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Beyond Oncology – Application of HPMA Copolymers in Non-cancerous Diseases

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Abstract

Macromolecular drug conjugates have been developed to improve the efficacy and safety profile of various therapeutic agents for many years. Among them, *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-drug conjugates are the most extensively studied delivery platforms for the effective treatment of cancer. In recent years, the applications of HPMA copolymers for the treatment of a broader range of non-cancerous diseases have also been explored. This review highlights the recent developments in the rational design, synthesis, and evaluation of novel HPMA copolymer-drug conjugates for non-cancerous diseases, such as musculoskeletal diseases, infectious diseases and spinal cord injury. The translation potential of these applications are also briefly discussed.

Keywords

HPMA copolymers; musculoskeletal diseases; infectious diseases; arthritis; bone-targeting; central nervous system (CNS)

1. Introduction

Thirty six years have passed since the design and synthesis of *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymers were first reported [1]. Initially designed as plasma expanders, these versatile water-soluble polymers have emerged as one of the key players in the field of macromolecular therapeutics and have been extensively used in the delivery of many anti-cancer therapeutic agents [2]. Clinically, at least five HPMA copolymer-drug conjugates have been evaluated in different phases of trials [3].

Championed by Dr. Jindřich Kopeček (the inventor of HPMA) and his associates, many important aspects of this legendary polymer drug carrier have been thoroughly investigated [2,4]. On the chemistry aspect, HPMA copolymer-based macromolecular therapeutics can be synthesized via polymer analogous reaction or copolymerization of HPMA and various functional monomers. The molecular weight (MW) and polydispersity of the copolymers can

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be controlled by using living free radical polymerizations such as ATRP or RAFT technologies [5,6]. The control of the copolymer chain termini functionality can be achieved via the use of functional chain transfer agents [7]. In addition to the classic linear construction, star-shaped and branched HPMA copolymer-drug conjugates have all been explored for additional benefits [8-11].

HPMA homopolymer is generally considered as non-toxic and non-immunogenic [12]. When conjugated to various therapeutic agents or imaging probes, however, the biocompatibility of the conjugates must be evaluated individually [2,13,14]. As a non-degradable water-soluble polymer, the *in vivo* clearance of HPMA copolymer carrier is mainly through renal glomerular filtration [15]. The single most studied pathological condition that impacts its *in vivo* biodistribution is the enhanced permeability and retention or "EPR" effect of solid tumor [16]. Cellular entry of the HPMA copolymer conjugates is mainly through endocytosis [4, 17]. Decoration of the conjugate with various targeting ligands would significantly enhance their organ specificity and the rate of their cellular uptake [18-20]. The high proteolytic activity and acidic pH in the endosomal/lysosomal compartments, where the internalized polymer conjugates reside provide the molecular mechanisms for the polymer-drug conjugates to be activated [21]. Peptide sequences and acid cleavable chemical structures have been specially designed and used in conjugation for adequate intracellular activation of different therapeutic agents [22,23].

Conjugation of therapeutic agents to polymeric carriers, such as HPMA copolymers would provide many advantages. First of all, the conjugation would render the low MW payload a much longer half-life in circulation with potentially better bioavailability. It helps to solubilize drugs, which are normally not soluble in water. Polymer conjugation would also help to protect the carried drug against premature metabolism before its distribution to the tissue targets. Compared to low MW compounds, incorporation of labeling or targeting moieties is very easy. Lastly, the large hydrodynamic volume of the macromolecular therapeutics could also capitalize on certain pathophysiological conditions (e.g. EPR effect) and facilitate passive targeting of the conjugates.

While these unique properties of polymer therapeutics would clinically benefit the treatment of many diseases, its main beneficiary at present is cancer treatment, with PEGylated biologics as an exception. For HPMA copolymer-drug conjugates that have been evaluated clinically, all of the payloads are cancer chemotherapeutic agents. Further, in a SciFinder literature analysis performed on 08/19/2009 (Figure 1), of all the papers that involve the keyword of HPMA copolymers (1226 hits), 32.5% of them are related to cancer or tumor. For the rest of papers, 11.3% are studies focusing on the non-cancerous diseases, which include skeletal diseases (4.2%), infection (4.1%), ophthalmic diseases (1.5%), dental diseases (1.5%), arthritis (1.1%), central nervous system (CNS) diseases (0.9%) and dermatitis (0.4%). Most of these studies, however, are basic or preclinical research, which are different from the more advanced clinical development of HPMA copolymer cancer chemotherapeutic agents. The prevalence of cancer and the nature of the chemotherapeutic treatments no doubt set it apart as the best disease target for polymer therapeutics application. This is certainly encouraged by the tremendous amount of government and private funding that supported the development of this field.

With the many successes and the momentum people have gained in the battle against cancer, one would wonder if we should look beyond oncology and extrapolate the benefit of polymer therapeutics to the treatment of other afflicting diseases. With an aging society and the staggering health care cost, it may be a good time to review the potential applications of HPMA copolymers in non-cancerous diseases, especially those associated with aging.

2. HPMA copolymers for the treatment of skeletal diseases

The initial development of bone-targeting HPMA copolymer conjugate for osteoporosis treatment derived from one of the authors' (DW) postdoctoral research project in Dr. Kopeček laboratory [24]. A thorough literature research found that most of the available drugs (except the bisphosphonates) for the treatment of skeletal diseases do not have tissue specificity to bone. For example, prostaglandins E_1, E_2 and their analogues were found by Jee and Miller's groups to be very potent bone anabolic agents [25,26]. But due to their non-skeletal adverse effects, most of the *in vivo* anabolic efficacy studies were performed in rodents.

Several attempts have been made to develop bone-specific prodrugs, but with very limited success [27-30]. Alternatively, HPMA copolymers were contemplated as the drug carrier to be used in the delivery of these compounds to the skeleton, because of its excellent safety profile and well-established conjugation chemistry. Not only would this strategy claim all aforementioned benefits of polymer therapeutics, the capacity of introducing multiple drug molecules and targeting moieties onto the same carrier would be advantageous as well.

2.1 The design and validation of targeting strategies for osteotropic HPMA copolymer conjugates

The noticeable physiological difference between the skeleton and all the other soft tissues and organs is the mineral content of the bone. The bone mineral or apatite has been described as a poorly crystallized, CO_3-containing, calcium deficient hydroxyapatite (HA) analog [31]. To render a delivery system osteotropic, a targeting moiety that strongly binds to hydroxyapatite would be a natural selection. In searching for such targeting ligands, the first candidate came to mind was tetracycline (TC), the notorious antibiotic that stains tooth enamel [32]. At a later time, we realized that this compound has been widely used as a bone formation marker for bone histomorphometric analysis in animal and human (transiliac bone biopsy) due to its regular clinical prescription and its strong osteotropicity [33]. Several investigators have used TC or modified TC for the synthesis of osteotropic prodrugs [27,30]. TC analogs with simpler structures have also been screened for bone-seeking capability. In the quest for HPMA copolymer-TC conjugate, 9-aminoanhydrotetracycline (ATC) [34] was synthesized and reacted with methacryl chloride to obtain the polymerizable TC-containing monomer. While its HPMA copolymer showed a good binding to hydroxyapatite, the initial *in vivo* testing of the conjugate (contains 20 % ATC) failed due to the death of the animals after a single bolus injection. Acute hypocalcemia was suspected as the cause of death.

This incident prompted the exploration of other potential bone-targeting moieties for HPMA copolymer conjugates. Anionic amino acids are major component of several bone-associated proteins [35]. They were found to be able to form negatively charged pocket to facilitate nucleation of bone apatite. Salivary proteins are found to be able to bind to dental enamel strongly, due to the presence of anionic amino acid sequences in their primary structure. Inspired by these natural biomineral-binding macromolecules, oligo Glu and Asp peptides were used to develop bone-seeking estradiol for the treatment of osteoporosis [29]. The evaluation of Asp hexapeptide-estradiol ($E_2{\cdot}3D_6$) in ovariectomized mice was very encouraging. At one-day post administration, the Asp hexapeptide-estradiol ($E_2{\cdot}3D_6$) concentration in bone is about 100 times higher than that of the estradiol (E_2). $E_2{\cdot}3D_6$ shows bone protection effect similar to E_2, but the hepatic hypertrophy with nonalcoholic steatohepatitis that often associated with E_2 treatment was absent for $E_2{\cdot}3D_6$.

In consideration of using the anionic peptides as bone-targeting moieties for HPMA copolymer carrier, D-Asp_8 was selected. Hou et al. reported that cathepsin K/chondroitin sulfate complex was critical for the collagenase activity of cathepsin K [36]. Long anionic peptides were suggested to be able to prevent the assembly the complex and abolish the collagenase activity

of cathepsin K. The octamer rather than hexamer of Asp was therefore selected as the targeting moiety, with the intent that it may have the dual functions of bone seeking and bone resorption prevention. The D-configuration was used to prevent premature degradation of the targeting moiety. Different from ATC, the incorporation of anionic D-Asp_8 into HPMA copolymer was proved to be challenging due to the poor solubility of methacrylated D-Asp_8 (MA-GG-D-Asp_8) in methanol, in which the HPMA copolymerization was routinely performed. Eventually a compromised DMSO/H_2O mixed solvent system was used for the copolymerization of MA-GG-D-Asp_8 and HPMA with relatively good conversion of the peptide and the overall polymer conjugate yield. The conjugate was labeled with fluorescein isothiocyanate (FITC) for the convenience of tracking.

The *in vitro* study showed that FITC-labeled HPMA copolymer D-Asp_8 conjugate (P-GG-D-Asp_8-FITC) could swiftly bind to HA upon exposure with more than 80 % of the conjugate immobilized after 1 h. In the initial *in vivo* test of the conjugate's osteotropicity, healthy balb/c mice were given tail vein administration of P-GG-D-$(Asp)_8$-FITC at a dose of 0.5 g/kg. The mice were euthanized after 24 h with long bones isolated. From the histomorphometric analysis, it was clear that most of the surface areas of the long bone, such as endosteum and periosteum of diaphyseal shaft were labeled with P-GG-D-$(Asp)_8$-FITC. The strongest binding of the conjugate happens at the high bone turnover sites such as primary spongiosa and metaphyseal areas (Figure 2) [37]. While this result was very encouraging, one critical question remained to be answered. Will this strong bone-targeting capability of P-GG-D-$(Asp)_8$-FITC be reproducible in an animal model of skeletal disease? To answer this question, ovariectomized (OVX) rat model of osteopenia was selected for evaluation. Using the same dose, P-GG-D-$(Asp)_8$-FITC was administered to rats that have been OVXed for 3 month. After histomorphometric processing of the isolated bones, it was pleasing to find that the conjugate could indeed bind to the bones of the osteopenic animal model (Figure 3) [38].

To quantitatively understand the pharmacokinetics (PK) and biodistribution (BD) of the HPMA copolymer-D-$(Asp)_8$ conjugate *in vivo*, tyrosine amide was introduced into the copolymer to allow ^{125}Iodine labeling. For this study, ^{125}I labeled HPMA copolymer-D-$(Asp)_8$ conjugates (P-GG-D-Asp_8-^{125}I) and the ^{125}I labeled control HPMA polymers without targeting moiety were intravenously administered to healthy balb/c mice. At different time points post administration, the mice were analyzed alive with single photon-emission computed tomography (SPECT) or with a gamma counter after euthanasia. The advantage of SPECT is that it could provide the panorama view of the conjugates' distribution in the entire body. The planar images of the mice at 24 h post P-GG-D-$(Asp)_8$-^{125}I administration (10-20 μCi) confirm the earlier histomorphometric finding that the conjugates seem to prefer the bone sites (e.g. distal femur and proximal tibia) with high turnover rate. The planar image also reveals a significant accumulation of the conjugate in the head, which later with tomographic analysis clarified as disposition to the incisor roots, another site with high turnover rate. For the invasive gamma counter analysis, mice were euthanized at different points post P-GG-D-$(Asp)_8$-^{125}I (0.05-0.1 μCi) administration. At 24 h, the accumulation of all three P-GG-D-$(Asp)_8$-^{125}I (24, 46 and 96 kDa) in the bone was significantly higher ($p < 0.05$) than their respective control HPMA polymers with no targeting moiety. The targeting strategy did not result in high accumulation in any other organs except the conjugate with MW of 96 kDa, which showed a significantly higher accumulation in the liver and spleen than the control of the similar molecular weight. PK analysis of the tested conjugates in circulation suggests that the MW has a significant impact on their clearance. The higher the MW of HPMA copolymer conjugate, the longer its elimination half life ($T_{1/2}$). Based on the result from this study, it was concluded that to achieve higher and very specific targeting to the skeleton, a HPMA copolymer with a medium MW (less than the renal glomerular filtration threshold) and high D-Asp_8 content would be ideal [39].

