1
Backbone Degradable and Coiled-Coil Based Macromolecular Therapeutics

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1.1
Introduction

To address the lack of specificity of low molecular weight drugs for malignant cells, the concept of targeted polymer–drug conjugates was developed in the 1970s. The major rationale for the use of water-soluble polymers as carriers of anticancer drugs is based on the mechanism of cell entry [1–3]. Whereas the majority of low molecular weight drugs enter the cell interior by diffusion through the plasma membrane, the entry of macromolecules is restricted to endocytosis [4]. Macromolecules captured by this mechanism are channeled to the lysosomal compartment of the cell. In addition, moieties that complement cell surface receptors or antigens of a subset of cells may be incorporated into the macromolecular structure and render the conjugate biorecognizable [5–9].

There are numerous reviews, which summarize the rationale, design, synthesis, evaluation, and development of macromolecular therapeutics [5–16]. In this chapter, polymeric carriers based on copolymers of *N*-(2-hydroxypropyl)methacrylamide (HPMA) are used as an example. However, the conclusions can be considered generally applicable to water-soluble carriers with other chemical structures.

When using nondegradable water-soluble polymer drug carriers, their molecular weight needs to be below the renal threshold to safeguard biocompatibility. Unfortunately, this leads to relatively short intravascular half-life and the accumulation of the conjugates in solid tumors (because of enhanced permeability and retention (EPR) effect) is suboptimal [17]. Consequently, new designs for macromolecular therapeutics are needed and are discussed.

Following a discussion of general design principles of water-soluble polymer–drug conjugates – this chapter focuses on two recent designs: (i) New, second generation anticancer nanomedicines based on biodegradable, high molecular weight HPMA copolymer – drug carriers containing enzymatically degradable bonds in the main chain (polymer backbone). (ii) New paradigm in drug delivery – drug-free macromolecular therapeutics. This approach is based on the biorecognition of complementary motifs at cell surface and crosslinking

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of receptors with concomitant initiation of apoptosis; no low molecular weight drug is involved.

1.2 Water-Soluble Polymers as Carriers of Anticancer Drugs

The conjugation of drugs to synthetic and natural macromolecules was initiated about 60 years ago – for reviews of early work see Refs. [2, 18]. Jatzkewitz [19] used a dipeptide (glycylleucine) spacer to attach a drug (mescaline) to polyvinylpyrrolidone in the early 1950s and Ushakov's [20–22] group in Leningrad (now St. Petersburg) synthesized conjugates of polyvinylpyrrolidone with various antibiotics in the 1960s and 1970s. Mathé *et al*. [23] pioneered the conjugation of drugs to immunoglobulins, setting the stage for targeted delivery. De Duve [1] (who received the Nobel Prize in 1974) discovered that many enzymes are localized in the lysosomal compartment of the cell and the lysosomotropism of macromolecules, which are an important phenomena for the design of polymer–drug conjugates. Finally, Ringsdorf [24] analyzed the research results of the field and presented a clear concept of the use of polymers as targetable drug carriers.

1.2.1 First Generation Conjugates – Design, Synthesis, and Activity

As mentioned in Section 1.1, the design of first generation conjugates was based on the lysosomotropism of macromolecules, on the binding of drugs to the carrier via an attachment/release point that is stable in the blood stream but susceptible to catalyzed hydrolysis in the lysosomal compartment of the cell [25, 26], and on optional attachment of a targeting moiety [5, 6]. Numerous conjugates were evaluated and several proceeded into clinical trials [27–35]. Clinical trials with polymer–drug conjugates demonstrated a decrease of adverse effects when compared to free drugs, but the increase of efficacy (when compared to unbound drugs) was considerably smaller than in animal models. The reasons were recently analyzed and new directions in macromolecular therapeutics research proposed [10].

1.2.2 Analysis of Design Factors That Need Attention

There are numerous design factors that could speed up the translation of basic research into the clinics [10]. They are briefly discussed in the following sections:

1.2.2.1 Design of Conjugates for the Treatment of Noncancerous Diseases

HPMA copolymer conjugates with the well-established bone anabolic agent (prostaglandin E_1; PGE_1) are being developed for the treatment of osteoporosis and other musculoskeletal diseases. The biorecognition of the conjugates by

the skeleton is mediated by an octapeptide of D-aspartic acid (D-Asp_8) or a bisphosphonate, alendronate (ALN) [36, 37]. The same approach is applicable for the treatment of bone cancer.

1.2.2.2 Combination Therapy Using Polymer-Bound Therapeutics

Combination therapy using polymer-bound therapeutics has been studied actively [5, 38]. The first combination therapy using polymer-bound drugs used a mixture of HPMA copolymer–DOX (doxorubicin) conjugate and HPMA copolymer – chlorin e_6 conjugate [38]. On two cancer models, Neuro 2A neuroblastoma [38] and human ovarian carcinoma OVCAR-3 xenografts in nude mice [39–41] it was shown that combination therapy produced cures that could not be obtained with either chemotherapy or photodynamic therapy alone (Figure 1.1). Incorporation of anti-CD47 antibodies [42] or Fab′ fragments [43, 44] to these conjugates further increased the therapeutic efficacy.

From the synthetic and scale-up point of view it is preferable to use a mixture of two conjugates, each containing one drug. However, Vicent *et al*. [45] have shown that for some drug combinations binding two drugs to the same macromolecule results in higher efficacy when compared to a mixture of two polymer drugs. Recently, a new therapeutic strategy for bone neoplasms using combined targeted polymer-bound angiogenesis inhibitors (two per macromolecule: ALN and antiangiogenic TNP-470) was developed. The bispecific

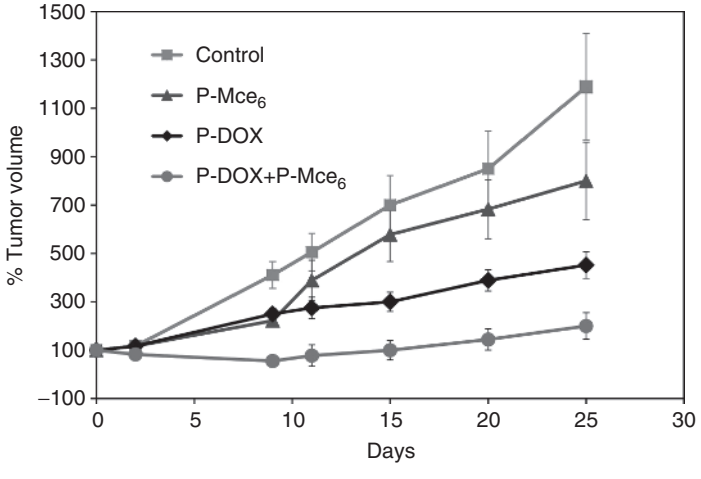


Figure 1.1 Combination of chemotherapy and photodynamic therapy of OVCAR-3 tumors heterotransplanted in nude mice treated with the HPMA copolymer-bound anticancer drugs, doxorubicin (DOX), and chlorin e_6 (Mce_6): control (vehicle); HPMA copolymer–Mce_6 conjugate ($P\text{-}Mce_6$; 1.5 mg kg^{-1} Mce_6 equivalent) with light; HPMA copolymer–DOX conjugate (P-DOX; 2.2 mg kg^{-1} DOX equivalent); combination therapy P-DOX + $P\text{-}Mce_6$ with light (2.2 mg kg^{-1} DOX equivalent plus 1.5 mg kg^{-1} Mce_6 equivalent). *Bars*, SE. (Adapted from Ref. [39].)

