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DESIGN OF SMART HPMA COPOLYMER-BASED NANOMEDICINES

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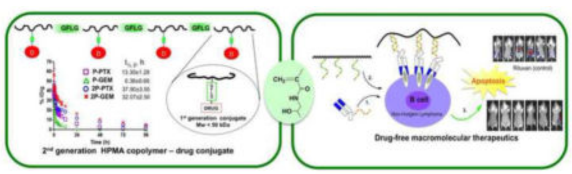
Abstract

The state-of-the art in water-soluble macromolecular therapeutics has been reviewed. First the design principles for polymer-drug conjugates are discussed followed by two recent developments in the field: a) The design, synthesis and properties of backbone degradable *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-drug conjugates. The enhanced intravascular half-life of such conjugates creates a concentration gradient (blood vs. tumor) for an extended time interval resulting in increased solid tumor accumulation by enhanced permeability and retention (EPR) effect with concomitant increase in efficacy. b) Drug-free macromolecular therapeutics is a new paradigm in macromolecular therapeutics. Apoptosis in malignant cell is induced by crosslinking of cell surface non-internalizing receptors. Crosslinking of receptors is mediated by the biorecognition of two nanoconjugates containing high-fidelity complementary motifs (peptides or oligonucleotides). Results for the treatment of B cell lymphomas in animal models and patient cells demonstrate the high translational potential of this aproach.

Graphical Abstract



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Conflict of interest

J.Y and J.K. are co-inventors on a pending US patent application related to backbone degradable HPMA copolymer-drug conjugates. J.K. has ownership in TheraTarget, Inc., which has licensed the technology from the University of Utah.
J.Y. and J.K. are co-inventors on a pending US patent application (PCT/US2014/023784; assigned to the University of Utah) related to drug-free macromolecular therapeutics. J.K. is Chief Scientific Advisor and J.Y. Scientific Advisor for Bastion Biologics. Otherwise, the authors declare no competing financial interests.

Keywords

Degradable polymeric carriers; drug-free macromolecular therapeutics; HPMA; oligonucleotides; coiled-coil peptides; self-assembly

1. Introduction and historical background

Polymers have been used in medicine for many years. The first application dates back to the 1940s when cellophane tubing used in sausages was utilized by Kolff to design the first artificial kidney. The tube was wrapped around a slated wooden drum and revolved in a tank filled with dialyzing solution [1]. In the 50s and 60s poly(vinyl alcohol) sponges (Ivalon) were used as breast implants [2]. Silicones have been used in several applications (tubings, shunts, urethra, joints) since the 1950s [3].

The first biomaterials rationally designed for human use were hydrogels. Lím synthesized hydrogels by crosslinking copolymerization of 2-hydroxyethylmethacylate (HEMA) [4] and Wichterle designed soft contact lenses using these materials [5]. They have been used in many applications in the clinic [6,7]. The rationale of design and the history of hydrogel discovery are reviewed in [6].

Sixty years ago, Jatzkewitz conjugated mescaline (drug) to polyvinylpyrrolidone (PVP) via a dipeptide spacer [8]. Ushakov's group in Leningrad (now St. Petersburg) conjugated numerous antibiotics to PVP and evaluated biological properties of the conjugates [9–11]. Mathé et al. was first to bind a drug to immunoglobulin [12]. De Duve discovered that numerous intracellular enzymes localize in the lysosomes (Nobel Prize 1974) and the lysosomotropism of macromolecules [13]. In 1975 Ringsdorf first clearly presented the concept of the use of polymers as targetable drug carriers [14]. The early results on polymer-drug conjugates are reviewed in ref. [15]. Another important use of polymers in therapeutics is protein modification. Davies and coworkers in the 1970s modified proteins with poly(ethylene glycol) to improve their intravascular half-life, immunogenicity, and stability [16]. The impact of the polymer structure on the properties of modified vesicular carriers was recently investigated [17].

New ideas on controlling the pharmacokinetics, pharmacodynamics, non-specific toxicity, immunogenicity, biorecognition, and efficacy of drugs were generated. These new strategies, often called drug delivery systems, are based on interdisciplinary approaches that combine polymer science, pharmaceutics, bioconjugate chemistry, and molecular biology. The combination of drugs with macromolecules created a new class of preparations – macromolecular therapeutics. Drug delivery systems based on water-soluble polymeric carriers, micelle-forming block copolymers, nanospheres, microspheres, liposomes, dendrimers, and hydrogels are examples of this strategy. Hoffman's review provides an excellent perspective on a new paradigm in drug delivery, the design, synthesis and evaluation of drug-free macromolecular therapeutics. the historical development of drug delivery systems [18].

This review summarizes the design principles of water-soluble polymer-drug conjugates and highlights the advantages of polymer therapeutics when compared to low molecular weight drugs. After briefly mentioning the differences in conjugates' therapeutic activity in animal models vs. human clinical trials, two new designs [19] that have the potential to overcome the limitations and speed up the translation into clinics are discussed: a) 2nd generation high molecular weight backbone degradable HPMA copolymer-drug conjugates synthesized by the combination of RAFT polymerization and chain extension via click reactions; and b) a new paradigm in drug delivery, the design, synthesis and evaluation of drug-free macromolecular therapeutics. This design is free of toxins and immune activation; apoptosis is initiated by crosslinking of receptors mediated by biorecognition of complementary motifs at cell surface.

2. Design principles for water-soluble polymer-drug conjugates

2.1 Polymer carriers: synthetic vs. natural

Polymers used as drug carriers must be well characterized; their structure should provide drug attachment/release sites for the incorporation of drugs. The carrier and all metabolic products should be nontoxic and nonantigenic. Likewise, the carriers should display the ability to be directed to predetermined cell types, and, they should be biodegradable or eliminated from the organism after fulfilling their function [20].

Macromolecules as drug carriers may be divided into naturally occurring and synthetic types. Frequently, it is assumed that polymeric drug carriers derived from natural products (polysaccharides, poly(amino acids), proteins) break down in the organism into small, easily eliminated fragments. However, the substitution of natural macromolecules with covalently linked drug molecules generally hampers the host's ability to effectively enzymatically degrade the polymeric carrier, because enzymes that cleave peptide or saccharide bonds have considerably large active sites that accommodate several amino acid or saccharide "monomer" units. Substitution along the macromolecular backbone renders the formation of the enzyme-substrate complex energetically less favorable. Therefore, drug substitution of a polymeric carrier, such as poly(amino acids) [21] or polysaccharides [22], may result in the inability of a naturally occurring, normally biodegradable macromolecule, to be degraded into small fragments able to cross the lysosomal membrane [23]. The larger fragments may accumulate in the lysosomes and increase the osmotic pressure with a potentially negative impact on biocompatibility.

Synthetic macromolecules can be tailor-made to have properties matching the biological situation [24]. However, to enhance elimination via glomerular filtration, the entire molecular weight distribution must be under the renal threshold – about 50 kDa for neutral hydrophilic random coils. Further, to prevent the nonspecific reuptake of the macromolecule after being released into the bloodstream following cell death, its structure must be designed in such a way that internalization occurs by fluid-phase pinocytosis. The absence of nonspecific interactions with plasma membranes will minimize the accumulation of the carrier in nontargeted cells and thus increase the biocompatibility of the carrier.

2.1.1 Impact of molecular weight of the carrier on fate and efficiency— *Molecular weight* has an important impact on the in vivo fate of soluble polymers. The higher the molecular weight, the longer the intravascular half-life and the slower the elimination of polymers from the organism as shown in the fate of polyHPMA [25–31], polyvinylpyrrolidone [32], dextran and pullulan [33], and other structures (reviewed in [34,35]).

