Journal of Controlled Release 161 (2012) 446–460

Contents lists available at SciVerse ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Review

Administration, distribution, metabolism and elimination of polymer therapeutics

Ela Markovsky, Hemda Baabur-Cohen, Anat Eldar-Boock, Liora Omer, Galia Tiram, Shiran Ferber, Paula Ofek, Dina Polyak, Anna Scomparin, Ronit Satchi-Fainaro *

Department of Physiology and Pharmacology, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel

ARTICLE INFO

Article history:
Received 6 September 2011
Accepted 16 December 2011
Available online 29 December 2011

Keywords:
Polymer therapeutics
Angiogenesis
ADME
Biodistribution
Angiogenesis-dependent diseases
Cancer

ABSTRACT

Polymer conjugation is an efficient approach to improve the delivery of drugs and biological agents, both by protecting the body from the drug (by improving biodistribution and reducing toxicity) and by protecting the drug from the body (by preventing degradation and enhancing cellular uptake). This review discusses the journey that polymer therapeutics make through the body, following the ADME (absorption, distribution, metabolism, excretion) concept. The biological factors and delivery system parameters that influence each stage of the process will be described, with examples illustrating the different solutions to the challenges of drug delivery systems in vivo.

Contents

1. Introduction . . . 446
1.1. Administration . . . 447
1.2. Distribution . . . 449
1.2.1. Travelling to the site of action . . . 449
1.2.2. Disease site microenvironment . . . 452
1.2.3. Cell internalization . . . 453
1.3. Metabolism and degradation . . . 455
1.3.1. Biodegradable polymers . . . 455
1.3.2. Semi-degradable polymer backbones . . . 455
1.4. Elimination of non-degradable polymers . . . 456
2. Summary . . . 456
Acknowledgments . . . 456
References . . . 457

1. Introduction

Many therapeutic agents are low-molecular weight compounds which are administered systemically and exhibit non-specific biodistribution profile, short plasma circulation time and rapid systemic elimination. Consequently, relatively small amounts of the drug reach the target site, and therapy is associated with side effects and low efficacy. The use of macromolecules as carriers was suggested over 50 years ago, mainly in order to avoid or improve those downsides. Jatzkewitz attached a drug to the water-soluble polymer polyvinylpyrrolidone (PVP) in the 1950s [1,2] and Ushakov and his group synthesized more water soluble conjugates during the following years [2-4].

An excellent analysis of a complete polymer-bound drug delivery system was first proposed by Helmut Ringsdorf in 1975 [5]. This proposed model consists mainly of five components: (i) macromolecular polymeric backbone, (ii) drug, (iii) spacer, (iv) targeting moiety and (v) solubilizing agent. Macromolecular carriers chosen for the preparation of polymer therapeutics should ideally be water-soluble, non-toxic, non-immunogenic, and also biodegradable and/or being able to eliminate from the organism [6,7]. Finally, the macromolecular carrier should exhibit suitable functional groups for attaching the

* Corresponding author. Tel.: +972 3 640 7427; fax: +972 3 640 9113.
E-mail address: ronitsf@post.tau.ac.il (R. Satchi-Fainaro).

0168-3659/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jconrel.2011.12.021

respective drug or spacer. The drug can be conjugated directly or via a degradable or non-degradable linker onto the polymer backbone to allow the release of the active drug from the conjugate at the target site [8].

The choice of polymeric backbone for the conjugate has great implications on the pharmacokinetics and pharmacodynamics of the drug. The polymer characteristics, such as molecular weight, polydispersity, architecture, charge and hydrophilicity, define the drug solubility and loading, its biodistribution, body excretion and the interaction with the immune system. The polymeric backbone of the conjugate can be biodegradable, non-biodegradable, or semi-biodegradable.

For a suitable conjugation to chemical and biopharmaceutical drugs, many polymers have been proposed as carriers, including N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers, poly(ethyleneimine) (PEI), linear polyamidoamines, polyvinylpyrrolidone (PVP), polyglutamic acid (PGA), polyacrylamide (PAM), polydimethylacrylamide (PDMA), polyvinyl alcohol (PVA), chitosan and dextran. It is important to note that polyethylene glycol (PEG) has significant contribution specifically in the field of polymer–protein conjugates. PEGylation has been proven to be one of the most straightforward procedures for enhancing the therapeutic and biotechnological potential of peptides and proteins [8]. The most common types of polymeric carriers used in the field of polymer therapeutics are presented in Fig. 1 and the most common polymer backbones are presented in Fig. 2.

Many reviews had been published discussing different aspects of the growing field of polymer therapeutics. The descriptor 'polymer therapeutics', defined by R. Duncan, is an umbrella term used to describe polymeric drugs, polymer–drug conjugates, polymer–protein conjugates, polymeric micelles to which drug is covalently bound, and multicomponent polyplexes that are being developed as non-viral vectors [9]. In this review, we will focus on the journey that polymer therapeutics make through the body, following the ADME (absorption, distribution, metabolism, excretion) concept (Fig. 3). We will start by discussing the different methods of administration; continue with the factors that govern the biodistribution of these conjugates, their internalization to the target organs, their degradation and the release of free drug within the target cells. Finally, we will present the issue of polymers' metabolism and their ways of elimination. We will describe the biological factors and delivery system parameters that influence each stage of the process, and present examples illustrating the different solutions to the challenges of drug delivery systems in vivo. It is our hope that the concept presented in this review will help advance the rational design of polymer therapeutics.

1.1. Administration

The vast majority of polymer therapeutics that are being developed are designed to be administered by injection, usually intravenous (IV). The IV administration is the most straightforward method and its advantages are obvious, as it enters the bloodstream directly and is distributed throughout the body within seconds.

Several formulations in the market today are designed to be administered subcutaneously (SC) and intramuscularly (IM) (Peg-intron®, Pegasys®, Adagen® and more). These routes of administration allow for slow release of the drug from the injection site to the bloodstream, and therefore less frequent injections are required.

Following evaluation of the biological fate of PVA and PEG of various molecular weights injected via different routes (intraperitoneal (IP), SC, and IM), Yamaoka et al. found the elimination rate to be IP > SC > IM, with lower molecular weight polymers eliminated sooner, for both polymer types [10].

The injection administration methods hold many drawbacks for the patient and therefore lead to poor patient compliance. When designing a treatment intended for long-term therapy, a more convenient administration method should be considered.

Oral administration is the best route in terms of patient compliance. However, an orally-administered drug needs to pass several barriers on its way to systemic circulation, such as acidic environment in the stomach, proteases in the gut lumen and brush border membrane (BBM), tightly-bound intestinal epithelial cells (enterocytes) and metabolism by liver enzymes (the "first-pass effect"). All these factors may considerably decrease the bioavailability of the drug. Nevertheless, several successful oral formulations of polymer–drug conjugates have been developed. In the field of oral drug delivery, conjugation to polymers can be used to: 1) protect sensitive drugs from degradation in stomach and gut lumen [11]; 2) enhance absorption in the intestine by increasing water solubility [12,13]; 3) overcome drug resistance mechanisms (MDR) by altering the absorption pathway from transcellular to paracellular or transcytosis routes [12-14] (topic of drug resistance is addressed in detail below); 4) protect the drug from first pass effect degradation by liver enzymes [11-14].

NKTR-118 is an orally-administered formulation of the anti-opiate naloxone conjugated to PEG, currently in Phase III clinical trial [15]. PEGylation of naloxone alters its pharmacokinetic properties, distribution, and metabolism. It reduces the first-pass effect, increases its bioavailability and limits its capacity to enter the CNS, while the opioid antagonist characteristics are retained [14].

Polymer–drug conjugates can cross the interstinal epithelium either between the enterocytes (paracellular route) or through the cells, by

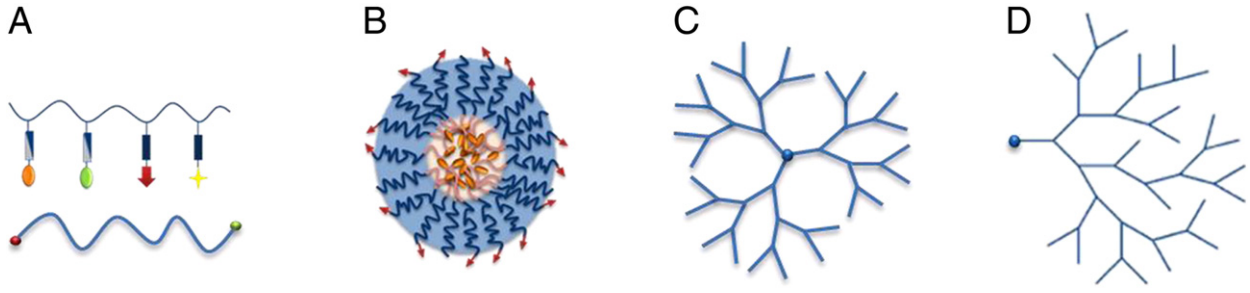

Fig. 1. Commonly used polymeric drug carriers. A: Multivalent (upper) or divalent (lower) linear polymer. Linear polymer carriers are composed of a single polymer backbone, forming a random coil structure. B: Micelle. Micelles are self-assembled vehicles, commonly constructed from diblock and triblock copolymers, where the hydrophilic block constitutes the shell of the micelle that interacts with the aqueous surroundings and the hydrophobic block forms the micellar core. Drugs can be encapsulated in the hydrophobic region of the aggregate or conjugated to the amphiphilic polymeric molecules. C: Dendrimer. A typical dendrimer comprises a central core, branched units and surface groups, all covalently attached. The repeated layers are termed "generations" (G) and are related to the number of steps in the synthesis, i.e. the number of brunching points between the core and the surface. Drugs can be encapsulated at the inner cavity of the dendrimers, of attached to the surface groups covalently or electrostatically. D: Hyperbranched polymers. Hyperbranched polymers are comprised of a randomly branched structure. Their ease of synthesis (typically using one-pot reaction) makes them relatively cheaper to produce than perfect dendrimers, though results in polydispersity.

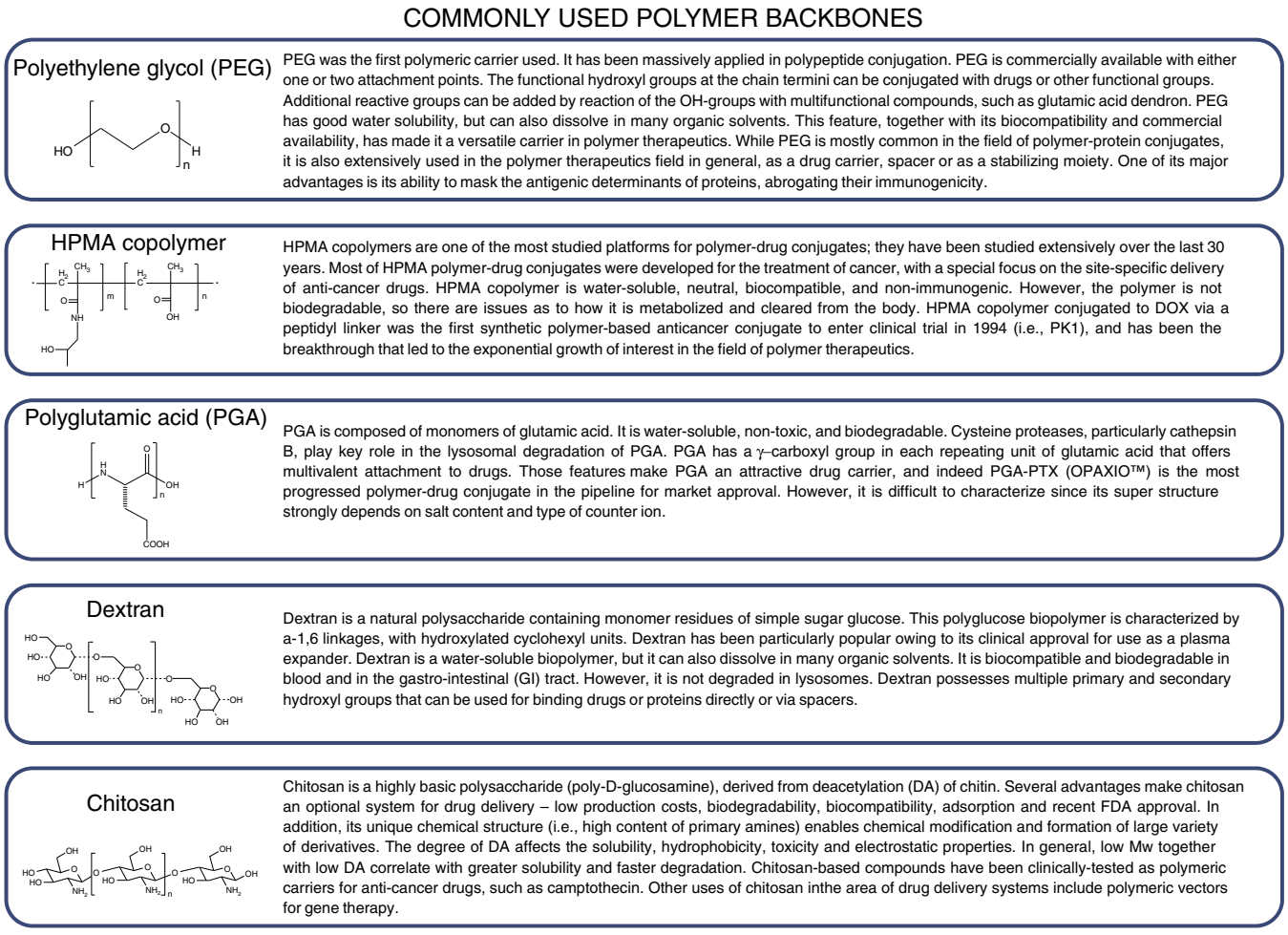

Fig. 2. Commonly used polymer backbones.

transcytosis mechanism. Free drugs can also enter by diffusing through the cells (transcellular route) (Fig. 4).

Chitosan is frequently used to enhance oral delivery of drugs. It is a highly mucoadhesive polymer and is capable of opening tight junctions of intestinal epithelium to facilitate paracellular transport. Low molecular weight chitosan (LMWC) form possesses even more benefits, such as improved water solubility, lower toxicity, and a narrower molecular weight distribution [11].

