POLYMERIC BIOMATERIALS AND NANOMEDICINES

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ABSTRACT

This overview intends to demonstrate the close relationship between the design of smart biomaterials and water-soluble polymer-drug conjugates. First, the discovery and systematic studies of hydrogels based on crosslinked poly(meth)acrylic acid esters and substituted amides is described. Then, the lessons learned for the design of water-soluble polymers as drug carriers are highlighted. The current state-of-the-art in water-soluble, mainly poly[*N*-(2-hydroxypropyl)methacrylamide (HPMA), polymer-drug conjugates is shown including the design of backbone degradable HPMA copolymer carriers. In the second part, the modern design of hybrid hydrogels focuses on the self-assembly of hybrid copolymers composed from the synthetic part (backbone) and biorecognizable grafts (coiled-coil forming peptides or morpholino oligonucleotides) is shown. The research of self-assembling hydrogels inspired the invention and design of drug-free macromolecular therapeutics – a new paradigm in drug delivery where crosslinking of non-internalizing CD20 receptors results in apoptosis *in vitro* and *in vivo*. The latter is mediated by biorecognition of complementary motifs; no low molecular weight drug is needed.

Keywords:

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1. Introduction

In this overview we intend to demonstrate the close relationship between the design of biomaterials and the design of nanomedicines as experienced in our research. One of us (JK) was a graduate student at a laboratory where hydrogels, the first rationally designed biomedical polymers, were discovered by Drahošlav Lím [1,2] and soft contact lenses designed by O. Wichterle [3] in the 1950s. These discoveries initiated biomaterial and nanomedicine research worldwide and for many remain an inspiration today.

Original hydrogels were synthesized by traditional radical copolymerization of vinyl and divinyl (crosslinker) compounds. The first hydrogels were based on hydrophilic esters of methacrylic acid – e.g. the first soft contact lenses were a copolymer of 2-hydroxyethyl methacrylate (HEMA) with ethylene dimethacrylate (EDMA). Numerous hydrogel structures followed [2] and a detailed study of the relationship between the composition of hydrogels and their biocompatibility [4] was the driving force for the design of water-soluble polymeric carriers based on *N*-substituted methacrylamides and development of polymer-drug conjugates, one of the most promising nanomedicines.

Our recent hydrogel research focuses on the self-assembly of hydrogels from hybrid block or graft copolymers driven by the interaction of complementary biorecognition motifs [5]. Both peptide/protein [6,7] and oligonucleotide [8] motifs have been used in hydrogel design. For example, two distinct pentaheptad peptides (CCE and CCK) were designed to create a dimerization motif and serve as physical crosslinkers. Indeed, graft copolymers, P-CCE and P-CCK (P is the *N*-(2-hydroxypropyl)methacrylamide (HPMA) backbone), self-assembled into hybrid hydrogels. The hydrogel formation was mediated by the formation of antiparallel coiled-coil CCK/CCE heterodimers [6,9]. This research was the motivation for the design of „drug-free macromolecular therapeutics“ [10]. Formation of coiled-coil heterodimers at B-cell surface resulted in the crosslinking of CD20 (non-internalizing) receptors and initiation of apoptosis [10,11].

The above two examples indicate the close relationship between biomaterials research and the design of nanomedicines. In this report we shall try to make this connection more clear.

2. Traditional hydrogels and water-soluble polymeric drug carriers

2.1. *Discovery, early research and clinical applications of hydrogels*

Hydrogels were systemically studied by Lím and Wichterle in the 1950s in Prague. They have chosen methacrylated derivatives because the structure of the polymer reflects a pivalic (trimethylacetic) acid structure. The latter is stable to pure hydrolysis and no similar structure in the nature was known, making enzyme-catalyzed hydrolysis less probable [12]. After trying the methacrylated polyvinyl alcohol and partially substituted mannitol [13], Lím hit the jackpot when he left the transesterification of methyl methacrylate with triethylene glycol overnight in the middle of the work-up; he added water to separate the triethylene glycol dimethacrylate layer from water soluble components. However, during night the water layer turned into a clear hydrogel. Obviously it was a copolymer of triethylene glycol monomethacrylate and triethylene glycol dimethacrylate [12]. A detailed evaluation of similar crosslinked copolymers from monoglycol and diglycol led to the selection of monoglycol (copolymer of HEMA and EDMA) for the synthesis of first soft contact lenses [1,14].

Parallel with the development of soft contact lenses other medical applications commenced – glaucoma microcapillary drains [15], augmentation of vocal cords [16], restoration of detached retina [17], preventing scar formation after surgery [18], and covering for perforated ear drums [19]. There are numerous excellent reviews that describe the early work on hydrogels [2,14,20-24].

2.1.1 Structure – biocompatibility relationship

Healing-in of hydrogel implants depends on the chemical structure, physical structure (porosity), and surface microarchitecture of hydrogels [25]. A systematic study of the biocompatibility of hydrogels based on esters and/or *N*-substituted amides of (meth)acrylic acid revealed no significant differences in the healing-in of hydrogels of different chemical compositions [4,26-29]. In contrast, significant differences have been observed for hydrogels with different porosity [30,31].