Kopeček and coworkers demonstrated the *impact of the molecular weight* of HPMA copolymer-doxorubicin (DOX) conjugates *on solid tumor treatment*. To verify the hypothesis that therapeutic efficacy of conjugates will be molecular weight dependent, they designed and synthesized branched, water-soluble HPMA copolymer DOX conjugates containing lysosomally degradable oligopeptide sequences as crosslinks and side-chains terminated in DOX [30]. Four conjugates with Mw of 22, 160, 895, and 1230 kDa were prepared. Their biodistribution and treatment efficacy were evaluated in nu/nu mice bearing s.c. human ovarian OVCAR-3 carcinoma xenografts [31]. The half-life of HPMA copolymer – DOX conjugate (Mw = 1230 kDa) in blood was up to 28 times longer, and the elimination rate from the tumor was 25 times slower than that of free DOX. The results clearly demonstrated that Mw of conjugates has a significant effect on solid tumor treatment. The higher the molecular weight of the HPMA copolymer – DOX conjugates, the higher the tumor accumulation with *concomitant increase in therapeutic efficacy* [31].

2.1.2 Impact of structural factors on the fate of polymer carriers—Lammers et al. [36] evaluated the effect of physicochemical modification on the biodistribution and tumor accumulation of HPMA copolymers. Similar to other authors, they observed that increasing the molecular weight of HPMA copolymers resulted in prolonged circulation times and enhanced tumor concentration. Interestingly, they found that modification of the structure with carboxyl and hydrazide groups, or attachment of oligopeptide spacers terminated in drug decreased the intravascular half-life; consequently, lower levels of polymer were found in tumor and all organs except kidney. Importantly, tumor to tissue ratio did not change, indicating that functionalization did not affect the targetability of the conjugates [36].

Macromolecular therapeutics cannot cross the phospholipid bilayer by diffusion; they enter cells by endocytic pathways [37]. Most common classification schemes of endocytosis are based on protein machinery that facilitates the process, such as clathrin-mediated endocytosis, and clathrin independent endocytosis [38–41]. Clathrin independent endocytosis is further categorized as caveolae-mediated endocytosis and clathrin- and caveolin-independent endocytosis [38,40] or dynamin dependent and dynamin independent endocytosis [39,40]. In addition, macropinocytosis is a distinct pathway of pinocytosis [42]. 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. Since 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 [43] and mitochondrial targeting mediated by delocalized hydrophobic cations [44–47]. Of particular interest, the experiments of Murphy et al. used terminally functionalized triphenylphosphonium (TPP) to target peptide nucleic acids (PNA) into the mitochondria of isolated organelles and whole intact cells in vitro [46,47]. Attachment of TPP to HPMA copolymer resulted in enhanced mitochondrial localization following microinjection and incubation experiments with ovarian carcinoma cells [44,45]. Similar concept (attachment of TPP) was used for dendrimers [48,49], liposomes [50], gold nanoparticles [51] and blended biodegradable nanoparticles based on glycolic acid, lactic acid, and polyethylene glycol [52].

Nuclear entry of macromolecules: Macromolecules (without subcellular targeting moieties) are typically excluded from entering membrane-limited organelles, with the exception of nucleus whose membrane possesses channels that allow the passive uptake of intermediate-sized macromolecules. The NPC (nuclear pore complex) of the nuclear envelope is composed of about 30 different nucleoporin proteins and is the conduit for both nuclear import and export of macromolecules, such as proteins and nucleic acids. In active transport, cargo as large as 40 nm possessing NLS (nuclear localization sequence) or NES (nuclear export sequence) signaling peptides are guided through the channel after binding to nuclear transport receptor proteins [53]. For smaller macromolecules below 10 nm, however, NPCs have been shown to act as *non-specific pores* that allow exchange between the nucleus and cytoplasm by diffusion [54]. As a conduit for non-biological macromolecules, the NPCs have been shown to transmit PEG-coated gold colloid particles 4–7 nm in diameter [55]. The basic physico-chemical properties that determine the distribution and fate of synthetic macromolecules in living cells were characterized using fluorescently-labeled HPMA copolymers [56]. Twelve different classes of water-soluble copolymers were created by incorporating eight different functionalized comonomers. These comonomers possessed functional groups with positive or negative charges, or contained short hydrophobic peptides (1ure 1). The copolymers were fractionated to create 10 fractions of narrow polydispersity with molecular weights ranging from 10 to 200 kDa. The intracellular distributions were characterized for copolymer solutions microinjected into the cytoplasm of cultured MDAH2774 ovarian carcinoma cells. Even the highest molecular weight HPMA copolymers were shown to quickly and evenly diffuse throughout the cytoplasm and remain excluded from membrane-bound organelles, regardless of composition. The exceptions were the strongly cationic copolymers, which demonstrated a pronounced localization to microtubules. For all copolymers, nuclear entry was consistent with passive transport through the nuclear pore complex (NPC). Nuclear uptake was shown to be largely dictated by the molecular weight of the copolymers, however, detailed kinetic analyses showed that nuclear import rates were moderately, but significantly, affected by differences in comonomer composition. HPMA copolymers containing amide-terminated phenylalanine-glycine (FG) sequences, analogous to those found in the NPC channel protein, demonstrated a potential to regulate import to the nuclear compartment. Kinetic analyses showed that 15 kDa copolymers containing GG**FG**, but not those containing GGL**FG**, peptide pendant groups altered the size-exclusion characteristics of NPC-mediated nuclear import [56]. One possible explanation is that the GGFG moieties were able to weakly bind to FG-domain crosslinks in a way that altered the dynamics of a putative nucleoporin hydrogel structure, whereas GGLFG peptides would be expected to bind more strongly and not allow a rapid transfer of crosslinks in the hydrogel-like structure of the nucleopore proteins [57].

The same set of polymers was used to study the relationship between the polymer structure and uptake and subcellular trafficking into cultured C4-2 prostate cancer cells [37]. The copolymer charge was the predominant physicochemical feature in terms of cellular uptake. Fast and efficient uptake occurred in positively charged copolymers due to non-specific adsorptive endocytosis, whereas slow uptake of negatively charged copolymers was observed (Figure 1). The uptake of copolymers was also molecular weight dependent. The copolymers were internalized into the cells through multiple endocytic pathways: positively charged copolymers robustly engaged clathrin-mediated endocytosis, macropinocytosis and dynamin-dependent endocytosis, while weakly negatively charged copolymers weakly employed these pathways; strongly negatively charged copolymers only mobilized macropinocytosis. HPMA copolymer possessing 4 mol% of moderately hydrophobic functional groups did not show preferential uptake. All copolymers ultimately localized in late endosomes/lysosomes via early endosomes; with varying kinetics among the copolymers [37].

Conformation and aggregation: Anticancer drugs are usually hydrophobic and their binding to hydrophilic water-soluble polymers will impact the solution properties of the polymer-drug conjugates. As a result, drug conjugates may associate intramolecularly and intermolecularly, affecting the conformation of single chains and yielding formation of aggregates, respectively [58–60]. This effect gains importance in multifunctional conjugates as described below.

2.1.3 Multifunctional issues—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 [61]. 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 exert some influence on the other components [59,62–64]. Therefore, awareness of the complexities caused by the introduction of each component is needed to design multi-component drug carriers [65]. For example, higher amounts of the hydrophobic drug prostaglandin E_1 bound to polyHPMA macromolecules resulted in a lower rate of drug release [62]. Ding et al. studied the self-association of HPMA copolymers containing an amphipathic CD21-binding heptapeptide (YILIHRN) using fluorescence resonance energy transfer (FRET), light scattering, and size exclusion chromatography (SEC) [66]. 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 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. A better understanding of the relationship between the self-association of polymer conjugates [67] and biological significance is a prerequisite for the rational design of polymeric drug delivery systems.

2.1.4 Architecture—Polymer architecture has an important impact on the activity of the conjugates. 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 [reviewed in 68]. Szoka and Fréchet performed careful studies on the impact of molecular architecture (hydrodynamic volume, conformation, flexibility, and branching) on the fate of polymers in the organism. They concluded 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 [reviewed in 69].