HPMA copolymer ALN-TNP-470 is the first antiangiogenic conjugate that targets both the tumor epithelial and endothelial compartments, warranting its use on angiogenesis-dependent calcified neoplasms such as osteosarcomas and bone metastases [46, 47].

1.2.2.3 New Targeting Strategies

New targeting strategies involve identification of targeting moieties that can enhance the efficacy of macromolecular therapeutics. One example is the selection of peptides using combinatorial approaches, phage display [48], and the chemical combinatorial technique, one-bead one-compound (OBOC) method [49]. However, the first selection usually produces peptides with a low binding constant and the use of a second, directed library is needed. This is time consuming, but may produce an optimization of the structure and enhancement of the binding constant by several orders of magnitude [50].

Another important aspect for the future development of anticancer nanomedicines is the *targeting of cancer stem cells* (CSCs) [8, 9, 51]. Cancer cells are biologically and functionally heterogeneous, in terms of phenotype, proliferation, tumorigenesis, invasiveness, and so on. Noticeably, cancer cells are present in various differentiation statuses, with relatively undifferentiated CSCs maintaining the hierarchical organization of the tumor mass, similar to the role of normal stem cells (NSCs) in healthy tissues [52, 53]. Moreover, the CSC theory suggests that the often-observed treatment failures are largely because of the failure of conventional cytotoxic anticancer therapies to eliminate CSCs. Therefore, targeting CSCs or in combination with traditional anticancer therapeutics represents a promising strategy to improve cancer patient survival [51]. Aiming to improve the outcome of prostate cancer treatments by targeting CSCs, we designed a CSC-specific nanomedicine. Cyclopamine, a hedgehog pathway inhibitor, was attached to the end of GFLG (glycylphenylalanylleucylglycyl) biodegradable tetrapeptide side chains of HPMA copolymer. We evaluated the CSC inhibitory effects of the HPMA copolymer-cyclopamine conjugate in an *in vitro* prostate cancer epithelial cell model using cells derived from a prostate cancer patient that were immortalized by transcription of human telomerase reverse transcriptase (RC-92a/hTERT), [54]. RC-92a/hTERT cells were chosen as the CD133+/integrin $\alpha 2\beta 1^{hi}$/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. Cell surface marker expression analysis and cytotoxicity studies following drug and conjugate treatments on RC-92a/hTERT cells supported the anti-CSC efficacy of the designed macromolecular therapeutics. The HPMA copolymer–cyclopamine conjugate, like free cyclopamine, showed selective inhibitory effect on prostate CSCs when compared with bulk cancer cells in the *in vitro* prostate cancer model. In contrast, docetaxel, a traditional chemotherapeutic agent for prostate cancer, showed preferential cytotoxicity to bulk cancer cells. These results suggest the treatment potential of a combination of macromolecular therapeutics targeting both bulk tumor cells and CSCs [51].

1.2.2.4 Relationship Between Detailed Structure of the Conjugates and Their Properties

Current research in drug delivery is not sufficiently focused on the analysis of the interplay of individual factors in multifunctional conjugates on the final properties. For example, an increase in the number of hydrophobic targeting peptides per macromolecule leads to enhanced avidity of the conjugate and better targetability. However, during internalization, the conformational changes of the macromolecule may lead to the association of side chains terminated in drug with concomitant decrease in the drug release rate [55, 56]. Combination of characterization techniques that include analysis of the conformation of the polymer conjugate by, for example, FRET (fluorescence resonance energy transfer) needs to be undertaken to optimize the structure of the conjugate. Increased amount of hydrophobic peptide side chain resulted in a more compact conformation of the polymer coil (Figure 1.2). Unsurprisingly, the rate of enzymatically catalyzed release of DOX from the compact coil decreased [55].

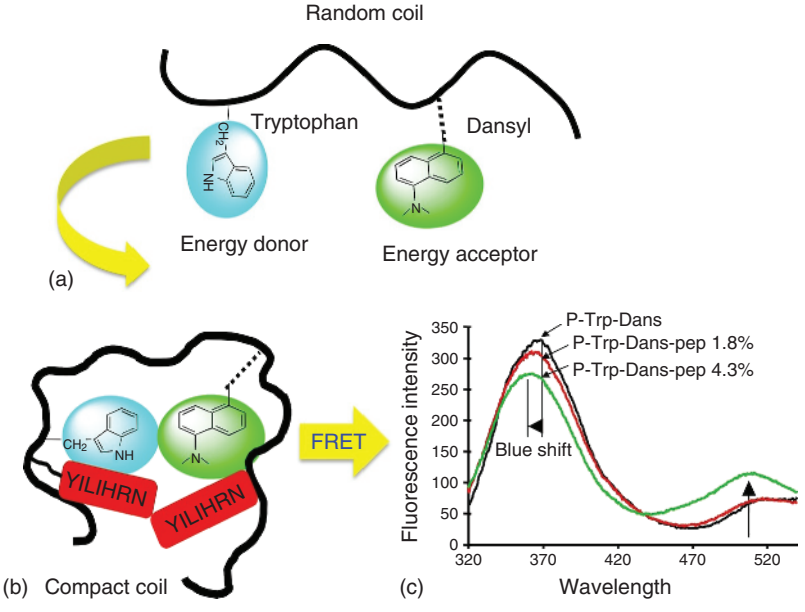


Figure 1.2 Evaluation of conformational changes in HPMA copolymer conjugates caused by intramolecular association of hydrophobic (YILIHRN) side chains. Fluorescence (Förster) resonance energy transfer (FRET) measurements using covalently bound tryptophan as donor and dansyl as acceptor could distinguish conformation of HPMA copolymer containing donor and acceptor only (P-Trp-Dans), HPMA copolymer containing 1.8 mol% of YILIHRN side chains (P-Trp-Dans-pep 1.8%) and HPMA copolymer containing 4.3 mol% of YILIHRN side chains (P-Trp-Dans-pep 4.3%). (Adapted from Ref. [55].)

1.2.2.5 Impact of Binding a Drug to a Polymer on the Mechanism of Action

Free and polymer-bound drugs may activate different signaling pathways because of different internalization mechanisms. This hypothesis is based on the fact that low molecular weight drugs interact with recognition moieties located at cell surface; biorecognition may result in the initiation of a signaling pathway. In contrast, water-soluble polymer-bound drugs are internalized in membrane-limited organelles and thus restricted in the interactions with surface structures. They are released from the carrier in secondary lysosomes and usually enter the cytoplasm in the perinuclear region. Consequently, they will interact with different molecules and could initiate a different signaling pathway. Examples are in the literature on the differences in the mechanism of action of free and HPMA polymer-bound drugs, including DOX [57, 58] and geldanamycin (GA) [59].