Hydrogels prepared by crosslinking copolymerization of HEMA with EDMA are an excellent model for the study of the relationship between porosity and biocompatibility. Due to the fact, that the interaction parameter (χ) polymer-water for this system is 0.7-0.8 (depending on crosslinking density) [32], phase separation may occur during copolymerization, which depends on the amount of water in the feed. Manipulating the water to monomer ratio in the feed permits the formation of homogeneous (transparent) hydrogels (<50% water in the feed), microporous hydrogels (pores are not interconnected; 50-70% water in the feed), and macroporous spongy hydrogels with interconnecting channels (>70% water in the feed) [31]. Thus, the biocompatibility of hydrogels with identical chemical structure, but differing in porosity could be evaluated [30,31]. The implantation of both homogeneous and heterogeneous hydrogels resulted in fibrous capsule formation. However, following implantation of porous hydrogels, in contrast to homogeneous hydrogels, newly formed blood capillaries and an eosinophil containing exudate penetrated into the implant. The intensity and the area of penetration were greater with higher hydrogel porosity [30,31]. An investigation of calcium deposits using von Kóssa staining revealed the dependence of the extent and localization of calcium deposits on porosity. There was only sporadic calcification in the margin of the implant following implantation of homogeneous or microporous hydrogels; however, with an increase in porosity, calcification occurred. The site of the deposition moved from the margin of the implant toward its center with increasing porosity [31]. Early studies on the biocompatibility of hydrogels have been summarized in ref. [4].

These results were corroborated in clinical settings. Implantation of homogeneous HEMA-based hydrogels to treat nasal malformation resulted in minor calcification at the margin of the implant (about 50% of patients evaluated after 3-10 years). Apparently, with scalpel damaged surface (due to surgeons modifying the size of the hydrogel implants in the operation room) connective tissue accumulated and initiated calcium deposition. Minor calcium deposition did not affect the biocompatibility or the final cosmetic effect (Fig. 1) [33,34].

Figure 1

2.1.2 Stimuli-sensitive hydrogels

Stimuli-sensitive polymers exhibit sharp changes in behavior in response to an external stimulus, such as pH, temperature, solvents, salts, electrical field, and chemical or biochemical agents. Such polymers may be used in numerous applications, including phase separations, affinity precipitations, bioactive surfaces, permeation switches, bioreactors, medical diagnostics, and drug delivery systems [35].

Upon a change in the environment, hydrogels swell or collapse. Environmentally induced changes in the transport properties of pH-sensitive hydrogels [36,37] or temperature-sensitive hydrogels [38] were studied several decades ago. Dušek and Paterson [39] predicted that changes in external conditions might result in abrupt changes of the hydrogel degree of swelling (phase transition). Tanaka et al. [40] and others [41,42] have verified the theory by experimental observations.

The majority of temperature-sensitive polymer hydrogels have a LCST, i.e., the gels collapse as the temperature increases. The process is thought being driven by entropy, which is supported by the observation that LCST transitions are endothermic. One widely accepted mechanism is based on disruption and re-establishment of a balance between hydrophobic and electrostatic interactions. Below the LCST, water molecules form hydrogen bonds with polar groups on the polymer backbone and organize around hydrophobic groups as iceberg water. As temperature increases past LCST, bound water molecules are released to the bulk with a large gain in entropy, resulting in the collapse of the polymer network [43].

Incorporation of enzyme-degradable peptide sequences [44] as crosslinks renders the hydrogels enzymatically degradable. Interestingly, the degree of swelling decides if the degradation occurs from surface or is bulk degradation. In the latter case the swelling degree has to be high so that the enzyme can diffuse into the hydrogel structure [45].

2.2 Lessons learned from hydrogel research suitable for the design of water-soluble polymers as drug carriers

We studied the structure-properties relationship of hydrophilic esters and *N*-substituted amides of (meth)acrylic acid. While hydrophilic esters of methacrylic acid (monoesters of glycol [32], diglycol [46,47] and triglycol [48]) produced crosslinked polymers (hydrogels) with excellent properties from the application point of view, the *N*-substituted amides represented a group of polymers where properties could be easily manipulated by choosing the proper substituent on the amide nitrogen [4,49]. Hydrophilic esters (prepared by transesterification) always contained a small (variable) amount of diester as an impurity. This was not a problem for the synthesis of hydrogels, but prevented the synthesis of soluble polymers with reproducible molecular weight distribution. Thus the experience from hydrogels directed us to concentrate on *N*-substituted amides of (meth)acrylic acid in the design of soluble polymeric drug carriers. Based on the detailed studies of the relationship between the structure of hydrophilic polymers and their biocompatibility [4,26-29], we have chosen *N*-substituted methacrylamides as our target because the α -carbon substitution and the *N*-substituted amide bond ensured hydrolytic stability of the side-chains. We synthesized a series of compounds trying to identify a crystalline monomer for easy purification and reproducible synthesis. The first crystalline *N*-substituted methacrylamide we succeeded to synthesize, *N*-(2-hydroxypropyl)methacrylamide (HPMA), was chosen for future development. This ensured a reproducible synthesis of water-soluble polymeric carriers [55-57].

2.3 Advances in soluble polymer-drug conjugates

The validation of polyHPMA [55,56] as a drug carrier was based on evaluation of its biocompatibility using all test required for blood plasma expanders [50-54,58]. The development of HPMA copolymer – drug conjugates was based on basic biological principles that govern the **behavior** of macromolecules.