Lee et al. developed new oral delivery systems for paclitaxel and docetaxel by conjugation to LMWC [12,13]. Both are highly effective chemotherapeutic drugs of the taxane family, characterized by poor water solubility. They are currently administered IV in solubilizing formulations, which cause considerable side effects by themselves. The LMWC–paclitaxel conjugate system exhibited several favorable features for oral delivery, including 1) increased water solubility of paclitaxel by conjugation to water-soluble LMWC; 2) prolonged retention of the conjugate in the GI tract due to the mucoadhesive property of LMWC; 3) ability to bypass the P-glycoprotein-mediated efflux; and 4) an ability to bypass cytochrome P450-mediated metabolism, all of which led to dramatically enhanced bioavailability, lower side effects and comparable antitumor efficacy in vivo to that of IV-administered drugs.

Conjugation to polymers can provide a means to facilitate serosal transfer and to overcome the obstacles associated with oral delivery of "problematic" compounds. For example, the bioavailability of peptides, such as insulin, is very poor due to formidable barriers in the gastrointestinal (GI) tract such as acid hydrolysis in the stomach, enzymatic degradation by proteases in the lumen and BBM, and absorption through the intestinal wall.

Conjugation to LMWC also allowed for effective oral delivery of insulin [11]. LMWC–insulin conjugates reduced blood glucose levels in diabetic rats as opposed to native insulin and LMWC alone. The therapeutic effect was in proportion to the molecular weight of the conjugated LMWC. This molecular weight dependency may be attributed to the discrepancy in the ability to open tight junctions of intestinal epithelial cells and to the mucoadhesiveness of each LMWC molecule. It is anticipated that LMWC–insulin conjugate may also be able to escape hepatic enzymatic degradation once absorbed in blood, as demonstrated for oral delivery of taxanes in the form of LMWC conjugates.

Oral delivery using PAMAM dendrimers was evaluated using the everted rat intestinal sac model [16]. Anionic PAMAM dendrimer generations 2.5 and 3.5 showed rapid serosal transfer rates and had low tissue deposition, likely indicating a very efficient transcytotic transport pathway. These dendrimers provide, therefore, a platform for future development of oral polymeric drug delivery systems.

The adhesive properties of chitosan were also exploited for enhancement of delivery to other mucosal surfaces. For example, Slütter et al. developed N-trimethyl chitosan (TMC)–antigen conjugate for nasal vaccination. TMC serves both as an immunostimulatory adjuvant, to enhance immune response in the nasal mucosa and as mucoadhesive to prolong nasal residence time [17]. Conjugates also produced stronger immune response than previously used TMC nanoparticles, due to their smaller size and better penetration through the nasal epithelium.

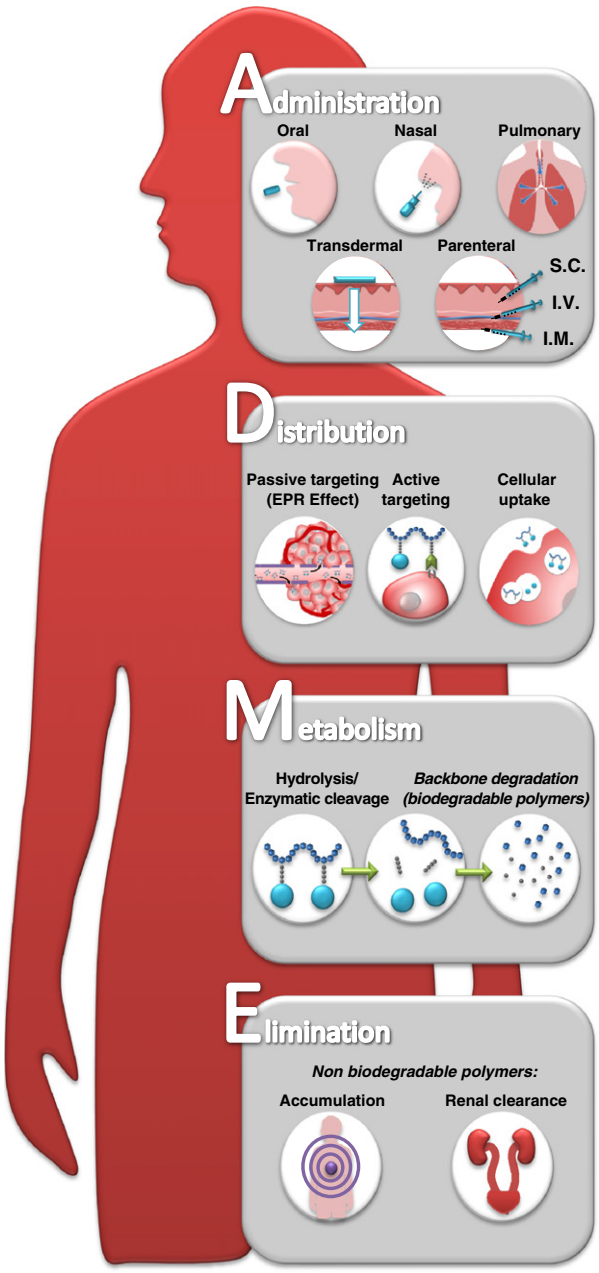

Fig. 3. Drug ADME route in the human body. Administration (different routes) → Distribution → Microenvironment → Internalization → Metabolism → Elimination (different routes).

Once the conjugate is absorbed through the intestinal wall, it enters the hepatic portal system and is carried via the portal vein into the liver, where many drugs undergo metabolism by liver enzymes (the first pass effect). After passing through the liver, the orally-administered conjugate enters the systemic circulation and is carried with the blood flow throughout the body, now sharing paths with its IV-administered counterpart.

It is noteworthy that many non-covalent polymeric oral delivery systems are also being developed, such as self-assembled micelles, nanoparticles etc. [18-22]. However, covering this broad topic is beyond the scope of this review.

Many other systemic and local administration methods of polymer–drug conjugates exist, such as intravitreal, vaginal, pulmonary, etc. However, the IV administration remains the gold standard, being the quickest and with the least amount of barriers for the conjugate to cross, and therefore the most effective. Indeed, most laboratories

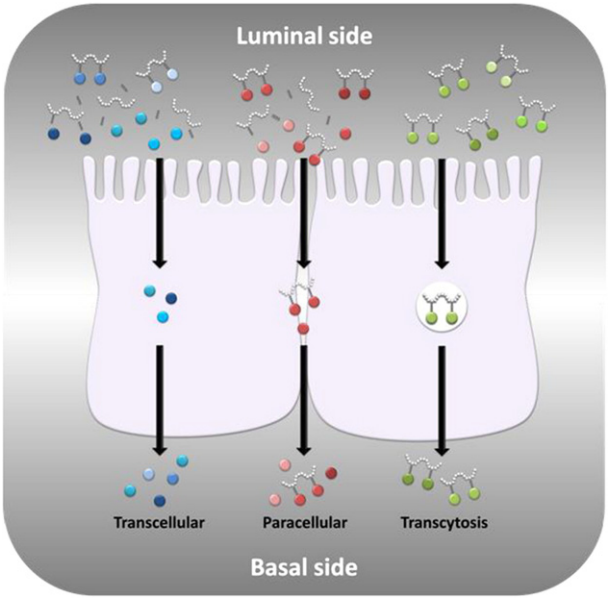

Fig. 4. Transepithelial delivery. Polymer–drug conjugates can cross the epithelium through either transcytosis (through the cells) or via paracellular (between the cells) route. Free drugs can also diffuse through the cells (transcellular route).

choose to design their delivery systems for IV administration, with examples being too numerous to list [23-26].

1.2. Distribution

1.2.1. Travelling to the site of action

1.2.1.1. Properties of the carrier. Once in the bloodstream, the size, shape, surface charge and decorations, and mechanical properties of the conjugate play key roles in its biodistribution, vascular dynamics, targeting, clearance, uptake, drug release kinetics and degradation. Naturally, interactions with the immune system and with serum proteins, if occur, have also great influence on these processes. The impact of size on biodistribution has largely been elucidated using spherically shaped particles. However, particle shape was indicated just as important, showing that shape has a significant impact on biodistribution [27,28].

Circulation time of polymer–drug conjugates is significantly longer than that of low molecular weight drugs, since the endothelium of normal blood vessels is typically impermeable to macromolecules. The disorganized vasculature that characterizes angiogenic tumors and other pathologies, with its discontinuous endothelium, leads to hyperpermeability to circulating macromolecules, in addition to the ineffective lymphatic drainage. The subsequent macromolecular retention is defined as the enhanced permeability and retention (EPR) effect [29,30]. This selective distribution pattern of the drug carriers is referred to as “passive targeting”.

HPMA copolymer conjugated to the chemotherapeutic drug doxorubicin (i.e. PK1) is the first example of passive targeting to the tumor site using the EPR effect and the first synthetic polymer-based anticancer conjugate to enter clinical trial in 1994 [31]. It presented a breakthrough that led to the exponential growth of interest in the field of polymer therapeutics.

While most examples of conjugates targeted by the EPR effect are related to treatment of solid tumors, angiogenesis is a crucial process not only in tumor growth and progression. Excessive angiogenesis also characterizes diseases such as diabetes, age-related macular degeneration, inflammation, rheumatoid arthritis (RA), psoriasis and more [32]. Although the lymphatic drainage in these tissues is usually

normal, neovascularization permeability is similar to that found in solid tumors [30], allowing the design of polymer therapeutics for passive targeting by an EPR-like effect [33,34].

The actual size of the gaps in tumor vasculature is dynamic and varies greatly between different tumor types and between vessels in the same tumor. Usually the molecular weight range of 20–200 kDa is used to take advantage of the EPR effect and to avoid rapid renal excretion. Particle size range of 20–100 nm was found as an optimum for prolonged circulation, accumulation in tumor tissue and enhanced diffusion within tissue [35-37].

The shape of the drug carrier also has significant influence on its distribution and performance. The study of Geng et al. [27] on filomicelles (long filamentous stable diblock copolymeric micelles of PEG-polyethylethylene or PEG-polycaprolactone) showed that filament structures persist in the circulation after intravenous injection considerably longer than rigid rods and flexible spheres ('stealth' polymersomes). Filomicelles of varied length loaded with paclitaxel shrank A549 tumors in nude mice, with greater efficacy for longer filomicelles (up to 8 μm). They attributed this phenomenon to the nanoparticles' behavior under flow; spherical and short filaments have shorter circulation time, because of better cellular uptake and blood vessel penetration. In contrast, flexible long filaments are extended by the blood flow and drift past the cells, exhibiting longer circulation time. Under static conditions, long filaments are relaxed and internalized by cells. Thus, the elongated filament shape, as well as filament flexibility, makes it a better delivery system. These in vitro experiments were conducted under flow velocity similar to that in the spleen. It should be interesting to conduct similar experiments under conditions resembling the sluggish flow of tumor vasculature. Since filomicelles are self-assembled polymer therapeutics and the conclusions in this study relied on the variations of shape of the filomicelles, we may extrapolate from this study to the whole field of polymer therapeutics, and examine it particularly for polymer–drug conjugates as well.

Saad et al. [38] conducted a comparative efficacy study of various drug nanocarriers. 30 nm linear PEG polymer, 5 nm PAMAM dendrimer and 100 nm liposome were used to deliver paclitaxel to H69 and A549 lung cancer cells and tumors in nude mice. All nanocarriers were fluorescently-labeled with Cy5.5 and a synthetic analog of LHRH (Luteinizing hormone-releasing hormone) peptide was attached for targeting. All treatments were given in suitable nanocarriers' concentrations to give an equivalent dose of paclitaxel of 2.5 mg/kg. It was found that all nanocarriers showed enhanced efficacy compared to the free drug. Without the targeting moiety of LHRH peptide, dendrimeric nanocarrier was the least effective, while PEG polymer nanocarrier was the most effective in suppressing tumor growth. This result correlates well with the theorem of Geng et al. [27] regarding better efficacy for long flexible filaments as nanocarriers. The most surprising result of Saad et al. [38] was received when targeting moieties were added to these nanocarriers. Targeting with LHRH peptide significantly enhanced antitumor activity of all nanocarriers, and leveled down the differences between them. This suggests that when using effective targeting, nanocarriers can be selected based on parameters such as type of therapeutic, solubility, electric charge, ease of preparation etc. rather than architectural parameters such as size and shape. This conclusion, derived from a first comprehensive comparative study, if verified in future analogous comparative studies of various delivery systems, holds great implications for rational design of drug delivery nanocarriers.

An important advantage of linear polymeric carriers over spherical particles is their flexible random coil structure, which allows them to "snake" into gaps smaller than their hydrodynamic diameter and penetrate into tissues better than the more structured spherical carriers. This, perhaps, makes them more effective in the conditions of angiogenic vasculature characterized by gaps of variable size and of dense tumor tissue, as illustrated by the studies mentioned above.

1.2.1.2. Active targeting. As mentioned above, passive targeting of polymeric nanocarriers is achieved by exploiting the EPR effect. However, there are several limitations to this approach, including variable vascular hyperpermeability among different tumor types and different areas of the heterogenic tumor tissue. Low cellular uptake of nanocarriers after extravasation can be another limitation, reducing the actual drug concentration within the tumor cells due to the stagnation around the tumor tissue. The passive localization associated with the EPR effect can be significantly improved by an active mechanism involving receptor–ligand interactions. Thus, a targeting moiety can be used in order to direct the molecule of interest to the target in a more specific way and thus overcome those limitations.

Active targeting combined with passive targeting became dominant in the design of drug delivery systems, especially in the field of cancer. Active targeting is expected to lead to higher and faster intra-tumor accumulation and, in the case of targeting with internalizing ligands, to increase intracellular concentrations of the drug. However, when designing a polymer therapeutic with a targeting moiety, the chemical properties of this additional molecule have to be carefully evaluated, because it may have an effect on the entire conjugate. For example, the attachment of folate to HPMA homopolymers led to aggregate formation [39].

A wide variety of targeting moieties is currently being examined. Practically any molecule overexpressed in the disease being targeted, either extra- or intracellularly, can be exploited. Examples include targeting to various markers expressed exclusively or overexpressed on tumor vasculature; such as VEGF receptor, $\alpha v\beta 3$ integrin, E-selectin [26,40-42] and on tumor cells; such as folate receptor, neural cell adhesion molecule, transferrin receptor [43-45]. Examples of targeting moieties include: peptides or other substrates which selectively bind cell surface receptors [46-48]; antibodies directed to antigens on cell surface [49]; materials with high affinity to compounds found at the extracellular matrix of the target site [50,51].