In addition to TC and D-Asp_8, other major bone-targeting moieties that have been explored for HPMA copolymer conjugation are the bisphosphonates (BPs). The BPs inhibit the precipitation of calcium carbonate and were initially used as water softeners and for the prevention of scaling in commercial water systems [40]. Later many BPs were found to be potent inhibitors of mineralization, bone resorption and mineral dissolution [41]. The most unique physical-chemical property of the BPs is their strong binding to calcium phosphate and bone apatite. It was estimated that after single i.v. infusion of alendronate (ALN, Fosamax, a BP clinically used for the treatment of osteoporosis), 50 % of the entire dose would distribute to the skeleton [42]. Due to this unique property, BPs have been explored in construction of osteotropic prodrugs [43].

The initial attempt to synthesize methacrylated ALN-containing monomer for copolymerization with HPMA failed due to the difficulty in separating ALN from its methacrylated derivatives using routine purification techniques (data not shown). An altered synthetic strategy was then employed by using a polymer analogous reaction between HPMA copolymer with pendent *p*-nitrophenyl ester (ONP) functional side chain and ALN to obtain the desired conjugate. Histomorphometric studies revealed that the HPMA copolymer-ALN conjugate labeled with FITC could indeed target to the skeleton, especially the bone sites with high turnover rates [37].

The issues associated with the polymer analogous reaction is its relatively slow reaction rate and low ALN incorporation ratio. To address this problem, Pan el al. later synthesized methacrylated ALN monomer (MA-Gly-Gly-Pro-Nle-ALN) with Gly-Gly-Pro-Nle spacer (purpose to be discussed in later sections) using thiazolidine-2-thione (TT) active group [44]. Again, the purification was challenging but it was resolved by using a preparative HPLC system, albeit with a relatively low yield. To evaluate the PK/BD profile of HPMA copolymer-ALN conjugate (P-Gly-Gly-Pro-Nle-ALN), MA-Gly-Gly-Pro-Nle-ALN and HPMA were copolymerized in the mix solvent of water/methanol using reversible addition–fragmentation chain transfer or RAFT technique. ALN-containing conjugates and their controls without targeting moiety were obtained with different molecular weights and rather low polydispersity. This is a significant step forward from the PK/BD study of HPMA copolymer-D-Asp_8 conjugate where the conjugates with low polydispersity were prepared by laborious column fractionation [39].

Examination of this study reveals that both the MW and ALN content affect the PK/BD profiles of the P-Gly-Gly-Pro-Nle-ALN, which is similar to the results found with P-GG-D-$(Asp)_8$. Increase of MW would generally increase the distribution of the P-Gly-Gly-Pro-Nle-ALN to bone as the result of reduced clearance. The distribution of the conjugate in liver and spleen increases as the MW and ALN content are raised. This may be explained by the fact that BPs are very strong chelator for calcium and the presence of high concentrations of BP *in vivo* may cause nanoprecipitation of calcium salts and the subsequent capture of the particles by the RES system. All ALN-containing conjugates with relatively low MW (can be efficiently cleared through urine) show higher binding to bone than D-$(Asp)_8$-containing conjugates, which may be explained by the stronger bone-binding capability of ALN than D-$(Asp)_8$. For the conjugates with much higher MW, the two targeting moieties seems to produce little difference, as the rather slow clearance of the conjugates has become the deciding factor for their in vivo disposition to bone.

The different binding potentials of ALN and D-$(Asp)_8$ has been noted before in previous histomorphometric analysis [37]. In the OVX rats that have received bone formation marker TC, it was found that HPMA copolymer-ALN conjugate seems to distribute to all bone surfaces regardless any functional domain (resorption, formation or quiescent). For P-Gly-Gly-D-$(Asp)_8$, however, the conjugate was found to preferentially deposit on the resorption domain

(eroded surface). Such difference in targeting must originate from specific differences between the functional domains, not of the morphology but rather the molecular structure of the bone apatite. It was known that certain disease would cause the alteration of bone apatite's size, shape and crystallinity [45,46]. It was also known that the bone apatite at the formation surface is immature and may contain more amorphous calcium phosphate, while the apatite at the resorption surface is mature with "closer-to-perfect" crystal structure. Then the question would be why D-$(Asp)_8$ can differentiate this variation but not ALN? Based on general observation, it was hypothesized that D-$(Asp)_8$ is a weaker binding moiety to bone apatite than ALN. As its binding force is lower, it is more "picky" of the surface that it would bind. To test this idea, D-$(Asp)_8$ and ALN were conjugated to the tip of AFM and used to perform a force measurement on a dental enamel surface (95% hydroxyapatite). The result shows that D-$(Asp)_8$'s adhesion force to the surface is around 100 pN. For ALN, the force has much wider range with an average around 190 pN. To validate if this adhesion force difference causes the targeting moieties' different binding to the functional domains, ALN and D-$(Asp)_8$ were incubated with synthetic hydroxyapatites of similar surface area but with very different crystallinity. Indeed, the amount of ALN bound to the two apatites was similar. But the binding percentage of D-$(Asp)_8$ was much different with the hydroxyapatite having higher crystallinity attains the higher amount of binding. This finding is very significant as it will provide the tools to help direct therapeutic or diagnostic agents not only to the skeleton as an general targeting organ but also more specifically to different dynamic functional domains, D-$(Asp)_8$ for resorption surface, TC and analogs for formation surface and BP for both domains [47].

2.2 Drug activation mechanism for the bone-targeting HPMA copolymers

A critical factor in the development of HPMA copolymer-anticancer drug conjugates is their interaction with cancer cells and the drug activation mechanisms to be employed [2]. As the bone-targeting HPMA copolymer drug carriers have been successfully developed, the next logical question to ask is how the bone cells would internalize them and how to attach and activate the therapeutic agent that the delivery platform carries.

Different from other tissues or organs in the body, the extracellular matrix of bone is mainly made of bone apatite and proteins such as type I collagen. Relative to the enormous size and weight of the matrix, the bone cells only constitute a very small, albeit critical fraction. There are generally three types of bone cells, osteoblasts, osteoclasts and osteocytes. Osteoblasts are derived from mesenchymal stem cell and responsible for bone formation. Osteoclasts are of hemopoietic stem cell origin and responsible for the resorption of bone matrix [48]. These two types of cells coexist as basic metabolic units (BMU) on bone surfaces that remodel bones at different locations through out the skeleton. The imbalance between the bone resorption and formation leads to various skeletal diseases such as osteoporosis. Osteocytes are the most abundant cell type of the bone. They are stellate shaped cells enclosed within the lacunocanalicular network of bone. As osteocytes are connected via long and slender cell processes, they are also known as bone "nerve cells". Functionally, they are responsible for the calcium/phosphate homeostasis and also act as mechanosensory cells for the skeleton [49].

Osteoclasts are multinucleated giant cells that resorb bone. Among the three cell types of bone, its biology is of critical importance for the development of drug activation mechanism of the bone-targeting HPMA polymeric conjugates. After proliferation in bone marrow, preosteoclasts migrate to bone surface and fuse with each other to form multinucleated mature osteoclasts [50]. As they are guided to the site for resorption, these giant cells polarize and attach the bone surfaces to form a sealing zone, which isolates the apical membrane of osteoclast from the surrounding environment. Through an H^+-ATP pump and exocytosis, acid (i.e. HCl) and bone-specific protease (i.e. cathepsin K) are secreted into the space between the

osteoclast apical membrane (ruffled border) and the bone surface. This space is termed resorption lacuna, which is considered by many as an extracellular lysosomal compartment of osteoclasts. Its strong acidity is maintained for the dissolution of bone apatite and the optimal condition for cathepsin K activity. The presence of cathepsin K is responsible for the cleavage and degradation of the organic components of the bone (e.g. type I collagen). When an osteoclast finishes the resorption, it detaches and migrates to the next site for resorption (Figure 4). For bone-targeting HPMA copolymer carrier to enter the resorption lacunae, there are at least two potential mechanisms. First, when a mature osteoclast attaches to the bone surface where the conjugate reside, it will directly locate in the resorption lacuna and be exposed to the proteolytic and acidic environment. Second, if the conjugate is in the bone fluid, it may enter the resorption lacunae by transcytosis via the basolateral membrane → endosome/lysosome → resorption lacuna pathway. Clearly, both cathepsin K and the acidity in the resorption lacuna can be used as local physiological factors for HPMA copolymer-drug conjugate activation in the bone. It is also important to emphasize that different from the acidity, which is universal for the endosomal/lysosomal compartment for different cell types, cathepsin K is highly specific for the osteoclast [51]. Therefore, by using cathepsin K as the drug activation mechanism, the bone-targeted HPMA copolymer-drug conjugate will claim strong specificity at tissue, cell and molecular levels.

Comparing to the extensive study in identification of cathepsin B-specific Gly-Phe-Leu-Gly tetrapeptide sequence (GFLG) for anticancer drug conjugation [22], the search for cathepsin K-specific peptide was rather simple. A literature research revealed that the substrate specificity profiles of cathepsin K have been determined using positional scanning libraries [52]. Among the substrates tested, the sequence Gly–Pro–Arg was identified to be a very specific substrate for cathepsin K. Due to the presence of the basic residue in the sequence, however, it may not be stable in the serum. To ensure the cathepsin K-specific spacer stable in circulation [53], Gly-Pro-Nle (another specific substrate for cathepsin K) was selected as an alternative.

2.3 The synthesis and in vitro evaluation of osteotropic HPMA copolymer-PGE_1 conjugate

Prostaglandin E_1 (PGE_1) was selected as the first testing compound to be conjugated to bone-targeting HPMA copolymer conjugate. It is one of the most potent bone anabolic agents but when given systemically, has undesirable side effects. This compound was selected for the following reasons. 1. In the treatment of osteoporosis, the development of antiresorptive agents has been very fruitful, but identifying suitable bone anabolic agents has been slower. 2. Prostaglandin E series are very potent anabolic agents with limiting side effects, and thus would greatly benefit from the bone-targeting delivery system. 3. One of the authors (SCM) has accumulated many years of experience with the biological properties of the prostaglandins.

The general design of osteotropic HPMA copolymer-PGE_1 conjugate is shown in Figure 5. To avoid potential steric hindrance of the polymer backbone, an additional Gly was added to the N end of the Gly-Pro-Nle sequence. As there is no amine in PGE_1, a self-eliminating 4-aminobenzyl alcohol structure was introduced to connect the tetrapeptide (Gly-Gly-Pro-Nle) with the C-1 COOH of PGE_1. When the peptide spacer is cleaved by cathepsin K, free PGE_1 will be released immediately via 1,6-elimination of the 4-aminobenzyl alcohol. Modification of C-1 COOH would contribute to the stabilization of the PGE_1 against metabolism. However, the use of an ester bond can cause potential susceptibility of the conjugate to circulating esterases. Polymerizable methacrylated monomer (MA-Gly-Gly-Pro-Nle-4AB-PGE1) containing PGE_1 and Gly-Gly-Pro-Nle spacer was obtained by solid phase peptide synthesis. To obtain the final bone-targeting polymer conjugate, a polymer analogous reaction was chosen to introduce the targeting moiety because the yields of the conjugate by copolymerization of all monomers including the polymerizable derivative of the targeting moiety (MA-GG-D-Asp_8) were low. Because of the low stability of PGE_1 at high temperature, photopolymerization

at room temperature was employed [54]. When tested against human cathepsin K, free unmodified PGE_1 was released from osteotropic HPMA copolymer-PGE_1 conjugate. The release of PGE_1 from the conjugate is slower than the release from MA-Gly-Gly-Pro-Nle-4AB-PGE_1, which may be due to the steric hindrance of the HPMA copolymer backbone. It was also found that the increase of the hydrophobic PGE_1 content would slow down the release, potentially due to the hydrophobic aggregation of the PGE_1 molecules.

It would be ideal if the osteotropic HPMA copolymer-PGE_1 conjugate will be stable in the circulation and be activated exclusively in the skeleton. Any significant premature activation would diminish the benefit of the delivery system. To answer this question, the conjugates were incubated in human, rat, and mouse plasma at 37°C in the presence and absence of specific esterase inhibitors. The testing against the rodent species are important as the OVX rat and a non-rodent animal model are recommended by the FDA for pre-clinical evaluation of drugs for the treatment of osteoporosis [55].

