-CD20 mAb (either approved by FDA or in clinical trials).

Importantly, the concept of drug-free macromolecular therapeutics could be expanded by using different components in the design. For example, the Fab' fragment can be replaced by antigen binding saccharides [99] or by peptides selected by phage display [221] or by combinatorial methods [222,223].

5. Future prospects

The advantages of polymer-bound drugs (when compared to low-molecular weight drugs) are [55,59]: a) active uptake by fluid-phase

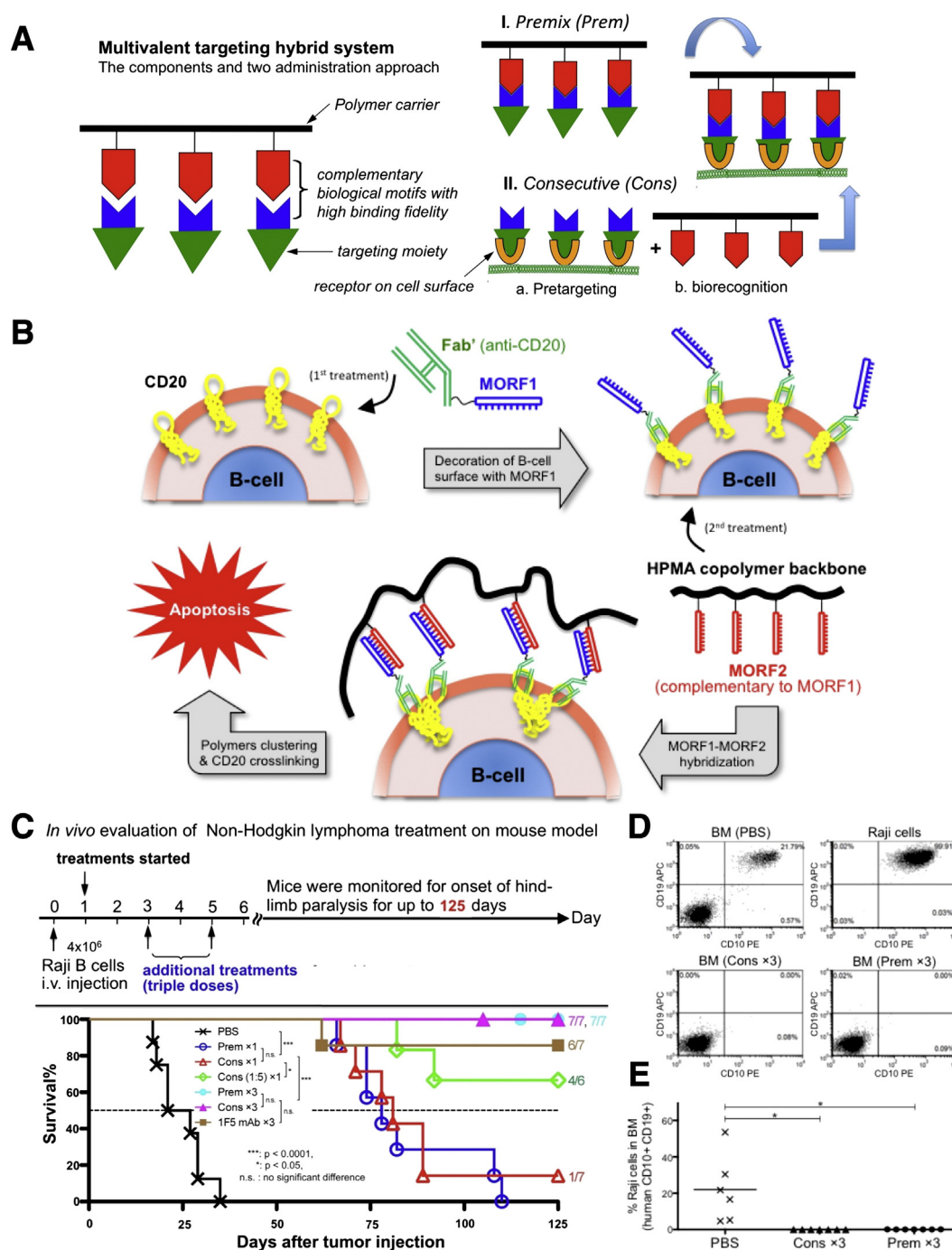


Fig. 9. Drug-free macromolecular therapeutics. (A) General design concept of the therapeutic platform; (B) apoptosis induction of B-cells by crosslinking of the CD20 antigens that is mediated by extracellular hybridization of complementary morpholino oligonucleotides (MORF1–MORF2); (C) treatment schedule and Kaplan–Meier plot with indication of numbers of long-term survivors (7 mice per group); (D) flow cytometry analysis of residual Raji cells in the bone marrow (BM) of the PBS-treated, paralyzed mice (PBS) and the nanomedicine-treated, surviving mice (Cons x3, Prem x3). Bone marrow cells isolated from the femur of mice and Raji cells from culture flasks (upper right panel) were stained with PE-labeled mouse anti-human CD10 and APC-labeled mouse anti-human CD19 antibodies. (E) Quantitative comparison of % Raji cells (human CD10+CD19+) in the bone marrow of control mice (PBS, n = 6) and the nanomedicine-treated mice (Cons x3 and Prem x3, n = 7 per group). Statistics was performed by Student's *t* test of unpaired samples (*: *p* < 0.05). Cons, consecutive administration of Fab'-MORF1 first followed 1 h later by P-MORF2; Prem, premixture was administered. Reprinted with permission from ref. [203].

pinocytosis (non-targeted polymer-bound drugs) or receptor-mediated endocytosis (targeted polymer-bound drug), b) increased passive accumulation of the drug at the tumor site by the EPR effect, c) increased active accumulation of the drug at the tumor site by targeting, d) long-lasting circulation in the bloodstream, e) decreased non-specific toxicity of the conjugated drugs, f) potential to overcome multidrug resistance, g) decreased immunogenicity of

the targeting moiety, h) immunoprotecting and immunomobilizing activities, and i) modulation of the cell signaling and apoptotic pathways. In addition to preclinical evaluation on animal cancer models, the benefits of macromolecular therapeutics over free (unbound) drugs have been demonstrated in preclinical and clinical arrangements [224,225]. Results from clinical trials with 1st generation HPMA conjugates indicated a significant decrease of adverse effects

when compared to small molecule drugs; however, the therapeutic efficacy did not match the data in preclinical animal studies.

Here we present data that show the advantage of the second generation conjugates over both, first generation conjugates and free drugs [198–200]. These new conjugates are backbone degradable and long-circulating; they are composed from enzymatically cleavable sequences and synthetic segments in an alternating arrangement [195–197]. These conjugates have a clear translational potential. In addition to enhanced efficacy, the synthesis of biodegradable diblock HPMa copolymer conjugates using a RAFT chain transfer agent that contains a degradable peptide sequence flanked by two dithiobenzoate groups, is clearly a scalable process.

The new paradigm in nanomedicine–drug-free macromolecular therapeutics [201–203] seems to address the challenges of treating blood borne cancers. It avoids the use of low molecular weight, potentially toxic drugs, is triggered by specific recognition at the molecular level and offers the possibility of pretargeting.

Thus on the design side the area of macromolecular therapeutics is well advanced. The major challenge, however, is to merge the knowledge of design principles of conjugates with the understanding of the biological features of cancer, including heterogeneity of cancer cells, tumor microenvironment and metastasis [58]. The design of conjugates targeting stem cells [143] and their use in combination with conjugates targeting differentiated cells as well as destabilization of stroma [153] surrounding the tumor tissue will enhance the efficiency of treatment. The development of non-invasive imaging technologies will undoubtedly contribute to progress [226–228]. Imaging can provide valuable feedback to fine tune the conjugate design and treatment protocol.

The scientific knowledge and experience from preclinical and clinical research accumulated in the last 30 years form the basis for the possible translation of macromolecular therapeutics into the clinics within the next decade.