When discussing active targeting to tumor cells, it should be kept in mind that tumors contain genetically unstable cell populations with different cell-surface receptors, marker enzymes and drug sensitivity. This heterogeneity within a tumor can be a major obstacle to the success of a treatment, and can facilitate development of drug resistance. This is particularly relevant for polymer–drug conjugates designed for active targeting of tumor cells or intracellular bioresponsive drug release. Overcoming this obstacle is possible if the targeted treatment will lead to toxicity also in the non-targeted neighboring cells, in a phenomenon known as the "bystander effect". The bystander effect is usually achieved locally, by diffusion or via gap junction transfer of the active drug, after its dissociation from the carrier, to surrounding tumor cells. Other mechanisms include: (1) endothelial cell bystander effect derived from anti-angiogenic potential of chemotherapeutic agents; (2) systemic bystander effect due to cell-mediated immune response towards drug-treated tumor cells, which can lead to an extended therapeutic effect on metastatic tumor cells.

1.2.1.3. Immune response. An additional factor exerting a critical effect on the biodistribution of polymer therapeutics is their recognition and uptake by the immune system. The uptake of the macromolecules by the mononuclear phagocyte system (MPS), also known as reticuloendothelial system (RES), is a multi-step process, and it usually starts in the bloodstream, with the opsonization, i.e. the recognition and binding of foreign material by opsonins. The opsonins can be blood serum proteins (mainly albumin and fibrinogen) but the most common are specific proteins of the immune system, such as immunoglobulins and complement proteins [52]. Many drug delivery systems and their components are immunogenic and elicit the formation of antibodies, responsible of the immunological adverse reactions and of the rapid clearance of the drug delivery systems from the bloodstream [53].

PEG, which is one the most commonly used polymers in the field of polymer therapeutics, is known to induce the formation of anti-PEG IgM antibodies. Although the IgM response is not considered clinically relevant [54], several studies recently reported a rapid clearance of PEGylated proteins in the presence of antibodies against PEG or against the PEG–protein conjugate [55-57]. In some cases, allergic reactions after treatment with PEGylated asparaginase have been reported [58]. Negligible antibody formation was observed against the homopolymer poly(HPMA) or its copolymers [59], although hypersensitivity reactions induced by (meth)acrylates might occur [60].

Following opsonization, the macromolecules undergo phagocytosis by macrophages and Kupffer cells. The uptake by reticuloendothelial cells takes place within seconds after opsonization [61], and it is responsible for the fast clearance of the drug delivery systems from the blood and their accumulation in the liver and spleen, and to a lesser degree in the lung, kidney and lymph nodes. After phagocytosis, the nanomaterials have different metabolism and excretion pathways according to their specific characteristics, such as biodegradability, size and other properties, as described below.

Among the drug delivery systems, conjugation to polymers is one of the main approaches to prolong the circulation time of therapeutic agents, in particular due to the steric hindrance of the polymer that reduces aggregation and to the hydrophobic interactions responsible for the uptake by the MPS [62]. Bioconjugates appear to be more effective than other drug delivery systems (e.g. liposomes) in reducing uptake by the liver and spleen. As highlighted by Duncan and Vicent [63], HPMA–drug conjugates usually have a hydrodynamic diameter lower than 20 nm, which allows the escape from the MPS clearance.

It has often been reported that the physicochemical characteristics of the polymers, such as molecular weight and charge, are the main determinants of the biodistribution of the conjugate. Cationic polymers are preferentially accumulated in the liver [64]. In general, the higher is the size of the drug delivery system, the faster is the uptake by macrophages. Nevertheless, PEGylation, which increases the size of the systems, is the main technique to avoid opsonization of the drug carriers, especially in the case of PEG–protein conjugates [62] or in the case of PEGylation of liposomes, making them “stealth liposomes” [65].

In order to evaluate the different behavior according to the composition and structure of the macromolecules, Caliceti et al. conjugated four neutral polymers (PVP, poly(N-acryloilmorpholine) (PAcM), linear and branched PEG) to uricase, and followed the biodistribution profile in healthy mice. Despite the similar physicochemical characteristics (neutrality, relatively low molecular weight and amphiphilicity), the four polymers confer different behavior to the conjugates. In particular, PAcM conjugate easily distributes in all the organs rich in reticuloendothelial cells, while PVP–uricase conjugate shows low tissue distribution, and only partially accumulates in the liver. Remarkably, the linear and branched PEG conjugates showed significant differences in the biodistribution profile, as the branched PEG accumulated in the liver and spleen, while the linear one did not. The study suggests that not only charge and molecular weight, but also chemical composition and structure have to be considered in the choice of the polymer for the protein conjugation [66].

Lammers et al. [67] reported the biodistribution profiles after IV injection of thirteen HPMA copolymers, with different MW, functional groups and drug loading, showing that spleen is the principal accumulation site and spleen macrophages are the main cells responsible for the clearance from the bloodstream of all the conjugates. The uptake by the liver and the other organs of the MPS varies according to the physicochemical characteristics of the polymers. In general, increase in MW (from 23 to 65 kDa) improved the biodistribution of the conjugate in tumor-bearing animals, decreasing the uptake by the MPS organs and increasing accumulation in the tumor tissue. Chemical modifications, such as the introduction of functional reactive groups, peptidic spacers and active drugs, increased the uptake of the conjugate in the liver and the lung.

PEG–Doxorubicin conjugates also show accumulation in the immune system organs according to their MW and structure (linear or branched). Veronese and colleagues reported that the PEG conjugates have a favorable biodistribution, with preferred accumulation in the tumor tissue, and liver uptake values similar to other polymer–drug conjugates, such as HPMA [68]. Other polymers, such as dextran, appear not adequate for the delivery of doxorubicin to tumors. Doxorubicin–dextran conjugate (DOX-OXD, AD-70) in Phase I clinical trial displayed very high toxicity, in particular hepatotoxicity, ascribed to uptake by the MPS [69]. Nevertheless, other conjugates that showed high accumulation in all the MPS organs, had better results in clinical trials. The PGA–Paclitaxel conjugate (Paclitaxel poliglumex, CT-2103), is already in phase III clinical trials despite its high accumulation rate in the liver and in other immune system organs [70].

Bioconjugation to polymers significantly decreased the uptake of camptothecin by the immune system organs. Following IV injection in tumor-bearing animals, camptothecin accumulated in the liver and spleen, but after conjugation with relatively high MW PEG (40 kDa) [71] or with polyacetal poly(1-hydroxymethylethylene hydroxymethylformal) 60 kDa (XMT-1001, phase I in clinical trial), the conjugates mainly accumulated in the tumor site, with reduced uptake by the MPS organs [72].

For most studies it was concluded that polymers were taken up by the liver, spleen and lung as organs related to the MPS. However, there is a lack of research on the accumulation of polymer therapeutics on the phagocytic level.

We realize that interaction of polymer–drug conjugates with the immune system is an immensely broad topic, and properly addressing it is beyond the scope of this review. The reader is referred to other excellent reviews that cover this field [53,73].

1.2.1.4. Distribution of low MW drugs. As opposed to macromolecules, which gain entry into tissues only through gaps in angiogenic vessels, low molecular-weight drugs are able to diffuse through normal vasculature to any tissue and internalize into cells, therefore causing undesirable systemic side effects. The main benefit of polymer–drug conjugates is the reduction in toxicity, mainly due to improved selectivity for target tissues. Coupling a low molecular-weight drug to a polymer results in altered biodistribution compared with the free drug. Not surprisingly, the first conjugates synthesized were coupled with orthodox anticancer drugs (i.e. doxorubicin, cyclophosphamide, methotrexate, etc.).

Angiogenesis inhibitors are relatively less toxic than conventional chemotherapy and have a lower risk of drug resistance. Nevertheless, most angiogenesis inhibitors are low molecular-weight-compounds that are delivered systemically and consequently exhibit a non-specific biodistribution, short plasma circulation times and rapid systemic elimination [74]. Consequently, relatively small amounts of the drug reach the target site, and therapy is associated with side effects and low efficacy [75]. The poor pharmacokinetics and limited therapeutic effect of anti-angiogenic compounds can be improved by conjugation of these agents with polymeric delivery systems. HPMA copolymer conjugated to the potent anti-angiogenic drug TNP-470 (Caplostatin), synthesized by Satchi-Fainaro et al., is the first example of anti-angiogenic polymer–drug conjugate [76]. In addition to the conjugate's selective tumor accumulation, it does not cross the blood–brain barrier and does not induce the neurotoxicity associated with the treatment with unconjugated TNP-470. Caplostatin is highly effective on a large variety of cancer types and can be administered over a dose range more than ten-fold that of the original TNP-470 without any toxicity [76-78].

1.2.1.5. Distribution of biological drugs. Despite their high therapeutic potential, the use of biomolecules (e.g. RNAi, proteins and peptides) as therapeutic agents is hindered by their low bioavailability. Crossing biological barriers, such as cell membranes and blood vessel walls, is a

major obstacle in the clinical application of biomolecules. When administered into the circulation, biomolecules are subject to degradation by proteolytic enzymes in the blood and to rapid renal clearance. The delivery of proteins is limited to cell surface and extracellular targets due to the problematic internalization of proteins to the cell, because of their large size and negative charge [79]. A large variety of carriers have been developed for delivery of biomolecules, including viruses, liposomes, nanoparticles and polymeric conjugates. Conjugation of the polymeric carrier to a biomolecule holds several benefits. Conjugation protects the biomolecule from degradation by masking it from nucleases and peptidases [80-83]. Moreover, multivalent polymers can concomitantly attach both a targeting moiety and the biomolecule, allowing its internalization by receptor-mediated endocytosis, a fast and efficient mechanism (discussed below).

Enhanced efficacy, increased safety, reduced immunogenicity and improved delivery can be achieved by conjugation of biomolecules, such as proteins, to polymeric carriers [9,84-87]. As mentioned above, the most common polymer for protein conjugation is PEG, with 8 PEGylated proteins approved for clinical use since the first PEGylated adenosine deaminase (Adagen®, Enzon Pharmaceutical, USA) in 1990 and one PEGylated anti-vascular endothelial growth factor (VEGF) aptamer [88,89]. Certolizumab pegol, marketed as Cimzia® by UCB, is a novel PEGylated anti-TNFα monoclonal antibody for the treatment of RA [90]. It has demonstrated a fast and lasting effect on the signs and symptoms, inhibition of joint damage and improvement in physical function [91,92].

1.2.2. Disease site microenvironment

Following extravasation through the blood vessel walls, polymer–drug conjugates meet with the extracellular milieu of the disease site. The microenvironment in cancer and in various inflammatory diseases, such as RA, has been studied extensively. The major common characteristics are low pH, overexpression of various enzymes and oxidative stress.

The conjugated drug needs to be released at the target site, whether extra- or intracellularly, in order to exert its therapeutic action. Pathophysiological conditions in the extracellular environment of the disease site offer excellent targets that can be exploited for designing drug delivery systems [93,94]. Targeting can be achieved by conjugating the drug to a polymeric backbone through a linker, susceptible to a chemical shift or biological element overexpressed at the pathological site. Thus, the majority of linkers in the polymer therapeutics field are either enzymatically cleavable [95,96] (Tables 1 and 2) or pH-sensitive [97-99]. The choice of linker between the active agent and the polymer backbone has a significant role in achieving a selective release of the drug in the target site. The drug is essentially biologically inert when attached by a linker to the polymer. Only when the drug is released from the polymer backbone, it gains its activity. The linker should be selected based on unique characteristics of the microenvironment, and on the final target of the drug, i.e. where the drug should be released: inside or outside the cell, or in a specific organelle. Naturally, the linker should be stable in the bloodstream until the polymer conjugate reaches its destination [5,100,101]. Careful attention should be given to the choice of suitable linkers. The chosen chemical bond will affect the pH-dependent stability of the pro-drug, as well as the molecular weight of the carrier and the site attachment on the carrier.

A key factor in the microenvironment of cancer and inflammatory diseases is the acidic pH. Numerous studies have shown that the extracellular pH in tumors is consistently acidic and can reach pH values approaching 6.0. As solid tumors develop faster than their blood supply, a hypoxic microenvironment typically forms within the tumor mass, resulting in high glycolysis, the end product of which are metabolic acids [102,103]. In terms of molecular events and cellular behavior, many similarities exist between cancer and inflammation. For instance, leukocytes on their way to a site of inflammation and metastasizing cancer cells use similar proteases, such as matrix metallo-proteases (MMP), to degrade the extracellular matrix in order to be able cross endothelial basement membranes from or into the blood or lymph circulation. At another level, signaling molecules, such as cytokines and chemokines, regulate not only cell proliferation but also the balance between proteases and natural inhibitors in both inflammation and cancer [104]. Poor perfusion in tumors and inflammation sites not only reduces the ability to remove tumor-derived acids, but also leads to regional hypoxia, which can exacerbate fermentative metabolism. We can design our linker as an acid-labile trigger, thus releasing the drug in an environment with a lower pH than the plasma pH, such as the acidic surroundings of a tumor or the even more acidic environment in the endosome and lysosome. Linkers that can be used for pH-triggered release of the drug are N-cis-aconityl, hydrazone and carboxylic–hydrazone bonds, acetal, imine and trityl bonds.

Dexamethasone (Dex), a powerful anti-inflammatory glucocorticoid, was conjugated with an HPMA polymer to form HPMA copolymer–Dex conjugate for the treatment of RA [105]. Besides taking advantage of the EPR effect for accumulation in the inflamed joints, the conjugate was designed using a pH-sensitive hydrazone linker, allowing the release of the drug only at the acidic environment of the inflammation site. The therapeutic effect of HPMA copolymer–Dex conjugate in adjuvant-induced arthritis rat models was highly remarkable and systemic administration of the conjugate clearly showed a superior and long lasting anti-inflammatory effect, together with a profound bone and cartilage protection when compared with free Dex [33,34,105].

The extracellular microenvironment of many disease sites, including cancer, cardiovascular diseases and arthritis, is further characterized by increased expression and activity of various enzymes, the

Table 1
Extracellularly cleaved linkers.