For example, the gene expression profiles of human ovarian carcinoma A2780 cells were examined after exposure to free 17-(3-aminopropylamino)-17-demethoxygeldanamycin (AP-GA) and HPMA copolymer-bound AP-GA (P(AP-GA)) [59]. As P(AP-GA)-treated cells may exhibit delayed responses, longer exposure times (2 × IC50 dose for 6 and 12 h) were examined (Figure 1.3). The hierarchical clustering of the expression ratios of the selected 68 genes indicated considerable similarities in the gene expression profiles after AP-GA and P(AP-GA) treatments (Figure 1.3a). On the other hand, unlike the

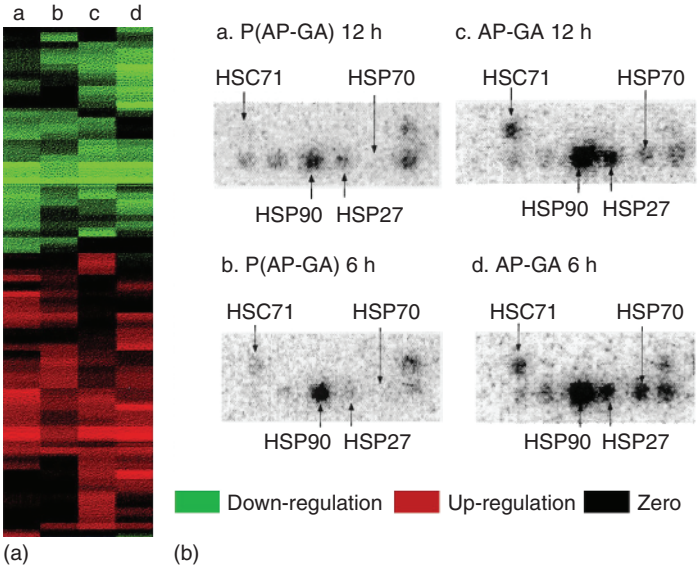


Figure 1.3 Gene expression in A2780 ovarian carcinoma cells exposed to free 17-(3-aminopropylamino)-17-demethoxygeldanamycin (AP-GA) and HPMA copolymer bound AP-GA (P(AP-GA)) at 2 × IC50 concentration for 6 and 12 h. (a) Hierarchical cluster analysis of the expression of selected 68 genes in the Atlas human 1.2 cDNA expression array. (b) Gene expression of cell stress response-related proteins. (Adapted from Ref. [59].)

AP-GA treatment, P(AP-GA) treatment induced little expression in stress response-related genes even after 12 h (Figure 1.3b). As GA-treated cells exhibited little expression in stress response-related genes, the elevated expression of stress response-related genes after exposure of cells to AP-GA may not be directly related to cell death mechanism induced by HSP90 inhibition. Therefore, it is probable that P(AP-GA) may suppress the expression of stress response-related genes activated by AP-GA following differences in its internalization mechanism, subcellular localization, and intracellular concentration gradients. Thus the results suggest that conjugation of AP-GA to HPMA copolymer may be able to modulate the cell stress responses induced by AP-GA following differences in its internalization mechanism, subcellular localization, and intracellular concentration gradients [59].

1.2.2.6 Mechanism of Internalization and Subcellular Trafficking

Macromolecular therapeutics cannot cross the phospholipid bilayer by diffusion; they enter cells by endocytic pathways [4]. Most common classification schemes of endocytosis are based on protein machinery that facilitates the process, such as clathrin-mediated endocytosis and clathrin-independent endocytosis [60–63]. Clathrin-independent endocytosis is further categorized as caveolae-mediated endocytosis and clathrin- and caveolin-independent endocytosis [60, 62] or dynamin-dependent and dynamin-independent endocytosis [62, 63]. In addition, macropinocytosis is a distinct pathway of pinocytosis [64]. The relationship between the detailed structure of the polymer–drug conjugate and its mechanism of internalization is important information, which provides feedback for the optimization of the conjugate structure.

Recently, research has been focusing on the identification of different routes of cell entry with the aim to deliver drugs into subcellular compartments different from lysosomes. As the activity of many drugs depends on their subcellular location, manipulation of the subcellular fate of macromolecular therapeutics may result in more effective conjugates. Approaches that seem to be effective are nuclear delivery of drugs mediated by steroid hormone receptors that shuttle between the cytoplasm and the nucleus [5, 65] and mitochondrial targeting mediated by delocalized hydrophobic cations [66–69]. Particularly, the experiments of Murphy *et al*. [68, 69] used terminally functionalized triphenylphosphonium (TPP) to target antisense peptide nucleic acid (PNA) into the mitochondria of isolated organelles and whole intact cells *in vitro*. Attachment of TPP to HPMA copolymer resulted in enhanced mitochondrial localization following microinjection and incubation experiments with ovarian carcinoma cells [66, 67]. Recently, Torchilin *et al*. used the same concept with TPP modified dendrimers [70] and liposomes [71].

1.2.2.7 Relationship Between the Molecular Weight of the Carrier and the Efficacy of the Conjugate

High molecular weight (long-circulating) polymer conjugates accumulate efficiently in tumor tissue because of the EPR effect [72]. Experimental data have shown that the higher the molecular weight of the conjugate, the higher the

accumulation in the tumor tissue with concomitant increase in therapeutic efficacy [73]. However, the renal threshold limits the molecular weight of the first generation of polymeric carriers to below ~50 kDa; this lowers the retention time of the conjugate in the circulation with concomitant decrease in pharmaceutical efficiency. Higher molecular weight drug carriers with a nondegradable backbone deposit and accumulate in various organs, impairing biocompatibility. To this end we designed the new second generation anticancer nanomedicines based on high molecular weight HPMA copolymer–drug carriers containing enzymatically degradable bonds in the main chain (polymer backbone) [74–76]. These multisegment block copolymers are synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization followed by click (alkyne–azide and/or thiol–ene) reactions (see following paragraph).

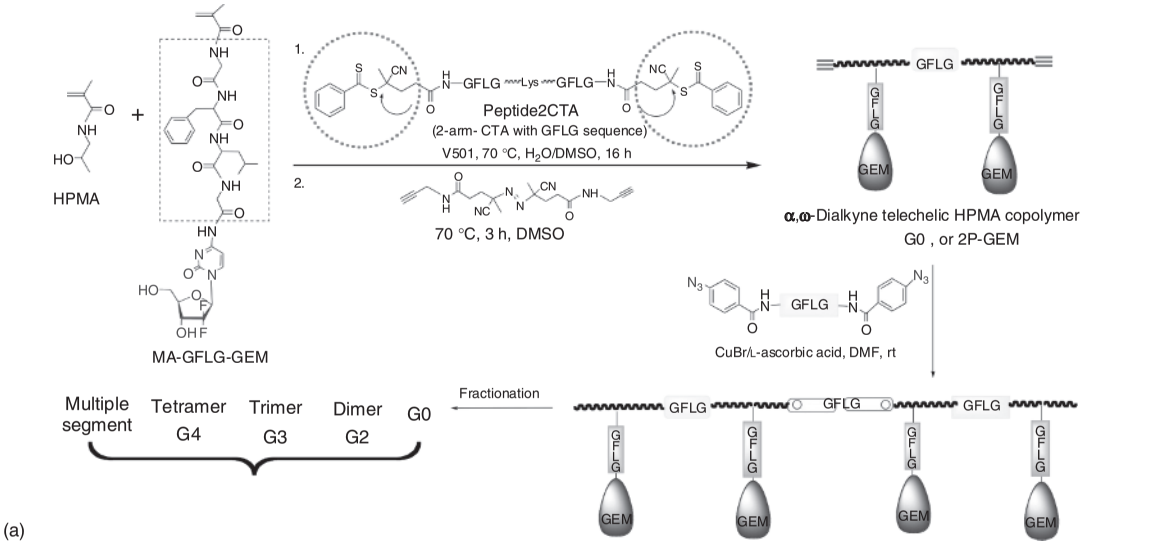
1.2.3 Design of Second Generation Conjugates – Long-Circulating and Backbone Degradable