Interestingly, it was found that the rate of PGE_1 release was strongly species dependent. Whereas the conjugate was stable in human plasma, the PGE_1 release in rat or mouse plasma was substantial. In rat plasma, the ester bond cleavage was mainly catalyzed by butyrylcholinesterase; in mouse plasma, in addition to butyrylcholinesterase, carboxylesterase also contributed to the cleavage [56]. These findings are very important but also present an interesting dilemma. While the eventual treatment objects are human, the preclinical evaluation of the conjugate must be performed in animal models. New conjugate designs that would avoid the use of ester bond are currently under development in Dr. Kopeček's laboratory.

After the *in vitro* evaluation of the osteotropic HPMA copolymer-PGE_1 conjugates against cathepsin K and serum of different species, the next logical step is to validate the cell mediated activation of the PGE_1 conjugate. For this purpose, the conjugate was introduced into the cultures of induced osteoclasts and osteoblasts, their precursors, and control non-skeletal cells. Confocal fluorescence microscopy result confirmed that the PGE_1-containing conjugates were taken up by cells via endocytosis with their ultimate localization in the lysosomal/endosomal compartments. For human cell lines, the release rate of PGE_1 were in the order of osteoclasts (FLG-OC)>osteoblasts (Saos-2-OB)≫osteoblast precursor (Saos-2), osteoclast precursor (FLG29.1), and control cells (HS-5). For murine cell lines, the order of release rates was osteoclasts (RAWOC)>osteoblasts (MC3T3-E1-OB)>osteoclast precursor (RAW264.7) ≫osteoblast precursor (MC3T3-E1) and control cells (C2C12). These data validated the design of the cathepsin K sensitive spacer. To investigate if other enzymes may also contribute to the release of PGE_1 from the conjugates, a series of potential enzyme inhibitors were introduced into the cultures. From this experiment, cathepsin L and lipases were identified as additional contributing enzymes [57].

2.4 Preliminary in vivo efficacy study of osteotropic HPMA copolymer-PGE_1 conjugate

The crowning experiment of this series of studies was the recent *in vivo* treatment study of aged OVX rats using the osteotropic PGE_1-containing HPMA copolymer conjugate. In this study, a single injection of the bone-targeting conjugate resulted in doubled histomorphometric indices {e.g. percent of double-labeled surface (dLS), percent mineralizing surface (MS), the corrected mineral appositional rate (cMAR), and the surface-referent bone formation rate (BFR)} of bone formation measured at 28 days after administration, compared to the non-targeting conjugate. The FITC-labeled conjugates were observed being buried in bone, frequently on prior eroded surfaces that had reversed and were undergoing bone formation (Figure 6). This indicated a remodeling reversal (from bone resorption to bone formation), perhaps due to the PGE_1 treatment. Another important observation in this study is that greater effects on bone formation were observed over a 4-week period when a total amount of PGE_1 at 0.6 mg per rat was administered as a single injection in 10 mg of the osteotropic PGE_1-

containing HPMA copolymer conjugate. This dose is much lower than the systemic doses ranging from of 0.3–6.0 mg PGE_2/kg given daily for up to several months to achieve anabolic responses in bone or the doses of PGE_1 ranging from 0.5–2.0 mg/week for 3 weeks were given by local infusion to stimulate periodontal tissue and alveolar bone formation. Importantly, no apparent adverse event that often associated with PGEs' administration was found in the rat treated with osteotropic PGE_1-containing HPMA copolymer conjugate [38].

Clearly, additional long-term treatment studies are needed to further establish this novel anabolic therapy for the treatment of osteoporosis. As bone-targeting HPMA copolymer conjugate is designed as a platform, loading of other therapeutic agents (e.g. anti-angiogenesis drugs) onto this conjugate can also be very beneficial [58].

3. HPMA copolymers for the treatment of rheumatoid arthritis (RA)

As evident in the development of bone-targeting HPMA copolymer conjugate, patho-physiological feature (e.g. mineral content of the bone) of the targeted tissue are often exploited to introduce tissue selectivity into a delivery system. Inflammation, as a pathological condition is associated with a large pool of different diseases (e.g. arthritis, asthma, atherosclerosis, etc.) [59]. It is the basic process whereby tissue of the body responds to injury. The key components are alterations in local blood flow along with the accumulation and activation of inflammatory cells of hematopoietic origin, followed by removal of foreign organisms and cells debris and of the inflammatory cells themselves and then stimulation of repair. Though being a normal defense mechanism of the body, failure of the inflammation resolution would result in variety of chronic inflammatory diseases and associated permanent tissue damage and loss of functions. It is critical, therefore, that therapeutic intervention can be delivered specifically to the inflammatory sites to assist the inflammation resolution. Of the four orchestrated patho-physiological events occurring in the inflammation process, the alteration of blood flow, or angiogenesis and vasculature leakage is similar to what one would observe in solid tumors [16]. Thus, it is logical to speculate if the EPR effect would also apply to inflammation. As noted by Dr. Maeda, however, the lymphatic drainage in inflammatory tissue is normal in most cases [60]. So to apply HPMA copolymer to the treatment of inflammatory diseases, a retention mechanism must be identified.

3.1. Arthrotropism of macromolecules in arthritic animal model

In our first attempt to evaluate the potential of macromolecular therapy for inflammatory diseases, rheumatoid arthritis (RA) was chosen as the disease target due to its strong prevalence (affecting 0.8% of the population worldwide) [61]. RA is a chronic inflammatory disease, which involves the destruction of joints. It is considered by many to be an autoimmune disorder, though the exact cause is still unknown. It is a systemic disease characterized by synovial inflammation. The inflammatory cells in the synovium tissue invade and damage articular bone and cartilage, leading in significant pain and loss of movement. Other symptoms may include stiffness, warmth, redness and swelling [62].

An adjuvant-induced arthritis (AIA) rat was selected as the working animal model for the study. It was selected because of its robustness and simplicity in establishment [63]. To induce arthritis in the male Lewis rats, heat-killed Mycobacterium tuberculosis H37Ra in mineral oil needs to be administered subcutaneously to the base of the tail. Nine days after the induction, inflammation starts to be observed in all the limbs (especially the hind). The swelling plateau appears around day 14. To increase the successful rate of the animal model, *N*,*N*-dioctadecyl-*N*',*N*'-bis(2-hydroxyethyl)-1,3-propanediamine (LA) was synthesized according to literature [63]. The addition of LA to the adjuvant was found to increase the model successful rate from 70 % to more than 90%.

As the first proof-of-concept experiment, Evan's blue (EB) infused into AIA rat intravenously. EB is a dye that can bind to plasma albumin strongly. Because of this unique property, it has been used frequently to label the protein and evaluate vascular permeability [65], as evident in Maeda's original work on EPR effect. It is shown in Figure 7, profound accumulation of the blue color was observed in the inflammatory hind limbs of the AIA rats 4 h after the infusion, which indicating extensive extravasation of the EB labeled plasma albumin due to the enhanced vascular permeability of the arthritic joint.

Encouraged by this finding, low MW magnetic resonance imaging agent DOTA/Gd^{3+} was conjugated to HPMA copolymer and administered to the AIA rats to explore if this versatile drug carrier would also be able to selectively target the arthritic joint. As can be seen in the MIP images of RA and healthy control animals (Figure 8), before the injection of polymeric contrast agent conjugate, the intestine and stomach of the animal are clearly visible likely due to fluid retention. Signals observed in the lower abdomen can be attributed to i.p. injection site (s) of anesthetic agents. No significant MR signal was observed at the hind limbs. At 8 h post HPMA copolymer DOTA/Gd^{3+} conjugate injection, the MR contrast signal was very high in the ankle joint and metatarsal region compared to the rest of the body, which suggests the preferential accumulation of the HPMA copolymer in this region. When the animal was evaluated at 48 h post injection, the enhancing effect of the injected contrast agent-labeled HPMA copolymer was still detectable in the hind ankle joints and paws, with the signal intensity in the rest of the body return to the level of the baseline. Contrast to the observation in the AIA rats, the clearance of the DOTA/Gd^{3+}-labeled HPMA copolymer in the healthy rats is very fast. Its distribution was confined in the circulation system and the kidneys [66].

The result from this MRI imaging study is critical as it confirms the arthrotropism of HPMA copolymer in AIA rats. This result agrees with previous finding that stealth liposomes could selectively distribute to the arthritic joint, albeit coincide with large deposition in the RES system [67]. Given the fact that most of the anti-rheumatic drugs were not designed with joint specificity, it was a natural step to consider conjugation of these compounds to HPMA copolymer carrier and empower them with arthrotropicity, consequently with enhanced therapeutic efficacy and a potentially improved safety profile.

3.2. Development of HPMA copolymer-dexamethasone conjugate (P-Dex) for RA treatment

Currently, there are three classes of medications for the treatment and management of RA, which include nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids and disease-modifying antirheumatic drugs (DMARDs). NSAIDs, such as aspirin and its derivatives are traditionally used for the control of pain and inflammation of RA. Their therapeutic effects are mediated primarily through inhibition of prostaglandin G2/H2 synthase (PGG2/H2S, also known as cyclooxygenase, or COX). However, they will not prevent joint destruction and only show a small impact on inflammation. Due to the ubiquitous expression of the enzyme, application of NSAIDs is limited by side effects, especially GI and renal toxicity [68]. Many specific inhibitors for isozyme COX-2 have been developed to overcome side effects associated with traditional NSAIDs. While this strategy is successful to a certain extent, other severe toxicity problems have been observed [69].

Corticosteroids, or glucocorticoids (GCs) are potent immunosuppressive and anti-inflammatory agents that are widely used for controlling the symptoms of inflammatory diseases. GCs may also have disease-modifying effects in addition to their anti-inflammatory actions (considered by many as DMARDs) [70]. Unfortunately, long term treatment with GCs have been linked with a series of adverse events involving the endocrine, cardiovascular, gastrointestinal, nervous, hematopoietic and musculoskeletal systems [71]. Secondary osteoporosis is one of the major problems associated with long-term GC therapy in RA patients causing increased morbidity [72]. Therefore, only low dose GCs are recommended for early

stage of RA management. Considerable research has focused on the molecular mechanism of GCs' diverse biological functions. It is now becoming clear that GCs' actions are mediated through two distinct pathways: transactivation and transrepression, with the latter being responsible for the anti-inflammatory activity [73]. Compounds that may specifically activate the transrepression pathway have been developed [74,75]. However, such specificity is relative and the dose related side effects may still exist.

Joint inflammation and destruction in RA are complicated processes. DMARDs are agents that could interfere with multiple steps of these disease development cascades. Early intervention with DMARDs is currently recommended for effective RA treatment [76,77]. DMARDs, especially the newly developed biological DMARDs have very specific molecular targets (e.g. IL-1, TNF-α). However, due to ubiquitous biodistribution of these drugs and their molecular target in vivo, systemic side effects (e.g. liver toxicity for methotrexate (MTX) and leflunomide) may develop [77,78].

In our first attempt to conjugate an anti-rheumatic drug to HPMA copolymer, dexamethasone (Dex, a glucocorticoid) was selected, as this group of drug may best benefit from arthrotropic delivery due to its high anti-inflammation potency and high toxicity. As we analyze the chemical structure of Dex, the most obvious attachment site for its chemical conjugation to HPMA copolymer is its primary hydroxyl group. It can be used conveniently to link to the polymer side chain via an ester bond. Concerns were raised at the time if the ester bond would be stable enough to survive the circulation once administered *in vivo*. Ironically, however, a later literature search revealed that when Dex was conjugated to polyvinylpyrrolidone (PVP) via the ester bond, it was very stable against hydrolysis *in vivo* [79]. To allow proper activation of the conjugated Dex, we turned to the two carbonyl groups in Dex. By reacting them with hydrazine, the Dex molecule can be conjugated to HPMA copolymer via a hydrazone bond, which is acid-cleavable (Figure 9).

Acid-cleavable linkages have been extensively investigated in the development of polymeric drug delivery systems for cancer chemotherapy [23]. Hydrazone is one of the most widely used low pH cleavable structures. Another acid-cleavable bond commonly used in polymer-drug conjugation is the cis-aconityl linkage [80]. The rational for the applications of these structures as drug releasing mechanism is based on the understanding that polymeric drug carriers are lysosomotropic and they enter the cells via endocytosis. Once the polymer-containing early endosome becomes lysosome, the acidity within the lysosome increases, which will trigger the cleavage of these bonds and release the conjugated payload. Due to the cellular composition of the synovium includes macrophage-like and fibroblast-like cells, which show accelerated endocytosis activity after activation, the HPMA copolymer-Dex conjugate can be quickly internalized by the synoviocytes via endocytosis and release free Dex in the lysosome after its distribution to the arthritic joints. Another underlining mechanism being proposed to trigger the extracellular release of Dex is acidosis, a pathological condition of low pH often associated with arthritic joint. It is well known that the local inflammatory reaction in and around joint tissues promotes an acidic environment. This is partially due to the low levels of oxygen in the synovial fluid, which appears to induce a shift towards anaerobic glycolysis and lactate formation [81,82]. The pH values of synovial fluid had been reported as low as 6.0. Much lower pH values (4.4-5.6) in the synovial tissue have also been reported [83-85].