Acknowledgments

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References

- [1] J. Kopeček, Soluble biomedical polymers, *Polim. Med.* 7 (1977) 191–221.
- [2] Blood Substitutes, Preparation, Physiology, and Medical Applications, in: K.C. Lowe (Ed.), VCH Weinheim, ISBN: 3-527-26556-2, 1988.
- [3] A. Billiau, J.J. Muyembe, P. De Somer, Interferon-inducing polycarboxylates: mechanism of protection against virus infection in mice, *Infect. Immun.* 5 (1972) 854–857.
- [4] E.B. Tazulakhova, O.V. Parshina, T.S. Guseva, F.I. Ershov, Russian experience in screening, analysis, and clinical application of novel interferon inducers, *J. Interferon Cytokine Res.* 21 (2001) 65–73.
- [5] K.F. Mück, H. Rolly, K. Burg, Herstellung und antivirale Wirksamkeit von Polyacrylsäure und Polymethacrylsäure, *Makromol. Chem.* 178 (1977) 2773–2784.
- [6] G. Pasut, F.M. Veronese, State-of-the-art in PEGylation: the great versatility achieved after forty years of research, *J. Control. Release* 161 (2012) 461–472.
- [7] V. Levy, M.S. Herschfield, C. Fernandez-Mejia, S.H. Polmar, D. Scudieri, M. Berger, R. U. Sorensen, Adenosine deaminase deficiency with late onset or recurrent infections: response to treatment with polyethylene glycol modified adenosine deaminase, *J. Pediatr.* 113 (1988) 312–317.
- [8] L.M. Graham, Pegasparase: a review of clinical studies, *Adv. Drug Deliv. Rev.* 55 (2003) 1293–1302.
- [9] K.R. Reddy, M.W. Modi, S. Pedder, Use of peginterferon $\alpha 2a$ (40 kD) (Pegasys®) for the treatment of hepatitis C, *Adv. Drug Deliv. Rev.* 54 (2002) 571–586.
- [10] Y.S. Wang, S. Youngster, M. Grace, J. Bausch, R. Borden, D.F. Wyss, Structural and biological characterization of pegylated recombinant interferon $\alpha 2b$ and its therapeutic implications, *Adv. Drug Deliv. Rev.* 54 (2002) 547–570.
- [11] O. Kinstler, G. Moulinex, M. Treheit, D. Ladd, C. Gegg, Mono-N-terminal poly(ethylene glycol)–protein conjugates, *Adv. Drug Deliv. Rev.* 54 (2002) 477–485.
- [12] A.M. Nesbitt, S. Stephens, E.K. Chartash, Certolizumab pegol: a PEGylated antitumor necrosis factor alpha biological agent, in: F.M. Veronese (Ed.), *PEGylated Protein Drugs: Basic Science and Clinical Applications*, Birkhäuser Verlag, Basel, Switzerland, 2009, pp. 229–254.
- [13] M.R. Sherman, M.G. Saifer, F. Perez-Ruiz, PEG-uricase in the management of treatment-resistant gout and hyperuricemia, *Adv. Drug Deliv. Rev.* 60 (2008) 59–68.
- [14] Z.-R. Lu, P. Kopečková, J. Kopeček, Semitelechelic poly[N-(2-hydroxypropyl)-methacrylamide] for biomedical applications, in: R.M. Ottenbrite, S.W. Kim (Eds.), *Polymeric Drugs & Delivery Systems*, Technomic Publishing Co., Lancaster, PA, 2001, pp. 1–14.
- [15] Z.-R. Lu, P. Kopečková, Z. Wu, J. Kopeček, Functionalized semitelechelic poly[N-(2-hydroxypropyl)-methacrylamide] for protein modification, *Bioconjug. Chem.* 9 (1998) 793–804.
- [16] A. Lääne, A. Aaviksaar, M. Haga, V. Chytrý, J. Kopeček, Preparation of polymer-modified enzymes of prolonged circulation times. Poly[N-(2-hydroxypropyl)-methacrylamide] bound acetylcholinesterase, *Makromol. Chem. Suppl.* 9 (1985) 35–42.
- [17] V. Chytrý, A. Vrána, J. Kopeček, Synthesis and activity of a polymer which contains insulin covalently bound on a copolymer of N-(2-hydroxypropyl)-methacrylamide and N-methacryloylglycylglycine 4-nitrophenyl ester, *Makromol. Chem.* 179 (1978) 329–336.
- [18] J.H. Lee, G. Sabnis, A. Nan, Synthesis and *in vitro* characterization of semitelechelic poly[N-(2-hydroxypropyl)-methacrylamide]-trastuzumab conjugates targeted to breast cancer, *Macromol. Biosci.* 12 (2012) 55–60.
- [19] T.X. Viegas, M.D. Bentley, J.M. Harris, Z. Fang, K. Yoon, B. Dizman, R. Weimer, A. Mero, G. Pasut, F.M. Veronese, Polyoxazolones: chemistry, properties, and applications in drug delivery, *Bioconjug. Chem.* 22 (2011) 976–986.
- [20] K. Knop, R. Hoogenboom, D. Fischer, U.S. Schubert, Poly(ethylene glycol) in drug delivery: pros and cons as well as potential alternatives, *Angew. Chem. Int. Ed.* 49 (2010) 6288–6308.
- [21] T. Ishigara, T. Maeda, H. Sakamoto, N. Takasaki, M. Shigyo, T. Ishida, H. Kiwada, Y. Mizushima, T. Mizushima, Evasion of nanoparticle accelerated blood clearance phenomenon by coating of nanoparticles with various hydrophilic polymers, *Biomacromolecules* 11 (2010) 2700–2706.
- [22] A. Abuchowski, J.R. McCoy, N.C. Palczuk, T. van Es, F.F. Davis, Effect of covalent attachment of polyethylene glycol on immunogenicity and circulation time of bovine liver catalase, *J. Biol. Chem.* 252 (1977) 3582–3586.
- [23] S. Kamei, J. Kopeček, Prolonged blood circulation in rats of nanospheres surface-modified with semitelechelic poly[N-(2-hydroxypropyl)-methacrylamide], *Pharm. Res.* 12 (1995) 663–668.
- [24] T. Saffra, F. Muggia, S. Jeffers, D.D. Tsao-Wei, S. Groshen, O. Lyas, R. Henderson, G. Berry, A. Gabizon, Pegylated liposomal doxorubicin (Doxil): reduced cardiotoxicity in patients reaching or exceeding cumulative doses of 500 mg/m², *Ann. Oncol.* 11 (2000) 1029–1033.
- [25] M.T. Peracchia, E. Fattal, D. Desmaele, M. Besnard, J.P. Noël, J.M. Gomis, M. Appel, J. d'Angelo, P. Couvreur, Stealth PEGylated polycyanoacrylate nanoparticles for intravenous administration and splenic targeting, *J. Control. Release* 60 (1999) 121–128.
- [26] Y. Ma, Q. Yang, L. Wang, X. Zhou, Y. Zhao, Y. Deng, Repeated injections of PEGylated liposomal topotecan induces accelerated blood clearance phenomenon in rats, *Eur. J. Pharm. Sci.* 45 (2012) 539–545.
- [27] J. Kopeček, Polymer–drug conjugates: origins, progress to date and future directions, *Adv. Drug Deliv. Rev.* 65 (2013) 49–59.
- [28] J. Kopeček, Soluble polymers in medicine, in: D.F. Williams (Ed.), *Systemic Aspects of Biocompatibility*, Vol. II, CRC Press, Boca Raton, Florida, 1981, pp. 159–180.
- [29] H. Jatzkewitz, Peptamin (glycyl-L-leucyl-mescaline) bound to blood plasma expander (polyvinylpyrrolidone) as a new depot form of a biologically active primary amine (mescaline), *Z. Naturforsch.* 10b (1955) 27–31.
- [30] N.I. Givetal, S.N. Ushakov, E.F. Panarin, G.O. Popova, Experimental studies on penicillin polymer derivatives (in Russian), *Antibiotiki* 10 (1965) 701–706.
- [31] K.I. Shumikina, E.F. Panarin, S.N. Ushakov, Experimental study of polymer salts of penicillins (in Russian), *Antibiotiki* 11 (1966) 767–770.
- [32] E.F. Panarin, S.N. Ushakov, Synthesis of polymer salts and amidopenicillins (in Russian), *Khim. Pharm. Zhur.* 2 (1968) 28–31.
- [33] G. Mathé, T.B. Loc, J. Bernard, Effect sur la leucémie L1210 de la souris d'une combinaison par diazotation d'a méthoptérine et de yoglobulines de hamsters porteurs de cette leucémie par hétérogreffe, *CR Acad. Sci.* 3 (1958) 1626–1628.
- [34] C. De Duve, T. De Barys, B. Poole, A. Trouet, P. Tulkens, F. van Hoof, Lysosomotropic agents, *Biochem. Pharmacol.* 23 (1974) 2495–2531.
- [35] H. Ringsdorf, Structure and properties of pharmacologically active polymers, *J. Polym. Sci. Polym. Symp.* 51 (1975) 135–153.
- [36] L. Šprinc, J. Vacić, J. Kopeček, D. Lím, Biological tolerance of poly(N-substituted methacrylamides), *J. Biomed. Mater. Res.* 5 (1971) 197–205.
- [37] J. Kopeček, L. Šprinc, H. Bažilová, J. Vacić, Biological tolerance of poly(N-substituted acrylamides), *J. Biomed. Mater. Res.* 7 (1973) 111–121.
- [38] L. Šprinc, J. Kopeček, D. Lím, Effect of porosity of heterogeneous poly(glycol monomethacrylate) gels on the healing-in of test implants, *J. Biomed. Mater. Res.* 5 (1971) 447–458.
- [39] L. Šprinc, J. Vacić, J. Kopeček, D. Lím, Biological tolerance of ionogenic hydrophilic gels, *J. Biomed. Mater. Res.* 7 (1973) 123–136.
- [40] K. Ulbrich, L. Šprinc, J. Kopeček, Biocompatibility of poly(2,4-pentadiene-1ol), *J. Biomed. Mater. Res.* 8 (1974) 155–161.

