Macromolecular Journals

Macromolecular Bioscience

www.mbs-journal.de

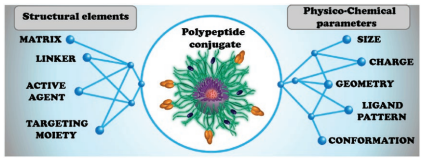
mabi.201600316

Review

Polypeptide-Based Conjugates as Therapeutics: Opportunities and Challenges

O. Zagorodko, J. José Arroyo-Crespo, V. J. Nebot,* María J. Vicent*

Macromol. Biosci. **2016**, *xx*, ooo–ooo

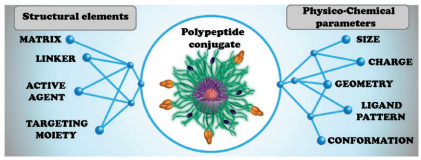


Polypeptide-based conjugates are emerging as excellent platforms to deliver various therapeutic/diagnostic agents in a controlled and selective manner. This review aims to describe the complex interplay of key design parameters which influence the desired therapeutic activity according to current thinking in this constantly evolving field. Furthermore, the authors highlight the existing opportunities and challenges facing the design of polypeptide-based conjugates.

Polypeptide-Based Conjugates as Therapeutics: Opportunities and Challenges

Oleksandr Zagorodko, Juan José Arroyo-Crespo, Vicent J. Nebot,* María J. Vicent*

Synthetic polypeptides or polyamino acids have become a useful and multifunctional platform in advanced drug delivery studies. Nonetheless, the full potential of these systems has yet to be achieved. The final structure of polypeptide conjugates and their in vivo behavior are dependent on an extraordinarily complex pattern of interconnected physico-chemical and structural parameters, making sophisticated directional design of such systems difficult and often unachievable. In this review, the authors aim to discuss the role of these parameters in the successful design of different drug delivery architectures and to delineate some basic correlations between structure, properties, and the biological behavior of polypeptide-based conjugates.



1. Introduction

Advances in synthetic chemistry, characterization techniques, as well as a growing understanding of the complex interactions between nanomaterials and biological interfaces, have facilitated the design of efficient nanometric systems with applications in many different biomedical fields. Polymer Therapeutics is a distinct class of nanomedicines representing a consistently growing market with a number of US food and drug administration-approved products.[1] These include polymeric drugs,[2] polymer-drug conjugates,[3] and polymer-protein conjugates.[4,5] Additionally, Polymer Therapeutics of other classes, such as polymeric micelles[2,6] and polyelectrolyte complexes as non-viral vectors[7] are in advanced clinical trials. In these later families, Prof. Kataoka's research group is one of the major references.[6–8]

The development of the first polymer-drug conjugates was realized in 1955, when German scientist Horst Jatzkewitz synthesized a polyvinylpyrrolidone-mescaline conjugate with different linkers[9] and was further reinforced by Ringsdorf in the 1970s.[10] Since then, a plethora of polymers have found use as drug delivery systems, including polymers of different origin (synthetic, semisynthetic or natural),[11,12] electrostatic nature,[7] topology (linear, branched, star, dendrimeric etc.),[13,14] morphology,[15] and degradability.[16] The use of polymeric compounds as drug delivery systems confers unique properties to the active agent, including modified pharmacokinetics, controlled biodistribution, and/or sustained release. The key advantage of polymeric drug delivery systems is an elevated functionality compared to free drugs. By careful functionalization, it is possible to create systems based on an identical polymeric matrix and suitable for diverse purposes, whether it be passive or active targeting, single or combination therapy, or theranostics.