Overexpressed enzyme	Substrate (linker)	Disease	Ref
Cathepsin K	GGPNle	Breast cancer; metastatic bone disease; osteoporosis, Psoriasis, multiple sclerosis, rheumatoid/osteoarthritis,	[51]
MMP (matrix metalloproteases):	CG LDD/GPLGV/PLGMTS/GPLGAG/ CDGR/GPLGVRGC	Inflammation, lung cancer, heart failure after myocardial infarction, chronic obstructive pulmonary diseases, Ocular diseases, diseases of the gut characterized by ulceration (MMP- 1, -2, -3, -7, -9, -13 and -14 are overexpressed in colorectal cancer)	[174,175]
MMP-2	PLGVR/PLGLYL/PLGLYAL/GPLGIAGQ/PVGLIG/GPLGMLSQ/GPLGLWAQ/ GPLGVRGK/HPVGLLAR/GGPLGLWAGG/AAAPLGLWA/GGPLGVRGG	Arthritis, tumor invasion and metastasis, angiogenesis, cerebral ischemia	[174,175]
MMP-9	PLGLYL/PLGLYAL/AALGNVA/PVGLIG/GPLGMLSQ/GPLGLWAQ/GGPLGLWAGG	Tumor invasion and metastasis, angiogenesis, cerebral ischemia	[174]
MMP-7	GVPLSLTMGC/RPLALWRS		[175]
MMP-13	GPLGMRGLGK		[175]

Table 2
Intracellularly cleaved linkers.

Overexpressed enzyme	Substrate (linker)	Disease	Reference
Cathepsins (cysteine proteases)	NEVA/KK		[176]
Cathepsin B	FK/VR/GFLG/FR/6-E-8-D	Cancer	[106,176]
Cathepsin D	GPIC(Et)FFRL/GPICFFRLISKC/GFLGF	Cancer metastasis	[175,177,178]
Cathepsin L	FR (cross-activation with cat. B)	Cancer	[106]
Cathepsin H		Hepatoma metastasis	[179]
Cathepsin S	LR	Rheumatoid arthritis, Psoriasis, Autoimmune diseases, multiple sclerosis, rheumatoid/osteoarthritis, osteoporosis	[175]
Legumain	CBZAAN/NEVA/6-E-8-D	Cancer	[151,176,180]
HDAC: histone deacetylases	K(Ac)	Cancer	[149,181]
HDAC1, 2, 3, 6	K(Ac)	Prostate, gastric, colon, breast and cervical tumors	[10,11]
HDAC8	K(Ac)/K(Ac)-K(Ac)/Aoda-Aoda sequence		[10,11]
Caspases (proteases):	NEVA	Apoptosis imaging	[176]
Caspase 3	NEVA/6-E-8-D/	Hypoxia	[176,182]
	DEVD/		
	DEVDAPK/GDEVDGSGC/DEVDC/SGDEVDSG		[175,183]
Caspase 7	NEVA	Traumatic brain injury	[176,184]
Caspase 9		Hypoxia	[182]
KLK6 Kallikrein-related peptidase 6—encodes a trypsin-like serine protease	GARRRG/WARRS/WARKR/KRKRW/AKRRG/WKKKR	Colon and gastric cancer	[185-187]
PIM (PIM1, PIM2, PIM3) serine/threonine kinases	(K/R)3-XS/TX X-not basic or large hydrophobic residue Consensuses sequence: ARKRRRHPS-GPPTA	Hematologic malignancies and solid cancers	[188]

most prominent types being matrix metallo-proteases (MMP) and cathepsins [106]. MMP, also referred to as matrixins, are a family of approximately 24 human zinc-containing endopeptidases that are together capable of degrading all components of the extracellular matrix (ECM) and many other proteins. Though MMP were discovered as ECM degrading enzymes, an emerging view is that matrix degradation is not the only (or even primary) function of these enzymes, and their activity has been extended to cell growth, signaling, migration, differentiation, and apoptosis [107-109]. Overactivity of MMP-1, -3, -7, -9, and -13 has been implicated in arthritis and represents a potential therapeutic target in this disease [110,111]. Increased or misregulated levels of many MMP are observed in many pathologies associated with inflammation [112,113].

Enzymatically-cleavable linkers are commonly used for selective targeting of polymer–drug conjugates. For example, for extracellular release linkers degradable by an extra-cellular enzyme, such as cathepsin K (described below), are often used. Many other examples of such linkers are summarized in Table 1.

Another important element of the tumor and inflammation microenvironment is the increased oxidative stress, namely over-production of reactive oxygen species (ROS). During oxidative phosphorylation ROS including superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) are produced as by-products [114]. Hydrogen peroxide, that is abundant in the environment of inflammation and cancer [115], can cleave boronic acid or ester linkers, or even the ion Fe(II) used with the substrate trioxolane ring to target malaria [116].

1.2.3. Cell internalization

The usual uptake route of untargeted polymer-bound drugs to most cells is through fluid phase pinocytosis [117]. Internalization kinetics and efficacy are dependent on several factors, such as particle size, shape and surface charge. In a study done on HPMA copolymer, it was found that internalization was more efficient for smaller MW polymers, and there was virtually no internalization of polymer molecules above 400 kDa [118]. A recent study on PEG-based particles showed that rod-shaped nanoparticles are internalized faster than spherical particles [119], suggesting that linear or hyperbranched polymers might internalize faster than other spherical carriers. Actively-targeted drugs mostly undergo internalization through receptor-mediated endocytosis, in which macromolecules bind to complementary receptors on the cell surface and enter the cell as receptor-macromolecule complex in clathrin-coated vesicles. This process increases the efficiency of internalization of particular macromolecules more than 1000-fold compared with ordinary pinocytosis [120,121]. The size of clathrin-coated vesicles depends on the size of its cargo, with an observed upper limit of about 200 nm external diameter, which should be taken into account when designing any polymer therapeutic [122,123].

Following internalization by pinocytosis or by receptor-mediated endocytosis the conjugates are carried to the early endosome (EE). In the early endosome a slightly acidic pH (6.0–6.8) is maintained. The endosome serves as a sorting compartment, from which the molecules can be recycled back to the plasma membrane or routed to the late endosome (LE) and lysosome for degradation [124]. Here, ligands are degraded by the even lower pH (about 5.0) and the highly concentrated lysosomal enzymes. Recycling from lysosomes occurs relatively slowly, which explains why cells are capable of accumulating large amounts of internalized material.

Unconjugated small molecule drugs usually enter cells by passive diffusion through the cell membrane, a rapid process that takes minutes, as opposed to the slow processes of endocytosis and linker cleavage of the conjugates [117,125]. This should be kept in mind when comparing the efficacy of the conjugated drug against the free drug. This is especially true for in vitro experiments, where the lack of selective distribution leaves little advantage for the conjugated drug.

One of the major obstacles in cancer chemotherapeutics is the acquisition of multidrug resistance (MDR) by cancer cells. In this phenomenon, resistance to chemically-unrelated drugs occurs due to active transport of these drugs out of the cell. Overexpression of the ATP-binding cassette (ABC) transporters, mainly P-glycoprotein (Pgp) [126], multidrug resistance-associated proteins (MRP) [127] and breast cancer resistance proteins (BCRP) [128], is one of the primary mechanisms of MDR. Once these transporters bind a substrate in the inner membrane, the substrate is subsequently expelled into the extracellular space. This reduces the intracellular levels of cytotoxic drugs below lethal thresholds, making the drug ineffective. Conjugation of cytotoxic agents with polymers changes the path of drug internalization from diffusion to endocytosis (Fig. 4), thus minimizing drug interaction with MDR transporters, leading to increased intracellular accumulation and enhanced efficacy of the drug in resistant cells [129-132].

A series of studies on HPMA–copolymer doxorubicin (Adriamycin, ADR) conjugate addresses the issue of multi-drug resistance in sensitive and resistant ovarian carcinoma cells (A2780). These studies have shown increased uptake of the conjugated adriamycin into ADR-resistant ovarian carcinoma cells (A2780/AD) compared with the free drug. Furthermore, HPMA copolymer–adriamycin did not induce multidrug resistance in A2780 cells after repeated exposure, as demonstrated by low expression levels of the MDR1 gene. This data indicates that the conjugate is able to overcome the ATP-driven Pgp efflux pump [129-132].

Nucleic acids are not readily taken-up by cells due to their strong negative charge. Conjugation of a biomolecule with a polymeric carrier shields or neutralizes the negative charge and promotes internalization in the target cell either by pinocytosis or by receptor-mediated endocytosis, depending on the carrier. The end results of the use of such carriers, i.e. successful transfections by pDNA and RNAi, suggest that an endosomal escape event from the early endosome takes place, avoiding fusion with the lysosome and ultimately elimination via the Golgi system [80-83,133]. However, comprehensive monitoring of the endosomal escape has not been done. The leading hypothesis today, suggested by J.P. Behr in 1995 [134], is that endosomal escape occurs by the "proton sponge" effect [133-136]. The transition from the early endosome to the late endosome and finally fusion with the lysosome for degradation are accompanied with rapid drop in pH from neutral to approximately pH 6 in the early endosome and pH 5 in the lysosome. According to the "proton sponge" effect theory, different groups at the polymer backbone, such as amine groups, have the ability to protonate under acidic pH, reducing the free proton concentration, which disrupts the drop in pH. Reduction in the free proton concentration leads to proton and chloride influx, osmotic swelling and eventually rupturing of the endosome and release of polymer and nucleic acids to the cytoplasm [135,137] (Fig. 5).

When administering synthetic small interfering RNA (siRNA) to the cell, in addition to the therapeutic effect, silencing of unwanted genes can also occur [138]. This off-target effect might be caused by hybridization of the siRNA with non-specific mRNA strands. siRNA is further recognized by several immune-related cytoplasmic proteins, such as transmembrane toll-like receptors (TLRs), that ultimately stimulate the inflammatory response [139-141]. However, off-target effects can be restrained by different modifications of the siRNA and the conjugate [139,142-144].

The specific characteristics of both intracellular and extracellular environments can be used for targeting of drug delivery systems. Acid-labile linkers described above are usually intended for release of the conjugated drug in the lysosome. It should be kept in mind though, that pH-cleavable linkers can also be degraded, at least partially, in the low pH of the disease site microenvironment.

The intracellular environment of cancer cells is characterized by overexpression of various enzymes, such as cathepsins, histone deacetylases and legumain.

Cathepsins are classified based on their structure and catalytic type into serine (cathepsins A and G), aspartic (cathepsins D and E), and cysteine cathepsins. Cysteine cathepsins constitute the largest cathepsin family, with 11 proteases in humans referred to as cathepsins B, C (also known as cathepsin J and dipeptidyl-peptidase 1), F, H, K (also known as cathepsin O2), L, O, S, W, V (also known as cathepsin

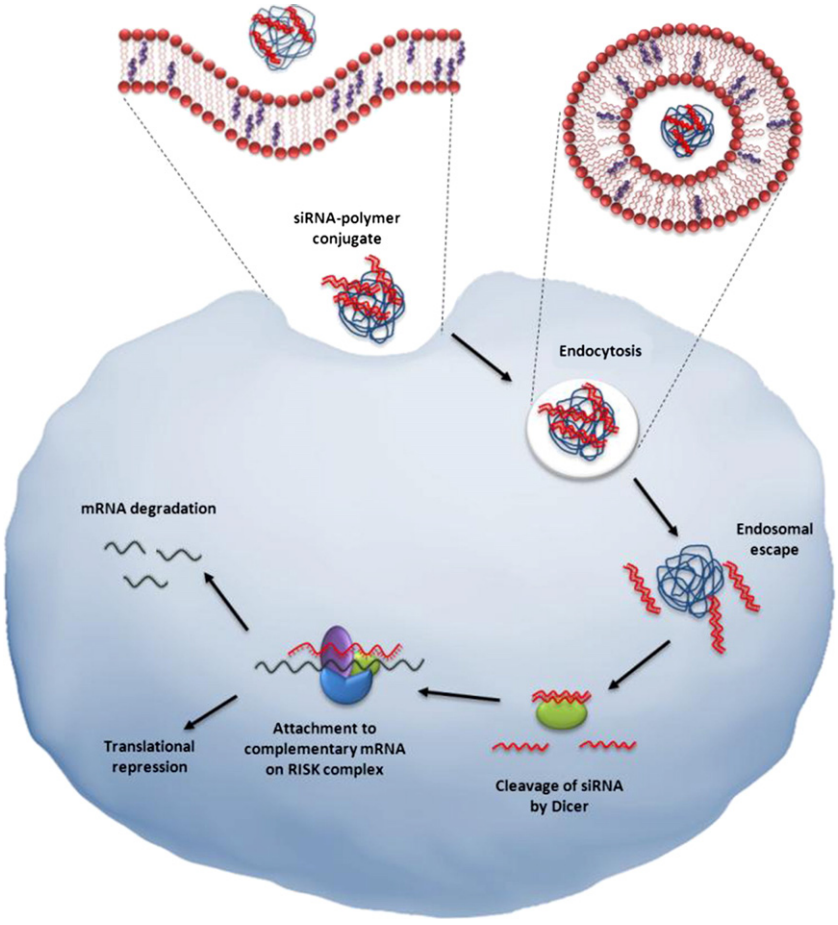

Fig. 5. RNA interference by siRNA–polymer conjugate. The siRNA–polymer conjugate enters the cell by endocytosis. The siRNA and polymer molecules are released into the cytoplasm, presumably after endosome rupturing by the proton "sponge effect". The double stranded siRNA molecules are cleaved into single strands by the RNAse III enzyme Dicer. The sequence specific binding to the corresponding target mRNA, which is mediated by the RNA-induced silencing complex (RISC), eventually leads to cleavage of mRNA and translational repression.

L2), and Z (also known as cathepsin X and cathepsin P) [145]. Most cathepsins become activated at the low pH found in lysosomes. Thus, the activity of this family lies almost entirely within those organelles, although there are exceptions. For example, cathepsin K works extracellularly after secretion by osteoclasts in bone resorption. In general, the cysteine cathepsins are stable in acidic cellular compartments, i.e., in lysosomes and endosomes, and capable of efficiently cleaving a wide variety of substrates. The roles of cathepsins in many physiologic and disease processes have been covered by recent comprehensive reviews [146-148]. Histone deacetylases (HDACs) and histone acetyltransferases (HATs), located in the nucleus, are two kinds of enzymes, which can, by reversible deacetylation and acetylation, modify the structure and function of chromatin histones that are involved in the regulation of gene expression, as well as many non-histone proteins that regulate cell function in eukaryotes. HDACs have attracted more and more attention over the past few years due to their relationship to cancer and several other diseases. Many HDAC inhibitors (HDACi) have even entered pre-clinical or clinical research as anti-cancer agents and have shown satisfying effects. HDACs, including 18 members at least, are subdivided into 4 classes that generally have high structure similarity and related substrate specificity (Lys(Ac)) but have divergent sequence and different functions even within classes [149]. An additional protease which expression has been reported to be pathology-related is legumain, a member of the C13 family of cysteine proteases. Legumain is overexpressed in the majority of human solid tumors; it promotes cell migration and is associated with enhanced tissue invasion and metastases. Due to its unique functional properties and high level of expression in many human tumors, it represents an attractive candidate for prodrug activation [150,151].