As the initial step, copolymer of HPMA and methacryl glycylglycine (P-Gly-Gly-OH) was synthesized via routine free radical polymerization. A simple polymer analogues reaction was then used to couple Dex to the copolymer via hydrazone bond [86]. A challenging issue, however, is that the content of Dex in the copolymer conjugate obtained using this polymer analogous reaction is variable from batch to batch. To overcome this problem, a new acid-cleavable Dex-containing monomer (MA-Gly-Gly-NHN=Dex) was synthesized. Direct

copolymerization of this monomer with HPMA allows easy and precise control of Dex loading in the conjugate. No unreacted pendent functionalities would exist in the drug conjugate. Reversible addition-fragmentation transfer (RAFT) polymerization was used to copolymerize the monomer with HPMA. This method provides much better control of the MW and polydispersity (PDI ≈ 1.3) of the polymer-drug conjugate. The P-Dex with MW of 34 kDa was used for the subsequent *in vitro* and *in vivo* study [87].

To evaluate if the P-Dex conjugate obtained can indeed to activated in acidic conditions, the conjugate was subjected to *in vitro* releasing study. It was found that at pH values of 7.4 and 6.0, the release of Dex is almost negligible. On the other hand, when the pH value of the releasing medium was set at 5.0, P-Dex would release free Dex in a close to zero-order fashion at a rate of 1% per day during the entire testing period (14 days). It needs to be noted that this release testing was done *in vitro* using buffers as releasing media. Many factors *in vivo*, such as the presence of various enzymes may alter the releasing profile dramatically [86].

3.3. In vivo evaluation of P-Dex

On 14 days post RA induction, rats with established arthritis were selected and randomly assigned into P-Dex, free Dex, and saline groups (6-7/group). A healthy untreated group was included as another control. P-Dex was given intravenously to the first AIA rat group on day 14. An equivalent dose of free Dex was divided into four aliquots (2.5 mg/kg of free Dex) and was administered to the second group on days 14, 15, 16, and 17. Saline was given similarly to the third group of AIA rats. The clinical measurements and grading of ankle joints were performed daily. At the end point, bone mineral density measurement and histological grading of the ankle joints were performed [86,87].

The therapeutic effect of P-Dex was very impressive. Upon the administration of the conjugate, the joint inflammation resolution of the AIA rats was immediate. The animals were more mobile and active. The ankle size and clinical grading of the animals were decreased after the first day, which is similar to the rats' response to free Dex treatment. The effect of P-Dex is long lasting. At 10 days post administration, the ankle joint size and clinical score were still very low. For the free Dex group, however, the cessation of the treatment immediately led to inflammatory flare. Long-term joint inflammation often leads to the deterioration of the bone and cartilage. To verify if P-Dex treatment would lead to any preservation of the joint bone, the bone mineral density (BMD) from distal tibia to the phalanges of the foot of all animals was performed at the end of the study. The mean BMD values were found with the following order: saline group < free Dex group < P-Dex group < healthy control group. Similarly, healthy control was found with the best histological score and the saline group had the worst. The score for P-Dex is similar to the healthy and better than free Dex. As shown in the photomicrographs of representative slides (Figure 10) from each group, severe bone destruction of the distal tibia and cartilage erosion of the joint are present in saline and the free Dex group, inflammatory cell infiltration were present in mild to moderate levels. On the contrary, P-Dex treatment demonstrates the best anti-inflammatory effect with profound bone and cartilage protection. Cell infiltration into periarticular soft tissue and bone destruction at the distal tibia are absent in most cases [86, 87].

Clearly, a single P-Dex treatment has a long-lasting inflammation resolution effect. This result is critical and indirectly supports the presence of a drug retention mechanism in the inflammatory joint. Apparently this is a mechanism different from the classical EPR effect as the intimal lymphatic is intact in most of the RA joints. Rather, we suspect that this retention is probably mediated by the activated synoviocytes, which internalize and store P-Dex in their lysosomes after the polymer conjugate was delivered to the joints. These subcellular vesicles would act as individual drug depot, which gradually release free Dex to control the local inflammation. As the slowly released free Dex reaches the circulation, it will be diluted

dramatically to a level that may not cause significant side effect to the patients. Currently, cell culture study and *in vivo* safety study are being performed to validate these hypotheses.

It is important to note that in this series of studies, Dex was used as a prototype inflammation resolution drug. Other anti-inflammation or immunosuppressant drugs may also be used. As an example, cyclosporines have been successfully conjugated to HPMA copolymer using various linkages [88-90]. So far they have been primarily used in the treatment of various cancers and have been found to be able to overcome multidrug resistance mediated by P-glycoprotein. These conjugates can be easily adapted for applications in RA treatment.

4. HPMA copolymers for the treatment of infectious diseases

In addition to musculoskeletal diseases, HPMA copolymers have also been explored for applications in the treatment of other disorders. Among those listed in Figure 1, infectious diseases, such as visceral leishmaniasis and hepatitis B virus (HBV) infection may be a group of illnesses that can benefit from the application of HPMA copolymers.

4.1. Lysosomotropic HPMA copolymer conjugates for the treatment of visceral leishmaniasis

Leishmaniasis is an infectious disease caused by obligate intra-macrophage protozoa of the genus Leishmania, which consists of four main clinical syndromes: cutaneous leishmaniasis; muco-cutaneous leishmaniasis (also known as espundia); visceral leishmaniasis (VL; also known as kala-azar); and post-kala-azar dermal leishmaniasis (PKDL). Among them, visceral leishmaniasis (VL) is the most severe form of this disease, which is caused primarily by Leishmania donovani and is potentially fatal if untreated [91]. Treatment of VL relies on specific anti-leishmanial drugs. The pentavalent antimonials sodium stibogluconate and meglumine antimoniate have been the first-line treatment of VL for more than 70 years. Amphotericin B has been developed as a replacement for antimonials. Applications of these drugs, however, have been hampered by their severe systemic side effects [92]. This problem is partially attributed to the lack of desirable tissue/organ distribution, which also plagues the development of new drugs for VL treatment [93,94]. Therefore, the most recent advance in this area has been focused on development of drug delivery strategies to optimize their body and subcellular distribution, which may be translated into improved therapeutic efficacy and safety profile.

VL directly results from multiplication of Leishmania in the lysosomal compartment of the macrophages of the host reticuloendothelial system (RES) [95]. The intracellular location of these microorganisms protects them from the therapeutic agents that are unable to penetrate the cells, which explains the difficulty involved in treating VL [96,97]. Colloidal systems have been employed to deliver antileishmanial drugs to the cells of the RES [98-100]. These systems have demonstrated improved therapeutic efficacy against VL and reduced toxicity compared to the free drugs [97]. Nevertheless, the spontaneous release of free drugs from colloidal carriers (e.g. liposome), could still cause significant side effects [93,102,103].

Different from liposome formulation, the loading of therapeutic agent to polymeric drug delivery system, such as HPMA copolymers is through covalent conjugation [1,104]. Such drug conjugates are stable in circulation and biologically inactive, but can be gradually activated via cleavage of the enzyme specific peptide linkers or acid cleavable linkers in lysosomes [2]. Major glycoproteins on the leishmanial parasite cell surfaces contain terminal mannose residues [105] that can be recognized by the mannose receptors on the resident liver macrophages thorough mannose-dependent-receptor-mediated endocytosis [106]. Receptor-mediated antileishmanial drug delivery using sugar grafted liposomes have been shown to increase efficacy and decrease the toxicity of antileishmanial drugs, when compared to regular liposome-encapsulated drug [100]. Based upon these principles, Ghandehari's group

developed a series of targeted and nontargeted antileishmanial agent - HPMA copolymer conjugates (PDs) in order to improve the current liposome-based treatment for VL (Figure 11) [107,108]. The delivery system is composed of four basic units: (a) HPMA, the copolymer backbone; (b) antileishmanial drug, 8-[(4-amino-1-methylbutyl)amino]-5-[3,4-dichlorophenoxy]-6-methoxy-4-methylquinoline (NPC1161); (c) GFLG, a lysosomally degradable oligopeptide linker between the drug and the polymer backbone; (d) N-acetyl mannosamine (ManN), a mannose-dependent-receptor targeting moiety to the macrophages at the side chains of the copolymers. Preliminary cytotoxicity test showed that all conjugates were nontoxic towards the mammalian cells. The initial antileishmanial an efficacy of these PDs was performed in L. donovani infected mice. The results showed that these PDs caused greater than 99% inhibition of the leishmania parasites at 5 mg drug equivalent dose, which were as effective as the free drug at this dose. At a dose equivalent of 2 mg, the antileishmanial activity of the conjugate with 2 mol % targeting sugar moiety provide 91% inhibition activity, which were significantly higher than the free drug alone (67% inhibition). While the *in vitro* studies indicated that adjusting ManN amount (5% → 20%) could significantly increase the macrophage uptake of PDs [108], the *in vivo* study showed that the conjugate without targeting moiety also had very good antileishmanial activity (99% inhibition at 5 mg equivalent dose and 85% inhibition at 2 mg equivalent dose), which is just slightly less effective than the targeted conjugate. This observation may be attribute to the well-documented lysosomotropic nature of HPMA copolymers [109-110]. Importantly at 1 mg/kg equivalent antileishmanial drug the effect of targeting moiety was pronounced and polymer-drug conjugates with 20 mole % mannosamine showed nearly twice the activity compared to the conjugates without the targeting moiety [108]. With increasing content of ManN in the polymeric conjugates, it was shown that it is possible to deliver lower dosages of anti-leishmanial drugs (in this case 1 mg/kg drug equivalent) to the macrophages, while maintaining activity similar to the free drug. This can potentially result in lower toxicity of the drug while maintaining efficacy.

Amphotericin B (AmB) is a polyene antibiotic with potent activity against Leishmania spp, and is widely used for the treatment of VL. The major clinical drawbacks of conventional amphotericin B (Fungizone®) treatment are its toxic side effects. The liposomal formulation of AmB (AmBisome®) has been used as first-line treatment of VL in Europe and the United States [101]. Its cost, however, was rather high. Inspired by Ghandehari group's work, Nicoletti et al. developed HPMA-GFLG-AmB and HPMA-GFLG-ManN-AmB copolymer conjugates for effective VL treatment (Figure 12) [112]. These conjugates displayed decreased cytotoxicity against mammalian cells. When tested *in vivo* with BALB/c mice infected with amastigotes, HPMA-GFLG-AmB copolymer conjugate showed excellent antileishmanial activity, which is close to that of AmBisome®. The dose–response experiments also confirmed that HPMA-GFLG-AmB copolymer conjugates has potent antileishmanial activity *in vivo*, which is similar to AmBisome®. Importantly, no signs of *in vivo* toxicity were found for HPMA-GFLG-AmB and HPMA-GFLG-ManN-AmB copolymer conjugates even at 3 mg/kg dose, which is above the LD50 Fungizone® [113].

To summarize the data reported in these studies, significantly improved therapeutic efficacy and safety profiles were observed with the ManN-targeting and non-targeting HPMA copolymer antileishmanial drug conjugates. This demonstrates the critical importance of lysosomotropic delivery of the drugs. Comparing to the liposome delivery systems, the activation of the HPMA copolymer – drug conjugates via cathepsin B provided an additional level of control, which can be crucial for the safety of antileishmanial drugs with high toxicity.

4.2. HPMA copolymer conjugates for the treatment of Hepatitis B virus (HBV) infection

Hepatitis B virus (HBV) infection is the world's most prevalent chronic virus infectious disease with over 350 million HBV carriers worldwide. Approximately one million deaths occur

annually due to HBV-induced liver diseases [114,115]. At present, only immunomodulator interferon-α (IFN-α) and nucleoside analogs lamivudine or adefovir are approved for chronic HBV treatment [116]. As a novel therapeutic approach, free antisense oligonucleotides have been developed for HBV treatment [116-118]. Their *in vivo* efficacies largely depend on the proper delivery of antisense oligonucleotides to the cytoplasm and nucleus with sufficient concentration to inhibit the expression of the target protein. Endocytosis appears to be the major pathway by which oligonucleotides enter most cells [119]. To facilitate the intracellular trafficking, Jensen et al. proposed to deliver antisense oligonucleotides into the lysosome using HPMA copolymer conjugate [120]. The degradable GFLG linker was used to conjugate oligonucleotide to HPMA copolymer.