HPMA copolymer-anticancer drug conjugates have been evaluated in clinical trials, but no product has entered clinical use yet. Questions have been raised whether HPMA conjugates "have a future as clinically useful nanomedicines?" [14]. In the following sections, we summarize our efforts in the design, development, and evaluation of second generation backbone degradable HPMA copolymer–anticancer drug conjugates. The *in vitro* and *in vivo* data suggest a great potential of these new nanomedicines.

Backbone degradable HPMA copolymer–drug conjugates were designed and synthesized by combination of RAFT copolymerization with either alkyne–azide or thiol–ene click chemistry [74–76]. An example, the synthesis of HPMA copolymer–gemcitabine (GEM) conjugates, is shown in Figure 1.4. Compared with previously evaluated HPMA, copolymer conjugates entered early clinical trials but were discontinued; the hallmark of the new second generation conjugates is the biodegradability of the HPMA linear backbone because of multiblock structure composed of alternating HPMA copolymer blocks and enzyme-cleavable oligopeptide segments. This permits to use high molecular weight long-circulating conjugates without impairing biocompatibility. Another remarkable synthetic feature is the utilization of polymerizable drug derivatives and controlled polymerization chemistry rather than attachment of drugs via polymer-analogous reactions. The drug content and average Mw can be tailored by the variation of feed ratio and polymerization conditions. The process results in the narrow distribution of molecular weights and minimal heterogeneity in chemical composition of the conjugates. In addition, the amount of free drug in the conjugates is minimized.

1.2.3.1 RAFT Copolymerization for the Synthesis of Conjugates

The advances in controlled ("living") radical polymerization contributed to the improved designs of nanomedicines by permitting the synthesis of well-defined



	G0 (2P-GEM)	After click/fractionation		
		G2	G3	G4
Mw, kDa	110	213	314	427
Mw/Mn	1.12	1.06	1.17	1.06
Gem%	5.6	5.5	4.8	–

Figure 1.4 HPMA copolymer–gemcitabine conjugates. (a) (i) Synthesis of backbone degradable multiblock conjugates via three steps: (1) RAFT copolymerization using peptide2CTA as RAFT agent; (ii) chain end modification to obtain clickable telechelic diblock drug conjugates; and (iii) chain extension by alkyne–azide click reaction. (2) Synthesis of first generation conjugate (P-GEM). (b) Characterization of narrow molecular weight fractions of HPMA copolymer–gemcitabine conjugates: G0, diblock HPMA copolymer conjugate prepared in one step using the new CTA containing a GFLG degradable sequence; G2, tetrablock (dimer of dimer) isolated by fractionation; G3, hexablock (trimer of diblock) isolated by fractionation. (c) Size exclusion chromatograms of G0 and G2 and G3 fractions. (d) Following incubation with papain, the molecular weight of 2P-GEM decreased to half of the original value. (Adapted from Ref. [77].)

bioconjugates that possess narrow distribution of molecular weights and a low degree of compositional (chemical) heterogeneity. The use of RAFT polymerization with dithiobenzoate or trithiocarbonate chain transfer agents (CTAs) [78] is an excellent approach for water-soluble polymer–anticancer drug conjugates, including the (co)polymerization of HPMA [74–76, 79]. The use of CTAs that contain an enzymatically degradable sequence in their structure results in polymer chains with inserted degradation point. Yang *et al*. synthesized an enzyme-sensitive, alkyne functionalized CTA, N^{α}-(4-pentynoyl)-N^{δ}-(4- cyano-4-(phenylcarbonothioylthio)pentanoylglycylphenylalanylleucylglycyl)-lysine(CTA-GFLG-alkyne), and used it in the synthesis of polyHPMA that contained an alkyne attachment point connected via an enzymatically degradable bond (Figure 1.5). Postpolymerization modification with 4,4′-azobis(azidopropyl 4-cyanopentoate) resulted in the formation of heterotelechelic HPMA copolymers containing terminal alkyne and azide groups [74].

Similarly, Pan *et al*. synthesized an enzyme-sensitive bifunctional CTA, N^{α}, N^{ϵ}-bis(4-cyano-4-(phenylcarbonothioylthio)pentanoylglycylphenylalanylleucyl-glycyl)lysine (Peptide2CTA). During RAFT polymerization the HPMA monomers incorporated at both dithiobenzoate groups. 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; this suggests that the monomers inserted into both dithiobenzoate groups of the Peptide2CTA with identical efficiency (Figure 1.4). The postpolymerization aminolysis of the terminal dithiobenzoate moieties results in telechelic α,ω-dithiol-polyHPMA [76].

1.2.3.2 Click Reactions for Chain Extension into Multiblock Copolymers

The RAFT copolymerization of HPMA with polymerizable derivatives of anticancer drugs and subsequent postpolymerization end-group modification results in heterotelechelic or homotelechelic copolymers that can be chain extended into high molecular weight multiblock backbone degradable copolymers. However, the chain extension is a condensation reaction that widens the distribution of molecular weights. Consequently, the product needs to be fractionated by size exclusion chromatography to create fractions with narrow polydispersity. For example, the heterotelechelic HPMA copolymers described above (Figure 1.5) can be chain extended just by exposing them to a catalyst facilitating the alkyne–azide reaction [74]. Similarly, telechelic α,ω-dialkyne-polyHPMA can be chain extended by a diazide containing an enzymatically degradable sequence [75]. The telechelic α,ω-dithiol-polyHPMA can be chain extended by dimaleic anhydride compounds, for example, bis-[1,13-(3-maleimidopropionyl)amido]-4,7,10-trioxatridecane (Bis-MAL-dPEG3) in methanol at room temperature for 24 h [76]. The schematic of chain extension reactions is shown in Figure 1.6.

1.2.3.3 Biological Properties of Long-Circulating Macromolecular Therapeutics

The improved therapeutic efficacy of second generation, backbone degradable HPMA copolymer conjugates has been recently evaluated in several studies.