The major rationale for the use of water-soluble polymers as carriers of anticancer drugs is based on the mechanism of cell entry [54,58,59]. Whereas the majority of low molecular weight drugs enter the cell by diffusion across the plasma membrane, the entry of macromolecules is restricted to endocytosis [60,61]. Macromolecules captured by this mechanism are usually channeled to the lysosomal compartment of the cell. Moieties incorporated into the macromolecular structure that complement cell surface receptors or antigens of a subset of cells, render the macromolecule biorecognizable [62,63]. For efficiency, targetable polymer – drug conjugates should be biorecognizable at two levels: at the plasma membrane, eliciting selective recognition and internalization by a subset of target cells [62,64], and intracellularly, where lysosomal enzymes induce the release of drug from the carrier [65]. The latter is a prerequisite for transport of the drug across the lysosomal membrane into the cytoplasm and translocation into the organelle decisive for biological activity.

There are numerous reviews that summarize the rationale, design, synthesis, and evaluation of macromolecular therapeutics [44,58,59,66-77]. Here, we shall discuss just four important topics in water-soluble nanomedicines: combination therapy, multidrug resistance, backbone degradable polymer carriers, and importance of targeting.

2.3.1 Combination therapy

Photodynamic therapy is a paradigm in anticancer therapy that involves activation of specific compounds, called photosensitizers. Illumination of these compounds results in the generation of free radicals and singlet oxygen, which cause cell damage and ultimately cell death. A *combination of chemotherapy and photodynamic therapy* may result in a synergistic response, resulting in a better cure rate than monotherapy. On two cancer models, Neuro 2A neuroblastoma [78] and human ovarian carcinoma heterotransplanted in the nude mice [79], we have shown that combination therapy with HPMA copolymer - anticancer drug [DOX and meso chlorin e_6 mono(*N*-2-aminoethylamide) {Mce₆}] conjugates showed tumor cures that could not be obtained with either chemotherapy or photodynamic therapy alone. Cooperativity of the action of both drugs contributed to the observed effect [80]. Based on biodistribution data [81], we hypothesized that combination therapies of s.c. human ovarian carcinoma OVCAR-3 xenografts in nude mice using multiple doses/irradiation of P(GFLG)-Mce₆ (P is the HPMA copolymer backbone) and P(GFLG)-DOX may acquire low effective doses without sacrificing the therapeutic efficacy. Indeed, 10 out of 12 tumors exhibited complete responses in the group of mice receiving multiple PDT plus multiple chemotherapy [82].

Employing *targeted combination chemotherapy and photodynamic therapy* using OV-TL16-targeted HPMA copolymer-DOX and HPMA copolymer-mesochlorin e_6 conjugates results in enhanced tumor accumulation and treatment efficacy. OV-TL16 antibodies are complementary to the OA-3 antigen (CD47) present on the majority of ovarian cancers.

The immunoconjugates (Figure 3) preferentially accumulated in human ovarian carcinoma OVCAR-3 xenografts in nude mice with a concomitant increase in therapeutic efficacy when compared with non-targeted conjugates [64]. The targeted conjugates suppressed tumor growth for the entire length of the experiment (> 60 days).

Other combination systems were quantitatively evaluated by combination index (CI) analysis in A498 renal carcinoma cells [83] and in OVCAR-3 ovarian carcinoma cells [84]. The results demonstrated synergistic effects of HPMA copolymer-drug (SOS thiophene, DOX, and chlorin e_6) conjugate combinations in a wide range of concentrations. Recently, the regions of synergism for the combination of backbone degradable HPMA copolymer-gemcitabine and HPMA copolymer-paclitaxel conjugates [85] and backbone degradable HPMA copolymer-gemcitabine and HPMA copolymer-platinum conjugates [86] have been identified.

A detailed comparison of the efficacy of combination therapy of ovarian carcinoma with 1st and 2nd generation HPMA copolymer – PTX and GEM conjugates clearly demonstrated the advantage of long circulating 2nd generation conjugates - favorable pharmacokinetics, dramatically enhanced inhibition efficacy on tumor growth, and absence of adverse effects [87]. The second generation of backbone degradable HPMA copolymer carriers is discussed below.

From the synthetic and scale-up point of view it is preferable to use a mixture of two polymer-conjugates, each containing one drug. However, Vicent et al. 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 [88]. 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 bi-specific 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 [89,90].

2.3.2 Targeting stem cells

Another important aspect for the future development of anticancer nanomedicines is the targeting of cancer stem cells [66,73,91,92]. Cancer cells are biologically and functionally heterogeneous, in terms of phenotype, proliferation, tumorigenesis and invasiveness, etc. Noticeably, cancer cells are present in various differentiation statuses, with relatively undifferentiated Cancer Stem Cells (CSCs) maintaining the hierarchical organization of the tumor mass, similar to the role of normal stem cells (NSCs) in healthy tissues [93,94]. Moreover, the CSC theory suggests that the often-observed treatment failures are largely due to the failure of conventional cytotoxic anti-cancer therapies to eliminate CSCs. Therefore, targeting CSCs or in combination with traditional anticancer therapeutics represents a promising strategy to improve cancer patient survival [95]. 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, RC-92a/hTERT cells, with stem cell properties [96]. RC-92a/hTERT cells were chosen since 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 than on 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 macromolecular therapeutics targeting both bulk tumor cells and CSCs [91].