- [41] J. Kopeček, L. Šprinc, Relationship between the structure and biocompatibility of hydrophilic gels, *Polim. Med.* 4 (1974) 109–117.
- [42] J. Kopeček, L. Šprinc, D. Lím, New types of synthetic infusion solutions. I. Investigation of the effect of solutions of some hydrophilic polymers on blood, *J. Biomed. Mater. Res.* 7 (1973) 179–191.
- [43] L. Šprinc, J. Exner, O. Štěrbá, J. Kopeček, New types of synthetic infusion solutions. III. Elimination and retention of poly[N-(2-hydroxypropyl)methacrylamide] in a test organism, *J. Biomed. Mater. Res.* 10 (1976) 953–963.
- [44] E. Paluska, J. Činát, L. Korčáková, O. Štěrbá, J. Kopeček, A. Hrubá, J. Nezvalová, R. Staněk, Immunosuppressive effect of a synthetic polymer–poly[N-(2-hydroxypropyl)methacrylamide] (Duxon), *Folia Biol.* 26 (1980) 304–311.
- [45] E. Paluska, A. Hrubá, O. Štěrbá, J. Kopeček, Effect of a synthetic poly[N-(2-hydroxypropyl)methacrylamide] (Duxon) on haemopoiesis and graft versus host reaction, *Folia Biol.* 32 (1986) 91–102.
- [46] J. Kopeček, H. Bažilová, Poly[N-(2-hydroxypropyl)methacrylamide]. 1. Radical polymerization and copolymerization, *Europ. Polym. J.* 9 (1973) 7–14.
- [47] M. Bohdanecký, H. Bažilová, J. Kopeček, Poly[N-(2-hydroxypropyl)methacrylamide]. II. Hydrodynamic properties of diluted polymer solutions, *Europ. Polym. J.* 10 (1974) 405–410.
- [48] J. Kopeček, K. Ulbrich, J. Vacík, J. Strohalm, V. Chytrý, J. Drobňík, J. Kálal, Copolymers based on N-substituted acrylamides, N-substituted methacrylamides and N,N-disubstituted acrylamides and the method of their manufacturing, U.S. Patent 4,062,831 (Dec.13,1977).
- [49] J. Drobňík, J. Kopeček, J. Labský, P. Rejmanová, J. Exner, J. Kálal, Preparation of biologically active substances bearing NH₂ groups in a form releasable by enzymatic cleavage, U.S. Patent 4,097,470 (June 27, 1978).
- [50] P. Rejmanová, J. Kopeček, R. Duncan, J.B. Lloyd, Stability in rat plasma and serum of lysozymally degradable oligopeptide sequences in N-(2-hydroxypropyl)methacrylamide copolymers, *Biomaterials* 6 (1985) 45–48.
- [51] P. Rejmanová, J. Pohl, M. Baudyš, V. Kostka, J. Kopeček, Polymers containing enzymatically degradable bonds. 8. Degradation of oligopeptide sequences in N-(2-hydroxypropyl)methacrylamide copolymers by bovine spleen cathepsin B, *Makromol. Chem.* 184 (1983) 2009–2020.
- [52] B. Řihová, J. Kopeček, Biological properties of targetable poly[N-(2-hydroxypropyl)methacrylamide]–antibody conjugates, *J. Control. Release* 2 (1985) 289–310.
- [53] B. Řihová, P. Kopečková, J. Strohalm, P. Rossmann, V. Větvíčka, J. Kopeček, Antibody directed affinity therapy applied to the immune system: *in vivo* effectiveness and limited toxicity of daunomycin conjugates to HPMa copolymers and targeting antibody, *Clin. Immunol. Immunopathol.* 46 (1988) 100–114.
- [54] Z.-R. Lu, J.-G. Shiah, S. Sakuma, P. Kopečková, J. Kopeček, Design of novel bioconjugates for targeted drug delivery, *J. Control. Release* 78 (2002) 165–173.
- [55] J. Kopeček, P. Kopečková, Design of polymer–drug conjugates, in: F. Kratz, P. Senter, H. Steinhagen (Eds.), *Drug Delivery in Oncology*, Vol. 2, Wiley-VCH, Weinheim, Germany, 2012, pp. 485–512.
- [56] T.M. Allen, Ligand-targeted therapeutics in anticancer therapy, *Nat. Rev. Cancer* 2 (2002) 750–763.
- [57] R. Duncan, Polymer therapeutics as nanomedicines: new perspectives, *Curr. Opin. Biotechnol.* 22 (2011) 492–501.
- [58] Y. Zhou, J. Kopeček, Biological rationale for the design of polymeric anti-cancer nanomedicines, *J. Drug Target.* 21 (2013) 1–26.
- [59] J. Kopeček, P. Kopečková, HPMa copolymers: origins, early developments, present, and future, *Adv. Drug Deliv. Rev.* 62 (2010) 122–149.
- [60] B.S. Tucker, B.S. Sumerlin, Poly(N-(2-hydroxypropyl) methacrylamide)-based nanotherapeutics, *Polym. Chem.* 5 (2014) 1566–1572.
- [61] J. Nakamura, N. Nakajima, K. Matsumura, S.H. Hyon, Water-soluble taxol conjugates with dextran and targets tumor cells by folic acid immobilization, *Anticancer Res.* 30 (2010) 903–909.
- [62] K. Inoue, E. Kumazawa, H. Kuga, H. Susaki, N. Masabuchi, T. Kajimura, CM-dextran-polyalcohol–camptothecin conjugate: DE-310 with a novel carrier system and its preclinical data, *Adv. Exp. Med. Biol.* 519 (2003) 145–153.
- [63] C. Li, R.A. Newman, Q.P. Wu, S. Ke, W. Chen, T. Hutto, Z. Kan, M.D. Brannan, C. Charnsangavej, S. Wallace, Biodistribution of paclitaxel and poly(L-glutamic acid)–paclitaxel conjugate in mice with ovarian OCa-1 tumor, *Cancer Chemother. Pharmacol.* 46 (2000) 416–422.
- [64] J.W. Singer, S. Shaffer, B. Baker, A. Bernareggi, S. Stromatt, D. Nienstedt, M. Besman, Paclitaxel polyglutex (XYOTAX; CT-2103): an intracellularly targeted taxane, *Anticancer Drugs* 16 (2005) 243–254.
- [65] P. Sabbatini, A. Aghajanian, D. Dizon, S. Anderson, J. Dupont, J.V. Brown, W.A. Peters, A. Jacobs, A. Mehdi, S. Rivkin, A.J. Eisenfeld, D. Spriggs, Phase II study of CT-2103 in patients with recurrent epithelial, ovarian, fallopian tube, or primary peritoneal carcinoma, *J. Clin. Oncol.* 22 (2004) 4523–4531.
- [66] H. Ding, G. Helguera, J.A. Rodriguez, J. Markman, R. Luria-Pérez, P. Gangalum, J. Portilla-Arias, S. Inoue, T.R. Daniels-Wells, K. Black, E. Holler, M.L. Penichet, J.Y. Ljubimova, Polymeric acid nanobioconjugate for simultaneous immunostimulation and inhibition of tumor growth in HER2/neu-positive breast cancer, *J. Control. Release* 171 (2013) 322–329.
- [67] H. Ding, J. Portilla-Arias, R. Patil, K.L. Black, J.Y. Ljubimova, E. Holler, Distinct mechanisms of membrane permeation induced by two polymeric acid copolymers, *Biomaterials* 34 (2013) 217–225.
- [68] R. Tomlinson, J. Heller, S. Brocchini, R. Duncan, Polyacetal–doxorubicin conjugates designed for pH-dependent degradation, *Bioconjug. Chem.* 14 (2003) 1096–1106.
- [69] R.M. England, E. Masiá, V. Giménez, R. Lucas, M.J. Vicent, Polyacetal–stilbene conjugates – the first examples of polymer therapeutics for the inhibition of HIF-1 in the treatment of solid tumors, *J. Control. Release* 164 (2012) 314–322.