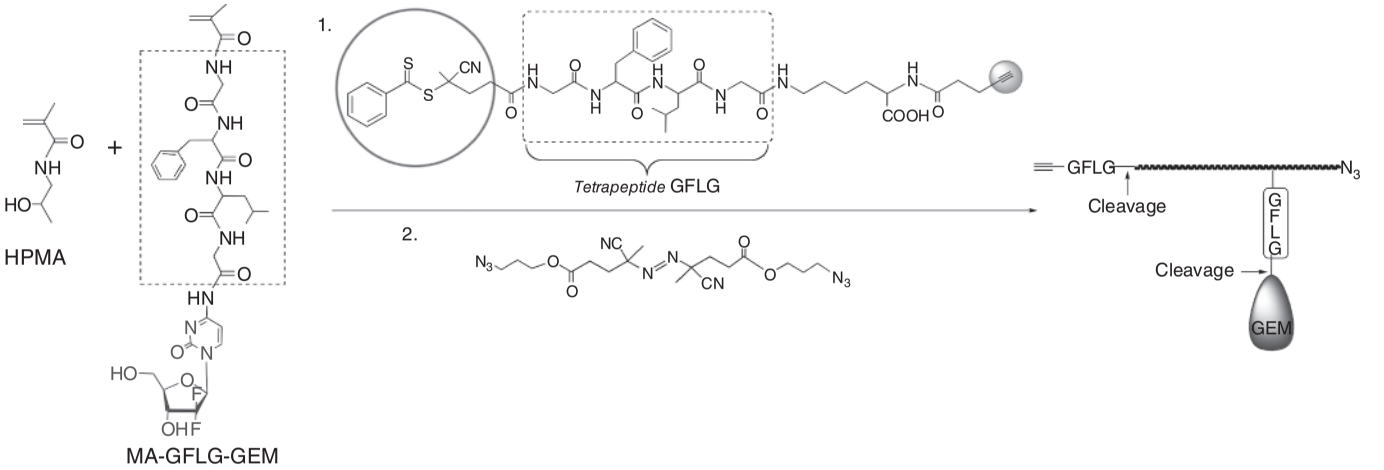


Figure 1.5 Synthesis of clickable heterotelechelic HPMA copolymer–gemcitabine conjugate via RAFT copolymerization using an enzyme-sensitive (degradable) alkyne functionalized chain transfer agent (CTA), N^{α}-(4-pentynoyl)-N^{δ}-(4-cyano-4-(phenylcarbonothioylthio)pentanoylglycyl-phenylalanylleucylglycyl)-lysine (CTA-GFLG-alkyne). Postpolymerization modification with 4,4′-azobis(azidopropyl 4-cyanopentoate) resulted in the formation of heterotelechelic HPMA copolymers containing terminal alkyne and azide groups and an enzymatically degradable GFLG sequence sequence. (Adapted from Ref. [74].)

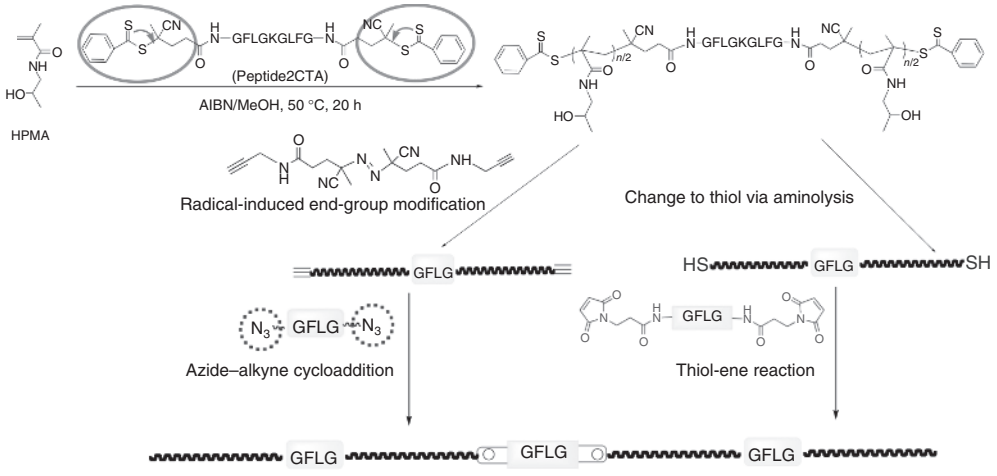


Figure 1.6 Alkyne–azide and thiol–ene chain extension reactions used in the synthesis of backbone degradable, long-circulating HPMA copolymer–drug conjugates. (Adapted from Refs. [74–76].)

Multiblock HPMA copolymer–DOX conjugates were synthesized by RAFT polymerization followed by chain extension via thiol–ene click reaction. The examination of molecular weight dependent antitumor activity toward human ovarian A2780/AD carcinoma xenografts in nude mice revealed enhanced activity of multiblock, second generation higher molecular weight conjugates (Mw = 93; 185; and 349 kDa) when compared to traditional HPMA copolymer–DOX conjugate (Mw = 20 kDa). The examination of body weight changes during treatment indicated the absence of nonspecific adverse effects [80].

In another study, a multiblock backbone degradable HPMA copolymer–paclitaxel conjugate (mP-PTX; Mw = 335 kDa) was synthesized by RAFT copolymerization, followed by chain extension. The *in vivo* efficiency of free PTX, HPMA copolymer–PTX conjugate with Mw of 48 kDa (P-PTX), and mP-PTX was determined on female nu/nu mice bearing orthotopic A2780 ovarian tumors. Pharmacokinetics study showed that high Mw mP-PTX was cleared more slowly from the blood than commercial PTX formulation and low Mw P-PTX. SPECT/CT imaging and biodistribution studies demonstrated biodegradability as well as elimination of mP-PTX from the body. The tumors in the mP-PTX treated group grew more slowly than those treated with saline, free PTX, and P-PTX (single dose at 20 mg PTX/kg equivalent). Moreover, mice treated with mP-PTX had no obvious ascites and body weight loss. Histological analysis indicated that mP-PTX had no toxicity in liver and spleen, but induced massive cell death in the tumor [81].

Second generation HPMA copolymer–GEM conjugates and combination of HPMA copolymer–GEM and HPMA copolymer–PTX conjugates have

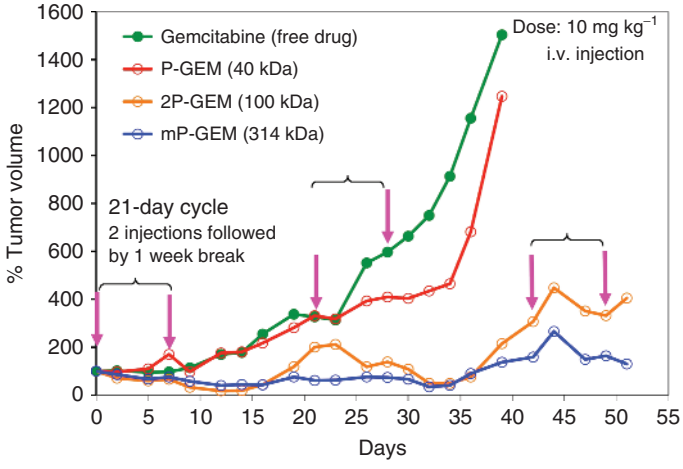


Figure 1.7 Molecular weight-dependent *in vivo* antitumor activity of free and HPMA copolymer-bound gemcitabine (GEM) against A2780 human ovarian carcinoma xenografts in nude mice. The mice received 10 mg kg^{-1} GEM equivalent on days 0, 7, 21, and 28. Reprinted from Ref. [77] with permission.