We also evaluated the *in vivo* tumor growth inhibitory effect in long-term. The combination of P-CYP (HPMA copolymer-cyclopamine conjugate) and P-DTX (HPMA copolymer-docetaxel conjugate), as well as the P-CYP or P-DTX single treatment all inhibited the PC-3 prostate tumor growth to certain extent compared to saline group, immediately after three-week treatment (Fig. 2) [92]. However, after stopping the treatment at day 21 only the combination of P-CYP and P-DTX showed persistent tumor growth inhibition, leading to the longest mice survival on average (Fig. 4A) [92,97].

Figure 2

2.3.3 Overcoming multidrug resistance

The acquired resistance of malignant tumors to therapeutics is one of the major causes of cancer therapy failure [98]. Membrane transporters from the ATP-Binding Casette (ABC) transport proteins families (P-glycoprotein, multidrug resistance-associated proteins and others) reduce the intracellular drug concentration. The elucidation of the function of P-glycoprotein [99], other ATP-driven efflux pumps [100], as well as other mechanisms of multidrug resistance [101] have had a major impact on the understanding of multidrug resistance in human tumors. The exclusion of nanomedicines, including polymer-drug conjugates, from the cytoplasm of the cell, through intracellular trafficking in membrane-limited organelles, renders the efflux pumps less efficient [102]. Subcellular trafficking along the endocytic pathway from the plasma membrane to the perinuclear region changes the gradient of distribution of drugs inside cells [103-105]. The concentration gradient of free drugs is directed from the plasma membrane to the perinuclear region (in the direction of diffusion); conversely, in polymer-bound drugs, the drug is released from the carrier in the lysosomal compartment located in the perinuclear region, have a concentration gradient in the opposite direction. Consequently, the interaction/recognition of the released drug by the P-glycoprotein efflux pump is minimized [104]. Quantitative determination of intracellular DOX concentration following exposure of human ovarian carcinoma cells to free and HPMA copolymer-bound DOX showed an enhanced intracellular accumulation of HPMA copolymer-bound DOX [106]. Efficient bypassing of multidrug resistance was detected for other drug delivery systems internalized by endocytosis, namely lipid/polymer particle assemblies, dendrimers, and micelles [102].

2.3.4 Backbone degradable long circulating conjugates

It is well known that high molecular weight (long-circulating) polymer conjugates accumulate efficiently in solid tumor tissue due to the EPR (enhanced permeability and retention) effect [71,72,107]. To achieve substantial accumulation of the polymer-drug conjugate in solid tumors (due to the EPR effect) a sustained concentration gradient is

needed. The concentration in the blood stream depends on the administered dose and on the molecular weight of the carrier. However, higher molecular weight drug carriers with a nondegradable backbone deposit and accumulate in various organs, impairing biocompatibility.

Previous attempts to design and synthesize long-circulating conjugates produced branched, partially crosslinked copolymers with enzymatically degradable sequences [108]. The synthetic process and the polymer structure were difficult to control; consequently, the process would be difficult to scale-up. Nevertheless, the results proved that a higher molecular weight of polymer carriers transfers into higher accumulation of drugs in the tumor tissue with concomitant enhancement of efficacy [109].

The advances in controlled radical polymerization [110,111] and click chemistry [112-114] offered new vistas for the design and synthesis of long-circulating biocompatible polymer-drug conjugates. To this end we designed new, **second-generation anticancer nanomedicines** based on high molecular weight HPMA copolymer carriers containing enzymatically degradable bonds in the main chain (polymer backbone) [115-117]. The proposed new design permits tailor-made synthesis of well-defined backbone degradable HPMA copolymers. The synthetic process consists of two main steps: first, the synthesis of a telechelic HPMA copolymer by reversible addition-fragmentation chain transfer (RAFT) polymerization, followed in the second step by chain extension using alkyne-azide [115,116] or thiol-ene [117] click reactions. In addition, we synthesized a new RAFT chain transfer agent (CTA), *N*^α,*N*^ε-bis(4-cyano-4-(phenylcarbonothioylthio)pentanoylglycylphenylalanylleucylglycyl) lysine (Peptide2CTA), containing an enzymatically degradable (GFLG) spacer capped at both ends with 4-cyano-4-(phenylcarbonothioylthio)pentanoate [117]. During RAFT polymerization the HPMA monomers incorporate at both dithiobenzoate groups of the Peptide2CTA with identical efficiency. When the final polymer was incubated with papain, a thiol proteinase with similar specificity as lysosomal proteinases, the molecular weight decreased to half of the original value. Thus it is possible to prepare a degradable diblock copolymer with narrow molecular weight distribution in one step, eliminating the chain extension reaction [117].

Multiblock polyHPMAs with Mw as high as 300 kDa and containing degradable GFLG sequences were obtained by chain extension followed by fractionation using size exclusion chromatography (SEC). The exposure of the multiblock HPMA copolymer to model enzyme papain or lysosomal cathepsin B (pH 6, 37 °C) resulted in complete degradation of GFLG segments and decrease of the molecular weight of the carrier to primary chains below the renal threshold [115-117]. *These data support our hypothesis and bode well for the success of the proposed design of backbone degradable* HPMA copolymers composed of alternating segments of HPMA copolymer, with molecular weight below the renal threshold, and lysosomally degradable GFLG containing oligopeptides.