Polyamino acids (PAA or polypeptide)-based materials have gained much attention in the field of biomedicine over recent decades.[17–19] PAAs mimic natural proteins and demonstrate remarkable biocompatibility and biodegradability due to the endogenous nature of the building monomers. Development of polymerization techniques (mainly *N*-carboxyanhydride ring-opening polymerization (NCA-ROP))[20] and synthetic chemistry have permitted the production of polypeptides with narrow polydispersity, minimal side product formation,

O. Zagorodko, J. J. Arroyo-Crespo, Dr. V. J. Nebot, Dr. M. J. Vicent
Polymer Therapeutics Laboratory
Centro de Investigación Príncipe Felipe
Valencia 46012, Spain
E-mail: mjvicent@cipf.es; vjnebot@cipf.es
Dr. V. J. Nebot, Polypeptide Therapeutic Solutions SL
Centro de Investigación Príncipe Felipe, Valencia 46012, Spain

Macromol. Biosci. **2016**, DOI: 10.1002/mabi.201600316

high reproducibility, and precise functionalization of the polypeptide backbone.[21]

The suitability of polypeptide-based materials for drug delivery applications is also confirmed through the steady increase in the number of polypeptide-based compounds reaching preclinical studies and clinical trials.[18] Within the following review, we will mainly focus on polypeptide drug-conjugates and their key design features; further reading on polypeptide-based materials can be found in the literature.[18,22,23]

Applicability of synthetic polypeptide-based conjugate development has reached multiple areas of medicine, including the elaboration of anti-microbial,[24–27] anti-virus,[28] anti-cancer,[29–36] anti-diabetic,[37] anti-apoptotic,[38] and anti-tuberculosis drugs,[39] magnetic resonance imaging (MRI) agents,[40] and theranostic agents.[41] The great advantage of polypeptides is their structural versatility which allows the generation of a myriad of architectures with differential physico-chemical features, allowing high loading rates and the possibility to conjugate drugs of different origin, polarity, and hydrophilicity. Poly-L-glutamate (P(L-Glu)), for example, has been used for the delivery of highly lipophilic drugs (paclitaxel,[33] 4-(*N*-hydroxyphenyl)retinamide,[42] camptothecin (CPT),[43] *N*,*N*-dimethylsphingosine),[44] hydrophilic drugs (dopamine),[45] peptoids,[46] metal complexes (Gd(III)-DO3A,[47] VO,[48] and Cu[49]), and nucleic acids.[50] Structural versatility of polypeptide conjugates is also exemplified by the wide range of delivery systems in which P(L-Asp), P(L-Glu), or P(L-Lys), for example, are present.[27,36,51]

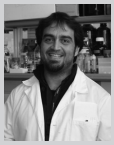
Engineering polypeptide complexity is an accepted approach in order to mimic nature and cross many biological barriers in a target pathology. This has led to the origination of interconnected systems selected from a rather wide, complex, and diverse toolbox. Advanced polypeptide conjugates are complex systems with several structural levels and diverse architectures. Even though each conjugate is a unique system, its final physico-chemical properties comply with strictly explicit and determined trends.

Existing key design features can be described at three distinct levels: primary, selection of structural elements before the synthesis of the polymer (e.g., polymer matrix nature and architecture, linker design and responsiveness, active molecule nature); secondary, optimization of the physico-chemical properties of the synthesized conjugates (e.g., size, charge, conformation, geometry, topology); tertiary, modulating the biological properties through fine tuning of the previous two levels in reiterative design cycles.

However, precise design of advanced polypeptide-based conjugates toward the pursued biological output remains a serious challenge due to several related points:

Oleksandr Zagorodko obtained his degree in polymer chemistry in the National university of "Kiev-Mohyla academy" (Ukraine) and his master's degree in Advanced spectroscopy in chemistry in a joint program between Lille University of Science and Technology (France) and the University of Helsinki (Finland). In November 2015, he joined the Polymer Therapeutics laboratory in the CIPF. His research is a part of ERC project "MyNano" and is focused on the development of the family of tumor-targeted polymer nanoconjugates for the treatment of advanced stage breast cancer with an emphasis on personalized treatment.

Juan José Arroyo Crespo obtained his degree in Chemistry at the University of Salamanca (Spain) in 2011 in the field of epigenetics, studying DNA methylation. In 2012 he received his MSc in Supramolecular Chemistry at University of Salamanca. From late 2012, he has been a predoctoral researcher in the Polymer Therapeutics laboratory in the CIPF. His research is focused on the development of polyglutamate-based combination therapies for the treatment of breast cancer. He is involved in the synthesis and identification of physico-chemical descriptors of polymer-drug conjugates in different physiological environments and the study of their biological behavior.