shown excellent activity toward human ovarian carcinoma xenografts [77, 82]. The evaluation of HPMA copolymer–GEM conjugates demonstrated the enhanced activity of the diblock (100 kDa) and multiblock (314 kDa) conjugates when compared to the first generation conjugate (40 kDa) (Figure 1.7) [77]. *In vivo* behavior of a combination of diblock backbone degradable HPMA copolymer–drug conjugates (2P-PTX and 2P-GEM) was investigated using pharmacokinetics, biodistribution, and SPECT/CT imaging studies. In parallel, the antitumor efficacy of combination treatment of 2P-PTX and 2P-GEM was evaluated and compared with free drugs (PTX and GEM) and first generation low Mw conjugates (P-PTX and P-GEM) in nu/nu mice bearing A2780 tumor xenografts. Compared to first generation low Mw HPMA copolymer conjugates, high Mw backbone biodegradable HPMA copolymer carriers significantly prolonged the intravascular half-life of drugs (PTX and GEM) in mice. The biodistribution and SPECT/CT imaging results demonstrated accumulation of conjugates 2P-PTX and 2P-GEM in the tumors and the degradation of new generation conjugates in mice. Notably, the tumors treated with combination of 2P-PTX and 2P-GEM were more effectively repressed, when compared to free drug combination and first generation (low Mw) conjugates combination (Figure 1.8). The histological analysis indicated that the combination treatment had no toxicity in major organs [82]. Furthermore, the backbone degradable HPMA copolymer–GEM conjugate exhibited excellent antitumor activity toward PANC1 pancreatic adenocarcinoma xenografts in nude mice [83].

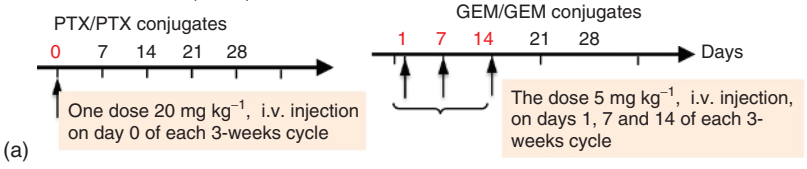
Tumor model and inoculation

(4×10^6)/100 µl A2780 cells s.c. injected to 6-8 week old female nude mice

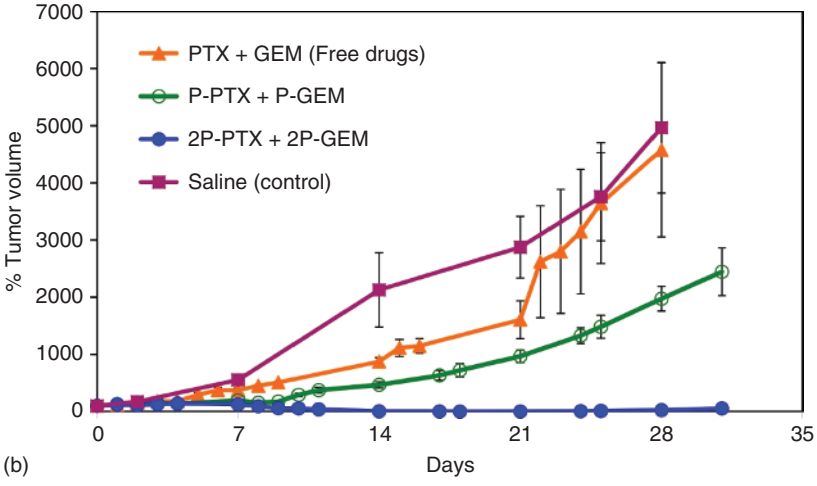
Administration:

Sequential combination treatment (paclitaxel (PTX) followed by gemcitabine) was given when tumor grew till ~ 50 mm^2. The procedure and doses:

i. PTX → GEM
ii. P-PTX → P-GEM
iii. 2P-PTX → 2P-GEM
iv. Control (saline)



(a)



(b)

Figure 1.8 (a, b) Treatment of s.c. A2780 human ovarian carcinoma xenografts by combination therapy of GEM and PTX and their HPMA copolymer conjugates [82]. Characterization of conjugates (Mw in kDa/polydispersity/content of drug in wt%): P-PTX 50/1.08/8.5; 2P-PTX 146/1.41/8.2; P-GEM 32/1.07/7.7; 2P-GEM 89/1.07/7.9. Dosing: 20 mg kg^{-1} PTX equivalent on day 0 followed by 5 mg kg^{-1} GEM equivalent on days 1, 7, and 14. (Adapted from Ref. [82].)

1.2.4 Summary of Part 2 and Future Prospects

The development of backbone degradable, linear HPMA copolymers as drug carriers is an important one in the area of water-soluble polymeric drug carriers. It permits to manipulate the intravascular half-life of polymer–drug conjugates in a wide range without impairing biocompatibility. The development of such carriers was possible following the development of new polymerization techniques, namely RAFT [78] and ATRP (atom transfer radical polymerization) [84].

In combination with click reactions a rational design of the second generation conjugates could be achieved.

Of particular interest is the development of RAFT CTAs that contain enzymatically degradable sequences in their structure. The possibility to prepare diblock copolymers that contain an enzymatically degradable sequence in one operation is important for the scale-up of the process. A biocompatible polymer carrier with a molecular weight of twice the renal threshold might be sufficient for augmenting the efficacy of water-soluble polymer–anticancer drug conjugates, as experimental results suggest [77, 80–83].

In general, the research in the area of water-soluble polymer–drug conjugates is on good track. The biocompatibility of the polymer carriers as well as the decrease of adverse effects have been proven in clinical trials. The design principles for more efficient conjugates have been identified. Employing modern imaging techniques [85–89] that permit noninvasive monitoring of the fate of conjugates will undoubtedly contribute to a more rational design of polymer therapeutics and theranostics. The major challenge of the field, however, is the translation into clinical application. The FDA approval of a conjugate that will be successful in clinical use will stimulate research and ultimately produce numerous new conjugates.

1.3
Drug-Free Macromolecular Therapeutics – A New Paradigm in Drug Delivery

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) motifs at cell surface, formation of heterodimers (e.g., antiparallel coiled-coils), crosslinking of noninternalizing receptors, and initiation of apoptosis [90–92].

1.3.1
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 may produce materials with properties superior to individual components. The peptide domain may insert 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 [90]. We have designed hybrid systems composed from hydrophilic HPMA copolymer backbone grafted with two pentaheptad oligopeptide sequences with opposite charge (CCE and CCK). 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 [93, 94].