The improved therapeutic efficacy of second generation, backbone degradable HPMA copolymer conjugates has been evaluated in several studies. The examination of multiblock HPMA copolymer – doxorubicin (DOX) conjugates demonstrated molecular weight dependent antitumor activity toward human ovarian A2780/AD carcinoma xenografts in nude mice. The study revealed enhanced activity of multiblock, second-

generation higher molecular weight conjugates ($M_w = 93$; 185; and 349 kDa) when compared to traditional HPMA copolymer-DOX conjugate ($M_w = 20$ kDa) [118].

Similarly, a multiblock backbone degradable HPMA copolymer – paclitaxel conjugate (mP-PTX; $M_w = 335$ kDa) possessed an enhanced blood circulation time when compared to 1st generation conjugates. 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 (1st generation conjugate, $M_w = 48$ kDa; single dose at 20 mg PTX/kg equivalent was administered). Histological analysis indicated that mP-PTX had no toxicity in liver and spleen, but induced massive cell death in the tumor [119].

Combination of 2nd generation HPMA copolymer – gemcitabine and HPMA copolymer – paclitaxel conjugates has shown excellent activity toward human ovarian carcinoma xenografts [87]. In vivo behavior of a combination of diblock (two polyHPMA blocks connected by a peptide degradable sequence) 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 M_w conjugates (P-PTX and P-GEM) in nu/nu mice bearing A2780 tumor xenografts. Compared to first-generation low M_w HPMA copolymer conjugates, high M_w 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 higher 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 M_w) conjugates combination (Fig. 3). The histological analysis indicated that the combination treatment had no toxicity in major organs [87].

Figure 3

2.3.4 Treatment of other diseases

Polymeric drug carriers are suitable also for the treatment of other diseases, e.g., musculoskeletal diseases [120-123]. Interestingly, bone-targeted long-circulating backbone degradable HPMA copolymer - prostaglandin E_1 conjugates had higher accumulation on bone tissue and greater indices of bone formation in an ovariectomized rat osteoporosis model when compared to 1st generation conjugates (Fig. 4) [124].

Figure 4

2.3.5 Colon delivery

Most of water-soluble polymer-drug conjugates are administered intravenously. However, the colon delivery is a suitable alternative. To achieve degradation in the colon, spacers that combine a reducible aromatic azobond and a peptide group [125] or azobond and a 1,6-elimination 4-aminobenzylcarbamate group [126] have been used. Such HPMA copolymer – 9-aminocamptothecin conjugates have been successful in the treatment of experimental orthotopic and subcutaneous HT29 cancer models (Fig. 5)

[127-129]. However, when using the oral route one has to be aware of the size limitation of transport [130].

Figure 5

3. Self-assembled hydrogels and crosslinking of receptors

3.1. Coiled-coil forming peptides

The coiled-coil is one of the folding patterns of native proteins. It consists of two or more right-handed α -helices winding together and forming a slightly left-handed super-helix [131]. The primary structure of the coiled coil motif is characterized by a heptad repeating sequence designated as *a, b, c, d, e, f, g*, 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. The distinctive feature of coiled coils is the specific spatial recognition, association, and dissociation of helices, making it an ideal model of protein biomaterials in which the higher structure 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. Depending on their detailed structure, α -helices may associate as homodimers, heterodimers in parallel or antiparallel alignments, or form higher order (e.g., tetramer) aggregates. Binding coiled-coil forming sequences to linear water-soluble polymers enhances their secondary structure slightly [132,133]. Formation of coiled-coil mediates the assembly of graft copolymers into 3D hydrogels. [2,5-7,9,20,43,134-138].

Crosslinking of polymer precursors with genetically engineered protein domains. A new strategy of hybrid hydrogel synthesis entails the non-covalent attachment of genetically engineered coiled-coil protein motifs to hydrophilic synthetic HPMA copolymer backbone. The physical crosslinking was established by self-assembly of the coiled-coil domains. A temperature-induced hydrogel collapse was observed that corresponded to the structural transition of the coiled-coil domains from an elongated helix to an unfolded state. Hydrogels formed by crosslinking of HPMA copolymer precursors with coiled-coil modules underwent dramatic volume transitions (de-swelling up to 10-fold) at the melting temperature of the coiled-coil modules [134]. This is a new temperature response mechanism for hydrogels that can be tuned over a wide temperature range by assembling gels with coiled-coils that have different melting temperatures [139]. These results seemed to indicate that the *properties of a well-defined coiled-coil protein motif can be imposed onto a hybrid hydrogel* containing synthetic polymer-based primary chains. Given the immense potential of tailoring material properties with genetically engineered proteins this strategy adds a new dimension to the field of “smart” hydrogel-based biomaterials [20,140].

Hydrogels self-assembled from graft copolymers via formation of coiled-coil antiparallel heterodimers Recently, self-assembly of graft copolymers into hybrid hydrogels was demonstrated [6,9]. A novel hybrid hydrogel system based on HPMA copolymers consisted of a hydrophilic polymer backbone and a pair of oppositely charged peptide grafts. Two distinct pentaheptad peptides (CCE and CCK) were designed to create a dimerization motif and serve as physical crosslinkers. Consequently, the graft copolymers, CCE-P (P is the HPMA copolymer backbone) and CCK-P, self-assembled

into hybrid hydrogels in situ; the process was modulated by the formation of antiparallel heterodimeric coiled-coils [6,9]. This approach possesses the advantage of decreased steric hindrance of the polymer backbone due to the “in-register” alignment of the peptide grafts. Equimolar mixtures of the graft copolymers, CCE-P/CCK-P, have been observed to self-assemble into hydrogels in PBS (phosphate buffer) solution at neutral pH at concentrations as low as 0.1 wt.%. The formation of these hybrid hydrogels was reversible [9]. The excellent CCE/CCK biorecognition was used by Lv *et al.* for the development of tandem modular protein-based hydrogels [141].