The subcellular fate of the free oligonucleotides and oligonucleotide-HPMA copolymer conjugates were studied in Hep G2 cells by confocal microscopy. The free oligonucleotides appeared to enter Hep G2 cells via endocytosis and accumulate in the cytoplasm and nucleus, which is consistent with previous report [121]. The oligonucleotide-HPMA copolymer conjugate with nondegradable dipeptide GG spacer (P1) restricted the oligonucleotides from entering the cytoplasm and nucleus of the cells; and remained in the endosomes/lysosomes. However, the subcellular distribution of the oligonucleotide-HPMA copolymer conjugate with lysosomally degradable GFLG spacer (P2) appeared to be a combination of that of free oligonucleotide and the oligonucleotide-HPMA copolymer P1. The oligonucleotide released from copolymer P2 was observed in the cytoplasm and nucleus similar to that of the free oligonucleotide. Furthermore, the half-life of the oligonucleotide conjugated to HPMA copolymer in isolated rat liver lysosomal enzymes (tritosomes) is much longer than that of free oligonucleotide. These results suggest that conjugation of the oligonucleotides to the HPMA copolymers via degradable spacers could increase the stability of oligonucleotide and prevent its inactivation before escaping the lysosome.

The antiviral activities of the oligonucleotide-HPMA copolymer conjugate (P2) was then tested in hepatitis B virus-producing human hepatoma cells, 2.2.15, against free oligonucleotide. The oligonucleotide-HPMA copolymer conjugate could inhibit 50% and 90% of the viral activity at 1.7 and 8.3 μM of oligonucleotide concentrations, respectively, indicating the protection HPMA copolymer provided to the oligonucleotide before its escape into the cytoplasm. The free oligonucleotide, however, did not show any antiviral activity at the concentrations studied (0.01–10 μM), which suggests degradation of the oligonucleotide in the cell media or endosome/lysosome. The high concentration of oligonucleotide-HPMA conjugates needed for antiviral activity also suggests that the design of this HPMA copolymer conjugate should be further optimized.

It was reported that galactose-terminating glycoproteins could accumulate in hepatocytes *in vitro* and *in vivo* as a result of a specific, receptor-mediated pinocytic uptake mechanism [122]; and lactosaminated albumin has been used to target antiviral drugs to mouse liver [123]. As the organ target for this therapy is liver, development of oligonucleotide-HPMA copolymer conjugate bearing pendent galactosamine residues would enhance the hepatotropicity of the therapy for better efficacy and reduced side effects.

4.3. HPMA copolymer conjugates for the treatment of wound infection

As discussed in the Introduction, one advantage of using water-soluble polymers (e.g. HPMA copolymers) for drug conjugation is to improve the water solubility of the drug. Gramicidin S (GS) is a cyclodecapeptide secreted by Bacillus brevis, with the primary structure of [cyclo-(Val-Orn-Leu-D-Phe-Pro)$_2$] [120]. It is a powerful antimicrobial agent against a broad spectrum of Gram-negative and Gram-positive bacteria as well as on several pathogenic fungi [124]. GS has historically been employed as a topical antibiotic for the treatment of infections from superficial wounds [125]. However, GS is insoluble in water, and exhibits a rather non-

specific activity and a high hemolytic activity, which limit its use as an antibiotic for topical applications [126]. To address this problem, Solovskij et al. developed water-soluble polymeric derivatives of GS based on HPMA copolymers [127]. HPMA copolymers containing reactive p-nitrophenoxycarbonyl groups were first synthesized. GS was then coupled to the polymer via aminolysis. Unfortunately, the polymer amides of GS only exhibited weak antimicrobial activity to *Staphylococcus* and *coli bacilli*, probably due to the slow cleavage of GS from HPMA copolymer. The authors then changed their strategy and prepared copolymers of HPMA and acrylic acid (AA), which form polymer salt derivatives with GS. The antimicrobial activity of these water-soluble polymer salts of GS is much higher than the GS polymer conjugate, but is still lower than the free GS. This polymer salt of GS is sufficiently stable and has the potential to be used for the topical treatment of wound infection.

5. HPMA copolymer-based hydrogels for the treatment of CNS diseases

In previous sections, various applications of HPMA copolymers as water-soluble drug carriers for non-cancerous diseases have been discussed. It is important to know, however, that this versatile polymer was invented and developed in a world-leading institute of hydrogel research. Therefore, it is no surprise to learn that HPMA copolymer also finds its application in treating CNS diseases in the form of hydrogels.

Physical injury to the central nervous system (CNS), such as traumatic spinal cord injury (SCI) often leads to loss of neuronal functions and disability [128,129]. Over the years, a wide variety of biomaterials have been studied as scaffolds for restoration of neurological functions [129]. One common approach is implantation of biomaterial scaffold (e.g. hydrogels) at the injury site to support the damaged CNS tissue. It is not only a matrix for local drug delivery, but also provides a bridging substrate for axon attachment and growth [129-131].

Among many different hydrogels that have been used for neural tissue regeneration, HPMA hydrogels have shown great promise. The preliminary *in vitro* study by Woerly et al. showed that HPMA hydrogels could support neuronal cell attachment and neurotic extensions [132]. Since aminosugar residues and arginine-glycine-aspartic acid (RGD) peptide sequences are critical in promoting tissue organization and regeneration through the ligand-receptor recognition [133,134], Plant et al. incorporated RGD peptide sequences and aminosugar sequences into HPMA hydrogels and evaluated their potentials in the treatment of CNS trauma [135]. The results show that the presence of the RGD peptide had a specific impact on astrocytic migration and adhesion. Furthermore, regenerative axonal growth was also seen within both RGD and aminosugar containing HPMA hydrogels. These findings suggest that RGD containing HPMA hydrogels (NeuroGel™) have the potential for facilitating tissue regeneration in the injured CNS such as spinal cord injury (SCI). The *in vivo* study by Woerly et al. further confirmed their *in vitro* finding and showed that the hydrogels were able to bridge tissue defects in the acutely injured spinal cord, and supported cellular ingrowth, angiogenesis, and axonogenesis within the structure of the hydrogel network, which were similar to those of the developing spinal cord [136]. The hydrogels also facilitated the movement of neuroactive factors and nutrients secreted by the chronically injured host tissue to support regenerative axonal growth. In addition, the hydrogels produced a reduction of necrosis and cavitation in the adjacent white and gray matter [137].

To investigate if the hydrogel system could restore the functions to the chronically injured spinal cord, NeuroGel™ was implanted into the cavity that formed in the rat spinal cord 3 months after a severe injury when the functional deficit had reached a plateau [138]. The results show that long-term (7 months) implantation not only reduce the volume of the cavity but also induced tissue repair and axonal regrowth with extended long distance throughout the graft, accompanied by gradual functional recovery. Another important finding is the presence of

many fibers myelinated by Schwann cells in the NeuroGel™, which may further contribute to tissue repair and functional recovery. Due to the permissive diffusion of some endogenous trophic factors into the hydrogel implant, this hydrogel also has a protective effect against Wallerian degeneration on the myelinated axons, which indirectly improved cell repair and slowed down the cascade of progressive tissue necrosis [136,137].

The spinal cord injury also leads to glial scar formation in and adjacent to the site of injury, which can constitute a diffusion barrier between the injury site and adjacent intact neural tissue by hindering free diffusion of ions, metabolites, and neuroactive molecules that contribute to repair of damaged neural tissue [139]. On the other hand, astroglial responses such as astrogliosis, has been shown to influence geometry of the extracellular space around neuronal cells and thus hinder access of neuroactive substances for neuronal survival and axonal growth [140]. Thus, prevention of gliotic scar formation and modulation of astroglial response have been recognized as a part of a local treatment of the CNS lesion. Woerly et al.'s *in vivo* study in cat spinal cord shows that NeuroGel™ not only promot axonal elongation, myelination and angiogenesis up to 21 months, but also significantly prevent scar formation, reduced the myelin degradation, and reduced the astroglial responses [141]. These results suggest that NeuroGel™ could offer both structural reconstruction and functional recovery by providing a favorable substrate for regeneration of transected spinal cord, reducing glial scar formation, and allowing angiogenesis.

It was reported that injection of recombinant neurotrophic factors such as brain derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF), into the CNS can prevent neuronal death after axotomy or deafferentation. However, in many cases the rescue from cell death was only transitory because of the short half-life of these factors [142]. An alternative strategy is to chronically deliver these genetically produced growth factors via cell grafts such as fibroblasts to promote neuron survival and axonal growth [143]. Loh et al. developed a novel RGD containing HPMA hydrogels combined with genetically engineered fibroblasts (to chronically produce BDNF or CNTF) for *in vivo* promoting axon regrowth across tissue defects in the injured CNS [144]. The results show that cografted fibroblasts in hydrogels could survive for at least 8 weeks *in vivo* which is significantly better than that of fibroblasts in media suspensions. More importantly, compared to control rats injected with untransfected fibroblasts, there was increased ingrowth of axons into implants in CNTF, BDNF and mixed BDNF/CNTF transfected fibroblast groups, with maximal axonal infiltration in the combined BDNF/CNTF group. Furthermore, hydrogels in these transfected fibroblast groups contained over 150 times as many axons as the control hydrogels with untransfected fibroblasts. Among all of the tested groups, the mixed BDNF/CNTF group showed the greatest axonal ingrowth distance in hydrogels. No retinal axon growth was found in hydrogels containing untransfected fibroblasts at this case.

Clearly, the HPMA copolymer-based NeuroGel™ is a simple and safe scaffold for neuronal regeneration. It allows the incorporation of different growth factors and cells to further potentiate their repair capability. All of the findings discussed above suggest that hydrogel system may be used clinically for the repair of CNS lesions, especially spinal cord injury in humans due to trauma or other diseases.

6. Perspective

It has been more than three decades since the HPMA copolymers were invented in Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences. While the majority of its medical applications are cancer related, we also witness its branching into the treatment of other diseases categories during the last decade.

As being discussed in this review, targeted delivery to skeleton using HPMA copolymers has emerged as a research area with great potential. For most of the metabolic bone diseases, maintaining the balance between bone resorption and formation is critical. As the first line anti-resorptive agent, many generic bisphosphonates have become available and occupied a large percentage of the anti-resorptive market share. What is desperately needed at present is the development of new bone anabolic agents for bone regeneration and repair. Currently, the only anabolic agent on the market is Eli Lilly's Teriparatide (Forteo|). Sclerostin antibodies, antagonists of the Wnt signaling pathway have also showed great promise as a robust bone anabolic agent, albeit with some oncogenic concerns [145,146]. Many potent and probably cheaper (compared to the aforementioned biologics) low MW bone anabolic agents (e.g. PGE receptors' agonists and statins) have also been identified [147,148]. Osteotropic delivery of these agents using HPMA copolymer would certainly potentiate their anabolic efficacy and minimize the distracting non-skeletal complications.

The development HPMA copolymer based arthrotropic dexamethasone prodrug for the treatment of rheumatoid arthritis is a very interesting project. In addition to its sustained anti-inflammatory and disease modifying effects, the macromolecular prodrug may also have reduced extra-articular side effects. A longer retention of the polymer conjugate at the inflammatory sites suggests a pathological condition similar to EPR effect. However, the retention mechanism is most likely not based on defective lymphatic drainage. Upon clear delineation of this mechanism, the delivery concept may also be extended into the treatment of many other inflammatory diseases.

The lysosomotropic nature of HPMA copolymers is critical for directing the anti-viral drug conjugate to the subcellular sites of infection. When coupled with hepatotropic ligands, the delivery system designed by Ghandehari's group is ideal for the treatment of liver-associated viral infections. As an extension to this application, other therapeutic agents may also be conjugated to this system for the treatment of infection or alcohol-induced liver fibrosis, which has a high prevalence in developing countries.

It is important to note that the development of these non-cancerous applications of HPMA copolymers is based upon the abundant knowledge and experience that have been accumulated in pursuing HPMA copolymer-based cancer chemotherapies. The time-proven outstanding biocompatibility, well-developed conjugation chemistry and multifunctionality are the major advantages that set the HPMA system apart from other polymeric carriers. These unique properties would effectively facilitate the translation of the non-cancerous applications of HPMA copolymer drug conjugates into clinical evaluation. And their applications will not be limited by the diseases that have been listed in this review. It is fair to say that this is just the beginning.