An interesting design is the combination of dendrimers with water-soluble polymer-drug conjugates. PAMAM dendrimers were modified with semitelechelic (ST) HPMA copolymer – DOX conjugates [70,71]. Ulbrich, Etrych and coworkers were very active in this area. They attached the ST HPMA copolymer-drug conjugates to PAMAM dendrimers via pH-sensitive hydrazone bonds to render the constructs biodegradable [72]. Additionally, they attached ST drug conjugates directly to immunoglobulin molecules via hydrazone [73] or disulfide linkages [74].

2.2 Drug attachment and conjugate stability

Macromolecules are lysosomotropic [13]. Due to limited permeability of lysosomal membrane [23], drugs have to be cleaved from the polymeric carrier in the lysosomal compartment either by enzymatically catalyzed hydrolysis or by pure hydrolysis at lower pH. The drug is usually bound to the polymeric carrier via spacers with different structures (see below). Drug incorporation into a polymer conjugate can be achieved by copolymerization with a polymerizable drug derivative or by polymeranalogous reaction, i.e., attaching the drug to a preformed polymer carrier [75,76]. If avoidance of the lysosomal compartment is needed due to instability of the drug (e.g., DNA based) destabilization of the prelysosomal (endosomal) membrane combined with the reduction of disulfide bonds between drug and carrier is a suitable design [77,78].

2.2.1 Enzymatically cleavable spacers—To ensure drugs released from the carriers inside lysosomes and translocated into the cytoplasm. the structure of the spacer has to provide stability of the bond between the drug and carrier in the blood stream [79] and interstitial space and match the specificity of lysosomal enzymes [80]. Based on detailed degradation studies of oligopeptide sequences attached to HPMA copolymers [reviewed in 81,82] with model enzymes [83–86], in rats in vivo [87] and with lysosomal enzymes [88,89], the sequence GFLG, specific for cathepsin B, was identified [90]; it has been widely used in preclinical [91–95] and clinical settings [96–99]. Another widely used lysosomally degradable sequence is valine-citruline [100,101]. The employment of matrix metalloproteinase (MMP) enzymes in the design of drug delivery systems has been demonstrated [102]. Efficient conjugates have been synthesized using a combination of MMP-2 cleavable bonds and tumor-homing and cell–penetrating peptide iRGD. These conjugates possess enhanced accumulation and penetration into tumors in a model system [93].

The design of enzymatically cleavable spacers is well covered in the literature so we shall avoid details here. We refer the reader to the following articles and references therein [80–90,103,104].

2.2.2 Hydrolytically cleavable spacers—Another design option is to use the pH difference between blood and lysosomes and bind the drug via pH-sensitive bonds [105,106], using hydrazo [107], cis-aconityl [108], or maleic [78] spacers. The area is well-reviewed in [105]. The effect of HPMA copolymer carrier modification with positively or negatively charged groups and hydrophobic substituents on the rate of release was recently evaluated [109]. The results revealed no change in the rate of DOX release when positively charged groups were introduced and 15–20% increase in release rate when carboxylic group containing comonomers were introduced. Introduction of oleyl group led to aggregation and decrease in the rate of release [109].

2.2.3 Reducible spacers—The difference in intracellular oxidoreductive conditions compared to the outside of the cell presents a unique opportunity that can be exploited for site-specific delivery. High concentrations of cystine (40 μM) and correspondingly low concentrations of reducing agents, cysteine (9.7 μM) and reduced glutathione (2.8 μM), produce an oxidative environment extracellularly [110]. On the other hand, the cytosol is reducing due to the high intracellular concentration of reduced glutathione (5 mM) as compared to oxidized glutathione, which may be present at a 100-fold lower concentration [111]. This highly reductive cytosolic condition is maintained by the action of glutathione reductase and NADPH [112,113]. In the endocytotic pathway, active accumulation of cysteine in lysosomes, due to cysteine transporters [114,115], may facilitate disulfide bond reduction. This may be enhanced by the action of reducing enzymes, such as Gamma-Interferon Inducible Lysosomal Thiol Reductase (GILT). This enzyme was the first reductive enzyme found in the lysosomes of antigen presenting cells and is optimally active at a pH less than neutral. The activity of GILT may require the presence of a reducing agent such as cysteine to maintain activity [116].

Disulfide bonds have been incorporated in the synthesis of cleavable delivery systems for DNA, RNA, and siRNA [117–121], antisense oligonucleotides [122], peptides [123], toxins [124] and anticancer drugs [125–127]. An interesting design is the dynamic conjugates that combine endosomolytic function with siRNA reductive release [77,128,129]. Such multifunctional conjugates contain comonomers with amino groups protected by pH-sensitive bonds, hydrophobic comonomers, and cargo (siRNA) bound via disulfide bonds. In the endosomes the amino group protection (usually a semitelechelic hydrophilic polymer) is cleaved off, the combination of electrostatic (via amino groups) and hydrophobic interaction destabilizes the endosomal membrane. The reductive environment then reduces the disulfide bonds and releases siRNA into the cytoplasm.

2.2.4 Self-immolative spacers—Katzellenbogen and coworkers introduced a new type of spacer [130] where the biologically active molecule (drug) is bound to the carrier via spacer (S) and a linker (L). Following enzymatic cleavage of the bond between S and L, a spontaneous rearrangement of the L structure results in the release of the free, unmodified drug (Figure 2). Typical linker groups employ benzyl eliminations for the release of drugs – 1,4- or 1,6-elimination. The aromatic structure contains a hydroxyl, amino or thiol group. When covalently bound these groups are stable; however, activation by e.g. enzymatic cleavage produces a nucleophilic compound that initiates self-immolation releasing the unmodified drug [100,131–133].

Self-immolative spacers have been used in the design of drug delivery systems, prodrugs, sensors for chemicals or enzymes, and in material science [131]; several examples follow. Dubowchik et al. developed immunoconjugates of BR96 antibody (Ab) with doxorubicin (DOX) containing dipeptide spacers (Val-Lys, Val-citrulin) cleavable in the lysosomal compartment, especially by cathepsin B [104]. To avoid steric crowding at the position P1 (S-P nomenclature according to Schechter and Berger [134]) and to achieve cleavage a p-aminobenzylcarbonyl (PABC) 1,6-elimination group was inserted between the Ab and Val-Cit-DOX.

Elongated spacers, where the enzymatically cleavable bond is separated from the drug by a self-eliminating 4-aminobenzylalcohol structure group, have been used for the design of oral drug delivery systems based on HPMA copolymer – 9-aminocamptothecin conjugates [135,136] and for binding prostaglandin to HPMA copolymer via a cathepsin K sensitive tetrapeptide (GGPNle) [62]. Putnam et al. synthesized oligopeptide spacers terminated in α-substituted glycine derivative of 5-fluorouracil (5-FU). Following enzymatically catalyzed release of 5-FU substituted glycine a spontaneous decomposition yielded 5-FU and a glyoxylate [137]. Shabat et al. used the self-immolative concept for the design of degradable materials (e.g. dendrimers) [138] and demonstrating that one trigger reaction may result in the release of numerous drug molecules at once [139].

2.3 Targeted conjugates

Numerous compounds complementary to target receptors can be used as targeting moieties, such as antibodies [74,140–143], antibody fragments [144,145], saccharides [146–148], lectins [149,150], peptides [61,66,151–156], aptamers [157], small molecule ligands [92], etc. The impact of a targeting moiety on the biodistribution and efficacy of the conjugate will depend on the type of tumor and structure of the conjugate [158,159]. As discussed in more detail in ref. [158] targeting for solid tumors may not be needed due to the enhanced permeability and retention (EPR) effect and beneficial manipulation of molecular weight of conjugates. In contrast, for the treatment of blood cancers, such as non-Hodgkin's lymphoma, targeting is beneficial.

Cancer cells are present in various differentiation statuses, such as cancer stem cells (CSC) and differentiated cells [160]. 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 [161–163].