Enzymatically-cleavable linkers are often employed for intracellular drug release. In the anticancer conjugate PK1 mentioned above, doxorubicin was conjugated to HPMA copolymer via the peptidyl linker Gly-Phe-Leu-Gly. The free doxorubicin has many dose limiting toxicities, mainly severe cardiac side effects and also nausea, vomiting, mucositis and neutropenia. The peptidyl linker allowed controlled release of the drug in tumors by cleavage by cathepsin B. The dose-limiting toxicities in phase I clinical study were neutropenia and mucositis, however nausea and vomiting were not a problem [31]. In phase II clinical studies for the treatment of breast, lung and colorectal cancer drug-related toxicities were generally tolerable, and similar for all three disease groups. Notably, no sign of cardiotoxicity was reported [152].

Examples of other linkers cleavable by intracellular enzymes are summarized in Table 2.

In this context, there are several parameters which need to be considered regarding the design of polymer-bound drugs. The efficacy of the designed polymer–drug conjugates correlates with the expression of those target enzymes in the tumor. Detailed knowledge of the expression of tumor-related proteases in individual tumor entities would certainly be helpful for the future development of cleavable polymer therapeutics [153], and possibly support the development of personalized medicine. One example is OPAXIO™ (paclitaxel poliglumex, CT-2103) which was designed to deliver paclitaxel preferentially to the tumor tissue. Once inside the tumor, the conjugated chemotherapeutic agent is activated and released by lysosomal proteases, particularly cathepsin B. It was found that estrogen modulates cathepsin B levels in normal tissues and also in estrogen receptor positive non-small cell lung cancer (NSCLC) cell lines [154]. Preclinical and clinical studies support that OPAXIO™ metabolism by lung cancer cells may be influenced by estrogen, which could lead to enhanced release of paclitaxel and efficacy in women with lung cancer compared to standard therapies [154,155]. However, since the enzymes that are used for pro-drug activation are also present in normal cells, activation of an enzymatically cleavable pro-drug can occur in healthy tissue too. Thus, there is a growing demand to improve tumor uptake through active or passive targeting and to study the overexpression and activity of those enzymes in the individual tumor clinically [25].

1.3. Metabolism and degradation

The polymer used for drug delivery should be eliminated from the body, either by excretion of non-degradable polymers, or by degradation of the biodegradable polymers.

1.3.1. Biodegradable polymers

Biodegradable polymers are polymers that can undergo cleavage of bonds in the polymeric backbone, either hydrolytically or enzymatically. Natural polymers such as collagen and hyaluronic acid undergo biodegradation with the degradation products, such as amino acids and saccharides, absorbed in the biochemical pathways of the body. Some synthetic polymers can undergo similar degradation. For example, Poly(L-glutamic acid) and poly(aspartic acid) are highly susceptible to degradation by lysosomal enzymes, producing monomeric amino acids as degradation products. The polysaccharide chitosan is mainly degraded by lysozyme through the hydrolysis of the acetylated residues [156]. Dextran is degraded by different dextranases, α-1-glucosidases, present in various organs, including liver, spleen, kidney, and the lower part of the GI tract [157].

1.3.2. Semi-degradable polymer backbones

A new emerging approach binds non-degradable polymer blocks with degradable linkers. Thus, high molecular weight copolymers, with prolonged circulation and increased tumor-to-organ accumulation ratios due to the EPR effect, are obtained. At the target site, the linkers are cleaved, releasing the active agent and at the same time causing the degradation of the backbone into small blocks which are excreted by the kidney. Recently, several reports on linear polymers were published, in which the monomer units were linked by acid-labile linkers, such as ketal, acetal, and cis-aconityl bonds. By incorporating pH-sensitive bonds into the backbone of the polymeric carrier, degradation of the polymer under the acidic conditions in the intracellular environment is achieved. Heffernan et al. synthesized a novel acid-labile poly-(1,4-phenyleneacetone dimethylene ketal) (PPADK), through acetal exchange reaction. Those polyketal nanoparticles bear ketal linkages in their backbone and degrade via acid-catalyzed hydrolysis into low Mw compounds that can be easily excreted [158]. This strategy still suffers from several disadvantages, such as low MW and absence of functional groups for attaching drugs, and thus requires further optimization [101].

Another example was developed by the Kopecek group, which converted the non-degradable HPMA copolymer to an enzymatically degradable one, which is still stable in circulation [159-161]. Pan et al. designed a new bifunctional RAFT chain transfer agent (CTA), which was added to two copies of lysosomally degradable Gly-Phe-Leu-Gly sequences, linked by Lys (i.e., peptide2CTA). Then, HPMA monomers were incorporated at both ends of the peptide2CTA with identical efficiency by RAFT polymerization, followed by thiol-ene chain extension. By doing so, they successfully linked 10–40 kDa linear polymer segments to enzymatically degradable oligopeptide sequences, which degradation products have MW distributions below the renal threshold. Both polyHPMA and HPMA-DOX were synthesized using this novel approach.

A novel type of partially degradable amphiphilic block copolymer of HPMA copolymer with poly(L-lactide) (PLLA) namely, P(HPMA)-b-P(LLA), was recently synthesized and characterized by Zentel and coworkers [162]. Their aim was to convert a known non-degradable copolymer structure into a degradable one, which generates segments of size smaller than the renal excretion cut-off. They combined two different mechanisms of controlled polymerization; ring-opening polymerization (ROP) and subsequent RAFT polymerization. Till now, only fluorescent markers have been attached to this novel copolymer,

but it represents a platform for the attachment of different drugs and/or targeting moieties.

1.4. Elimination of non-degradable polymers

Elimination of non-biodegradable polymers, such as PEG or HPMA copolymer, is hampered by their high molecular weight. Large molecules, such as the polymers discussed here, remain in the tissue after cellular death or undergo exocytosis. Then, they return to the bloodstream via the lymphatic circulation (which is usually impaired at the tumor site, making elimination slow [30]) and are eliminated by glomerular filtration in the kidney, provided they are below the glomerular threshold [163]. The molecular weight, the size and the shape of the polymer have major influence on its excretion and rate of glomerular filtration [164-166]. For example, it was demonstrated that star-shaped HPMA copolymer-bound doxorubicin conjugates were eliminated slower than classical hyperbranched conjugates [166]. In general, the rate of renal elimination is inversely correlated with the MW of the polymers [10,163,167]. The molecular weight thresholds for HPMA copolymer and for alginate were found to be about 45 kDa [163,168,169] and for PEG about 30 kDa [167] (however, the issue of PEG elimination is more complex, as discussed below).

Although many PEGylated compounds are currently marketed, concerns about the fate of PEG and potential toxic effects have been recently expressed [170]. The toxicity associated with PEGylated proteins is due to the pharmacology of the protein conjugated with PEG rather than due to PEG itself. However, this does not mean that PEG has no biological toxicity. Perhaps PEGylated biological products mask PEG's toxicity in clinical setting on one hand, but on the other hand, marketed PEGylated compounds are administered at low clinical doses so that PEG toxicity is unlikely to occur.

The available data from animal studies show that PEG exhibits toxicities after systemic administration at high doses [171]. The usual target organ is the kidney, as renal excretion is the predominant route of clearance for PEG [171]. In some preclinical studies it has been reported on organ specific vacuolation occurring in animal models. The formation of these PEG-containing intracellular vesicles was linked to the clearance mechanism of PEGylated proteins. TNF binding protein PEGylated with 20 kDa PEG was shown to cause kidney lesions characterized by the presence of single or multiple cytoplasmic vacuoles in cortical tubular epithelial cells (i.e. vacuolation) at low-chronic-parenteral administration and after higher single dose in rats. Correlation has been seen between dose and dosing regimen of PEG–protein conjugates and the size of vacuoles formed, moreover, morphological alternation occurred in affected cells. Vacuolation may be a result of fluid distension of lysosomes due to the hygroscopic nature of PEG. This vacuolation did not occur with >70 kDa PEG–protein conjugates and was more severe for lower molecular weight PEG [172]. A probable cause for this phenomenon is the uptake of PEG of molecular weight below the glomerular filtration threshold by the kidney tubular cells. Although vacuolation showed morphological changes in clearance-associated cells following administration of PEGylated protein, no evidence for functional disturbance was detected.

Although it seems that PEG itself does not cause adverse clinical effects [170], the progressive accumulation of the polymer upon repeated administration, and the possible associated morphological changes are certainly a cause for concern. Accumulation is a potential problem for all non-biodegradable polymers. Even when the average MW of the administered polymer is below the glomerular threshold, a certain amount of material will still be of higher molecular weight, since the polymers have a certain measure of polydispersity. Barz et al. presented detailed calculations illustrating this point [173]. Considering this, it is preferable that drug/protein-carrying polymers were biodegradable. Otherwise, an early thorough characterization of the nature and extent of their elimination routes should be carried out.

2. Summary

When a polymer conjugate is being designed, following the ADME route of the desired active entity can be a useful tool for successful rational design. The field of polymer therapeutics is constantly growing, and when attempting to assemble a new conjugate, the possibilities seem endless. The choice of drug should be the first step, mainly since it holds the therapeutic effect on the target cells, but also because knowing the drug limitations, whether it is a chemical drug suffering from low solubility, high toxicity and severe side effects, or a biological drug suffering from short half-life in the circulation, is crucial for further decisions regarding the polymeric backbone, the size and shape of the final conjugate and the required linker.

The administration route of the drug should be deduced both from the nature of the drug and the treated disease, but should also comply with the patient's use. Combination of the chosen drug and administration route should lead to the selection of the polymeric backbone.

Many polymers have been synthesized and examined for various applications. Some, such as PEG or dextran, have been approved for use in humans by the FDA, and others, such as HPMA and PGA, are in advanced clinical trials. The polymer MW, size and shape influence the distribution of the drug in the body, its accumulation in the target site and the drug release and hence should be taken into consideration.

The biodistribution of the conjugate and its accumulation in the target site should be used for determining the linker between the drug and the polymer. When the conjugate is designed for solid tumors, the EPR effect will be used for passive targeting. The same holds for other inflammation-related disease, where the hyperpermeability of the blood vessels can be exploited for extravasation of large conjugates to the tissue, while preventing its diffusion to other healthy organs. Addition of an active targeting moiety, such as a specific ligand or antibody, will help directing the conjugate to the relevant site and enhance its internalization into the target cells.

A well-designed linker between the drug and the polymer, will be cleaved selectively in the target site, and allow the release of the drug only within the target area. The linker can be designed to release the drug either near or inside the target cells and it can be cleaved by low pH, overexpressed enzymes or other conditions of the disease site microenvironment.

The metabolism of the polymer and its elimination from the body, are two very important features of a conjugate design, yet they are often overlooked. A successful conjugate will not only deliver the drug to its target and release it there, but it will also be eliminated from the body, preventing an iatrogenic illness, emerging from the accumulation of the polymer in the tissue or in the clearance organs of the body, the liver, kidneys or spleen. The use of a biodegradable polymer is preferable, but not always feasible, for it may not suit the other important factors of the conjugate, such as the required size or stability. A semi-degradable copolymer represents an excellent alternative, as it allows the use of a large polymer that can be degraded to smaller blocks that can be easily eliminated from the body.

Although the ADME concept is traditionally used to describe the route of a drug in the body, using this concept for rational design of a polymer therapeutic can offer many advantages. Following every step of the concept as early as in the design stage, allows monitoring of many of the biological factors and delivery system parameters that influence the successful creation of a new effective polymer therapeutic.

Acknowledgments

The Satchi-Fainaro laboratory is supported by Grant No. 5145-300000 from the Chief Scientist Office of the Ministry of Health, Israel, by the Israel Science Foundation (Grant No. 1309/10), a grant from

the Alon Foundation, the Israel Cancer Association, Grant No. 2007347 given by the United States–Israel Binational Science Foundation (BSF), the German–Israel Foundation (GIF), the Israel Cancer Research Fund (ICRF), Association for International Cancer Research (AICR), TAU Center for Nanoscience and Nanotechnology and the Swiss Bridge.