- [70] A. Kakinoki, Y. Kaneo, Y. Ikeda, T. Tanaka, K. Fujita, Synthesis of poly(vinyl alcohol)–doxorubicin conjugates containing cis-aconityl acid-cleavable bond and its isomer dependent doxorubicin release, *Biol. Pharm. Bull.* 31 (2008) 103–110.
- [71] A. Kakinoki, Y. Kaneo, T. Tanaka, Y. Hosokawa, Synthesis and evaluation of water-soluble poly(vinyl alcohol)–paclitaxel conjugate as a macromolecular prodrug, *Biol. Pharm. Bull.* 31 (2008) 963–969.
- [72] W.J. Kim, M.S. Kang, H.K. Kim, Y. Kim, T. Chang, T. Ohulchanskyy, P.N. Prasad, K.S. Lee, Water-soluble porphyrin–polyethylene glycol conjugate with enhanced cellular uptake for photodynamic therapy, *J. Nanosci. Nanotechnol.* 9 (2009) 7130–7135.
- [73] P. Chadna, J.J. Khandare, E. Ber, L. Rodriguez-Rodriguez, T. Minko, Multifunctional tumor-targeted polymer–peptide–drug delivery system for treatment of primary and metastatic cancers, *Pharm. Res.* 27 (2011) 2296–2306.
- [74] I. Conejos-Sánchez, I. Cardoso, M.J. Saraiva, M.J. Vicent, Targeting a rare amyloidotic disease through rationally designed polymer conjugates, *J. Control. Release* 178 (2014) 95–100.
- [75] S. Van, S.K. Das, X. Wang, Z. Feng, Y. Jin, Z. Hou, F. Chen, A. Pham, N. Jiang, S.B. Howell, L. Yu, Synthesis, characterization, and biological evaluation of poly(L-γ-glutamyl–glutamine)–paclitaxel conjugate, *Int. J. Nanomedicine* 5 (2010) 825–837.
- [76] P.A. Vasey, S.B. Kaye, R. Morrison, C. Twelves, P. Wilson, R. Duncan, A.H. Thomson, L.S. Murray, T.E. Hilditch, T. Murray, S. Burtles, D. Fraier, E. Frigerio, J. Cassidy, and on behalf of the Cancer Research Campaign Phase I/II Committee, Phase I clinical and pharmacokinetic study of PK1 [N-(2-hydroxypropyl)methacrylamide copolymer doxorubicin]: first member of a new class of chemotherapeutic agents–drug–polymer conjugates, *Clin. Cancer Res.* 5 (1999) 83–94.
- [77] L.W. Seymour, D.R. Ferry, D. Anderson, S. Hesselwood, P.J. Julyan, R. Poyner, J. Doran, A.M. Young, S. Burtles, D.J. Kerr, Hepatic drug targeting: phase I evaluation of polymer-bound doxorubicin, *J. Clin. Oncol.* 20 (2002) 1668–1676.
- [78] J.M. Rademaker-Lakhai, C. Terret, S.B. Howell, C.M. Baud, R.F. De Boer, D. Pluim, J.H. Beijnen, J.H. Schellens, J.P. Droz, A phase I and pharmacological study of the platinum polymer AP5280 given as an intravenous infusion once every 3 weeks in patients with solid tumors, *Clin. Cancer Res.* 10 (2004) 3386–3395.
- [79] J.B. Lloyd, Lysosomal membrane permeability: implications for drug delivery, *Adv. Drug Deliv. Rev.* 41 (2000) 189–200.
- [80] K. Ulbrich, V. Subr, Polymeric anticancer drugs with pH-controlled activation, *Adv. Drug Deliv. Rev.* 56 (2004) 1023–1050.
- [81] H. Nakamura, T. Etrych, P. Chytil, M. Ohkubo, J. Fang, K. Ulbrich, H. Maeda, Two step mechanism of tumor selective delivery of N-(2-hydroxypropyl) methacrylamide copolymer conjugated with piarubicin via an acid-cleavable linkage, *J. Control. Release* 174 (2014) 81–87.
- [82] T. Etrych, M. Jelinková, B. Řihová, K. Ulbrich, New HPMa copolymers containing doxorubicin bound via pH-sensitive linkage: synthesis and preliminary *in vitro* and *in vivo* properties, *J. Control. Release* 73 (2001) 89–102.
- [83] W.-C. Shen, H.J.-P. Ryser, Cis-aconityl spacer between daunomycin and macromolecular carriers: a model of pH-sensitive linkage releasing drug from a lysosomotropic conjugate, *Biochem. Biophys. Res. Commun.* 102 (1981) 1048–1054.
- [84] D.B. Rozema, K. Ekena, D.L. Lewis, A.G. Loomis, J.A. Wolff, Endosomolysis by masking of a membrane-active agent (EMMA) for cytoplasmic release of macromolecules, *Bioconjug. Chem.* 14 (2003) 51–57.
- [85] J. Kopeček, P. Rejmanová, Enzymatically degradable bonds in synthetic polymers, in: S.D. Bruck (Ed.), *Controlled Drug Delivery*, vol. I, CRC Press, Boca Raton, Florida, 1983, pp. 81–124.
- [86] J. Kopeček, Biodegradation of polymers for biomedical use, in: H. Benoit, P. Rempp (Eds.), *IUPAC Macromolecules*, Oxford, Pergamon Press, 1982, pp. 305–320.
- [87] J. Kopeček, P. Rejmanová, V. Chytrý, Polymers containing enzymatically degradable bonds 1. Chymotrypsin catalyzed hydrolysis of p-nitroanilides of phenylalanine and tyrosine attached to side-chains of copolymers of N-(2-hydroxypropyl) methacrylamide, *Makromol. Chem.* 182 (1981) 799–809.
- [88] K. Ulbrich, J. Strohalm, J. Kopeček, Polymers containing enzymatically degradable bonds. 3. Poly[N-(2-hydroxypropyl)methacrylamide] chains connected by oligopeptide sequences cleavable by trypsin, *Makromol. Chem.* 182 (1981) 1917–1928.
- [89] K. Ulbrich, E.I. Zacharieva, B. Obereigner, J. Kopeček, Polymers containing enzymatically degradable bonds. 5. Hydrophilic polymers degradable by papain, *Biomaterials* 1 (1980) 199–204.
- [90] J. Kopeček, Controlled degradability of polymers – a key to drug delivery systems, *Biomaterials* 5 (1984) 19–25.
- [91] J. Kopeček, I. Cifková, P. Rejmanová, J. Strohalm, B. Obereigner, K. Ulbrich, Polymers containing enzymatically degradable bonds. 4. Preliminary experiments *in vivo*, *Makromol. Chem.* 182 (1981) 2941–2949.
- [92] R. Duncan, H.C. Cable, J.B. Lloyd, P. Rejmanová, J. Kopeček, Polymers containing enzymatically degradable bonds. 7. Design of oligopeptide side-chains in poly[N-(2-hydroxypropyl)methacrylamide] copolymers to promote efficient degradation by lysosomal enzymes, *Makromol. Chem.* 184 (1983) 1997–2008.
- [93] V. Subr, J. Kopeček, J. Pohl, M. Baudyš, V. Kostka, Cleavage of oligopeptide side-chains in N-(2-hydroxypropyl)methacrylamide copolymers by mixtures of lysosomal enzymes, *J. Control. Release* 8 (1988) 133–140.
- [94] A. Nan, H. Ghandehari, C. Harbert, H. Siavash, N. Nikitakis, M. Reynolds, J.J. Sauk, Water-soluble polymers for targeted drug delivery to human squamous carcinoma of head and neck, *J. Drug Target.* 13 (2005) 190–197.
- [95] M. Manea, J. Tóvári, M. Tejeda, A. Schulz, B. Kapuvári, B. Voncze, G. Mezo, In-vivo antitumour effect of daunorubicin–GNRH-III derivative conjugates on colon carcinoma-bearing mice, *Anticancer Drugs* 23 (2012) 90–97.