2.4 Highlights of polymer-drug conjugate properties

Here we shall briefly discuss the advantages of polymer-bound drugs when compared to low molecular weight (unbound) drugs: *Enhanced stability of drugs.* Binding of a chemically labile drug to a polymer carrier results in its stabilization. For example, cytarabine (CYT) is the most active agent available for the treatment of acute myeloid leukemia (AML). However, the potency of CYT is limited by its low stability after intravenous administration due to the metabolism into the inactive and more soluble form by cytidine deaminase. When bound to HPMA copolymer via GFLG side chains improved human plasma stability was achieved. After 48 h, all free drug disappeared, whereas there was still close to 50% of the polymer-bound drug present indicating advantage of conjugation of CYT to a polymer carrier [94].

Different mechanism of cell entry—It is well know that in contrast to low molecular weight compounds that can cross the plasma membrane by diffusion, macromolecules (and polymer-drug conjugates) enter cells by endocytosis with the ultimate location in the lysosomal compartment of the cell [37–42]. The rational design of polymer-drug conjugates is based on these phenomena [160].

Changed pharmacokinetics—Attaching drugs to polymer carriers results in enhanced intravascular half-life and changed biodistribution [164,165]. The pharmacokinetic parameters can be manipulated by the molecular weight and the structure and conformation of the polymer carrier [36,166].

Overcoming multidrug resistance—The fact that macromolecules' subcellular trafficking occurs in membrane limited organelles renders the drug-efflux pumps, present in multidrug resistant cells, less efficient [167]. Free (unbound) drug is recognized by the membrane transporter (e.g., P-glycoprotein) when trying to enter the cell [168]. In contrast, lysosomes are located in the perinuclear region and the drug released from the polymer carrier will enter cytoplasm in this region out of reach of the P-glycoprotein. This rationale was validated for HPMA copolymer-DOX conjugates in vitro and in vivo [169–171].

Immunostimulatory effect—The long-term efforts to employ the immune system in the treatment of cancer [172] did not miss the drug delivery field. Říhová and coworkers realized the immunostimulatory potential of HPMA copolymer-drug conjugates more than 25 years ago. They have shown that HPMA copolymer-daunomycin conjugate was not myelotoxic, but stimulated bone marrow stem cells resulting in an increase in the number of colony-forming units in the spleen [173]. A following study [174] has demonstrated that HPMA copolymer bound anticancer drugs, in contrast to unbound drugs, did not induce Fas ligand expression in cancer cells and thus did not trigger the death of antitumor effector cells. The proposed mechanism of the combined cytostatic and immunostimulatory activity postulates that first chemotherapy-sensitive tumor cells are killed, releasing tumor antigens.

Then, the antitumor response will result in the elimination of chemotherapy-resistant cells [175,176]. The fact, that mice cured from EL4 T-cell lymphoma with HPMA copolymer-DOX conjugates survive re-transplantation without further treatment supports the hypothesis [177].

3. Need for new/improved designs

The advantages of polymer-bound drugs, briefly described above, are numerous [15,68,81,82,98,158,178–182]. They have been tested in several clinical trials [183–186], but the translation of laboratory results into the clinics has been slow [19,187]. The research directions possessing a potential to enhance the speed of translation were discussed recently [19]. The polymer-drug conjugates in clinical trials demonstrated a decrease of adverse effects when compared to the administration of free (unbound) drug. However, the enhancement of the efficacy of treatment was lower than in animal models. This is most probably related to the ratio of tumor volume to body volume. In humans the ratio (of tumor volume to body volume) is much lower than in animal models and not enough conjugate extravasates into the solid tumor when a conjugate with a short intravascular half-life is used. To achieve an effective accumulation of the conjugate into the solid tumor a concentration gradient for an extended time is needed. The design of conjugates that can achieve this – backbone degradable carriers of anticancer drugs will be used as the first example.

Another direction how to move the field forward is to design new, previously unused approaches. We invented a new paradigm in drug delivery - “drug-free macromolecular therapeutics”. This strategy is based on the induction of apoptosis by crosslinking of receptors mediated by the biorecognition of two nanoconjugates at the cell surface. Importantly, no low molecular weight drug is needed. This approach will be used as the example of new trend in macromolecular therapeutics research.

4. Backbone degradable long-circulating (2nd generation) conjugates

As mentioned above, molecular weight (Mw) and molecular weight distribution are important factors in the design of effective macromolecular therapeutics. The higher the molecular weight of the polymer carrier, the higher the accumulation of the polymer-drug conjugate in the tumor tissue with concomitant increase in therapeutic efficacy [31,82]. However, the renal threshold limited the molecular weight of the first generation (non-degradable) polymeric carriers to below 50 kDa [26]; this lowers the retention time of the conjugate in the circulation with simultaneous decrease in pharmaceutical efficiency. It is evident from previous research that for an efficient EPR effect a concentration gradient for an extended time is essential. Higher molecular weight drug carriers with a nondegradable backbone deposit and accumulate in various organs, impairing biocompatibility. Clearly, new designs are needed. To address the challenge, we designed backbone degradable HPMA copolymer carriers that possess a long circulating time while preserving biocompatibility. The new design of backbone degradable HPMA copolymer drug carrier is based on the state-of-the-art approaches – combination of controlled/living radical polymerization and click reactions [188–190]. The backbone degradable HPMA copolymer–drug conjugates provide long circulating water-soluble polymeric carriers that effectively accumulate in tumor tissue. They will be eliminated from the body after enzymatic cleavage of the oligopeptide segments in the backbone (producing polymer fragments below the renal threshold) and in side chains (releasing the drug). The synthesis procedures proposed are versatile; they provide a platform for the preparation of a large variation of polymer-drug conjugates with tailor-made properties, such as predetermined circulation time and composition.

4.1 Multiblock degradable polymeric carriers

Multiblock backbone degradable HPMA copolymers are prepared in two steps: first, a telechelic diblock copolymer is prepared by RAFT (reversible addition-fragmentation chain transfer) polymerization using a bifunctional CTA followed in the second step by chain extension using alkyne-azide [188,189] or thiol-ene [190] click reactions resulting in ***long circulating multiblock HPMA copolymer-drug conjugates***. Finally, the product needs to be fractionated by size exclusion chromatography (SEC). Figure 3 shows an example of synthesis of multiblock HPMA copolymer-drug conjugates using Peptide2CTA. Following this approach, various multiblock conjugates containing anticancer drugs paclitaxel [191,192], gemcitabine [192], doxorubicin [193] have been synthesized and evaluated in vitro and in vivo.

4.2 Diblock degradable polymeric carriers

Using an RAFT chain transfer agent (CTA) (Peptide2CTA) that contains an enzymatically degradable oligopeptide (GFLGKGLFG) flanked by two dithiobenzoate groups, **the synthesis of degradable diblock copolymers of narrow polydispersity can be done in one step** [190]. This feature makes the Peptide2CTA very attractive for scale-up of the synthetic procedures. During RAFT polymerization the 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 [190]. Degradable diblock copolymers extend the biocompatible molecular weight range to 100 kDa. The copolymers have a narrow distribution of molecular weights and the process is scalable.

4.3 Comparison of 2nd generation conjugates with original (1st generation) conjugates

The enhanced activity of 2nd generation of 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 [193], paclitaxel [191], gemcitabine [194] 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 [195].

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 [166,193]. Combination chemotherapy with 2nd generation backbone degradable diblock HPMA copolymer-paclitaxel and HPMA copolymer-gemcitabine conjugates against A2780 human ovarian carcinoma xenografts was recently evaluated and results compared with 1st generation conjugates and free drugs [166]. The 2nd generation degradable high-Mw conjugates showed distinct advantages, such as favorable pharmacokinetics (3–5×half-life when compared to the 1st generation), dramatically enhanced inhibition of tumor growth (complete tumor regression) by paclitaxel and gemcitabine conjugates combination, and absence of adverse effects (Figure 4). In addition, multimodality imaging studies of dual-labeled model conjugates confirmed the efficacy of 2nd generation conjugates by visualizing more than 5 times enhanced tumor accumulation, rapid conjugate internalization, and effective intracellular release of payload. Together, the results indicate that the 2nd generation degradable HPMA copolymer carrier can provide an ideal platform for the delivery of a range of antitumor compounds, which makes it one of the most attractive candidates for potential clinical application [166].