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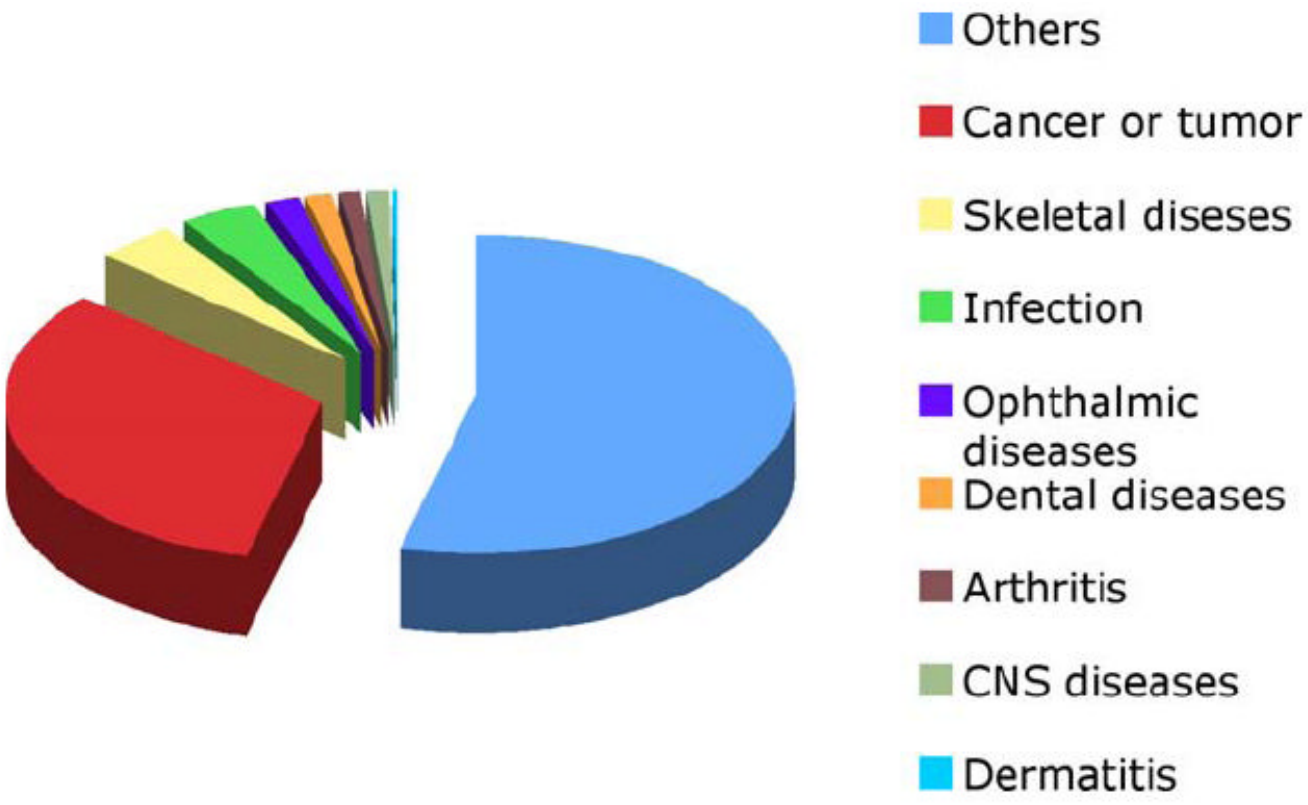

Figure 1.
Applications of HPMA copolymers in the treatment of different diseases.

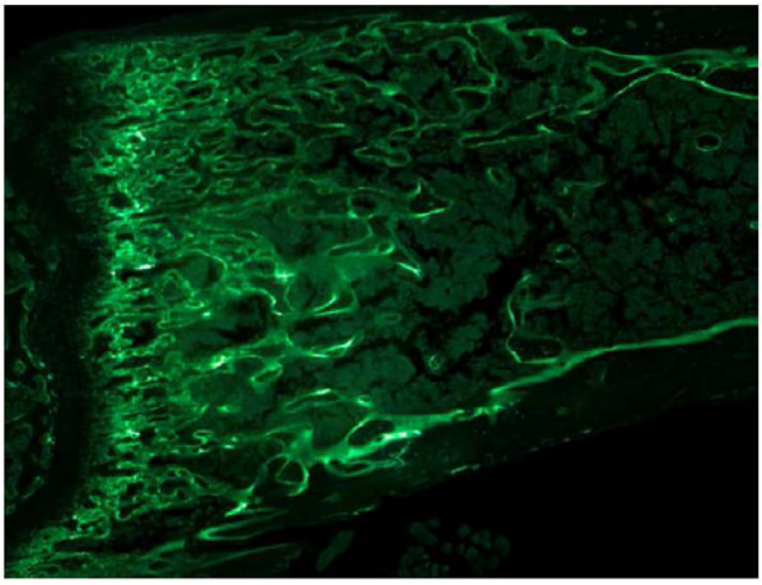

Figure 2.

Low power micrograph of a section of the proximal tibia from a mouse that was injected with P-GG-D-$(Asp)_8$-FITC. The uptake was most evident in areas of greatest bone formation and turnover, particularly in the metaphyseal cancellous bone region. Magnification = 30 ×.

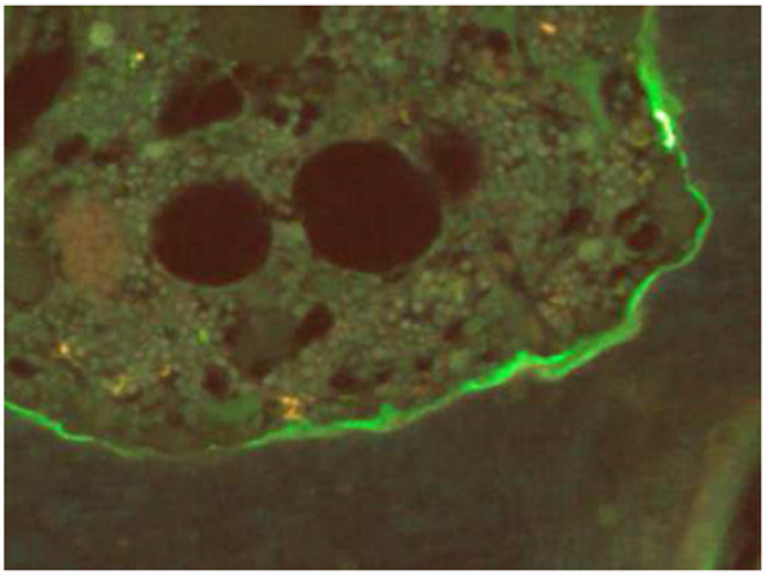

Figure 3.
Uptake of P-GG-D-$(Asp)_8$-FITC on cancellous bone surfaces in rats that were rendered estrogen deficient by ovariectomy. The uptake of the polymer (green label) was most evident on resorption (eroded) surfaces. Magnification = 200 ×.

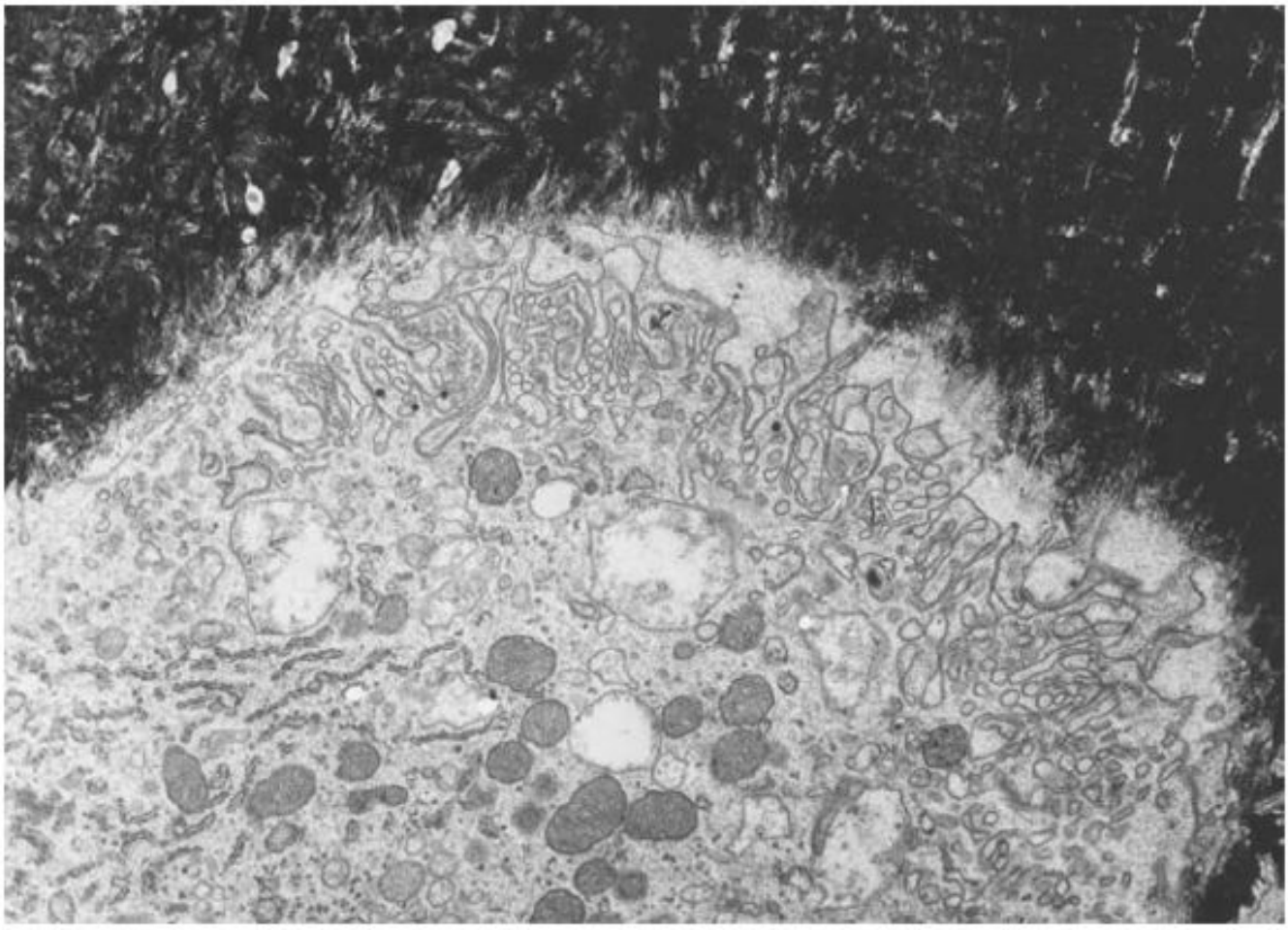

Figure 4.
Transmission electron micrograph of a portion of an osteoclast in a resorption pit (Howship's lacunae) in bone (black material). This osteoclast was actively resorbing bone as indicated by the extensive membrane invaginations of the ruffled border. Magnification = 2,500 ×.