Vicent J. Nebot finished his PhD in 2012 working in the design, study, and applications of supramolecular hydrogels based on amino acids. In 2012, he joined the Polymer Therapeutics laboratory in the CIPF as a post-doctoral research fellow to work in the development of new therapeutic polymers for the treatment of Spinal Cord Injury and the development of novel self-assembled Drug Delivery Systems. In 2014, he obtained a Spanish Torres Quevedo grant (MINECO) to work in the spin-off company, Polypeptide Therapeutic Solutions, where he is currently developing translational research in collaboration with the Polymer Therapeutics lab as R&D deputy director. His research focuses in the design, synthesis, and physico-chemical characterization of novel architectures to bypass biological barriers with potential applications in diverse pathologies.

María J. Vicent received her Ph.D. degree in 2001 in chemistry on solid supports, then she moved to more biomedically oriented research at the Centre for Polymer Therapeutics with Prof. Duncan after the award of a Marie Curie Postdoctoral Fellowship in 2002. Since 2006, María is Head of the Polymer Therapeutics Lab. at Centro de Investigación Príncipe Felipe (CIPF, Spain). Her research group focuses on the development of novel nanopharmaceuticals for different therapeutic applications and has been funded by national and European grants (including an ERC Consolidator grant "MyNano") from academia as well as industry. María co-authored 80 peer reviewed papers and seven patents, two of them licensed to the pharmaceutical industry and a third one used as foundation of the spin-off company 'Polypeptide Therapeutic Solutions SL' in 2012. Currently, she also coordinates the Drug Discovery strategy of the Valencian Community on Innovative medicines and the Advanced Therapies Program at CIPF.

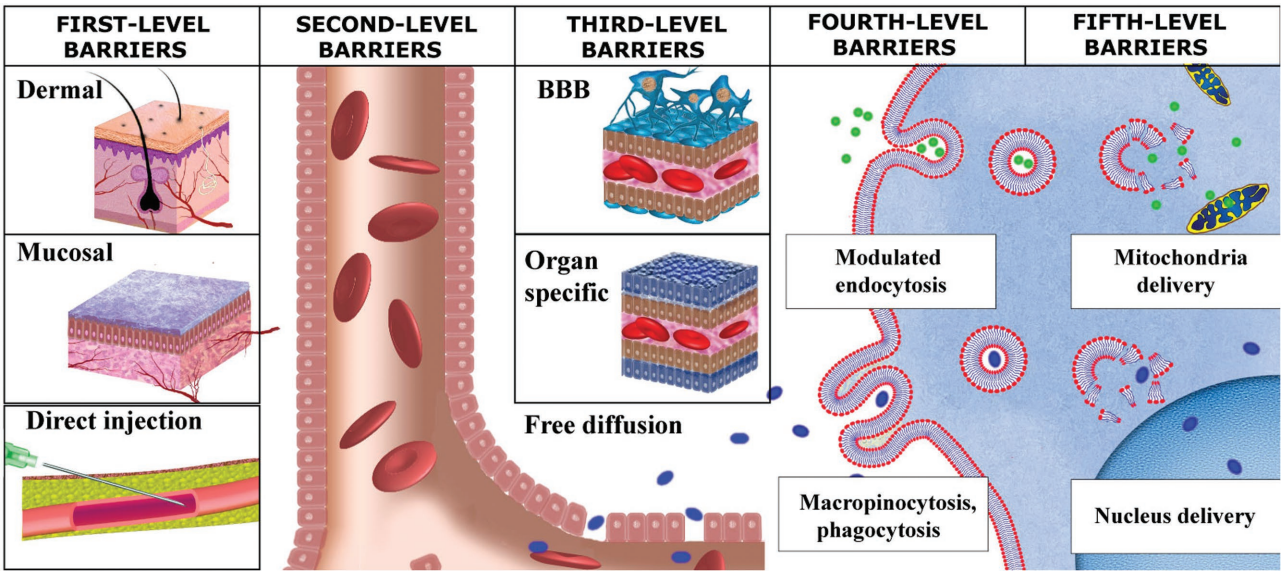


Figure 1. General representation of biological barriers present in the body.