5. New paradigm: Drug-free macromolecular therapeutics

5.1 Rationale and origin

The idea to design drug free therapeutics was based on the known mechanism of apoptosis induction in B cells via crosslinking of the CD20 receptor [196] and our studies on self-assembling hybrid graft HPMA copolymers [197]. It has been known that crosslinking of (non-internalizing) CD20 receptors initiates apoptosis in B cells. At the same time we found that two HPMA copolymers grafted with complementary coiled-coil forming peptides self-assemble into hydrogels [197,198]. The self-assembly was mediated by the biorecognition of the peptide grafts [199]. Thus we hypothesized that if we can crosslink graft copolymers with concomitant formation of hydrogels, we could crosslink cell surface (non-internalizing) CD20 receptors with simultaneous apoptosis induction. Indeed, we succeeded and the activity of this system both in vitro and in vivo was far better than we expected [reviewed in 200].

5.1.1 Non-Hodgkin's lymphoma and CD20—Non-Hodgkin's lymphoma (NHL) originates about 85% from B cells and the remaining 15% are mostly T-cell related. The approval of Rituximab, an anti-CD20 antibody revolutionized the field. However, large populations of patients exist that do not respond or develop resistance to therapy. CD20 is a 35–37 kDa integral membrane protein highly expressed on more than 95% of B-cell lymphomas [201]. Free CD20 antigen is not present in serum, and when bound to ligand (antibodies), it has a very low intracellular internalization rate [202]; CD20 is often considered a non-internalizing receptor. It functions as a store-operated calcium channel and a cell cycle regulator [203]. It is one of the most reliable biomarkers of B-lymphocytes, thus providing an ideal target for treatment of B-cell NHL [204]. CD20 is also expressed on normal B-cells; however, it is not expressed on stem cells or progenitor cells and mature or activated plasma cells [205]. Therefore, the "B-cell depletion" therapeutic approach is considered safe; normal numbers of B-cells can be restored after treatment [206]. Apoptosis initiated by anti-CD20 mAbs occurs by three cellular events: a) antibody-dependent cellular cytotoxicity (ADCC); b) complement-dependent cytotoxicity (CDC); and c) CD20-mediated apoptosis [207–209]. All of these mechanisms require immune effector cells to function

[209]. The third mechanism (c) is initiated by crosslinking of bound antibodies by immunocompetent cells via the Fc fragment.

Direct crosslinking of CD20 receptors leads to apoptosis. Studies with synthetically crosslinked Rituximab have shown enhanced induction of apoptosis and tumor clearance when compared with Rituximab alone [210,211]. Multivalent Fab' fragment – HPMA copolymer conjugates induce apoptosis in Raji B cells via crosslinking of CD20 receptors [212–214]. Similarly, multivalent rituximab lipid nanoparticles [215] and nanoparticles self-assembled from fusion between a single chain antibody and a soluble protein polymer [216] induce apoptosis. However, such constructs are difficult to synthesize and they do not permit to use pretargeting strategies. Binary systems, described below are suitable for such strategies [217].

The apoptotic pathways provided a roadmap for the design of the new treatment paradigm. Avoiding the Fc fragment and using Fab' antibody fragment one could decorate cells with a moiety bound to the Fab' fragment. Then, the crosslinking of receptors could be achieved by using a graft copolymer containing several copies of a complementary structure. This would avoid crosslinking via immunocompetent cells, ADCC and CDC mechanisms, and side effects of therapy associated with the Fc fragments [218]. Drug-free macromolecular therapeutics trigger direct and specific apoptosis of B-cell lymphomas without the help of effector cells (Figure 5). This is achieved by the design of synthetic effectors that reproduce the function of immune effector cells.

5.2 Coiled-coil peptide based design

Molecular self-assembly is becoming a popular route to new supramolecular structures and molecular materials. The inspiration for such structures is commonly derived from self-assembling systems in nature [199]. ***The coiled-coil*** is one of the basic folding patterns of native proteins. It consists of two or more α-helices winding together and forming a super-helix [219]. The primary structure of the coiled-coil motif is characterized by a heptad repeating sequence where the first and fourth are hydrophobic amino acid residues, while the others are polar. The distinctive feature of coiled-coils is the specific spatial recognition, association, and dissociation of the helices. Depending on their structure they may form homodimers, heterodimers with parallel or antiparallel orientation [199]. They provide an ideal model of protein biomaterials in which the higher structure may be predicted based on primary sequence. We designed two heptapeptad sequences with opposite charge (CCE and CCK) that self-assemble as antiparallel heterodimers (CCE: CYGG E VSALEKE VSALEKK NSALEKE VSALEKE VSALEK; CCK: CYGG K VSALKEK VSALKEE VSANKEK VSALKEK VSALKE) and synthesized HPMA copolymers grafted with these sequences, P-CCE and P-CCK [197]. As predicted, based on the design of peptides, the aqueous solutions of P-CCE or P-CCK alone did not form gels. In contrast, hydrogels self-assembled from equimolar mixtures of P-CCE/P-CCK within the full concentration range tested (0.1 – 1.0 wt.%). The formation of hydrogels at concentrations as low as 0.1 wt.% indicated a superior biorecognition of the two graft copolymers [197,198].

To prove the concept of drug-free macromolecular therapeutics, two nanoconjugates were synthesized: a) Fab' fragment of 1F5 anti-CD20 by P-(CCK)$_x$ initiated apoptosis due to crosslinking of the CD20 receptors mediated by antiparallel coiled-coil formation at the cell surface [220,221]. In vivo evaluation on SCID mice bearing systemically disseminated NHL Raji B cells the administration of two nanoconjugates (both consecutive or premixed) produced long-term survivors and eradicated lymphoma cells in blood and bone marrow [221].

Super-resolution imaging was used to quantify organizational changes in the plasma membrane after treatment with hybrid nanoconjugates, Fab'-CCE and P-$(CCK)_x$ [222]. Lipid rafts play a vital role in cell signaling, especially in apoptosis [223]. It is still unknown whether the nanoconjugates require lipid raft platforms to induce apoptosis and how the conjugates alter the lateral organizing of proteins in the membrane. We used methyl-β-cyclodextrin (MβCD), a cholesterol chelator, to extract cholesterol, a component of lipid rafts, from the cell membrane [224] and latrunculin B (LatB), an actin destabilizer, to disassemble cortical actin and prevent lipid raft formation [225]. In order to validate the lipid raft-clustering hypothesis, we imaged CD20 and lipid raft clusters after nanoconjugate treatment. – dSTORM (direct stochastic optical reconstruction microscopy) was used to provide super resolution images of Raji cellular membranes subjected to various treatments. Lipid rafts were tracked using Alexa Fluor 555 conjugated to cholera toxin B. CD20 distribution was tracked using fluorescently labeled Fab'-MORF1-AF647. Lipid raft cluster size correlated with apoptosis induction after treatment with the nanoconjugates. Clusters greater than 100 nm in radii did not form in cells pre-treated with lipid raft disrupting molecules. Super-resolution images provided precise molecule location coordinates that could be used to determine density of bound conjugates, cluster size, and number of molecules per cluster [222].

In another study, multiple fluorescence imaging studies, including 2-channel FMT, 3D confocal microscopy, and 4-color FACS, were performed to prove that two conjugates (Fab'-CCE and P-$(CCK)_x$) can assemble at cell surface [226]. Confocal microscopy showed co-localization of two fluorescently labeled conjugates on NHL Raji cell surface, indicating "two-step" targeting specificity. The fluorescent images also revealed that these two conjugates could disrupt normal membrane lipid distribution and form lipid raft clusters, leading to cancer cell apoptosis. This "two-step" biorecognition capacity was further demonstrated in a NHL xenograft model, using fluorescent images at whole-body, tissue and cell levels [226].