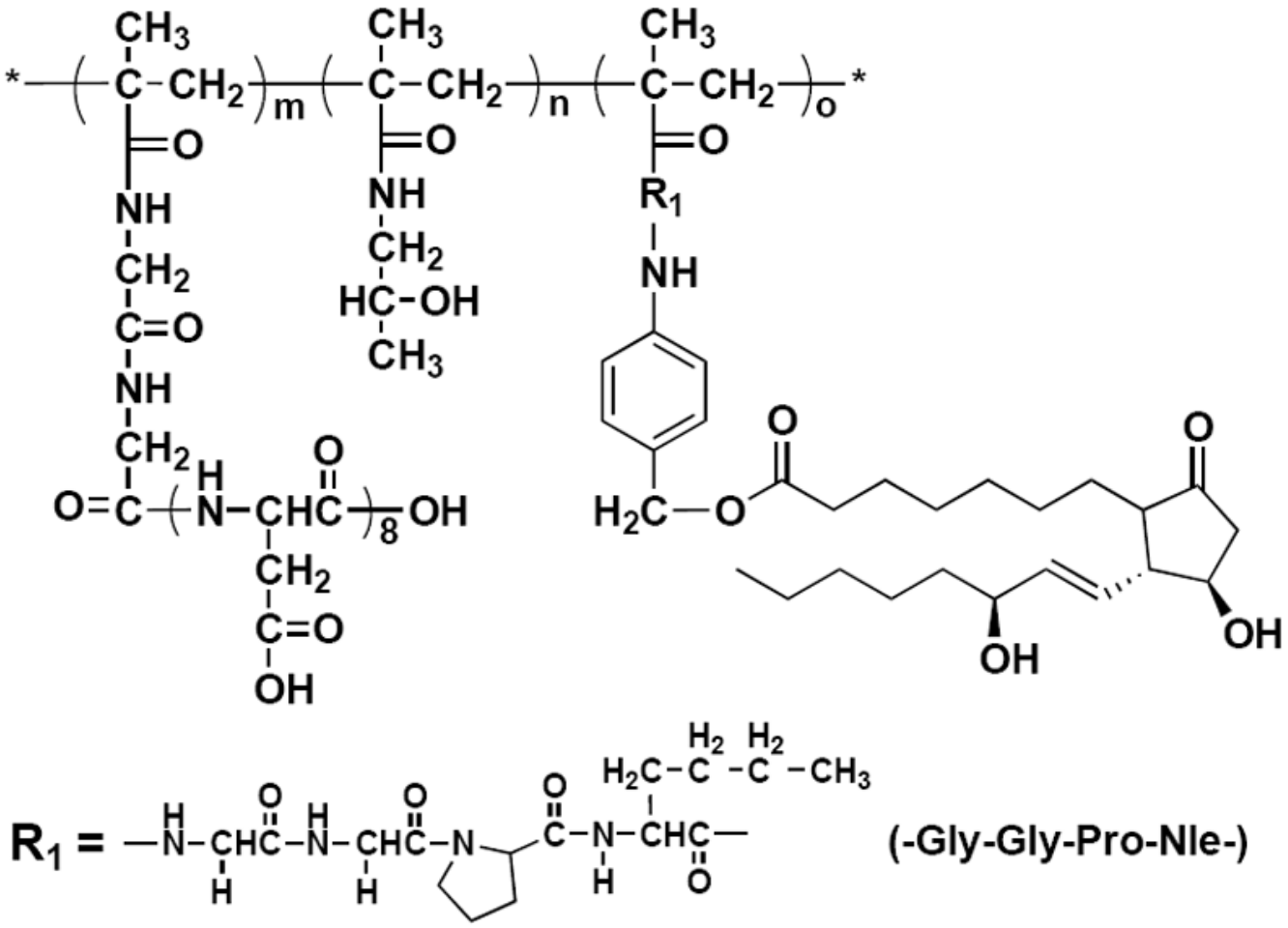

Figure 5.
Structure of osteotropic HPMA compolymer-PGE_1 conjugate. Adapted from reference [54].

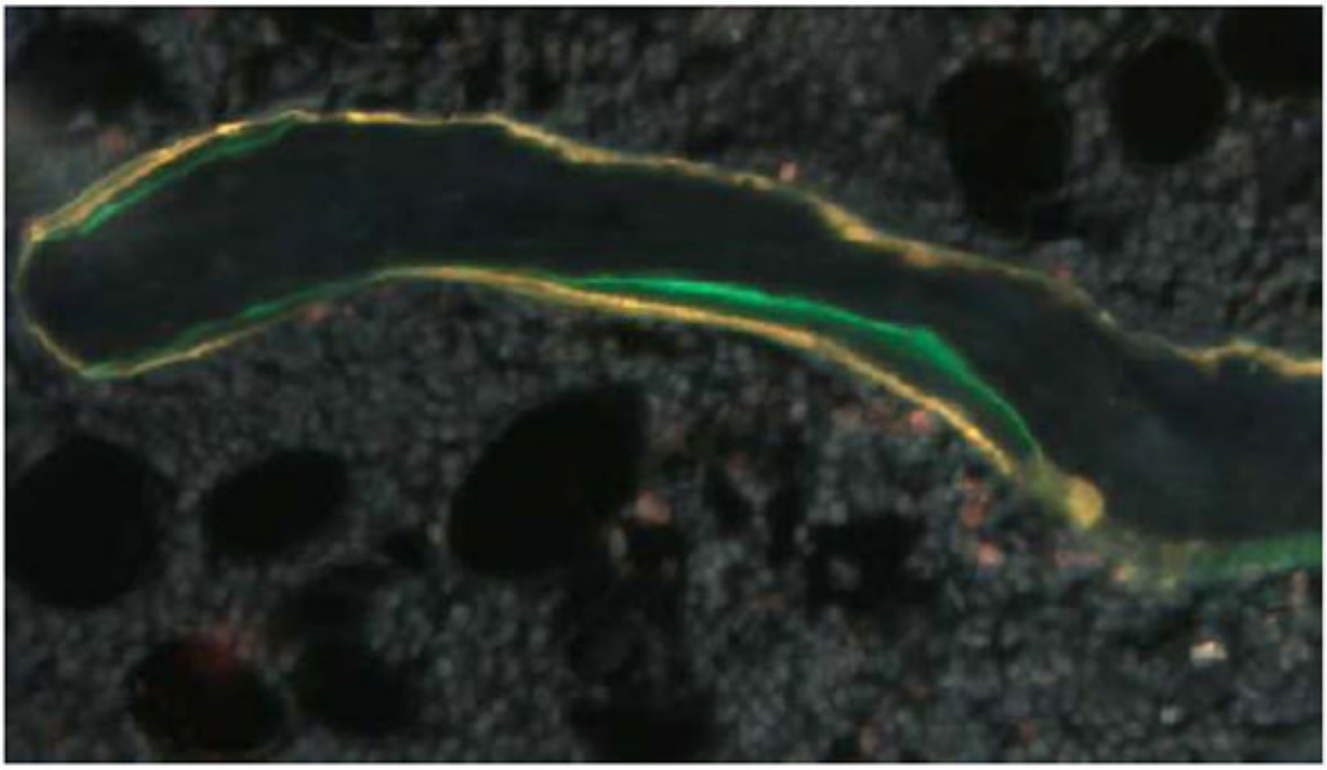

Figure 6.
A bone trabeculae from a rat at 4 weeks after the administration of HPMA copolymers conjugated with PGE_1 and FITC. The conjugate (green label) is buried in the bone matrix with new bone formation often occurring over the same region, as indicated by the tetracycline marker (orange yellow) for bone formation. Magnification = 150 ×.

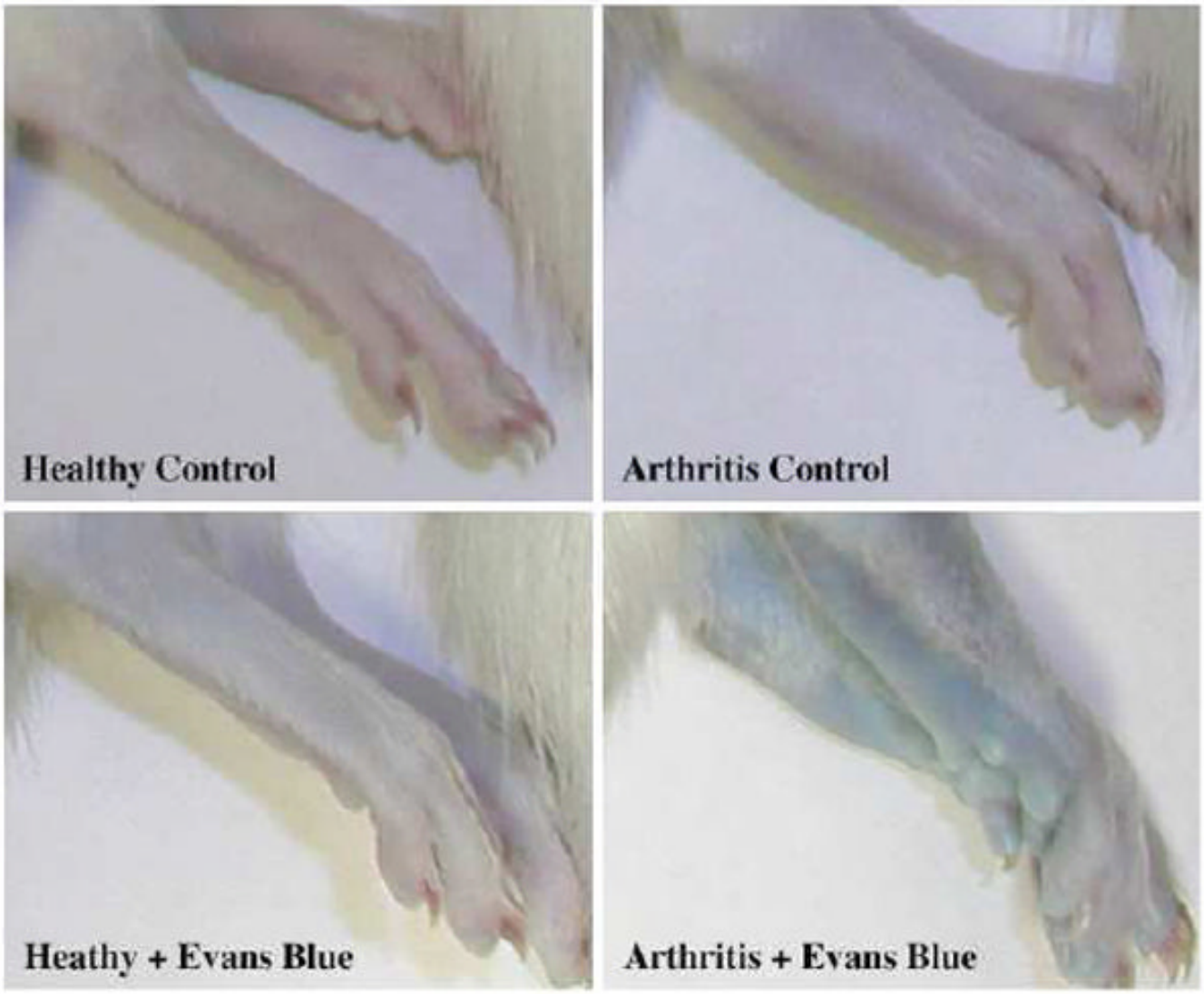

Figure 7.
A healthy rat and an arthritic rat injected with Evans Blue. The uptake of the dye is evident in the arthritic rat compared to healthy control.

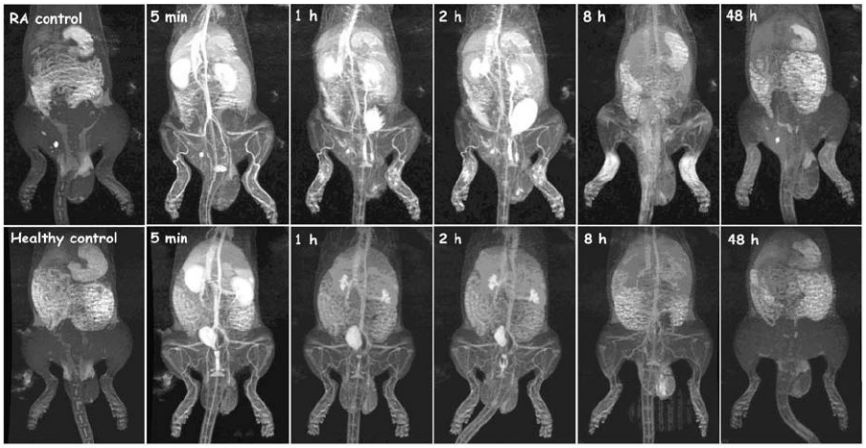

Figure 8.

MR images of arthritis (RA, top panel) and healthy (bottom panel) rats at different time points post DOTA/Gd^{3+}-labeled HPMA copolymer administration. The greater uptake and retention of the polymer in the ankle joints is evident in the RA rats compared to the healthy control. Adapted from reference [66].

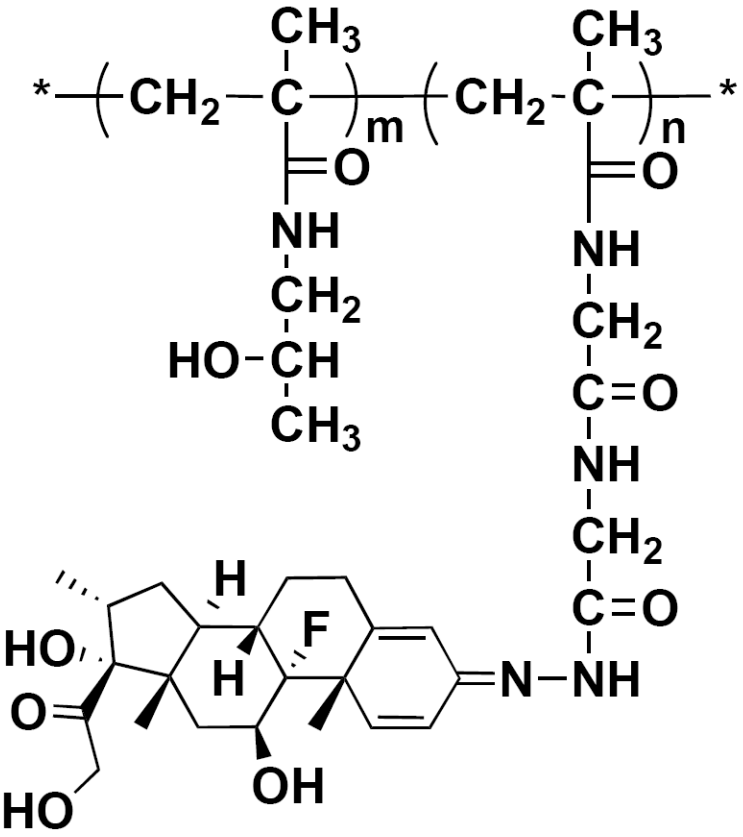

Figure 9.
The structure of acid-cleavable HPMA copolymer-Dex conjugate. Adapted from reference [87].