- [96] Z.H. Peng, M. Sima, M.E. Salama, P. Kopečková, J. Kopeček, Spacer length impacts the efficacy of targeted docetaxel conjugates in prostate-specific membrane antigen expressing prostate cancer, *J. Drug Target.* 21 (2013) 968–980.
- [97] S.O. Doronina, T.D. Bovee, D.W. Meyer, J.B. Miyamoto, M.E. Anderson, C.A. Morris-Tilden, P.D. Senter, Novel peptide linkers for highly potent antibody–auristatin conjugate, *Bioconjug. Chem.* 19 (2008) 1960–1963.
- [98] P.J. Burke, P.D. Senter, D.W. Meyer, J.B. Miyamoto, M. Anderson, B.E. Toki, G. Manikumar, M.C. Wani, D.J. Krol, S.C. Jeffrey, Design, synthesis, and biological evaluation of antibody–drug conjugates comprised of potent camptothecin analogues, *Bioconjug. Chem.* 20 (2009) 1242–1250.
- [99] P.L. Carl, P.K. Chakravarty, J.A. Katzenellenbogen, A novel connector linkage applicable in prodrug design, *J. Med. Chem.* 24 (1981) 479–480.
- [100] F.M. de Groot, W.J. Loos, R. Koelsch, L.W. van Berkum, G.F. Busscher, A.E. Seelen, C. Albrecht, P. de Bruijn, H.W. Scheeren, Elongated multiple electronic cascade and cyclization spacer systems in activatable anticancer prodrugs for enhanced drug release, *J. Org. Chem.* 66 (2001) 8815–8830.
- [101] B.E. Toki, C.G. Cerveny, A.F. Wahl, P.D. Senter, Protease-mediated fragmentation of p-amidobenzyl ethers: a new strategy for the activation of anticancer prodrugs, *J. Org. Chem.* 67 (2002) 1866–1872.
- [102] S. Gao, Z. Lu, B. Petri, P. Kopečková, J. Kopeček, Colon-specific 9-aminocamptothecin-HPMA copolymer conjugates containing a 1,6-elimination spacer, *J. Control. Release* 110 (2006) 323–331.
- [103] H.Z. Pan, P. Kopečková, D. Wang, J. Yang, S. Miller, J. Kopeček, Water-soluble HPMA copolymer–prostaglandin conjugates containing a cathepsin K sensitive spacer, *J. Drug Target.* 14 (2006) 425–435.
- [104] J.-G. Shiah, Y. Sun, P. Kopečková, C.M. Peterson, R.C. Straight, J. Kopeček, Combination chemotherapy and photodynamic therapy of targetable N-(2-hydroxypropyl) methacrylamide copolymer–doxorubicin/mesochlorin e₆–OV-TL16 antibody immunoconjugates, *J. Control. Release* 74 (2001) 249–253.
- [105] M. Mammen, S.K. Choi, G.M. Whitesides, Polyvalent interactions in biological systems: implications for design and use of multivalent ligands and inhibitors, *Angew. Chem. Int. Ed.* 3 (1998) 2754–2794.
- [106] R.N. Johnson, P. Kopečková, J. Kopeček, Synthesis and evaluation of multivalent branched HPMA copolymer–Fab' conjugates targeted to the B-cell antigen, *Bioconjug. Chem.* 20 (2009) 129–137.
- [107] T.W. Chu, J. Yang, J. Kopeček, Anti-CD20 multivalent HPMA copolymer–Fab' conjugates for the direct induction of apoptosis, *Biomaterials* 33 (2012) 7174–7181.
- [108] R.N. Johnson, P. Kopečková, J. Kopeček, Biological activity of anti-CD20 multivalent HPMA copolymer–Fab' conjugates, *Biomacromolecules* 13 (2012) 727–735.
- [109] A. David, P. Kopečková, A. Rubinstein, J. Kopeček, Enhanced biorecognition and internalization of HPMA copolymers containing multi- or multivalent carbohydrate side-chains by human hepatocarcinoma cells, *Bioconjug. Chem.* 12 (2001) 890–899.
- [110] A. David, P. Kopečková, T. Minko, A. Rubinstein, J. Kopeček, Design of multivalent galactoside ligand for selective targeting of HPMA copolymers–doxorubicin conjugates to human colon cancer cells, *Eur. J. Cancer* 40 (2004) 148–157.
- [111] A. Tang, P. Kopečková, J. Kopeček, Binding and cytotoxicity of HPMA copolymers to lymphocytes mediated by receptor-binding epitopes, *Pharm. Res.* 20 (2003) 360–367.
- [112] V. Cuchelkar, P. Kopečková, J. Kopeček, Novel HPMA copolymer-bound constructs for combined tumor and mitochondrial targeting, *Mol. Pharm.* 5 (2008) 696–709.
- [113] A. Rebuffat, A. Bernasconi, M. Ceppi, H. Wehrli, S. Brenz Verca, M. Ibrahim, B.M. Frey, F.J. Frey, S. Rusconi, Selective enhancement of gene transfer by steroid-mediated gene delivery, *Nat. Biotechnol.* 19 (2001) 1155–1161.
- [114] V. Cuchelkar, Ph.D. Dissertation, University of Utah, Department of Bioengineering, 2008.
- [115] R. Duncan, P. Rejmanová, J. Kopeček, J.B. Lloyd, Pinocytic uptake and intracellular degradation of N-(2-hydroxypropyl)methacrylamide copolymers. A potential drug delivery system, *Biochim. Biophys. Acta* 678 (1981) 143–150.
- [116] R. Duncan, H.C. Cable, P. Rejmanová, J. Kopeček, J.B. Lloyd, Tyrosinamide residues enhance pinocytic capture of N-(2-hydroxypropyl)methacrylamide copolymers, *Biochim. Biophys. Acta* 799 (1984) 1–8.
- [117] J. Liu, P. Kopečková, P. Bühler, P. Wolf, H. Pan, H. Bauer, U. Elsässer-Beile, J. Kopeček, Biorecognition and subcellular trafficking of HPMA copolymer–anti-PMSA antibody conjugates by prostate cancer cells, *Mol. Pharm.* 6 (2009) 959–970.
- [118] J. Callahan, P. Kopečková, J. Kopeček, The intracellular trafficking and subcellular distribution of a large array of HPMA copolymer conjugates, *Biomacromolecules* 10 (2009) 1704–1714.
- [119] M. Tijerina, P. Kopečková, J. Kopeček, Correlation of subcellular compartmentalization of HPMA copolymer–Mce₆ conjugates with chemotherapeutic activity in human ovarian carcinoma cells, *Pharm. Res.* 20 (2003) 728–737.
- [120] M. Tijerina, P. Kopečková, J. Kopeček, Mechanism of cytotoxicity in human ovarian carcinoma cells exposed to free Mce₆ or HPMA copolymer–Mce₆ conjugates, *Photochem. Photobiol.* 77 (2003) 645–652.
- [121] D.B. Rozema, D.L. Lewis, D.H. Wakefield, S.C. Wong, J.J. Klein, P.L. Roesch, S.L. Bertin, T.W. Reppent, Q. Chu, A.V. Blokhin, J.E. Hagstrom, J.A. Wolff, Dynamic polyconjugates for targeted *in vivo* delivery of siRNA to hepatocytes, *Proc. Natl. Acad. Sci. U. S. A.* 104 (2007) 12982–12987.
- [122] B.B. Lundy, A. Convertine, M. Miteva, P.S. Stayton, Neutral polymeric micelles for RNA delivery, *Bioconjug. Chem.* 24 (2013) 398–407.
- [123] K. Gunasekaran, T.H. Nguyen, H.D. Maynard, T.P. Davis, V. Bulmus, Conjugation of siRNA with comb-type PEG enhances serum stability and gene silencing efficiency, *Macromol. Rapid Commun.* 32 (2011) 654–659.
- [124] M.E. Fox, F.C. Szóka, J.M. Fréchet, Soluble polymer carriers for the treatment of cancer: the importance of molecular architecture, *Acc. Chem. Res.* 42 (2009) 1141–1151.