5.2.1 Evaluation of potential immunogenicity of CCE, CCK and their conjugates—

The aim of the study [227] was to a) assess general biocompatibility of the therapeutical system; and b) to compare the immune response to L- and D-CCE/CCK. In our previous research as well as in clinical trials it was demonstrated that short peptide spacers attached to HPMA copolymers do not exhibit immunogenicity [228,229]. However, the peptides used in coiled-coils contain 38 amino acid residues; this may be still not enough to initiate immune response when unbound, but these peptides may act as haptens when bound to HPMA copolymers or when associated with blood proteins. Consequently, we evaluated the immunogenicity in vitro and in vivo of free peptides, their conjugates with HPMA copolymer and with antibody fragment as well as their premixture (possible conformational epitopes). Both L- and D-peptides and conjugates were synthesized. The in vitro response was evaluated on RAW264.7 macrophages; the immunogenicity in vivo was performed in Balb/c mice following intravenous administration [227].

RAW264.7 cells were cultivated together with the tested compounds and cytokine production determined by ELISA (TNF-α, IL-1β, IL-6 and IL-10), and viability or changes in the surface markers (M1 vs. M2 polarization, activation markers) by flow cytometry. Lipopolysaccharide (LPS) served as positive control of activation (and M1 shift). Neither HPMA copolymer nor any peptide, either L- or D-, induced any response in murine macrophages. The component responsible for macrophage activation was the 1F5 Ab or its fragment. Interestingly, Press et al. treated 4 patients with 1F5 Ab and observed minimal treatment toxicities [230,231].

In vivo: the therapeutics based on L-peptides (MIX L, Fab‘-L-CCE/P-L-CCK) did not induce substantially different Ab response than those based on D peptides (MIX D; Fab‘-D-CCE/P-D-CCK). The titer and avidity of Ab induced by i.v. treatment with MIX L or MIX D were generally low, slightly lower in case of MIX D, except for anti-Fab‘-CCE IgM Ab. In general, there were detectable Abs, but no cellular response to the therapeutics administered i.v. The Ab response was predominantly directed against the Fab’ part of the therapeutics [227]. In summary, the immunogenicity is acceptable for translation; to be on the safe side, humanization of the Fab’ fragment is recommended before translation into the clinics.

5.3 Morpholino oligonucleotide based design

The biorecognition of oligopeptide sequences, CCE and CCK, resulted in the design of nanoconjugates with a robust anticancer effect. However, at physiological pH the peptide sequences did not possess a strong secondary structure. Their first contact was mediated by hydrophobic and electrostatic interactions. Once paired, they folded into highly organized antiparallel coiled-coil heterodimers. Consequently, an excess of the second nanoconjugate had to be used to achieve effective biorecognition in vivo [221].

With the aim to achieve excellent biorecognition at a 1:1 ratio of complementary moieties morpholino oligonucleotides have been selected based on their fast and efficient hybridization, stability in plasma, and water solubility [232,233].

Nucleic acid hybridization, such as the formation of a DNA double helix is composed of Watson-Crick base pairing, *i.e.*, hydrogen bonding of A/T and C/G, between two single-stranded polynucleotides with complementary sequences. The conformation is further stabilized by base stacking, *i.e.*, π-π interaction of neighboring bases on the same strand. DNA has been used as building blocks for biomaterials design [234,235] and functional nanostructures for drug delivery [236]. Over the years, a variety of artificial oligonucleotides with chemically modified backbones have been synthesized [237]. These non-phosphodiester backbones are nuclease resistant and stable in the body; thus, they are suitable for biopharmaceutical applications. We designed a pair of phosphorodiamidate morpholino (MORF) oligomers, MORF1 (5’-GAGTAAGCCAAGGAGAATCAATATA-linker-amine-3’) and MORF2 (5’-TATATTGATTCTCCTTGGCTTACTC-linker-amine-3’), as the biorecognition motifs for the second-generation “drug-free” therapeutic system [232].

The MORF oligonucleotides are charge neutral, resulting in significantly stronger binding than natural DNA and RNA [238]. Hybridization of MORF pairs has well-defined binding specificity, which prevents potential off-target effects [239]. In addition, MORF oligonucleotides have good aqueous solubility and favorable pharmacokinetics [240]. The sequences of MORF1 and MORF2 were designed to achieve optimal binding efficiency and minimal off-targets with human and murine mRNA, and to prevent self-complementarity [232]. This new therapeutic system is composed of two hybrid conjugates: (1) anti-CD20 Fab' linked to MORF1 (Fab'-MORF1), and (2) HPMA copolymers grafted with multiple MORF2 (P-(MORF2)$_x$). The two conjugates self-assemble via MORF1-MORF2 hybridization at the surface of CD20$^+$ B-cells, crosslink CD20 antigens and initiate apoptosis [232].

5.4 Advantages and general applicability of the new paradigm

The therapeutic system comprises a pretargeting component (anti-CD20 Fab' conjugated with MORF1) and a crosslinking component (*N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer grafted with multiple complementary MORF2). Consecutive treatment with the two components resulted in CD20 clustering on the cell surface and effectively killed malignant B-cells *in vivo*. To enhance therapeutic efficacy, a two-step pretargeting approach was employed. We showed that the time lag between the two doses can be optimized based on pharmacokinetics and biodistribution of the Fab'-MORF1 conjugate. In a mouse model of human non-Hodgkin lymphoma (NHL), increasing the time lag from 1 h to 5 h resulted in dramatically improved tumor growth inhibition and animal survival. When the 5 h interval was used, the nanotherapy was more efficacious than rituximab and led to complete eradication of lymphoma cells with no signs of metastasis or disease recurrence [241].

We further evaluated the nanomedicines using cells from 10 patients with chronic lymphocytic leukemia (CLL) [242]. Apoptosis and cytotoxicity were observed in 8 patients including two with the 17p13 deletion, a high-risk prognostic factor. Additionally, cells from patients with mantle cell lymphoma also responded to the drug-free therapy [241].

The drug-free system is a new paradigm in macromolecular therapeutics. Its main feature is the absence of a low molecular weight drug. Other favorable features are: a) Pretargeting – the two-step treatment permits to manipulate the pharmacokinetics by separating the targeting modality from the effector modality; b) Multivalency – the second nanoconjugate is multivalent and it was demonstrated that the efficiency relates to its valency [232]; c) The system is immune independent. The nanoconjugates directly induce apoptosis without the need for immune activation. The drug-free macromolecular therapy is distinct from mAb-based immunotherapy. Here synthetic effectors are operative that replace the function of immune effector cells. This approach may benefit patients that do not respond to immunotherapies [243].

6. Conclusions and future prospects

Scientifically, the design principles for polymer-based nanomedicines 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 [160]. The remarkable progress in imaging techniques that permit non-invasive monitoring of the fate of conjugates will undoubtedly contribute to a more rational design of polymer therapeutics and theranostics [244,245]. However, a qualitative change in the approach to design and treatment is needed for successful translation to the clinics.

The advantage of the second generation (backbone degradable) conjugates over both, first generation conjugates and free drugs was clearly demonstrated. These new conjugates are long-circulating; they are composed from enzymatically cleavable sequences and synthetic blocks in an alternating arrangement. 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 proces.

Drug-free macromolecular therapeutics is a new paradigm in polymer-based nanomedicines. It is an example of qualitatively new design approach. The absence of low molecular weight drugs and independence from immune activation render the method applicable in many areas. In addition to NHL therapy, it may be used for the treatment of other B cell related diseases – rheumatoid arthritis, idiopathic thrombocytopenic purpura, hemolytic anemia [246–247], and multiple sclerosis [249]. Moreover, in addition to CD20, other slowly internalizing receptors may be used as targets, such as CD45, death receptor 4, prostate cell antigen, and carcinoembryonic antigen [200].