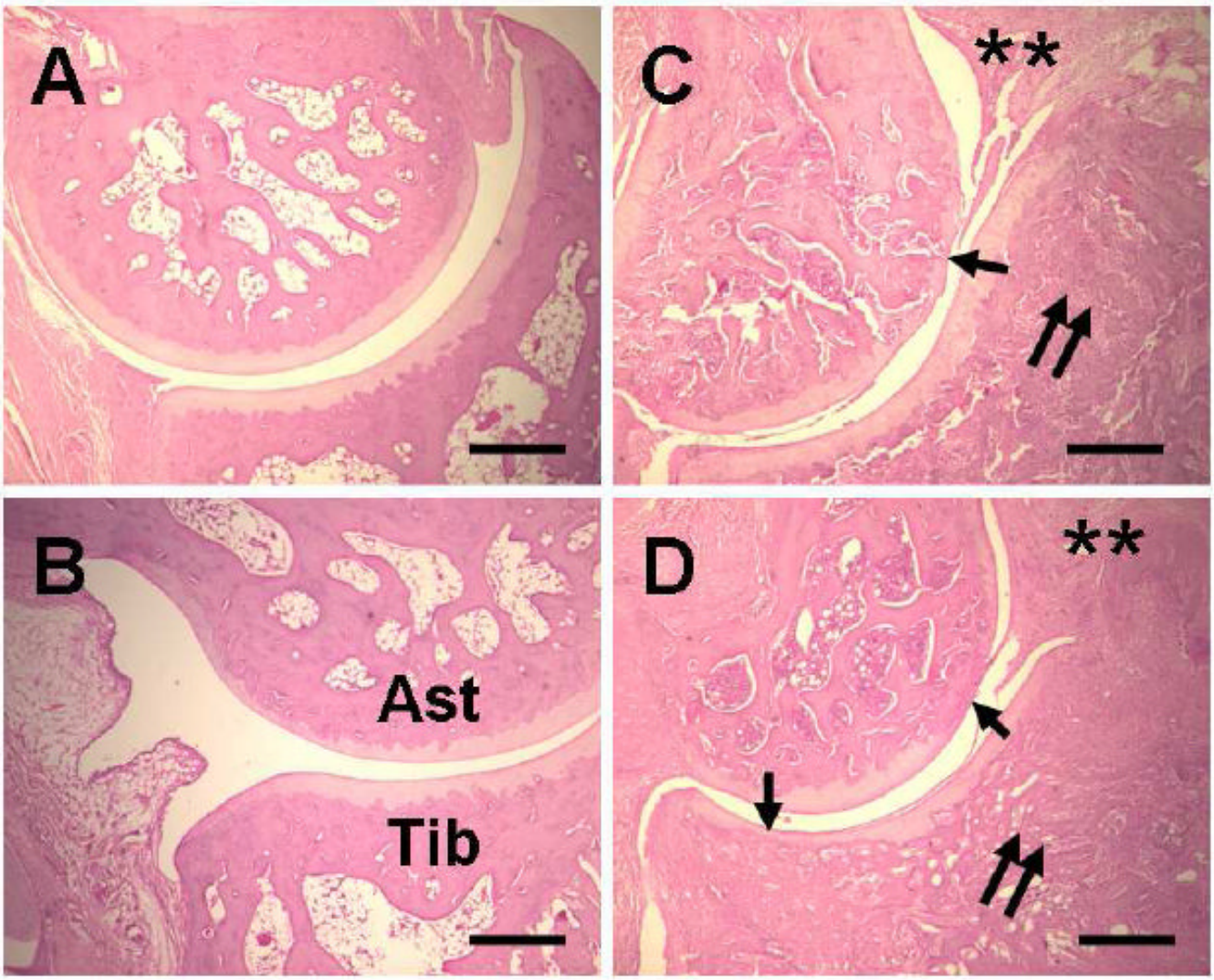

Figure 10.

Representative histology pictures of the ankle joints from the four animal groups. A. P-Dex; B. Healthy; C. Free Dex; D. Saline. Cartilage damage (single arrow), bone destruction (double arrow), and synovial cell lining and villous hyperplasia (**) are evident in free Dex and saline groups. Tib = Tibia, Ast = astrogalus. Bar = 0.5 mm. Adapted from reference [87].

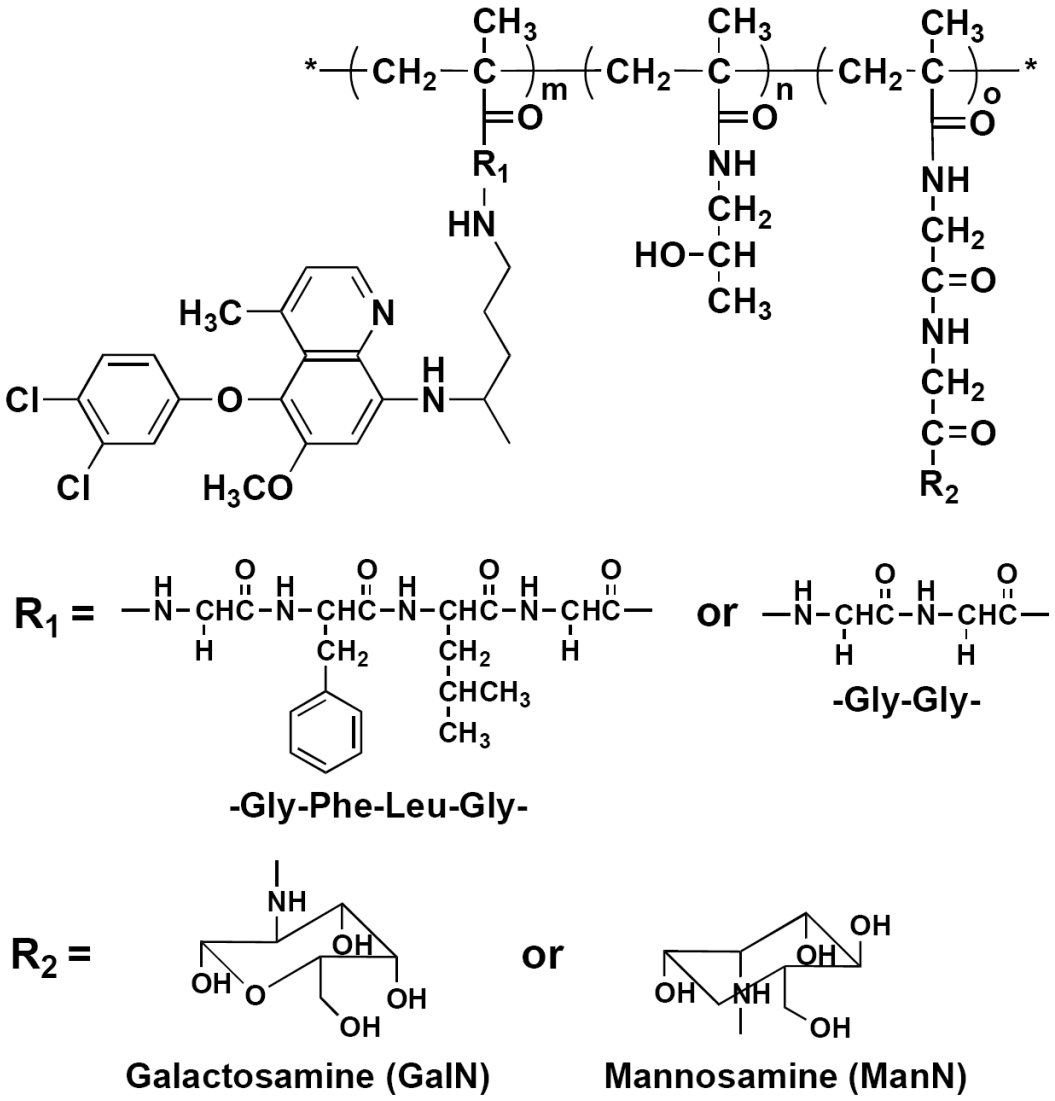

Figure 11.
The structures of HPMA copolymer-NPC1161 conjugates. Adapted from Reference [107].

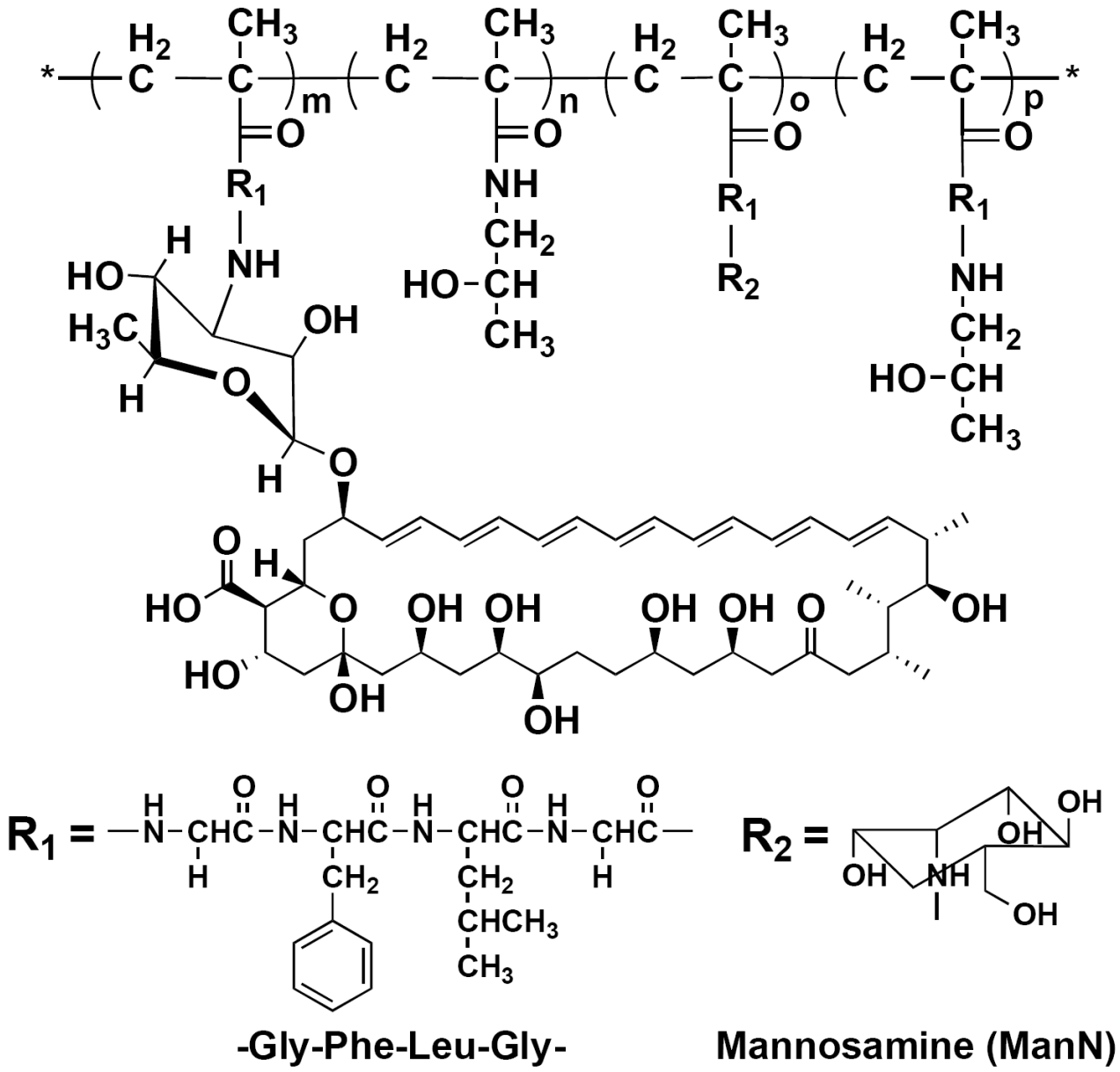

Figure 12.
Structure of HPMA copolymer - Amphotericin B conjugates. Adapted from reference [112].