3.2 *Beta-sheet forming peptides*

Beta-sheets are important structural elements in proteins. β -Strands are aligned adjacent to each other and are stabilized by hydrogen bonds between the carbonyl oxygen of an amino acid in one strand and the backbone nitrogen of a second amino acid in another strand. The strands (at least two, but frequently more) can arrange in parallel or antiparallel fashion to form the β -sheets. The stability of β -sheets depends on the interaction of side chains of neighboring amino acids. These interactions must compensate for the loss of translational and solvation energies of the peptide [142].

Hydrogels from graft copolymers containing beta-sheet peptides were thoroughly investigated [143]. For example, the I28 immunoglobulin (Ig)-like domain of human cardiac titin was used to crosslink acrylamide copolymers into temperature sensitive hydrogels [144]. HPMA hybrid graft copolymers were prepared by attachment of *N*-terminal CGG modified β -sheet peptide (CGGTTRFTWTFTTT) to polyHPMA precursor, which contained pendant maleimide groups. CD spectra showed that the strong tendency of the peptide to self-assemble into β -sheets was retained in the copolymers. In addition, β -sheet sensitivity to temperature and pH variations decreased due to polyHPMA shielding effect [145]. Finally, the ability of a hybrid hydrogel self-assembled from HPMA copolymers and complementary β -sheet grafts (TTRFTWTFTTT-NH₂ and TTEFTWTFETT-NH₂) to act as scaffolds for bone tissue engineering was explored [146]. The hydrogel displayed anisotropic porosity; thus, besides support for preosteoblast cells, it provided surfaces characterized by epitaxy that favored template-driven mineralization of hydroxyapatite.

3.3 *Proteins*

Protein mutants when used as crosslinkers in hydrogels may provide specificity based on binding of calcium or a low molecular weight compound. For example calmodulin undergoes two conformational changes from the native state: into a dumbbell-like conformation when bound to Ca⁺⁺, and into a more constrictive conformation when bound to phenothiazine. The hydrogel was responsive to both Ca⁺⁺ and phenothiazine; its incorporation into a simple microfluidic system demonstrated the hydrogel's potential to control flow [147-149]. Generally the conformational change of the protein, e.g. upon binding its substrate [150] translates into macroscopic motion.

3.4 *Oligonucleotides*

Matsuda and Nagahara [151] prepared self-assembling hydrogels by self-assembly of two poly(*N,N*-dimethylacrylamide) graft copolymers. One was grafted with oligodeoxythymilidate (oligoT₁₀), the other with oligodeoxyadenylate (oligoA₁₀).

3.5 *Lessons from hydrogels self-assembled from hybrid copolymers for the design of drug-free nanomedicines*

The results of self-assembly of graft copolymers into precisely defined 3D hydrogels provided several pathways for translation. First, it would be possible, under efficient mixing, to subcutaneously inject a mixture of two solutions, one containing a therapeutic protein. The pharmacokinetics of release could be controlled. However, this was abandoned because one can achieve similar results with cheaper polymers.

Then we formulated the following hypothesis on apoptosis induction in B cells (expressing a non-internalizing CD20 receptor) that is the base for our current research in drug-free macromolecular therapeutics:

If biorecognition of complementary coiled-coil forming peptides results in self-assembly of graft copolymers into crosslinked hydrogels
then
biorecognition of these peptides at cell surface should result in crosslinking of CD20 receptors and initiation of apoptosis.

In other words – if we know how to crosslink hydrogels we should know how to crosslink receptors if this is the mechanism for apoptosis initiation. As described in the next section, the approach was quite successful.

3.6 *Recent advances in drug-free macromolecular therapeutics*

3.6.1 *Coiled-coil-based system*

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 that self-assemble into antiparallel coiled-coil heterodimers were engaged in the design of a new CD20+ cell apoptosis induction system, called drug-free macromolecular therapeutics [10,11]. CD20 is an ideal target for immunotherapies. It is an integral membrane protein [152] that is expressed from pre-B cells to terminally differentiated plasma cells and is present on greater than 90% of B-cell malignancies [153]. 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 that either controls or functions as a store operated calcium channel. The protein forms dynamic dimers and tetramers [154] constitutively associated in lipid rafts of the cell membrane [155].

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 non-internalizing 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 and triggered the apoptosis of Raji B cells in vitro [10] and in a Non-Hodgkin lymphoma (NHL) animal model in vivo [11]. This is a new concept, where the biological activity of drug-free macromolecular therapeutics is based on the biorecognition of peptide motifs (Fig. 6).

Figure 6

To prove that two conjugates assemble at cell surface, multiple fluorescence imaging studies were performed, including 2-channel FMT, 3D confocal microscopy, and 4-color FACS [156]. Confocal microscopy showed co-localization of two fluorescently labeled nanoconjugates, Fab'-CCE and P-(CCK)₉ on non-Hodgkin's lymphoma (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. We also found that delaying injection of P-(CCK)_x could significantly enhance targeting efficacy. This high-specificity therapeutics provide a safe option to treat NHL and other B cell malignancies [156].