References

[1] H. Jatzkewits, Peptamin (glycyl-L-leucyl-mescaline) bound to blood plasma expander (polyvinylpyrrolidone) as a new depot form of a biologically active primary amine (mescaline), Z. NatuNaturforsch. 10b (1955) 27–31.
[2] J. Kopecek, P. Kopeckova, Design of polymer–drug conjugates, in: F. Kratz, P. Senter, H. Steinhagen (Eds.), Drug Delivery in Oncology, WILEY-VCH, 2011, pp. 485–512.
[3] N.I. Givental, S.N. Ushakov, E.F. Panarin, G.O. Popova, Experimental studies on penicillin polymer derivatives, Antibiotiki 10 (1965) 701–706.
[4] K.I. Shumikhina, E.F. Panarin, S.N. Ushakov, Experimental study of polymer salts of penicillins, Antibiotiki 11 (1966) 767–770.
[5] H. Ringsdorf, Structure and properties of pharmacologically active polymers, J. Polym. Sci. (1975) 135–153.
[6] A. Godwin, K. Bolina, M. Clochard, E. Dinand, S. Rankin, S. Simic, S. Brocchini, New strategies for polymer development in pharmaceutical science—a short review, J. Pharm. Pharmacol. 53 (2001) 1175–1184.
[7] M. Kovar, L. Kovar, V. Subr, T. Etrych, K. Ulbrich, T. Mrkvan, J. Loucka, B. Rihova, HPMA copolymers containing doxorubicin bound by a proteolytically or hydrolytically cleavable bond: comparison of biological properties in vitro, J. Control. Release 99 (2004) 301–314.
[8] R. Satchi-Fainaro, R. Duncan, C.M. Barnes, Polymer therapeutics for cancer: current status and future challenges, in: R. Satchi-Fainaro, R. Duncan (Eds.), Polymer Therapeutics II Polymers as Drugs, Conjugates and Gene delivery systems, Springer-Verlag, Heidelberg, Germany, 2006, pp. 1–65.
[9] R. Duncan, The dawning era of polymer therapeutics, Nat. Rev. Drug Discov. 2 (2003) 347–360.
[10] T. Yamaoka, Y. Tabata, Y. Ikada, Fate of water-soluble polymers administered via different routes, J. Pharm. Sci. 84 (1995) 349–354.
[11] E. Lee, J. Lee, S. Jon, A novel approach to oral delivery of insulin by conjugating with low molecular weight chitosan, Bioconjug Chem 21 (2010) 1720–1723.
[12] E. Lee, H. Kim, I.H. Lee, S. Jon, In vivo antitumor effects of chitosan-conjugated docetaxel after oral administration, J. Control. Release 140 (2009) 79–85.
[13] E. Lee, J. Lee, I.-H. Lee, M. Yu, H. Kim, S.Y. Chae, S. Jon, Conjugated chitosan as a novel platform for oral delivery of paclitaxel, J. Med. Chem. 51 (2008) 6442–6449.
[14] L. Diego, R. Atayee, P. Helmons, G. Hsiao, C.F. von Gunten, Novel opioid antagonists for opioid-induced bowel dysfunction, Expert Opin. Investig. Drugs 20 (2011) 1047–1056.
[15] Nektar corporation website, http://www.nektar.com/product_pipeline/cns_pain_oral_nktr-118and119.html2011.
[16] R. Wiwattanapatapee, B. Carreno-Gomez, N. Malik, R. Duncan, Anionic PAMAM dendrimers rapidly cross adult rat intestine in vitro: a potential oral delivery system? Pharm. Res. 17 (2000) 991–998.
[17] B. Slutter, S.M. Bal, I. Que, E. Kaijzel, C. Lowik, J. Bouwstra, W. Jiskoot, Antigen–adjuvant nanoconjugates for nasal vaccination: an improvement over the use of nanoparticles? Mol. Pharm. 7 (2010) 2207–2215.
[18] G. Gaucher, P. Satturwar, M.C. Jones, A. Furtos, J.C. Leroux, Polymeric micelles for oral drug delivery, Eur. J. Pharm. Biopharm. 76 (2010) 147–158.
[19] K. Nagpal, S.K. Singh, D.N. Mishra, Chitosan nanoparticles: a promising system in novel drug delivery, Chem. Pharm. Bull. (Tokyo) 58 (2010) 1423–1430.
[20] C.M. Lopes, P. Martins-Lopes, E.B. Souto, Nanoparticulate carriers (NPC) for oral pharmaceutics and nutraceutics, Pharmazie 65 (2010) 75–82.
[21] K. Phua, K.W. Leong, Microscale oral delivery devices incorporating nanoparticles, Nanomedicine (Lond.) 5 (2010) 161–163.
[22] T.W. Wong, Design of oral insulin delivery systems, J. Drug Target. 18 (2010) 79–92.
[23] E. Segal, R. Satchi-Fainaro, Design and development of polymer conjugates as anti-angiogenic agents, Adv. Drug Deliv. Rev. 61 (2009) 1159–1176.
[24] R. Gaspar, R. Duncan, Polymeric carriers: preclinical safety and the regulatory implications for design and development of polymer therapeutics, Adv. Drug Deliv. Rev. 61 (2009) 1220–1231.
[25] H. Baabur-Cohen, L. Omer, R. Satchi-Fainaro, Recent progress in polymer therapeutics as nanomedicines, in: D. Peer (Ed.), Handbook of Harnessing biomaterials in Nanomedicine: Preparation, toxicity and applications, Pan Stanford Publishing Pte. Ltd., Hackensack, NJ, USA, 2011, pp. 77–122.
[26] A. Eldar-Boock, D. Polyak, R. Satchi-Fainaro, Ligand-assisted vascular targeting of polymer therapeutics, in: F. Kratz, P. Senter (Eds.), Drug Delivery in Oncology—From Basic Research to Cancer Therapy, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, 2011.
[27] Y. Geng, P. Dalhaimer, S.S. Cai, R. Tsai, M. Tewari, T. Minko, D.E. Discher, Shape effects of filaments versus spherical particles in flow and drug delivery, Nat. Nanotechnol. 2 (2007) 249–255.
[28] R.A. Petros, J.M. DeSimone, Strategies in the design of nanoparticles for therapeutic applications, Nat. Rev. Drug Discov. 9 (2010) 615–627.
[29] J. Fang, H. Nakamura, H. Maeda, The EPR effect: unique features of tumor blood vessels for drug delivery, factors involved, and limitations and augmentation of the effect, Adv. Drug Deliv. Rev. 63 (2011) 136–151.
[30] Y. Matsumura, H. Maeda, A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs, Cancer Res. 46 (1986) 6387–6392.
[31] P.A. Vasey, S.B. Kaye, R. Morrison, C. Twelves, P. Wilson, R. Duncan, A.H. Thomson, L.S. Murray, T.E. Hilditch, T. Murray, S. Burtles, D. Fraier, E. Frigerio, J. Cassidy, Phase I clinical and pharmacokinetic study of PK1 [N-(2-hydroxypropyl)methacrylamide copolymer doxorubicin]: first member of a new class of chemotherapeutic agents–drug–polymer conjugates. Cancer Research Campaign Phase I/II Committee, Clin. Cancer Res. 5 (1999) 83–94.
[32] J. Folkman, Angiogenesis: an organizing principle for drug discovery? Nat. Rev. Drug Discov. 6 (2007) 273–286.
[33] X.M. Liu, S.C. Miller, D. Wang, Beyond oncology—application of HPMA copolymers in non-cancerous diseases, Adv. Drug Deliv. Rev. 62 (2010) 258–271.
[34] J. Sanchis, F. Canal, R. Lucas, M.J. Vicent, Polymer–drug conjugates for novel molecular targets, Nanomedicine (Lond.) 5 (2010) 915–935.
[35] F. Alexis, E. Pridgen, L.K. Molnar, O.C. Farokhzad, Factors affecting the clearance and biodistribution of polymeric nanoparticles, Mol. Pharm. 5 (2008) 505–515.
[36] T. Schluep, J. Cheng, K.T. Khin, M.E. Davis, Pharmacokinetics and biodistribution of the camptothecin–polymer conjugate IT-101 in rats and tumor-bearing mice, Cancer Chemother. Pharmacol. 57 (2006) 654–662.
[37] S.D. Perrault, C. Walkey, T. Jennings, H.C. Fischer, W.C.W. Chan, Mediating tumor targeting efficiency of nanoparticles through design, Nano Lett. 9 (2009) 1909–1915.
[38] M. Saad, O.B. Garbuzenko, E. Ber, P. Chandna, J.J. Khandare, V.P. Pozharov, T. Minko, Receptor targeted polymers, dendrimers, liposomes: which nanocarrier is the most efficient for tumor-specific treatment and imaging? J. Control. Release 130 (2008) 107–114.
[39] M. Barz, F. Canal, K. Koynov, R. Zentel, M.J. Vicent, Synthesis and in vitro evaluation of defined HPMA folate conjugates: influence of aggregation on folate receptor (FR) mediated cellular uptake, Biomacromolecules 11 (2010) 2274–2282.
[40] E. Simone, B.S. Ding, V. Muzykantov, Targeted delivery of therapeutics to endothelium, Cell Tissue Res. 335 (2009) 283–300.
[41] C. Fischbach, D.J. Mooney, Polymers for pro- and anti-angiogenic therapy, Biomaterials 28 (2007) 2069–2076.
[42] H. Kobayashi, K.C. Boelte, P.C. Lin, Endothelial cell adhesion molecules and cancer progression, Curr. Med. Chem. 14 (2007) 377–386.
[43] M. Jensen, F. Berthold, Targeting the neural cell adhesion molecule in cancer, Cancer Lett. 258 (2007) 9–21.
[44] Z.M. Qian, H. Li, H. Sun, K. Ho, Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway, Pharmacol. Rev. 54 (2002) 561–587.
[45] A. Gabizon, H. Shmeeda, H. Baabur, R. Satchi-Fainaro, Targeting the folate receptor with liposomes and polymer therapeutics, in: C. Leamon, A. Jackman (Eds.), Targeted Drug Strategies for Cancer and Inflammation, Springer-Verlag, Heidelberg, Germany, 2011, pp. 217–247.
[46] R. Pasqualini, E. Koivunen, R. Kain, J. Lahdenranta, M. Sakamoto, A. Stryhn, R.A. Ashmun, L.H. Shapiro, W. Arap, E. Ruoslahti, Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis, Cancer Res. 60 (2000) 722–727.
[47] M.D. Pierschbacher, E. Ruoslahti, Variants of the cell recognition site of fibronectin that retain attachment-promoting activity, Proc. Natl. Acad. Sci. U. S. A. 81 (1984) 5985–5988.
[48] L.W. Seymour, D.R. Ferry, D. Anderson, S. Hesslewood, P.J. Julyan, R. Poyner, J. Doran, A.M. Young, S. Burtles, D.J. Kerr, Hepatic drug targeting: phase I evaluation of polymer-bound doxorubicin, J. Clin. Oncol. 20 (2002) 1668–1676.
[49] K. Ulbrich, T. Etrych, P. Chytil, M. Jelinkova, B. Rihova, Antibody-targeted polymer–doxorubicin conjugates with pH-controlled activation, J. Drug Target. 12 (2004) 477–489.
[50] K. Miller, R. Erez, E. Segal, D. Shabat, R. Satchi-Fainaro, Targeting bone metastases with a bispecific anticancer and antiangiogenic polymer–alendronate–taxane conjugate, Angew. Chem. Int. Ed. Engl. 48 (2009) 2949–2954.
[51] E. Segal, H. Pan, P. Ofek, T. Udagawa, P. Kopeckova, J. Kopecek, R. Satchi-Fainaro, Targeting angiogenesis-dependent calcified neoplasms using combined polymer therapeutics, PLoS One 4 (2009) e5233.
[52] D.E. Owens III, N.A. Peppas, Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles, Int. J. Pharm. 307 (2006) 93–102.
[53] W. Jiskoot, R.M. van Schie, M.G. Carstens, H. Schellekens, Immunological risk of injectable drug delivery systems, Pharm. Res. 26 (2009) 1303–1314.
[54] A.W. Richter, E. Akerblom, Polyethylene-glycol reactive antibodies in man—titer distribution in allergic patients treated with monomethoxy polyethylene-glycol modified allergens or placebo, and in healthy blood-donors, Int. Arch. Allergy Immunol. 74 (1984) 36–39.
[55] J.K. Armstrong, G. Hempel, S. Koling, L.S. Chan, T. Fisher, H.J. Meiselman, G. Garratty, Antibody against poly(ethylene glycol) adversely affects PEG-asparaginase therapy in acute lymphoblastic leukemia patients, Cancer-Am. Cancer Soc. 110 (2007) 103–111.
[56] N.J. Ganson, S.J. Kelly, E. Scarlett, J.S. Sundy, M.S. Hershfield, Control of hyperuricemia in subjects with refractory gout, and induction of antibody against poly(-ethylene glycol) (PEG), in a phase I trial of subcutaneous PEGylated urate oxidase, Arthritis Res. Ther. 8 (2006).
[57] A.A. van der Eijk, J.M. Vrolijk, B.L. Haagmans, Antibodies neutralizing peginterferon alfa during retreatment of hepatitis C, N. Engl. J. Med. 354 (2006) 1323–1324.
[58] M. Pidaparti, B. Bostrom, Comparison of allergic reactions to pegasparaginase given intravenously versus intramuscularly, Pediatr. Blood Cancer (2011), doi: 10.1002/pbc.23380.