The scientific knowledge and experience from preclinical and clinical research are the basis for the possible translation of macromolecular therapeutics into the clinics within the next decade.

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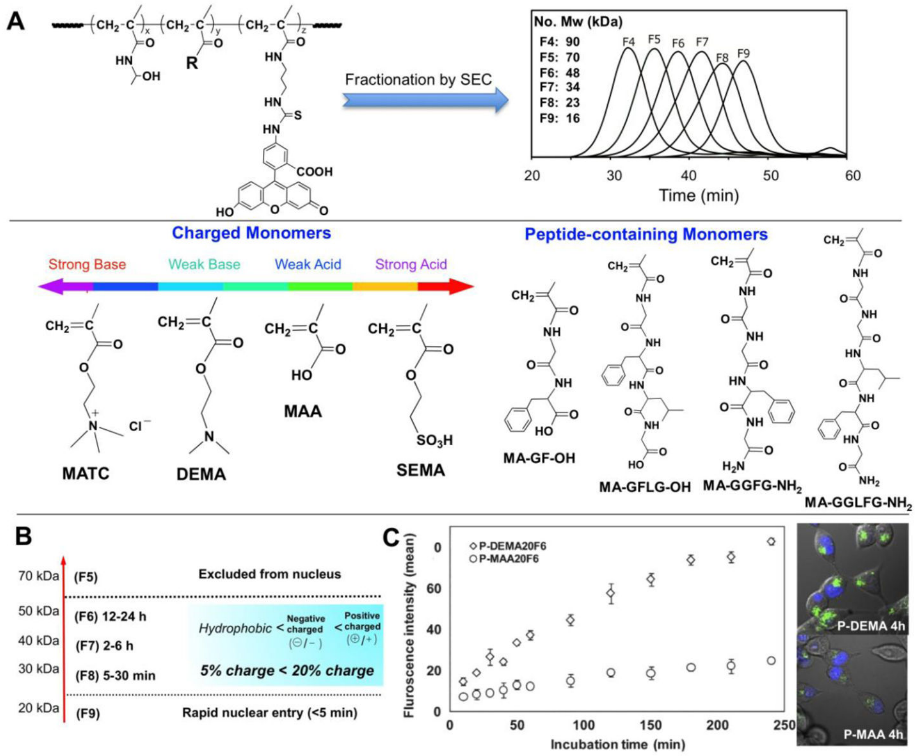

Figure 1.

A) Structure of monomers and polymers [37,56]; B) Impact of Mw and structural factors on transport of macromolecules from the cytoplasm into the nucleus [56]; C) Internalization of positively and negatively charged macromolecules into prostate cancer cells [37].

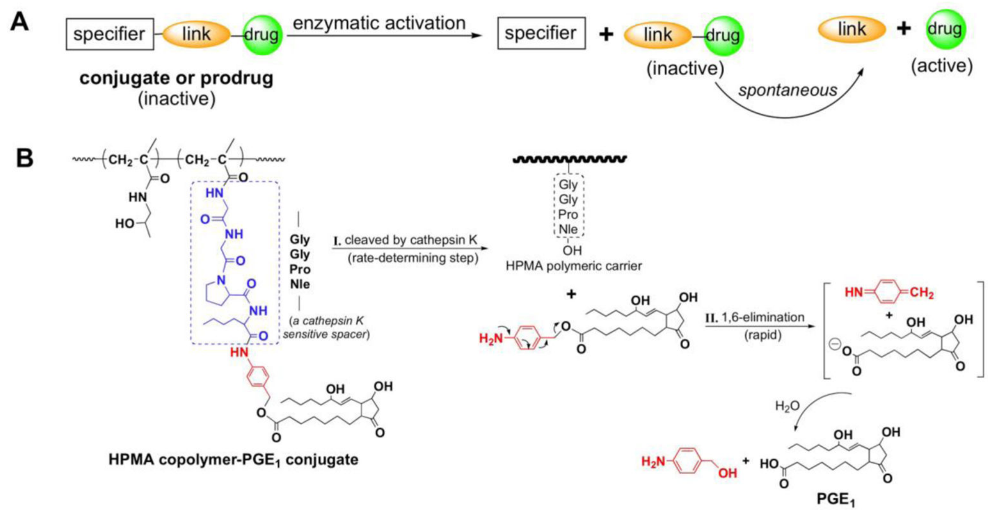

Figure 2.

Self-immolative spacers. a) Principle of design [130]; b) Scheme of release of unmodified prostaglandin (PGE_1) from HPMA copolymer–PGE_1 conjugates by a two-step process – rate controlling enzymatic cleavage followed by fast 1,6-elimination [62].

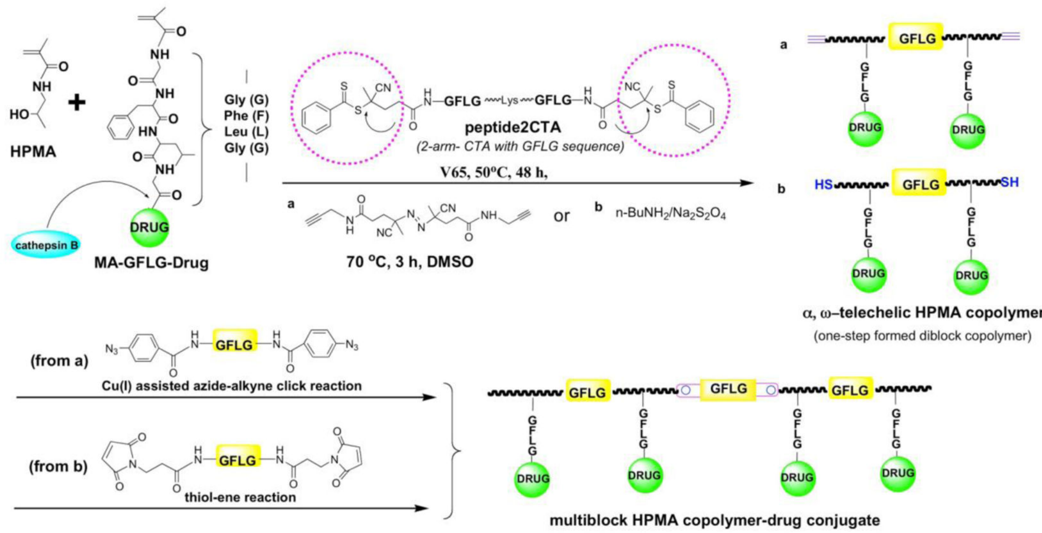

Figure 3.
Synthesis of multiblock degradable HPMA copolymer-drug conjugates [190–193]

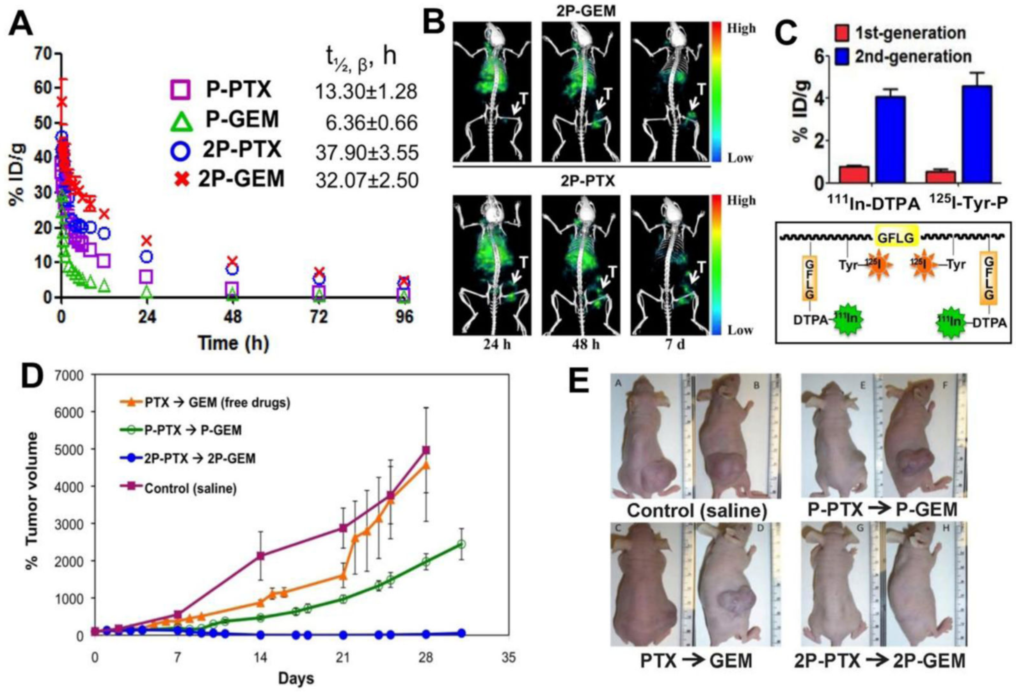

Figure 4.