We evaluated the *immunogenicity* of the coiled-coil based drug-free macromolecular therapeutics [157]. We synthesized enantiomeric peptides (L- and D-CCE and L- and D-CCK), HPMA copolymer-peptide conjugates and Fab' fragment-peptide conjugates. The immunological properties were evaluated in vitro on RAW264.7 macrophages and in vivo on immunocompetent BALB/c mice. There was no substantial difference in the ability of D-peptide and L-peptide conjugates to induce Ab response. HPMA copolymer and unconjugated peptides did not induce any response in RAW264.7 macrophages. *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 was detectable Ab, but no cellular response to the therapeutics administered i.v. The Ab response was predominantly directed against the Fab' part of the therapeutics [157]. Therefore, in spite of the fact that 1F5 antibodies were used in human with minimal toxicities [158,159] we suggest to humanize the Fab' fragment before moving to the clinics.

3.6.2. Other applications of the coiled-coil system

Biorecognition of coiled-coils has been used in many applications and several examples follow. Shen et al. controlled the accessibility of ligands by formation of coiled-coils at the surface [160]. Two peptide fragments of green fluorescent protein (GFP) were modified with coiled-coil forming peptides; following reassembly the GFP displayed its characteristic fluorescence [161]. Chelur and Chalfie demonstrated that co-expression of caspase 3 subunits generates constitutively active caspase activity that lead to cell death. The caspase activity occurred only when the subunits associated through binding of linked antiparallel coiled-coil domains [162]. Ryadnov and coworkers designed a cyclopeptide block consisting of two domains that oligomerize by forming a parallel coiled-coil heterodimer. Self-assembly leads to formation of hyperbranched fibrillar networks with nano- to micrometer size [163]. Coiled-coil formation between a peptide graft attached to a synthetic copolymer and a complementary graft containing a biologically active compound have been used for the design of novel drug delivery systems. Pechar and coworkers used coiled-coil peptides for the design of novel HPMA copolymer-based macromolecular therapeutics [164-166]. Apostolovic et al. evaluated the uptake and subcellular trafficking in B16F10 cells of HPMA copolymers containing K3 grafts dimerized with E3 peptides terminated in methotrexate [167,168]. Tirrell et al. pioneered the design of ABA block copolymers that assemble into hydrogels by

biorecognition of coiled-coil A blocks [169-171]. It is easy to modify the structure of A blocks to manipulate the properties of the resulting hydrogel [172].

3.6.3 Morpholino oligonucleotide based system

The coiled-coil based system worked well. However, the peptides do not have a strong secondary structure at neutral pH. They associate by hydrophobic and electrostatic interactions and then fold into a antiparallel coiled-coil heterodimer. To be efficient *in vivo*, we use large excess of the CCK peptide (~25x) bound as grafts to the HPMa copolymer. Therefore our aim was to identify a biorecognition pair that will bind strongly at an equimolar concentration. We selected morpholino oligonucleotides due to their fast hybridization, excellent binding affinity, stability in plasma and water-solubility [173,174].

To apply a similar approach for the design of drug-free nanomedicine, we designed a pair of phosphorodiamidate morpholino (MORF) oligomers (25 base pairs each), MORF1 and MORF2, as the biorecognition motifs for the second-generation “drug-free” therapeutic system [8]. The MORF oligonucleotides are charge neutral, resulting in significantly stronger binding than natural DNA and RNA [175]. 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 [8]. This new therapeutic system was composed of two hybrid conjugates: (1) anti-CD20 Fab’ linked to MORF1 (Fab’-MORF1), and (2) HPMa copolymers grafted with multiple copies of MORF2 (P-(MORF2)_x). The two conjugates self-assembled via MORF1-MORF2 hybridization at the surface of CD20⁺ B-cells, which crosslinked CD20 and initiated apoptosis [8,176].

To evaluate *in vivo* anticancer efficacy of the hybridization system we performed animal experiments using the same mouse model of systemic human B-NHL (Fig. 7). Mice were intravenously injected with Raji cells, followed by administration (i.v.) of the two conjugates. Results showed that, at equivalent doses, a single treatment of Fab’-MORF1 and P-(MORF2)₉ (MORF1:MORF2=1:1) was significantly more effective than a single treatment of Fab’-CCE and P-(CCK)₉ (CCE:CCK=1:25) on preventing lymphoma dissemination and on extending the animal survival (compare refs. 11 and 8). The efficacy of one dose treatment can be further improved by using a 5-time excess of P-(MORF2)_x (MORF1:MORF2=1:5). Moreover, the time lag in the consecutive treatment can be optimized based on biodistribution and pharmacokinetics of the Fab’-MORF1 conjugate [177]. The comparison between the coiled-coil and the oligonucleotide designs clearly indicates that the hybridization system is advantageous for the drug-free approach. This is likely due to a more direct and specific binding pattern of the oligonucleotide base pairing at physiological conditions, when compared to the binding of the coiled-coil forming peptides. We also believe that the therapeutic system composed of two nanoconjugates has a greater potential for activity and decrease of side effects than the one conjugate multivalent systems [178-183].