1.3.2
Coiled-Coils in Biomedical Systems

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 superhelix [90, 95–98]. The primary structure of the coiled-coil motif is characterized by a sequence of repeating heptads (motif of seven amino acids) designated as $[a, b, c, d, e, f, g]_x$, 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 of the coiled-coil (Figure 1.9). Depending on their detailed structure, α-helices may associate as homodimers or heterodimers in parallel or antiparallel alignments, or form higher order (e.g., tetramer) aggregates [99–101]. 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 autoantigens, have functional coiled-coil

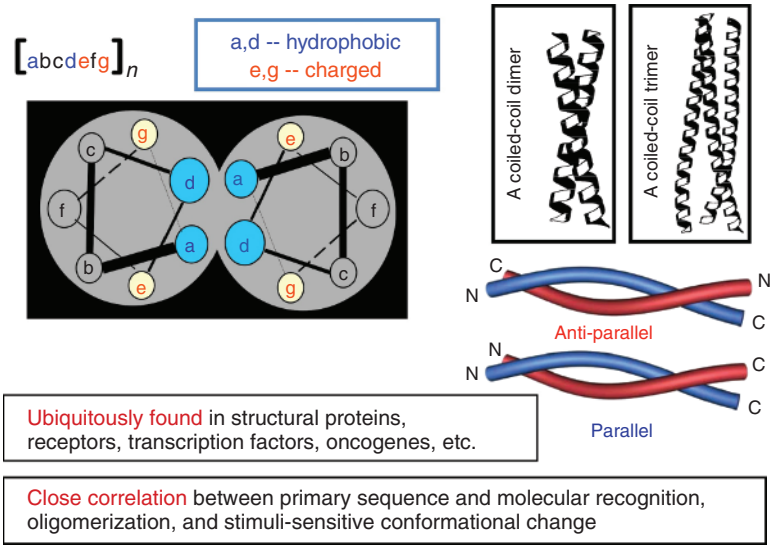


Figure 1.9 Coiled-coil is a common folding motif. The primary sequence of a typical coiled-coil is composed of seven-residue repeats, designated as heptads. The amino acid residues in a heptad are conventionally denoted as "a, b, c, d, e, f, g." Hydrophobic residues at positions "a" and "d" form an interhelical hydrophobic core, providing a stabilizing interface between the helices. Charged residues at positions "e" and "g" form electrostatic interactions, which contribute to coiled-coil stability and mediate specific association among helices. Depending on the primary structure, the helices may form homodimers or heterodimers and associate in a parallel or antiparallel arrangement. (Adapted from Refs. [90, 101].)

domains [102]. 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.

A typical α-helix is right-handed and 3.6 amino acid residues are needed to form a full turn. In a left-handed coiled-coil (composed of right-handed helices), one heptad forms exactly two turns (so-called 7/2 repeat – 7 amino acids per 2 turns). In nature, coiled-coils with different priodicities, for example, 11-residues periodicities, or with insertions of one or more residues into the heptad pattern, can be found (insertions of one residue are called skips, three-residue insertions stammers, and four residue insertions are stutters) [95, 103]. The versatility of the coiled-coil motif, especially the possibility to manipulate its stability and specificity by modifying the primary structure (up to 10^{-15} M stabilities may be achieved [104]), bodes well for their use in the successful design of new biomaterials.

1.3.3 Coiled-Coil Based Drug-Free Macromolecular Therapeutics: Design, *In Vitro,* and *In Vivo* Activity

As mentioned above, we designed two oppositely charged heptapeptad sequences, CCE and CCK, that form antiparallel coiled-coil heterodimers [93]. These sequences served as physical crosslinkers in the self-assembly of HPMA graft copolymers – P-CCK and P-CCE [93, 94] – and in the design of tandem modular proteins [105]. The excellent biorecognition of the peptide domains was an inspiration for the design of new nanomedicines; this created a bridge between the design of biomaterials and the design of nanomedicines.

CCK and CCE peptides were engaged in the design of a new CD20+ cell apoptosis induction system, called drug-free macromolecular therapeutics [91, 92]. CD20 is an ideal target for immunotherapies. It is an integral membrane protein [106] that is expressed from pre-B cells to terminally differentiated plasma cells and is present on greater than 90% of B cell malignancies [107, 108]. 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 [109] that either controls or functions as a store-operated calcium channel. The protein forms dynamic dimers and tetramers [110] constitutively associated with lipid rafts of the cell membrane [111].

Indeed, the biorecognition of CCE/CCK peptide motifs at the cellular surface was able to control apoptosis of CD20+ B cells. Exposure of Raji B cells to an anti-CD20 Fab′-CCE conjugate decorated the cell surface with CCE (CD20 is a noninternalizing receptor) through antigen–antibody fragment recognition. Further exposure of the decorated cells to CCK-P (grafted with multiple copies of CCK) resulted in the formation of CCE/CCK coiled-coil heterodimers at the cell surface. This second biorecognition induced the crosslinking of CD20 receptors

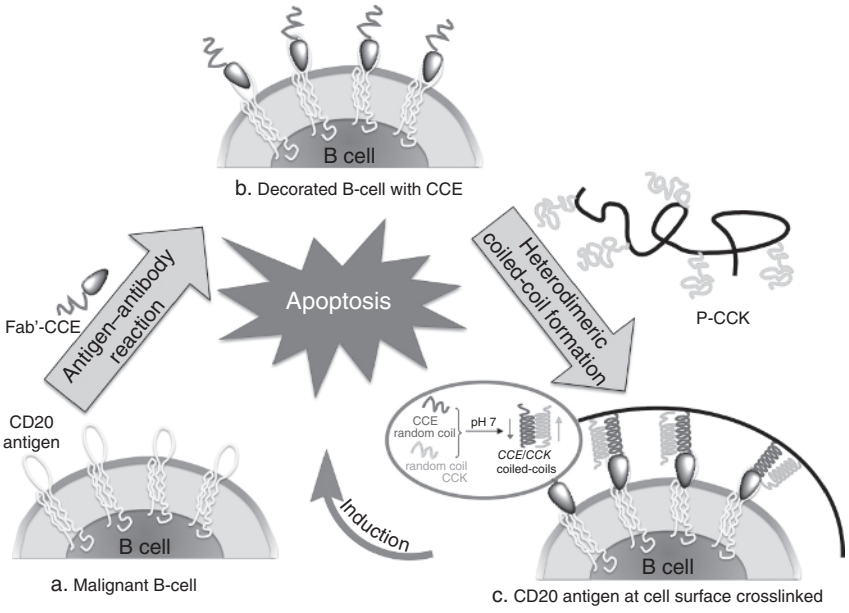


Figure 1.10 Drug-free macromolecular therapeutics. Cartoon of overall design and possible mechanism of treatment of NHL with conjugates of antiparallel coiled-coil forming peptides, CCE and CCK. Exposure of malignant B cells to anti-CD20 Fab′ fragment – CCE conjugate (Fab′-CCE) decorates the cells with the CCE peptide by biorecognition of the Fab′ fragment by the noninternalizing CD20 receptor. Further exposure of decorated cells to a HPMA copolymer grafted with several copies of complementary CCK peptide(P-CCK) results in the formation of antiparallel coiled-coil at the cell surface, crosslinking of CD20 receptors, and initiation of apoptosis. Inset: Formation of antiparallel coiled-coils by mixing equimolar amount of CCE (maleimide-YGG E VSALEKE VSALEKK NSALEKE VSALEKE VSALEK) and CCK (CYGG K VSALKEK VSALKEE VSANKEK VSALKEK VSALKE) peptides. Design of peptides from Ref. [93]; principle of drug-free macromolecular therapeutics. (Adapted from Ref. [91].)

and triggered the apoptosis of Raji B cells *in vitro* [91] and in a Non-Hodgkin lymphoma animal model *in vivo* [92]. This is a new concept, where the biological activity of drug-free macromolecular therapeutics is based on the biorecognition of peptide motifs (Figures 1.10 and 1.11).