[59] J. Kopecek, P. Kopeckova, T. Minko, Z.R. Lu, HPMA copolymer–anticancer drug conjugates: design, activity, and mechanism of action, Eur. J. Pharm. Biopharm. 50 (2000) 61–81.
[60] L. Kanerva, R. Jolanki, T. Estlander, 10 years of patch testing with the (meth)acrylate series, Contact Dermatitis 37 (1997) 255–258.
[61] Z. Panagi, A. Beletsi, G. Evangelatos, E. Livaniou, D.S. Ithakissios, K. Avgoustakis, Effect of dose on the biodistribution and pharmacokinetics of PLGA and PLGA–mPEG nanoparticles, Int. J. Pharm. 221 (2001) 143–152.
[62] G. Pasut, F.M. Veronese, PEG conjugates in clinical development or use as anticancer agents: an overview, Adv. Drug Deliv. Rev. 61 (2009) 1177–1188.
[63] R. Duncan, M.J. Vicent, Do HPMA copolymer conjugates have a future as clinically useful nanomedicines? A critical overview of current status and future opportunities, Adv. Drug Deliv. Rev. 62 (2010) 272–282.
[64] Y. Takakura, T. Fujita, M. Hashida, H. Sezaki, Disposition characteristics of macromolecules in tumor-bearing mice, Pharm. Res. 7 (1990) 339–346.
[65] A. Gabizon, F. Martin, Polyethylene glycol-coated (pegylated) liposomal doxorubicin. Rationale for use in solid tumours, Drugs 54 (1997) 15–21.
[66] P. Caliceti, O. Schiavon, F.M. Veronese, Biopharmaceutical properties of uricase conjugated to neutral and amphiphilic polymers, Bioconjug Chem 10 (1999) 638–646.
[67] T. Lammers, R. Kuhnlein, M. Kissel, V. Subr, T. Etrych, R. Pola, M. Pechar, K. Ulbrich, G. Storm, P. Huber, P. Peschke, Effect of physicochemical modification on the biodistribution and tumor accumulation of HPMA copolymers, J. Control. Release 110 (2005) 103–118.
[68] F.M. Veronese, O. Schiavon, G. Pasut, R. Mendichi, L. Andersson, A. Tsirk, J. Ford, G. Wu, S. Kneller, J. Davies, R. Duncan, PEG–doxorubicin conjugates: influence of polymer structure on drug release, in vitro cytotoxicity, biodistribution, and antitumor activity, Bioconjug Chem 16 (2005) 775–784.
[69] S. Danhauser-Riedl, E. Hausmann, H.D. Schick, R. Bender, H. Dietzfelbinger, J. Rastetter, A.R. Hanauske, Phase I clinical and pharmacokinetic trial of dextran conjugated doxorubicin (AD-70, DOX-OXD), Invest. New Drugs 11 (1993) 187–195.
[70] S.D. Chipman, F.B. Oldham, G. Pezzoni, J.W. Singer, Biological and clinical characterization of paclitaxel poliglumex (PPX, CT-2103), a macromolecular polymer–drug conjugate, Int. J. Nanomedicine 1 (2006) 375–383.
[71] A.V. Yurkovetskiy, R.J. Fram, XMT-1001, a novel polymeric camptothecin prodrug in clinical development for patients with advanced cancer, Adv. Drug Deliv. Rev. 61 (2009) 1193–1202.
[72] C.D. Conover, R.B. Greenwald, A. Pendri, C.W. Gilbert, K.L. Shum, Camptothecin delivery systems: enhanced efficacy and tumor accumulation of camptothecin following its conjugation to polyethylene glycol via a glycine linker, Cancer Chemother. Pharmacol. 42 (1998) 407–414.
[73] B. Rihova, Immunomodulating activities of soluble synthetic polymer-bound drugs, Adv. Drug Deliv. Rev. 54 (2002) 653–674.
[74] M.E. Eichhorn, S. Strieth, M. Dellian, Anti-vascular tumor therapy: recent advances, pitfalls and clinical perspectives, Drug Resist. Updat. 7 (2004) 125–138.
[75] R. Haag, F. Kratz, Polymer therapeutics: concepts and applications, Angew. Chem. Int. Ed. Engl. 45 (2006) 1198–1215.
[76] R. Satchi-Fainaro, M. Puder, J.W. Davies, H.T. Tran, D.A. Sampson, A.K. Greene, G. Corfas, J. Folkman, Targeting angiogenesis with a conjugate of HPMA copolymer and TNP-470, Nat. Med. 10 (2004) 255–261.
[77] L. Chesler, D.D. Goldenberg, I.T. Seales, R. Satchi-Fainaro, M. Grimmer, R. Collins, C. Struett, K.N. Nguyen, G. Kim, T. Tihan, Y. Bao, R.A. Brekken, G. Bergers, J. Folkman, W.A. Weiss, Malignant progression and blockade of angiogenesis in a murine transgenic model of neuroblastoma, Cancer Res. 67 (2007) 9435–9442.
[78] R. Satchi-Fainaro, R. Mamluk, L. Wang, S.M. Short, J.A. Nagy, D. Feng, A.M. Dvorak, H.F. Dvorak, M. Puder, D. Mukhopadhyay, J. Folkman, Inhibition of vessel permeability by TNP-470 and its polymer conjugate, caplostatin, Cancer Cell 7 (2005) 251–261.
[79] A.L. Marschall, A. Frenzel, T. Schirrmann, M. Schungel, S. Dubel, Targeting antibodies to the cytoplasm, MAbs 3 (2011) 3–16.
[80] I. Brasnjevic, H.W. Steinbusch, C. Schmitz, P. Martinez-Martinez, Delivery of peptide and protein drugs over the blood–brain barrier, Prog. Neurobiol. 87 (2009) 212–251.
[81] S.A. Cryan, Carrier-based strategies for targeting protein and peptide drugs to the lungs, AAPS J. 7 (2005) E20–E41.
[82] A. des Rieux, V. Fievez, M. Garinot, Y.J. Schneider, V. Preat, Nanoparticles as potential oral delivery systems of proteins and vaccines: a mechanistic approach, J. Control. Release 116 (2006) 1–27.
[83] Y. Dorsett, T. Tuschl, siRNAs: applications in functional genomics and potential as therapeutics, Nat. Rev. Drug Discov. 3 (2004) 318–329.
[84] P. Glue, J.W. Fang, R. Rouzier-Panis, C. Raffanel, R. Sabo, S.K. Gupta, M. Salfi, S. Jacobs, Pegylated interferon-alpha2b: pharmacokinetics, pharmacodynamics, safety, and preliminary efficacy data. Hepatitis C Intervention Therapy Group, Clin. Pharmacol. Ther. 68 (2000) 556–567.
[85] J.P. Magnusson, S. Bersani, S. Salmaso, C. Alexander, P. Caliceti, In situ growth of side-chain PEG polymers from functionalized human growth hormone—a new technique for preparation of enhanced protein–polymer conjugates, Bioconjug. Chem. 21 (2010) 671–678.
[86] W. Gao, W. Liu, T. Christensen, M.R. Zalutsky, A. Chilkoti, In situ growth of a PEG-like polymer from the C terminus of an intein fusion protein improves pharmacokinetics and tumor accumulation, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 16432–16437.
[87] W. Gao, W. Liu, J.A. Mackay, M.R. Zalutsky, E.J. Toone, A. Chilkoti, In situ growth of a stoichiometric PEG-like conjugate at a protein's N-terminus with significantly improved pharmacokinetics, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 15231–15236.
[88] S. Jevsevar, M. Kunstelj, V.G. Porekar, PEGylation of therapeutic proteins, Biotechnol. J. 5 (2010) 113–128.
[89] E.W. Ng, D.T. Shima, P. Calias, E.T. Cunningham Jr., D.R. Guyer, A.P. Adamis, Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease, Nat. Rev. Drug Discov. 5 (2006) 123–132.
[90] N. Goel, S. Stephens, Certolizumab pegol, MAbs 2 (2010) 137–147.
[91] E. Keystone, D. Heijde, D. Mason Jr., R. Landewe, R.V. Vollenhoven, B. Combe, P. Emery, V. Strand, P. Mease, C. Desai, K. Pavelka, Certolizumab pegol plus methotrexate is significantly more effective than placebo plus methotrexate in active rheumatoid arthritis: findings of a fifty-two-week, phase III, multicenter, randomized, double-blind, placebo-controlled, parallel-group study, Arthritis Rheum. 58 (2008) 3319–3329.
[92] J. Smolen, R.B. Landewe, P. Mease, J. Brzezicki, D. Mason, K. Luijtens, R.F. van Vollenhoven, A. Kavanaugh, M. Schiff, G.R. Burmester, V. Strand, J. Vencovsky, D. van der Heijde, Efficacy and safety of certolizumab pegol plus methotrexate in active rheumatoid arthritis: the RAPID 2 study. A randomised controlled trial, Ann. Rheum. Dis. 68 (2009) 797–804.
[93] P. Carmeliet, R.K. Jain, Angiogenesis in cancer and other diseases, Nature 407 (2000) 249–257.
[94] P. Vaupel, Pathophysiological and vascular characteristics of solid tumors in relation to drug delivery, Drug delivery in Oncology: From Basic Research to Cancer Therapy, Wiley, 2011.
[95] D. Putnam, J. Kopecek, Polymer conjugates with anticancer activity, Biopolymers II 122 (1995) 55–123.
[96] K. Ulbrich, J. Strohalm, V. Subr, D. Plocova, R. Duncan, B. Rihova, Polymeric conjugates of drugs and antibodies for site-specific drug delivery, Macromol. Symp. 103 (1996) 177–192.
[97] F. Kratz, U. Beyer, M.T. Schutte, Drug–polymer conjugates containing acid-cleavable bonds, Crit. Rev. Ther. Drug Carrier Syst. 16 (1999) 245–288.
[98] P.C.A. Rodrigues, T. Roth, H.H. Fiebig, C. Unger, R. Mulhaupt, F. Kratz, Correlation of the acid-sensitivity of polyethylene glycol daunorubicin conjugates with their in vitro antiproliferative activity, Bioorg. Med. Chem. 14 (2006) 4110–4117.
[99] K. Ulbrich, V. Subr, Polymeric anticancer drugs with pH-controlled activation, Advanced Drug Delivery Reviews 56 (2004) 1023–1050.
[100] G.M. Dubowchik, M.A. Walker, Receptor-mediated and enzyme-dependent targeting of cytotoxic anticancer drugs, Pharmacol. Therapeut. 83 (1999) 67–123.
[101] F. Kratz, I.A. Muller, C. Ryppa, A. Warnecke, Prodrug strategies in anticancer chemotherapy, ChemMedChem 3 (2008) 20–53.
[102] M.C. Brahimi-Horn, J. Chiche, J. Pouyssegur, Hypoxia signalling controls metabolic demand, Curr. Opin. Cell Biol. 19 (2007) 223–229.
[103] R.A. Gatenby, R.J. Gillies, Why do cancers have high aerobic glycolysis? Nat. Rev. Cancer 4 (2004) 891–899.
[104] J. Hu, P.E. Van den Steen, Q.X. Sang, G. Opdenakker, Matrix metalloproteinase inhibitors as therapy for inflammatory and vascular diseases, Nat. Rev. Drug Discov. 6 (2007) 480–498.
[105] D. Wang, S.C. Miller, X.M. Liu, B. Anderson, X.S. Wang, S.R. Goldring, Novel dexamethasone–HPMA copolymer conjugate and its potential application in treatment of rheumatoid arthritis, Arthritis Res. Ther. 9 (2007) R2.
[106] J.A.J. Carmela Palermo, Cysteine cathepsin proteases as pharmacological targets in cancer, Trends Pharmacol. Sci. 29 (2008) 22–28.
[107] J.D. Mott, Z. Werb, Regulation of matrix biology by matrix metalloproteinases, Curr. Opin. Cell Biol. 16 (2004) 558–564.
[108] T.H. Vu, Z. Werb, Matrix metalloproteinases: effectors of development and normal physiology, Genes Dev. 14 (2000) 2123–2133.
[109] J.F. Woessner Jr., MMPs and TIMPs—an historical perspective, Mol. Biotechnol. 22 (2002) 33–49.
[110] J. Martel-Pelletier, D.J. Welsch, J.P. Pelletier, Metalloproteases and inhibitors in arthritic diseases, Best Pract. Res. Clin. Rheumatol. 15 (2001) 805–829.
[111] J.A. Mengshol, K.S. Mix, C.E. Brinckerhoff, Matrix metalloproteinases as therapeutic targets in arthritic diseases: bull's-eye or missing the mark? Arthritis Rheum. 46 (2002) 13–20.
[112] B.K. Pilcher, J.A. Dumin, B.D. Sudbeck, S.M. Krane, H.G. Welgus, W.C. Parks, The activity of collagenase-1 is required for keratinocyte migration on a type I collagen matrix, J. Cell Biol. 137 (1997) 1445–1457.
[113] S.E. Dunsmore, U.K. Saarialho-Kere, J.D. Roby, C.L. Wilson, L.M. Matrisian, H.G. Welgus, W.C. Parks, Matrilysin expression and function in airway epithelium, J. Clin. Invest. 102 (1998) 1321–1331.
[114] J. Fang, T. Seki, H. Maeda, Therapeutic strategies by modulating oxygen stress in cancer and inflammation, Adv. Drug Deliv. Rev. 61 (2009) 290–302.
[115] T.P. Szatrowski, C.F. Nathan, Production of large amounts of hydrogen-peroxide by human tumor-cells, Cancer Res. 51 (1991) 794–798.
[116] S.S. Mahajan, E. Deu, E.M. Lauterwasser, M.J. Leyva, J.A. Ellman, M. Bogyo, A.R. Renslo, A fragmenting hybrid approach for targeted delivery of multiple therapeutic agents to the malaria parasite, ChemMedChem 6 (2011) 415–419.
[117] D. Putnam, J. Kopecek, Polymer conjugates with anticancer activity, Advances in Polymer Science, Springer-Verlag, Berlin Heidelberg, 1995, pp. 55–123.
[118] S.A. Cartlidge, R. Duncan, J.B. Lloyd, P. Rejmanová, J. Kopeček, Soluble, cross-linked N-(2-hydroxypropyl)methacrylamide copolymers as potential drug carriers: pinocytosis by rat visceral yolk sacs and rat intestine cultured in vitro. Effect of molecular weight on uptake and intracellular degradation, J. Control. Release 3 (1986) 55–66.
[119] S.E. Gratton, P.A. Ropp, P.D. Pohlhaus, J.C. Luft, V.J. Madden, M.E. Napier, J.M. DeSimone, The effect of particle design on cellular internalization pathways, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 11613–11618.
[120] D. Bray, B.D. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, P. Walter, Essential Cell Biology, Garland Publishing, New York, 1998.