Figure 7

We evaluated the drug-free approach in chronic lymphocytic leukemia (CLL) cells isolated from 10 patients [184]. Primary cells were treated with Fab’-MORF1 and P-(MORF2)_x, and apoptosis and cytotoxicity were observed in 8 samples, including 2 samples with the 17p13 deletion, an ultrahigh-risk prognostic factor [185]. The data suggest a p53-independent mechanism of apoptosis induction. This constitutes potential

treatment for chemoresistant malignancies [186] and may synergize with other therapies [187]. Similarly, the approach also worked in cells from patients with mantle cell lymphoma, an aggressive subset of B-NHL that is particularly difficult to treat [177]. When compared to anti-CD20 mAbs 1F5 and rituximab, drug-free macromolecular therapeutics showed significantly more potent apoptosis-inducing activity and cytotoxicity. These results highlight the promising potential of the drug-free approach for clinical translation, as novel treatments against NHL, CLL, and other B-cell associated malignancies.

In summary, these nanoconjugates performed extremely well on an animal model in vivo [8,177] and on cells from patients with chronic lymphocytic leukemia [184] and mantle cell lymphoma [177].

4. Conclusions

We hope that we demonstrated the close connection between the design of traditional hydrogels and the design of water-soluble polymer-drug conjugates. This connection led to the design of backbone degradable carriers of anticancer drugs that have a great translational potential. The synthesis of backbone degradable polymer carriers of drugs permits to use long-circulating conjugates without compromising their biocompatibility.

We also highlighted the connection between the design of self-assembled hybrid hydrogels and drug-free macromolecular therapeutics. The latter is a new paradigm in polymer therapeutics that also possesses an excellent application promise. This system does not use low molecular weight drugs; this decreases the probability of adverse effects. The onset of apoptosis is based on the crosslinking of non-internalizing receptors mediated by association of two complementary peptides or oligonucleotides. The nanoconjugate therapeutic system can be used not only for the treatment of blood cancers but also for other diseases, including rheumatoid arthritis [188], systemic lupus erythematosus [189], prostate cancer [190], and colon cancer [191].

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Captions

- Figure 1. The use of HEMA-based hydrogels (copolymers of HEMA with EDMA) in rhinoplasty. A) Patient before surgery; B) Patient after surgery. Reprinted from reference 33 with permission.
- Figure 2. Tumor growth inhibition by P-CYP, P-DTX, and combination of P-DTX and P-CYP in PC-3 tumor-bearing nude mice. Data are presented as mean $\pm$ SD. Reprinted from reference 92 with permission.
- Figure 3. Combination treatment of 2nd generation conjugates showed improved therapeutic efficacy in A2780 human ovarian carcinoma xenografts. (A) Blood activity-time profiles of ¹²⁵I-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 ¹²⁵I-labeled conjugates (2P-PTX, 2P-GEM). The representative images were acquired 24 h, 48 h, and 7 d after administration of conjugates. T, tumor. (C) Experimental schedules of treatment in mice bearing A2780 human ovarian tumor xenografts. 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. (D) A2780 tumor growth in mice treated with different formulation combinations (n=5). * $p < 0.01$. Note: in the orange (2P-PTX $\rightarrow$ 2P-GEM) line the error bars are hidden within the experimental points. (E) Photographs of A2780 tumors after treatment with different combinations. Reprinted from reference 87 with permission.
- Figure 4. Percentage of bone mineral density increase in OVX Sprague-Dawley rats following administration of Asp₈-targeted HPMA copolymer-PGE₁ conjugates. The BMD was measured on day -2 and day 33. Left columns – untreated controls (saline); middle columns - P-Asp₈-PGE₁ (1st generation conjugate, Mw 51.2 kDa); right columns - mP-Asp₈-PGE₁ (2nd generation multiblock backbone degradable conjugate, Mw 329 kDa). * $P < 0.05$ for mP-Asp₈-PGE₁ group compared to control. ** $P < 0.05$ for mP-Asp₈-PGE₁ group compared to P-Asp₈-PGE₁ and control. $n = 5$ per group. Data are means $\pm$ SD. Reprinted from reference 124 with permission.
- Figure 5. A) The structure of the HPMA copolymer – 9-AC conjugate (P-9-AC). B) Survival curves of mice bearing human colon carcinoma xenografts treated by 9-AC and P-9-AC at a dose of 3 mg/kg of 9-AC or 9-AC equivalent. Reprinted from reference 129 with permission.
- Figure 6. Therapeutic efficacy of drug-free macromolecular therapeutics based on coiled-coil peptides against systemically disseminated Raji B cell lymphoma in C.B.-17 SCID mice (7 mice per group). A) 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 CCK-P 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 (7 mice per group); B) Estimation of residual Raji B lymphoma cells in the bone marrow. Shown are results from representative mice that received the indicated treatment. Revealed are histograms of bone marrow cells isolated from mice (as indicated) followed by staining with PE mouse anti-human CD10 and APC mouse anti-human CD19. Reprinted from reference 11 with permission.

Figure 7. *In vivo* efficacy of drug-free macromolecular therapeutics based on morpholino oligonucleotides 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. *PBS*: mice injected with PBS ($n = 6$); *Cons 1h*: consecutive treatment of Fab'-MORF1 and P-MORF2, 1 h interval ($n = 7$); *Cons 5h*: consecutive treatment of Fab'-MORF1 and P-MORF2, 5 h interval ($n = 6$); *Rituximab* ($n = 6$); *1F5 mAb* ($n = 6$). (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 imaging. (C) Whole-body bioluminescence intensity of mice. Data are shown as mean $\pm$ SEM ($n = 6$ or 7). Statistics was performed by student's *t* test (**: $p < 0.005$). Black arrow: dose administration. Reprinted from reference 177 with permission.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7