- [125] B. Chen, D.G. van der Poll, K. Jerger, W.C. Floyd, J.M. Fréchet, F.C. Szóka, Synthesis and properties of star-comb polymers and their doxorubicin conjugates, *Bioconjug. Chem.* 22 (2011) 617–624.
- [126] K. Ulbrich, V. Šubr, Structural and chemical aspects of HPMA copolymers as drug carriers, *Adv. Drug Deliv. Rev.* 62 (2010) 150–166.
- [127] T. Etrych, V. Šubr, J. Strohalm, M. Šírová, B. Říhová, K. Ulbrich, HPMA copolymer–doxorubicin conjugates: the effects of molecular weight and architecture on biodistribution and *in vivo* activity, *J. Control. Release* 164 (2012) 346–354.
- [128] K. Ulbrich, Č. Koňák, Z. Tuzar, J. Kopeček, Solution properties of drug carriers based on poly[N-(2-hydroxypropyl)methacrylamide] containing biodegradable bonds, *Makromol. Chem.* 188 (1987) 1261–1272.
- [129] H. Ding, P. Kopečková, J. Kopeček, Self-association properties of HPMA copolymers containing an amphipathic heptapeptide, *J. Drug Target.* 15 (2007) 465–474.
- [130] M. Allmeroth, D. Mederegger, B. Biesalski, K. Koynov, F. Rösch, O. Thews, R. Zentel, Modifying the body distribution of HPMA-based copolymers by molecular weight and aggregate formation, *Biomacromolecules* 12 (2011) 2841–2849.
- [131] H. Maeda, Tumor-selective delivery of macromolecular drugs via the EPR effect: background and future prospects, *Bioconjug. Chem.* 21 (2010) 797–802.
- [132] Y. Matsumura, H. Maeda, A new concept for macromolecular therapeutics in cancer therapy: mechanism of tumorotropic accumulation of proteins and the antitumor agent SMANCS, *Cancer Res.* 46 (1986) 6387–6392.
- [133] L.W. Seymour, R. Duncan, J. Strohalm, J. Kopeček, Effect of molecular weight of N-(2-hydroxypropyl)methacrylamide copolymers on body distribution and rate of excretion after subcutaneous, intraperitoneal and intravenous administration, *J. Biomed. Mater. Res.* 21 (1987) 1341–1358.
- [134] Y. Noguchi, J. Wu, R. Duncan, J. Strohalm, K. Ulbrich, T. Akaike, H. Maeda, Early phase tumor accumulation of macromolecules: a great difference in clearance rate between tumor and normal tissues, *Jpn. J. Cancer Res.* 89 (1998) 307–314.
- [135] T. Etrych, J. Strohalm, B. Chytil, B. Říhová, K. Ulbrich, Novel star HPMA-based polymer conjugates for passive targeting to solid tumors, *J. Drug Target.* 19 (2011) 874–889.
- [136] J.P. Medema, Cancer stem cells: the challenges ahead, *Nat. Cell Biol.* 15 (2013) 338–344.
- [137] Y. Zhou, J. Yang, J. Kopeček, Cancer stem cells: potential target for anti-cancer nanomedicines, in: C. Scholz, J. Kressler (Eds.), Tailored polymer architectures for pharmaceutical and biomedical applications, ACS Symposium Series, 1135, American Chemical Society, Washington, D.C., 2013, pp. 127–149.
- [138] A. Dubrovskaja, J. Elliott, R.J. Salamone, S. Kim, L.J. Aimone, J.R. Walker, J. Watson, M. Sauveteur-Michel, C. Garcia-Echeverria, C.Y. Cho, V.A. Reddy, P.G. Schultz, Combination therapy targeting both tumor-initiating and differentiated cell populations in prostate carcinoma, *Clin. Cancer Res.* 16 (2010) 5692–5702.
- [139] Y. Zhou, J. Yang, J. Kopeček, Selective inhibitory effect of HPMA copolymer–cyclopamine conjugate on prostate cancer stem cells, *Biomaterials* 33 (2012) 1863–1872.
- [140] J. Gao, S. Graves, U. Koch, S. Liu, V. Jankovic, S. Buonamici, A.E.I. Andaloussi, S.D. Nimer, B.L. Kee, R. Taichman, F. Radtke, I. Aifantis, Hedgehog signaling is dispensable for adult hematopoietic stem cell function, *Cell Stem Cell* 4 (2009) 548–558.
- [141] I. Hofmann, E.H. Stover, D.E. Cullen, J. Mao, K.J. Morgan, B.H. Lee, M.G. Kharas, P.G. Miller, M.G. Cornejo, R. Okabe, S.A. Armstrong, N. Ghilardi, S. Gould, F.J. de Sauvage, A.P. McMahon, D.G. Gilliland, Hedgehog signaling is dispensable for adult murine hematopoietic stem cell function and hematopoiesis, *Cell Stem Cell* 4 (2009) 559–567.
- [142] Y. Gu, H. Li, J. Miki, K.H. Kim, B. Furusato, I.A. Sesterhenn, W.S. Chu, D.G. McLeod, S. Srivastava, C.M. Ewing, W.B. Isaacs, J.S. Rhim, Phenotypic characterization of telomerase-immortalized primary nonmalignant and malignant tumor-derived human prostate epithelial cell lines, *Exp. Cell Res.* 312 (2006) 831–843.
- [143] Y. Zhou, J. Yang, J.S. Rhim, J. Kopeček, HPMA copolymer-based combination therapy toxic to both prostate cancer stem/progenitor cells and differentiated cells induces durable anti-tumor effects, *J. Control. Release* 172 (2013) 946–953.
- [144] "Top story", *Prostate Cell News* 4:36 September 20, 2013.
- [145] J. Miki, B. Furusato, H. Li, Y. Gu, H. Takahashi, S. Egawa, I.A. Sesterhenn, D.G. McLeod, S. Srivastava, J.S. Rhim, Identification of putative stem cell markers, CD133 and CXCR4, in hTERT-immortalized primary nonmalignant and malignant tumor-derived human prostate epithelial cell lines and in prostate cancer specimens, *Cancer Res.* 67 (2007) 3153–3161.
- [146] G.D. Richardson, C.N. Robson, S.H. Lang, D.E. Neal, N.J. Maitland, A.T. Collins, CD133, a novel marker for human prostatic epithelial stem cells, *J. Cell Sci.* 117 (2004) 7180–7185.
- [147] K. Kemper, M.R. Sprick, M. de Bree, A. Scopelliti, L. Vermeulen, M. Hoek, J. Zeilstra, S.T. Pals, H. Mehmet, G. Stassi, J.P. Medema, The AC133 epitope, but not the CD133 protein, is lost upon cancer stem cell differentiation, *Cancer Res.* 70 (2010) 719–729.
- [148] B. Campos, C.C. Herold-Mende, Insight into the complex regulation of CD133 in glioma, *Int. J. Cancer* 128 (2011) 501–510.
- [149] P. Friedl, S. Alexander, Cancer invasion and the microenvironment: plasticity and reciprocity, *Cell* 147 (2011) 992–1009.
- [150] K.P. Olive, M.A. Jacobetz, C.J. Davidson, A. Gopinathan, D. McIntyre, D. Honess, B. Madhu, M.A. Goldgraben, M.E. Caldwell, D. Allard, K.K. Frese, G. Denicola, C. Feig, C. Combs, S.P. Winter, H. Ireland-Zecchini, S. Reichelt, W.J. Howat, A. Chang, M. Dhara, L. Wang, F. Rückert, R. Grützmann, C. Pilarsky, K. Izeradjene, S.R. Hingorani, P. Huang, S.E. Davies, W. Plunkett, M. Egorin, R.H. Hruban, N. Whitebread, K. McGovern, J. Adams, C. Iacobuzio-Donahue, J. Griffiths, D.A. Tuveson, Inhibition of

- hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer, *Science* 324 (2009) 1457–1461.
- [151] C. Whatcott, H. Han, R.G. Posner, D.D. Von Hoff, Tumor–stromal interactions in pancreatic cancer, *Crit. Rev. Oncog.* 18 (2013) 135–151.
 - [152] P.P. Provenzano, S.R. Hingorani, Hyaluronan, fluid pressure, and stromal resistance in pancreas cancer, *Brit. J. Cancer* 108 (2013) 1–8.
 - [153] P.P. Provenzano, C. Cuevas, A.E. Chang, V.K. Goel, D.D. Von Hoff, S.R. Hingorani, Enzymatic targeting of the stroma ablates physical barriers to treatment of pancreatic ductal adenocarcinoma, *Cancer Cell* 21 (2012) 418–429.
 - [154] M.A. Jacobetz, D.S. Chan, A. Neesse, T.E. Bapiro, N. Cook, K.K. Frese, C. Feig, T. Nakagawa, M.E. Caldwell, H.I. Zecchini, M.P. Lolkema, P. Jiang, A. Kultti, C.B. Thompson, D.C. Maneval, D.I. Jodrell, G.I. Frost, H.M. Shepard, J.N. Skepper, D.A. Tuveson, Hyaluronan impairs vascular function and drug delivery in a mouse model of pancreatic cancer, *Gut* 62 (2013) 112–120.
 - [155] L.C. Murtaugh, Pathogenesis of pancreatic cancer: lessons from animal models, *Toxicol. Pathol.* 42 (2014) 217–228.
 - [156] N. Cook, K.P. Olive, K. Frese, D.A. Tuveson, K-Ras-driven pancreatic cancer mouse model for anticancer inhibitor analyses, *Meth. Enzymol.* 439 (2008) 73–85.
 - [157] J.P. Morton, P. Timpson, S.A. Karim, R.A. Ridgway, D. Athineos, B. Doyle, N.B. Jamieson, K.A. Oien, A.M. Lowy, V.G. Brunton, M.C. Frame, T.R. Evans, O.J. Sansom, Mutant p53 drives metastasis and overcomes growth arrest/senescence in pancreatic cancer, *Proc. Natl. Acad. Sci. U. S. A.* 107 (2010) 246–251.
 - [158] A. Jimeno, G. Feldmann, A. Suarez-Gauthier, Z. Rasheed, A. Solomon, G.M. Zou, B. Rubio-Viqueira, E. García-García, F. López-Ríos, W. Matsui, A. Maitra, M. Hidalgo, A direct pancreatic cancer xenograft model as a platform for cancer stem cell therapeutic development, *Mol. Cancer Ther.* 8 (2009) 310–314.
 - [159] B. Buckway, Y. Wang, A. Ray, H. Ghandehari, Overcoming the stromal barrier for targeted delivery of HPMA copolymers to pancreatic tumors, *Int. J. Pharm.* 456 (2013) 202–211.
 - [160] M.M. Gottesman, T. Fojo, S.F. Bates, Multidrug resistance in cancer: role of ATP-dependent transporters, *Nat. Rev. Cancer* 2 (2002) 48–58.
 - [161] J.I. Fletcher, M. Haber, M.J. Henderson, M.D. Norris, ABC transporters in cancer: more than just drug efflux pumps, *Nat. Rev. Cancer* 10 (2010) 147–156.
 - [162] S.G. Aller, J. Yu, A. Ward, S. Chittaboina, R. Zhuo, P.M. Harrell, Y.T. Trinh, Q. Zhang, I.L. Urbatsch, G. Chang, Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding, *Science* 323 (2009) 1718–1722.
 - [163] V. Omelyanenko, P. Kopečková, C. Gentry, J. Kopeček, Targetable HPMA copolymer–adriamycin conjugates. Recognition, internalization, and subcellular fate, *J. Control. Release* 53 (1998) 25–37.
 - [164] T. Minko, P. Kopečková, J. Kopeček, Efficacy of chemotherapeutic action of HPMA copolymer-bound doxorubicin in a solid tumor model of ovarian carcinoma, *Int. J. Cancer* 86 (2000) 108–117.
 - [165] N.L. Krinick, Y. Sun, D. Joyner, J.D. Spikes, R.C. Straight, J. Kopeček, A polymeric drug delivery system for the simultaneous delivery of drugs activatable by enzymes and/or light, *J. Biomater. Sci. Polym. Ed.* 5 (1994) 303–324.
 - [166] C.M. Peterson, J.M. Lu, Y. Sun, C.A. Peterson, J.-G. Shiah, R.C. Straight, J. Kopeček, Combination chemotherapy and photodynamic therapy with N-(2-hydroxypropyl)methacrylamide copolymer-bound anticancer drugs inhibit human ovarian carcinoma heterotransplanted in nude mice, *Cancer Res.* 56 (1996) 3980–3985.
 - [167] J.-G. Shiah, Y. Sun, C.M. Peterson, R.C. Straight, J. Kopeček, Antitumor activity of HPMA copolymer–meso chlorin e₆ and adriamycin conjugates in combination treatments, *Clin. Cancer Res.* 6 (2000) 1008–1015.
 - [168] J. Hongrapipat, P. Kopečková, S. Prakongpan, J. Kopeček, Enhanced antitumor activity of combinations of free and HPMA copolymer-bound drugs, *Int. J. Pharm.* 351 (2008) 259–270.
 - [169] J. Hongrapipat, P. Kopečková, J. Liu, S. Prakongpan, J. Kopeček, Combination chemotherapy and photodynamic therapy with Fab' fragment targeted HPMA copolymer conjugates in human ovarian carcinoma cells, *Mol. Pharm.* 5 (2008) 696–709.
 - [170] N. Larson, J. Yang, A. Ray, D.L. Cheney, H. Ghandehari, J. Kopeček, Ovarian cancer combination therapy using biodegradable multiblock poly[N-(2-hydroxypropyl)methacrylamide] gemcitabine and paclitaxel conjugates, *Int. J. Pharm.* 454 (2013) 435–443.
 - [171] A. Duangjai, K. Luo, Y. Zhou, J. Yang, J. Kopeček, Combination cytotoxicity of backbone degradable HPMA copolymer gemcitabine and platinum conjugates toward human ovarian carcinoma cells, *Eur. J. Pharm. Biopharm.* (2014) (<http://dx.doi.org/10.1016/j.ejpb.2013.11.008>).
 - [172] R. Zhang, J. Yang, M. Sima, Y. Zhou, J. Kopeček, Sequential Combination Therapy of Ovarian Cancer with Backbone Degradable HPMA Copolymer Paclitaxel and Gemcitabine Conjugates, 2014. (submitted for publication).
 - [173] S.A. Low, J. Kopeček, Targeting polymer therapeutics to bone, *Adv. Drug Deliv. Rev.* 64 (2012) 1189–1204.
 - [174] D. Wang, S. Miller, P. Kopečková, J. Kopeček, Bone-targeting macromolecular therapeutics, *Adv. Drug Deliv. Rev.* 57 (2005) 1049–1076.
 - [175] X.M. Liu, S.C. Miller, D. Wang, Beyond oncology — application of HPMA copolymers in non-cancerous diseases, *Adv. Drug Deliv. Rev.* 62 (2010) 258–271.
 - [176] F. Yuan, L.D. Quan, L. Cui, S.R. Goldring, D. Wang, Development of macromolecular prodrug for rheumatoid arthritis, *Adv. Drug Deliv. Rev.* 64 (2012) 1205–1219.
 - [177] A. Nan, N.P. Nanayakkara, L.A. Walker, V. Yardley, S.L. Croft, H. Ghandehari, N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers for targeted delivery of 8-aminoquinoline antileishmanial drugs, *J. Control. Release* 77 (2001) 233–243.
 - [178] A. Nan, S.L. Croft, V. Yardley, H. Ghandehari, Targetable water-soluble polymer–drug conjugates for the treatment of visceral leishmaniasis, *J. Control. Release* 94 (2004) 115–127.
 - [179] S. Nicoletti, K. Seifert, I.H. Gilbert, N-(2-hydroxypropyl)methacrylamide–amphotericin B (HPMA–AmB) copolymer conjugates as antileishmanial agents, *Int. J. Antimicrob. Agents* 33 (2009) 441–448.
 - [180] S. Nicoletti, K. Seifert, I.H. Gilbert, Water-soluble polymer–drug conjugates for combination chemotherapy against visceral leishmaniasis, *Bioorg. Med. Chem.* 18 (2010) 2559–2565.
 - [181] D. Wang, S.C. Miller, M. Sima, D. Parker, H. Buswell, K.C. Goodrich, P. Kopečková, J. Kopeček, The arthrotropism of macromolecules in adjuvant-induced arthritis rat model: a preliminary study, *Pharm. Res.* 21 (2004) 1741–1749.
 - [182] L.D. Quan, P.E. Purdue, X.M. Liu, M.D. Boska, S.M. Lele, G.M. Thiele, T.R. Mikuls, H. Dou, S.R. Goldring, D. Wang, Development of a macromolecular prodrug for the treatment of inflammatory arthritis: mechanism involved in arthrotropism and sustained therapeutic efficacy, *Arthritis Res. Ther.* 12 (2010) R170, <http://dx.doi.org/10.1186/ar3130>.
 - [183] L. Quan, Y. Zhang, B.J. Crielgaard, A. Dusat, S.M. Lele, C.J. Rijcken, J.M. Metsalaar, H. Kostková, T. Etrych, K. Ulbrich, F. Kiessling, T.R. Mikuls, W.E. Hennink, G. Storm, T. Lammers, D. Wang, Nanomedicines for inflammatory arthritis: head-to-head comparison of glucocorticoid-containing polymers, micelles, and liposomes, *ACS Nano* 8 (2014) 458–466.
 - [184] J. Kopeček, Biomaterials and drug delivery — past, present, and future, *Mol. Pharm.* 7 (2010) 922–925.
 - [185] J. Yang, C. Xu, C. Wang, J. Kopeček, Refolding hydrogels self-assembled from HPMA graft copolymers by antiparallel coiled-coil formation, *Biomacromolecules* 7 (2006) 1187–1195.
 - [186] J. Yang, K. Wu, Č. Koňák, J. Kopeček, Dynamic light scattering study of the self-assembly of HPMA hybrid graft copolymers, *Biomacromolecules* 9 (2008) 510–517.
 - [187] J. Kopeček, J. Yang, Smart, self-assembled hybrid hydrogel materials, *Angew. Chem. Int. Ed.* 51 (2012) 7396–7417.
 - [188] M. Dvořák, P. Kopečková, J. Kopeček, High-molecular weight HPMA copolymer–adriamycin conjugates, *J. Control. Release* 60 (1999) 321–332.
 - [189] J.-G. Shiah, M. Dvořák, P. Kopečková, Y. Sun, C.M. Peterson, J. Kopeček, Biodistribution and antitumor efficacy of long-circulating N-(2-hydroxypropyl)methacrylamide copolymer–doxorubicin conjugates in nude mice, *Eur. J. Cancer* 37 (2001) 131–139.
 - [190] G. Moad, E. Rizzardo, S.H. Thang, Toward living radical polymerization, *Acc. Chem. Res.* 41 (2008) 1133–1142.
 - [191] C.W. Scales, Y.A. Vasilieva, A.J. Convertine, A.B. Lowe, C.L. McCormick, Direct, controlled synthesis of the nonimmunogenic, hydrophilic polymer, poly(N-(2-hydroxypropyl)methacrylamide) via RAFT in aqueous media, *Biomacromolecules* 6 (2005) 1846–1850.
 - [192] C.D. Hein, X.M. Liu, D. Wang, Click chemistry, a powerful tool for pharmaceutical sciences, *Pharm. Res.* 25 (2008) 2216–2230.
 - [193] J.C. Jewett, C.R. Bertozzi, Cu-free click cycloaddition reactions in chemical biology, *Chem. Soc. Rev.* 39 (2010) 1272–1279.
 - [194] C.E. Hoyle, C.N. Bowman, Thiol–ene click chemistry, *Angew. Chem. Int. Ed.* 49 (2010) 1540–1573.
 - [195] J. Yang, K. Luo, H. Pan, P. Kopečková, J. Kopeček, Synthesis of biodegradable multiblock copolymers by click coupling of RAFT-generated heterotelechelic polyHPMA conjugates, *React. Funct. Polym.* 71 (2011) 294–302.
 - [196] K. Luo, J. Yang, P. Kopečková, J. Kopeček, Biodegradable multiblock N-(2-hydroxypropyl)methacrylamide copolymers via reversible addition–fragmentation chain transfer polymerization and click chemistry, *Macromolecules* 44 (2011) 2481–2488.
 - [197] H. Pan, J. Yang, P. Kopečková, J. Kopeček, Backbone degradable multiblock N-(2-hydroxypropyl)methacrylamide copolymer conjugates via reversible addition–fragmentation chain transfer polymerization and thiol–ene coupling reaction, *Biomacromolecules* 12 (2011) 247–252.
 - [198] H. Pan, M. Sima, J. Yang, J. Kopeček, Synthesis of long-circulating, backbone degradable HPMA copolymer–doxorubicin conjugates and evaluation of molecular-weight-dependent antitumor efficacy, *Macromol. Biosci.* 13 (2013) 155–160.
 - [199] R. Zhang, K. Luo, J. Yang, M. Sima, Y. Sun, M.M. Janát-Amsbury, J. Kopeček, Synthesis and evaluation of a backbone biodegradable multiblock HPMA copolymer nanocarrier for the systemic delivery of paclitaxel, *J. Control. Release* 166 (2013) 66–74.
 - [200] H. Pan, M. Sima, S.C. Miller, P. Kopečková, J. Yang, J. Kopeček, Efficiency of high molecular weight backbone degradable HPMA copolymer–prostaglandin E1 conjugate in promotion of bone formation in ovariectomized rats, *Biomaterials* 34 (2013) 6528–6538.
 - [201] K. Wu, J. Liu, R.N. Johnson, J. Yang, J. Kopeček, Drug-free macromolecular therapeutics: induction of apoptosis by coiled-coil-mediated cross-linking of antigens on the cell surface, *Angew. Chem. Int. Ed.* 49 (2010) 1451–1455.
 - [202] K.G. Wu, J. Yang, J. Liu, J. Kopeček, Coiled-coil based drug-free macromolecular therapeutics: *in vivo* efficacy, *J. Control. Release* 157 (2012) 126–131.
 - [203] T.W. Chu, J. Yang, R. Zhang, M. Sima, J. Kopeček, Cell surface self-assembly of hybrid nanoconjugates via oligonucleotide hybridization induces apoptosis, *ACS Nano* 8 (2014) 719–730.
 - [204] D.A. Parry, R.D. Fraser, J.M. Squire, Fifty years of coiled-coils and α -helical bundles: a close relationship between sequence and structure, *J. Struct. Biol.* 163 (2008) 258–269.
 - [205] J.Y. Su, R.S. Hodges, C.M. Kay, Effect of chain length on the formation and stability of synthetic α -helical coiled coils, *Biochemistry* 33 (1994) 15501–15510.
 - [206] M.G. Oakley, J.J. Hollenbeck, The design of antiparallel coiled-coils, *Curr. Opin. Struct. Biol.* 11 (2001) 450–457.
 - [207] Y.B. Yu, Coiled-coils: stability, specificity, and drug delivery potential, *Adv. Drug Deliv. Rev.* 54 (2002) 1113–1129.