Combination treatment of 2nd generation backbone degradable diblock conjugates showed improved therapeutic efficacy in A2780 human ovarian carcinoma xenografts. (A) Blood activity-time profiles of ^{125}I-labeled conjugates in mice. The data represent the mean radioactivity expressed as a percentage of the injected dose per gram of blood from mice (n=5). (B) SPECT/CT images of mice bearing subcutaneous A2780 human ovarian carcinoma in right flank after intravenous injection of ^{125}I-labeled diblock conjugates (2P-PTX, 2P-GEM). The representative images were acquired 24 h, 48 h, and 7 d after administration of conjugates. T, tumor. (C) Tumor uptake of dual-labeled conjugates (^{111}In-DTPA as model drug and ^{125}I-Tyr-P as polymer carrier) in mice bearing subcutaneous A2780 tumor 48 h after injection of first- or second-generation conjugates. Data are plotted as percentage of injected dose per gram of tissue (% ID/g). All of the data are expressed as mean ± SD (n = 4–5). (D) A2780 tumor growth in mice treated with different formulation combinations (n=5). * p<0.01. Female nude mice received one dose of PTX or HPMA copolymer-PTX conjugate (20 mg/kg PTX equivalent) on day 0 and 3 doses of GEM or HPMA copolymer-GEM conjugate (5 mg/kg GEM equivalent) on days 1, 7, and 14. Note: in the orange (2P-PTX→2P-GEM) line the error bars are hidden within the experimental points. (E) Photographs of A2780 tumors after treatment with different combinations. Mw of conjugates: P-GEM – 40 kDa; 2P-GEM – 110 kDa; P-PTX – 50 kDa; 2P-PTX -115 kDa. Adapted from ref. [166].

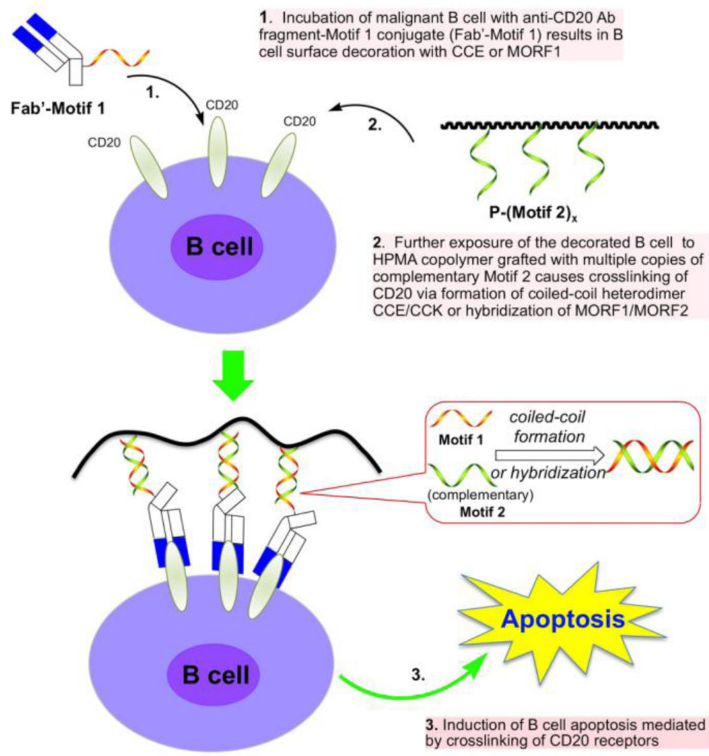

Figure 5.

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 or morpholino oligonucleotides, MORF1 and MORF2. The induction of apoptosis in human Burkitt's NHL Raji B cells was triggered by crosslinking of the surface CD20 antigen mediated by the biorecognition of antigen-antibody and complementary motifs [220,221,232,241].

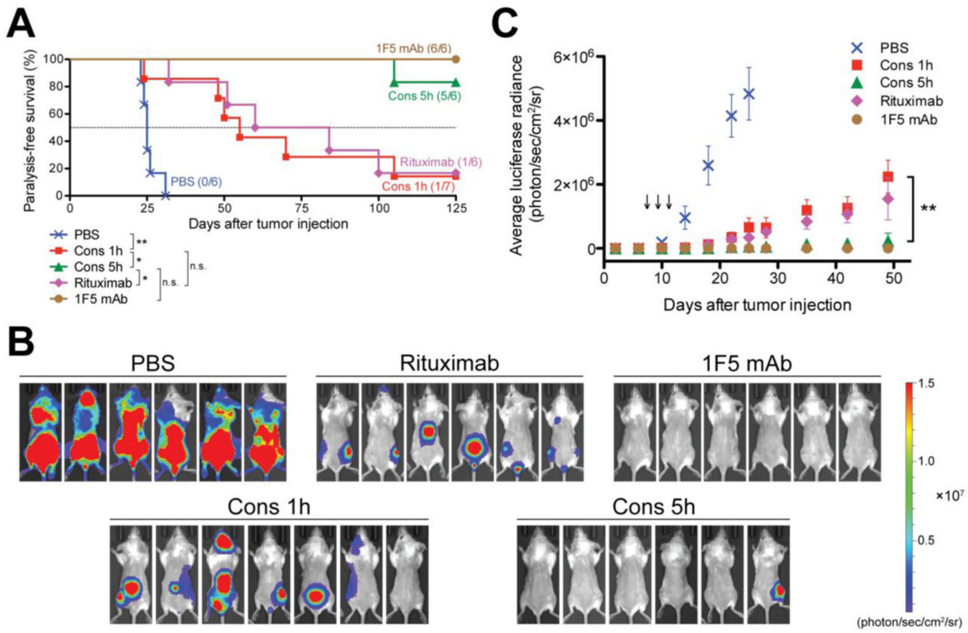

Figure 6.

In vivo efficacy of drug-free macromolecular therapeutics against systemic B-cell lymphoma. SCID mice were injected with luciferase-expressing Raji cells (4×10^6) via the tail vein on day 0. Three doses of each treatment were administered on days 7, 9, and 11 (n = 6 or 7). *PBS*: mice injected with PBS; *Cons 1h*: consecutive treatment of Fab'-MORF1 and P-MORF2, 1 h interval; *Cons 5h*: consecutive treatment of Fab'-MORF1 and P-MORF2, 5 h interval; *Rituximab; 1F5 mAb*. (**A**) Paralysis-free survival of mice presented in a Kaplan-Meier plot. Numbers of long-term survivors in each group are indicated. Statistics was performed with log-rank test (*: $p < 0.05$, **: $p < 0.005$, n.s.: no significant difference). (**B**) *In vivo* bioluminescence images at 25 days post-tumor injection. Mice were i.p. injected with 3 mg firefly D-luciferin 15 min prior to imaging. (**C**) Whole-body bioluminescence intensity of mice. Data are shown as mean ± SEM (n = 6 or 7). Statistics was performed by student's t test (**: $p < 0.005$). Black arrow: dose administration. Reprinted with permission from ref. [241].