[121] S.S. Dharap, B. Qiu, G.C. Williams, P. Sinko, S. Stein, T. Minko, Molecular targeting of drug delivery systems to ovarian cancer by BH3 and LHRH peptides, J. Control. Release 91 (2003) 61–73.
[122] M. Ehrlich, W. Boll, A. Van Oijen, R. Hariharan, K. Chandran, M.L. Nibert, T. Kirchhausen, Endocytosis by random initiation and stabilization of clathrin-coated pits, Cell 118 (2004) 591–605.
[123] H.T. McMahon, E. Boucrot, Molecular mechanism and physiological functions of clathrin-mediated endocytosis, Nat. Rev. Mol. Cell Biol. 12 (2011) 517–533.
[124] I. Mellman, Endocytosis and molecular sorting, Annu. Rev. Cell Dev. Biol. 12 (1996) 575–625.
[125] J. Khandare, T. Minko, Polymer–drug conjugates: progress in polymeric prodrugs, Prog. Polym. Sci. 31 (2006) 359–397.
[126] A. Di Pietro, G. Dayan, G. Conseil, E. Steinfels, T. Krell, D. Trompier, H. Baubichon-Cortay, J. Jault, P-glycoprotein-mediated resistance to chemotherapy in cancer cells: using recombinant cytosolic domains to establish structure–function relationships, Braz. J. Med. Biol. Res. 32 (1999) 925–939.
[127] S.P. Cole, G. Bhardwaj, J.H. Gerlach, J.E. Mackie, C.E. Grant, K.C. Almquist, A.J. Stewart, E.U. Kurz, A.M. Duncan, R.G. Deeley, Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line, Science 258 (1992) 1650–1654.
[128] L.A. Doyle, W. Yang, L.V. Abruzzo, T. Krogmann, Y. Gao, A.K. Rishi, D.D. Ross, A multidrug resistance transporter from human MCF-7 breast cancer cells, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 15665–15670.
[129] T. Minko, P. Kopeckova, J. Kopecek, Chronic exposure to HPMA copolymer-bound adriamycin does not induce multidrug resistance in a human ovarian carcinoma cell line, J. Control. Release 59 (1999) 133–148.
[130] T. Minko, P. Kopeckova, V. Pozharov, J. Kopecek, HPMA copolymer bound adriamycin overcomes MDR1 gene encoded resistance in a human ovarian carcinoma cell line, J. Control. Release 54 (1998) 223–233.
[131] A. Nori, J. Kopecek, Intracellular targeting of polymer–bound drugs for cancer chemotherapy, Adv. Drug Deliv. Rev. 57 (2005) 609–636.
[132] V. Omelyanenko, P. Kopeckova, C. Gentry, J. Kopecek, Targetable HPMA copolymer–adriamycin conjugates. Recognition, internalization, and subcellular fate, J. Control. Release 53 (1998) 25–37.
[133] V. Russ, H. Elfberg, C. Thoma, J. Kloeckner, M. Ogris, E. Wagner, Novel degradable oligoethylenimine acrylate ester-based pseudodendrimers for in vitro and in vivo gene transfer, Gene Ther. 15 (2008) 18–29.
[134] O. Boussif, F. Lezoualc'h, M.A. Zanta, M.D. Mergny, D. Scherman, B. Demeneix, J.P. Behr, A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine, Proc. Natl. Acad. Sci. 92 (1995) 7297–7301.
[135] J.P. Behr, The proton sponge: a trick to enter cells the viruses did not exploit, Chimia 51 (1997) 34–36.
[136] N.D. Sonawane, F.C. Szoka, A.S. Verkman, Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine–DNA polyplexes, J. Biol. Chem. 278 (2003) 44826–44831.
[137] S.D. Li, L. Huang, Targeted delivery of siRNA by nonviral vectors: lessons learned from recent advances, Curr. Opin. Investig. Drugs 9 (2008) 1317–1323.
[138] A.L. Jackson, S.R. Bartz, J. Schelter, S.V. Kobayashi, J. Burchard, M. Mao, B. Li, G. Cavet, P.S. Linsley, Expression profiling reveals off-target gene regulation by RNAi, Nat. Biotechnol. 21 (2003) 635–637.
[139] A.D. Judge, V. Sood, J.R. Shaw, D. Fang, K. McClintock, I. MacLachlan, Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA, Nat. Biotechnol. 23 (2005) 457–462.
[140] H. Kato, O. Takeuchi, S. Sato, M. Yoneyama, M. Yamamoto, K. Matsui, S. Uematsu, A. Jung, T. Kawai, K.J. Ishii, O. Yamaguchi, K. Otsu, T. Tsujimura, C.S. Koh, C. Reis e Sousa, Y. Matsuura, T. Fujita, S. Akira, Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses, Nature 441 (2006) 101–105.
[141] M. Sioud, RNA interference and innate immunity, Adv. Drug Deliv. Rev. 59 (2007) 153–163.
[142] A.L. Jackson, J. Burchard, D. Leake, A. Reynolds, J. Schelter, J. Guo, J.M. Johnson, L. Lim, J. Karpilow, K. Nichols, W. Marshall, A. Khvorova, P.S. Linsley, Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing, RNA 12 (2006) 1197–1205.
[143] A.L. Jackson, J. Burchard, J. Schelter, B.N. Chau, M. Cleary, L. Lim, P.S. Linsley, Widespread siRNA "off-target" transcript silencing mediated by seed region sequence complementarity, RNA 12 (2006) 1179–1187.
[144] A. Judge, I. MacLachlan, Overcoming the innate immune response to small interfering RNA, Hum. Gene Ther. 19 (2008) 111–124.
[145] N.D. Rawlings, F.R. Morton, C.Y. Kok, J. Kong, A.J. Barrett, MEROPS: the peptidase database, Nucleic Acids Res. 36 (2008) D320–D325.
[146] K. Brix, A. Dunkhorst, K. Mayer, S. Jordans, Cysteine cathepsins: cellular roadmap to different functions, Biochimie 90 (2008) 194–207.
[147] S.P. Lutgens, K.B. Cleutjens, M.J. Daemen, S. Heeneman, Cathepsin cysteine proteases in cardiovascular disease, FASEB J. 21 (2007) 3029–3041.
[148] M.M. Mohamed, B.F. Sloane, Cysteine cathepsins: multifunctional enzymes in cancer, Nat. Rev. Cancer 6 (2006) 764–775.
[149] Y.J. Zhang, H. Fang, J. Jiao, W.F. Xu, The structure and function of histone deacetylases: the target for anti-cancer therapy, Curr. Med. Chem. 15 (2008) 2840–2849.
[150] C. Liu, C.Z. Sun, H.N. Huang, K. Janda, T. Edgington, Overexpression of legumain in tumors is significant for invasion/metastasis and a candidate enzymatic target for prodrug therapy, Cancer Res. 63 (2003) 2957–2964.
[151] L. Stern, R. Perry, P. Ofek, A. Many, D. Shabat, R. Satchi-Fainaro, A novel antitumor prodrug platform designed to be cleaved by the endoprotease legumain, Bioconjug. Chem. 20 (2009) 500–510.
[152] L.W. Seymour, D.R. Ferry, D.J. Kerr, D. Rea, M. Whitlock, R. Poyner, C. Boivin, S. Hesslewood, C. Twelves, R. Blackie, A. Schatzlein, D. Jodrell, D. Bissett, H. Calvert, M. Lind, A. Robbins, S. Burtles, R. Duncan, J. Cassidy, Phase II studies of polymer–doxorubicin (PK1, FCE28068) in the treatment of breast, lung and colorectal cancer, Int. J. Oncol. 34 (2009) 1629–1636.
[153] F. Kratz, K. Abu Ajaj, A. Warnecke, Anticancer carrier-linked prodrugs in clinical trials, Expert Opin. Investig. Drugs 16 (2007) 1037–1058.
[154] K.S. Albain, C.P. Belani, P. Bonomi, K.J. O'Byrne, J.H. Schiller, M. Socinski, PIONEER: a phase III randomized trial of paclitaxel poliglumex versus paclitaxel in chemotherapy-naive women with advanced-stage non-small-cell lung cancer and performance status of 2, Clin. Lung Cancer 7 (2006) 417–419.
[155] P. Bonomi, Paclitaxel poliglumex (PPx, CT-2103): macromolecular medicine for advanced non-small-cell lung cancer, Expert Rev. Anticancer 7 (2007) 415–422.
[156] L.S. Nair, C.T. Laurencin, Biodegradable polymers as biomaterials, Prog. Polym. Sci. 32 (2007) 762–798.
[157] R. Mehvar, Dextrans for targeted and sustained delivery of therapeutic and imaging agents, J. Control. Release 69 (2000) 1–25.
[158] V. Knorr, M. Ogris, E. Wagner, An acid sensitive ketal-based polyethylene glycol-oligoethylenimine copolymer mediates improved transfection efficiency at reduced toxicity, Pharm. Res. 25 (2008) 2937–2945.
[159] K. Luo, J. Yang, P. Kopeckova, J. Kopecek, Biodegradable multiblock poly[N-(2-hydroxypropyl)methacrylamide] via reversible addition-fragmentation chain transfer polymerization and click chemistry, Macromolecules 44 (2011) 2481–2488.
[160] H. Pan, J. Yang, P. Kopeckova, J. Kopecek, Backbone degradable multiblock N-(2-hydroxypropyl)methacrylamide copolymer conjugates via reversible addition-fragmentation chain transfer polymerization and thiol-ene coupling reaction, Biomacromolecules 12 (2011) 247–252.
[161] J. Yang, K. Luo, H. Pan, P. Kopeckova, J. Kopecek, Synthesis of biodegradable multiblock copolymers by click coupling of RAFT-generated heterotelechelicPolyHPMA conjugates, React. Funct. Polym. 71 (2011) 294–302.
[162] M. Barz, F.K. Wolf, F. Canal, K. Koynov, M.J. Vicent, H. Frey, R. Zentel, Synthesis, characterization and preliminary biological evaluation of P(HPMA)-b-P(LLA) copolymers: a new type of functional biocompatible block copolymer, Macromol. Rapid Commun. 31 (2010) 1492–1500.
[163] L.W. Seymour, R. Duncan, J. Strohalm, J. Kopecek, Effect of molecular weight (Mw) of N-(2-hydroxypropyl)methacrylamide copolymers on body distribution and rate of excretion after subcutaneous, intraperitoneal, and intravenous administration to rats, J. Biomed. Mater. Res. 21 (1987) 1341–1358.
[164] M.P. Bohrer, W.M. Deen, C.R. Robertson, J.L. Troy, B.M. Brenner, Influence of molecular configuration on the passage of macromolecules across the glomerular capillary wall, J. Gen. Physiol. 74 (1979) 583–593.
[165] W.M. Deen, M.P. Bohrer, B.M. Brenner, Macromolecule transport across glomerular capillaries: application of pore theory, Kidney Int. 16 (1979) 353–365.
[166] M. Kovar, J. Strohalm, T. Etrych, K. Ulbrich, B. Rihova, Star structure of antibody-targeted HPMA copolymer-bound doxorubicin: a novel type of polymeric conjugate for targeted drug delivery with potent antitumor effect, Bioconjug. Chem. 13 (2002) 206–215.
[167] T. Yamaoka, Y. Tabata, Y. Ikada, Distribution and tissue uptake of poly(ethylene glycol) with different molecular-weights after intravenous administration to mice, J. Pharm. Sci. 83 (1994) 601–606.
[168] L.W. Seymour, Y. Miyamoto, H. Maeda, M. Brereton, J. Strohalm, K. Ulbrich, R. Duncan, Influence of molecular weight on passive tumour accumulation of a soluble macromolecular drug carrier, Eur. J. Cancer 31A (1995) 766–770.
[169] A. Al-Shamkhani, R. Duncan, Radioiodination of alginate via covalently-bound tyrosinamide allows monitoring of its fate in vivo, J. Bioact. Compat. Polym. 10 (1995) 4–13.
[170] R. Webster, V. Elliott, B.K. Park, D. Walker, M. Hankin, P. Taupin, PEG and PEG conjugates toxicity: towards an understanding of the toxicity of PEG and its relevance to PEGylated biologicals, in: F.M. Veronese (Ed.), PEGylated Protein Drugs: Basic Science and Clinical Applications, Birkhauser Verlag, Switzerland, 2009, pp. 127–146.
[171] R. Webster, E. Didier, P. Harris, N. Siegel, J. Stadler, L. Tilbury, D. Smith, PEGylated proteins: evaluation of their safety in the absence of definitive metabolism studies, Drug Metab. Dispos. 35 (2007) 9–16.
[172] A. Bendele, J. Seely, C. Richey, G. Sennello, G. Shopp, Short communication: renal tubular vacuolation in animals treated with polyethylene-glycol-conjugated proteins, Toxicol. Sci. 42 (1998) 152–157.
[173] M. Barz, R. Luxenhofer, R. Zentel, M.J. Vicent, Overcoming the PEG-addiction: well-defined alternatives to PEG, from structure–property relationships to better defined therapeutics, Polym. Chem.-Uk 2 (2011) 1900–1918.
[174] D.G. Vartak, R.A. Gemeinhart, Matrix metalloproteases: Underutilized targets for drug delivery, J. Drug Target. 15 (2007) 1–20.
[175] S. Lee, J. Xie, X. Chen, Activatable molecular probes for cancer imaging, Curr. Top Med. Chem. 10 (2010) 1135–1144.
[176] L.E. Edington, A.B. Berger, G. Blum, V.E. Albrow, M.G. Paulick, N. Lineberry, M. Bogyo, Noninvasive optical imaging of apoptosis by caspase-targeted activity-based probes, Nat. Med. 15 (2009) 967–974.
[177] C. Tung, U. Mahmood, S. Bredow, R. Weissleder, In vivo imaging of proteolytic enzyme activity using a novel molecular reporter, Cancer Res. 60 (2000) 4953–4958.
[178] R. Duncan, H.C. Cable, J.B. Lloyd, P. Rejmanová, J. Kopecek, Polymers containing enzymatically degradable bonds, 7) design of oligopeptide side-chains in poly [N-(2-hydroxypropyl)methacrylamide] copolymers to promote efficient degradation by lysosomal enzymes, Macromol. Chem. Phys. 184 (1983) 1997–2008.
[179] S. Wu, Y. Huang, C. Yeh, M. Tsai, C. Liao, W. Cheng, W. Chen, K. Lin, Cathepsin H regulated by the thyroid hormone receptors associate with tumor invasion in human hepatoma cells, Oncogene 30 (2011) 2057–2069.
[180] C. Liu, C. Sun, H. Huang, K. Janda, T. Edgington, Overexpression of legumain in tumors is significant for invasion/metastasis and a candidate enzymatic target for prodrug therapy, Cancer Res. 63 (2003) 2957–2964.

[181] C.A. Olsen, M.R. Ghadiri, Discovery of potent and selective histone deacetylase inhibitors via focused combinatorial libraries of cyclic α3β-tetrapeptides, J. Med. Chem. 52 (2009) 7836–7846.
[182] J.G. Kiang, K.T. Tsen, Biology of hypoxia, Chin. J. Physiol. 49 (2006) 223–233.
[183] Z. Zhang, J. Fan, P.P. Cheney, M. Berezin, Y., W.B. Edwards, W.J. Akers, D. Shen, K. Liang, J.P. Culver, S. Achilefu, Activatable molecular systems using homologous near-infrared fluorescent probes for monitoring enzyme activities in vitro, in cellulo, and in vivo., Mol. Pharm. 6 (2009) 416–427.
[184] S.F. Larner, D.M. McKinsey, R.L. Hayes, K.K.W. Wang, Caspase 7: increased expression and activation after traumatic brain injury in rats, J. Neurochem. 94 (2005) 97–108.
[185] H. Nagahara, K. Mimori, T. Utsunomiya, G. Barnard, M. Ohira, K. Hirakawa, M. Mori, Clinicopathologic and biological significance of kallikrein 6 overexpression in human gastric cancer, Clin. Cancer Res. 11 (2005) 6800–6806.
[186] J.T. Kim, E.Y. Song, K.S. Chung, M.A. Kang, J.W. Kim, S.J. Kim, Y.I. Yeom, J.H. Kim, K.H. Kim, H.G. Lee, Up-regulation and clinical significance of serine protease kallikrein 6 in colon cancer, Cancer 117 (2011) 2608–2619.
[187] N. Sharma, K. Oikonomopoulou, K. Ito, B. Renaux, E.P. Diamandis, M.D. Hollenberg, D.E. Rancourt, Substrate specificity determination of mouse implantation serine proteinase and human kallikrein-related peptidase 6 by phage display, Biol. Chem. 389 (2008) 1097–1105.
[188] C.G. Laurent Brault, Franz Bracher, Kilian Huber, Stefan Knapp, Jürg Schwaller, PIM serine/threonine kinases in the pathogenesis and therapy of hematologic malignancies and solid cancers, Haematologica 95 (2010) 1004–1015.