1.3.4 Potential, Limitations, and Future Prospect of Drug-Free Macromolecular Therapeutics

The design of drug-free macromolecular therapeutics is a truly novel approach. It builds on the design of new self-assembling biomaterials [90, 93, 94, 112, 113] and translates the biorecognition principles to nanomedicine [91, 92, 114]. The first design of this system was tailored for the noninternalizing CD20 receptor and NHL. The preference for noninternalizing receptors is the limitation of the design.

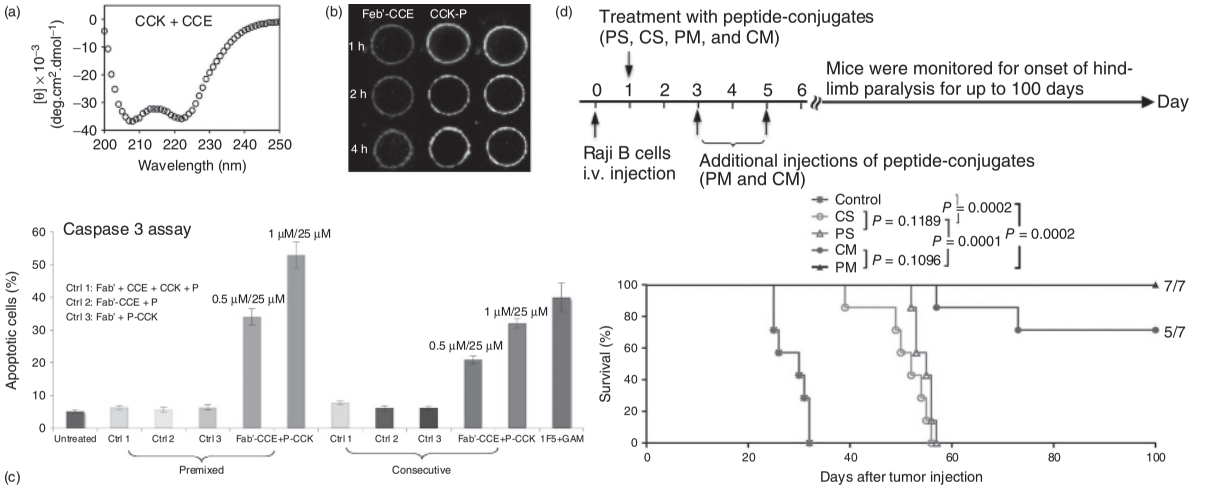


Figure 1.11 Coiled-coil mediated induction of apoptosis in Raji B cells *in vitro* and *in vivo*. (a) CD spectra of equimolar mixture of CCE and CCK; (b) biorecognition of Fab′-CCE (Rhodamine Red labeled) and P-CCK (FITC labeled) on Raji B surface: first column – red channel, second column – green channel, third column – overlay of red and green channels; (c) induction of apoptosis as determined by caspase 3 assay (1F5 + GAM is positive control – exposure to 1F5 Ab followed by goat antimouse secondary Ab; and (d) Therapeutic efficacy of drug-free macromolecular therapeutics against systemically disseminated Raji B cell lymphoma in C.B.-17 SCID mice (7 mice per group). Top panel shows timeline for the *in vivo* efficacy study. Four million Raji B cells were injected into the tail vein on day 0 to initiate the disseminated disease. The incidence of hind-limb paralysis or survival of mice was monitored until day 100. Five groups of animals were evaluated: untreated controls; consecutive administration of single dose (CS); premixed administration of single dose (PS); consecutive administration of three doses at days 1, 3, and 5 (CM); and premixed administration of three doses at days 1, 3, and 5 (PM). *Consecutive administration* involved the i.v. injection of 50 μg/20 g Fab′-CCE first and 1 h later the i.v. administration of 324 μg/20 g P-CCK conjugate; For *premixed administration*, the two conjugates were mixed together 1 h before injection via the tail vein. Bottom panel shows survival rate of tumor-bearing mice that received above treatments. The curve was presented in a Kaplan–Meier plot with indication of numbers of long-term survivors. (*in vitro* data adapted from Ref. [91; *in vivo* data adapted from Ref. [92].)

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 nonreversible impact on normal immune function [115]. 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 [116], by peptides selected by phage display [48] or by combinatorial methods [49]. The peptide part can be replaced by complementary oligonucleotides that hybridize at cell surface [114].

1.4 General Summary and Outlook

The advantages of polymer-bound drugs (when compared to low molecular weight drugs) are [5–8]: (i) active uptake by fluid-phase pinocytosis (nontargeted polymer-bound drug) or receptor-mediated endocytosis (targeted polymer-bound drug), (ii) increased *passive* accumulation of the drug at the tumor site by the EPR effect, (iii) increased *active* accumulation of the drug at the tumor site by targeting, (iv) long-lasting circulation in the bloodstream, (v) decreased nonspecific toxicity of the conjugated drugs, (vi) potential to overcome multidrug resistance, (vii) decreased immunogenicity of the targeting moiety, (viii) immunoprotecting and immunomobilizing activities, and (ix) modulation of the cell signaling and apoptotic pathways. In addition to preclinical evaluation on animal cancer models, these advantages were recognized in numerous clinical trials of water-soluble polymer–drug conjugates [reviewed in 35]. However, the translation of laboratory research into the clinics has been slow. The approaches to enhance the development and translation have been reviewed here.

The field of water-soluble polymer–drug conjugates is at the crossroads. Scientifically, the design principles for bioconjugates are well defined; the challenge is to combine the efficient design of the conjugates with the understanding of the biological features of cancer, including heterogeneity of cancer cells, tumor microenvironment, and metastasis [8, 9].

The progress will occur on several levels, including: (i) continuous progress of our knowledge resulting in the design of bioconjugates with higher activities. Some examples of these strategies were described above, such as the design of conjugates for the treatment of musculoskeletal diseases, combination

therapy using polymer-bound drugs, targeting CSCs, and backbone degradable long-circulating conjugates. (ii) The remarkable progress in imaging techniques that permits noninvasive monitoring of the fate of conjugates will undoubtedly contribute to a more rational design of polymer therapeutics and theranostics [85–89]. (iii) Qualitative changes in the approach to design and treatment. This includes manipulation of tumor microenvironment and new, nonconventional approaches to research. Examples of nonconventional approaches to research are the design of genetically engineered polymers capable of storing and propagating information [117, 118] and application of design principles from biomaterials to nanomedicines to create a cytotoxic system, drug-free macromolecular therapeutics, where the low molecular weight drug is not needed [91, 92, 114].

We certainly hope that a concerted effort of pharmaceutical chemists, molecular biologists, biomedical engineers, and physicians along these lines will achieve translation of water-soluble polymer–drug conjugates into the clinic within the current decade.

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