- [208] J. Walshaw, D.N. Woolfson, Socket: a program for identifying and analyzing coiled-coil motifs within protein structures, *J. Mol. Biol.* 307 (2001) 1427–1460.
- [209] CCE: CYGG E VSALEKE VSALEKK NSALEKE VSALEKE VSALEK; CCK: CYGG K VSALKEK VSALKEE VSANKEK VSALKEK VSALKE.
- [210] S. Lv, Y. Cao, H. Li, Tandem modular protein-based hydrogels constructed using a novel two-component approach, *Langmuir* 28 (2012) 2269–2274.
- [211] D.A. Einfeld, J.P. Brown, M.A. Valentine, E.A. Clark, J.A. Ledbetter, Molecular cloning of the human B cell CD20 receptor predicts a hydrophobic protein with multiple transmembrane domains, *EMBO J.* 7 (1988) 711–717.
- [212] O.W. Press, J. Howell-Clark, S. Anderson, I. Bernstein, Retention of B-cell-specific monoclonal antibodies by human lymphoma cells, *Blood* 83 (1994) 1390–1397.
- [213] J.T. Golay, E.A. Clark, P.C. Beverley, The CD20 (Bp35) antigen is involved in activation of B cells from the G0 to the G1 phase of the cell cycle, *J. Immunol.* 135 (1985) 3795–3801.
- [214] J.K. Bubien, L.J. Zhou, P.D. Bell, R.A. Frizzell, T.F. Tedder, Transfection of the CD20 cell surface molecule into ectopic cell types generates a Ca^{2+} conductance found constitutively in B lymphocytes, *J. Cell Biol.* 121 (1993) 1121–1132.
- [215] J.P. Deans, H. Li, M. Polyak, CD20-mediated apoptosis: signaling through lipid rafts, *Immunology* 107 (2002) 176–182.
- [216] MORF1: 5'-GAGTAAGCCAAGGAGAATCAATATA-linker-amine-3' (MW = 8630.5 Da); MORF2: 5'-TATATTGATTCTCCTGGCTTACTC-linker-amine-3' (MW = 8438.5 Da).
- [217] L.K. Boerner, Nanoconjugates trigger cell suicide, *Chem. Eng. News* (January 7, 2014) <http://cen.acs.org/articles/92/web/2014/01/Nanoconjugates-Trigger-Cancer-Cell-Suicide.html>.
- [218] J. Kopeček, Hydrogel biomaterials: a smart future? *Biomaterials* 28 (2007) 5185–5192.
- [219] J. Kopeček, Hydrogels: from soft contact lenses and implants to self-assembled nanomaterials, *J. Polym. Sci. A Polym. Chem.* 47 (2009) 5929–5946.
- [220] E. Kimby, Tolerability and safety of rituximab (MabThera), *Cancer Treat. Rev.* 31 (2005) 456–473.
- [221] H. Ding, W.M. Proding, J. Kopeček, Identification of CD21-binding peptides with phage display and investigation of binding properties of HPMA copolymer–peptide conjugates, *Bioconjug. Chem.* 17 (2006) 514–523.
- [222] H. Ding, W.M. Proding, J. Kopeček, Two-step fluorescence screening of CD21-binding peptides with one-bead one-compound library and investigation of binding properties of HPMA copolymer–peptide conjugates, *Biomacromolecules* 7 (2006) 3037–3046.
- [223] L. Peng, R. Liu, J. Marik, X. Wang, Y. Takada, K.S. Lam, Combinatorial chemistry identifies high-affinity peptidomimetics against $\alpha 4\beta 1$ integrin for *in vivo* tumor imaging, *Nat. Chem. Biol.* 2 (2006) 381–389.
- [224] C. Li, S. Wallace, Polymer–drug conjugates: recent development in clinical oncology, *Adv. Drug Deliv. Rev.* 60 (2008) 886–898.
- [225] R. Duncan, M.J. Vicent, Polymer therapeutics — prospects for 21st century: the end of the beginning, *Adv. Drug Deliv. Rev.* 65 (2013) 60–70.
- [226] Z.-R. Lu, Molecular imaging of HPMA copolymers: visualizing drug delivery in cell, mouse and man, *Adv. Drug Deliv. Rev.* 62 (2010) 246–257.
- [227] M.A. Pysz, S.S. Gambhir, J.K. Willman, Molecular imaging: current status and emerging strategies, *Clin. Radiol.* 65 (2010) 500–516.
- [228] M.J. Pittet, R. Weissleder, Intravital imaging, *Cell* 147 (2011) 983–991.