1. Multiple different parameters involved in the design can significantly alter the final properties of the conjugate.
2. Lack of scientific data devoted to the strategic and logical design of sophisticated polypeptide systems.
3. Lack of appropriate characterization tools and techniques to disclose the interaction of the nanocarriers with biological interfaces.[52]
4. Structural complexity and molecular mass inhomogeneity have made computer simulations of such systems highly challenging and so, only a few examples has been reported.[53] However, extensive practical work has revealed some correlations between structure of the conjugate and its properties.[54]
5. In this review, we will focus on the role of structural elements (especially stimuli responsive polymer-drug linkers) and the physico-chemical parameters that determine a given system biological performance and, therefore, their successful applicability and transferability. Throughout the following sections, we will endeavor to illustrate the role of different parameters affecting the final physico-chemical properties, the responsive nature, and the biological performance of the conjugate.

2. Rational Design of Polypeptide-Based Conjugates

The rational design of polypeptide-based therapeutics for the treatment of a given pathology must be holistically driven using a step-wise design following the pathway taken by the therapeutic through the body.[55] Therefore, the administration route, the biological barriers to be crossed, and the target site of action all require scrutiny for the successful selection and design of the conjugate with selected properties. The physico-chemical parameters of the drug delivery systems determine their inherent ability to cross the required biological barriers.[56] Another important aspect to be considered for appropriate biological activity is the specific response to the different physiological environments encountered in the body.[57] This knowledge would ensure an adequate overall stability, optimal response, and thus, delivery of the cargo in the target site of action at the desired timing. Furthermore, the administration route and the dose schedule in the target patient population are key issues for the successful translation of a drug delivery system.[58,59]

2.1. Biological Barriers and Administration Routes

"Biological barriers" is a generalized term for a group of specific protective mechanisms present throughout the body. All the known biological barriers can be classified into five hierarchical levels.

(a) Level 1 (absorption: reaching the bloodstream). Independent of the administration route, the first obstacle for most conjugates is the bloodstream to allow adequate body distribution and to reach the selected molecular target. Design of effective conjugates must ensure their chemical stability and integrity during blood circulation at physiological conditions (ionic strength, redox potential, pH, presence of proteases, etc.) until arrival at the desired site of action. The easiest administration strategy for a given drug conjugate is intravenous injection, which represents the most widely used route due to rapid onset and high bioavailability. For

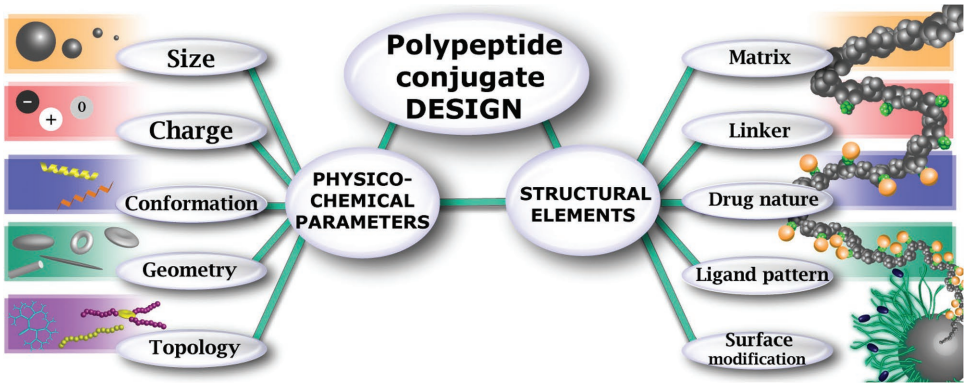


Figure 2. Schematic representation of the key design features related to structural levels and physico-chemical properties in the design of polypeptide therapeutics.

polypeptide-based therapeutics, alternative routes of administration include topical,[60,61] oral,[62] and pulmonary,[63] delivery through different mucus barriers (buccal, nasal, vaginal, ocular, etc.),[64–66] and through direct injection to the site of disease (intraocular, intraperitoneal etc.). However, in this context, these administration routes have not yet been fully exploited.

(b) Level 2 (circulatory barriers). In the bloodstream, the immune, reticuloendothelial (RES), and hepatic systems are the main obstacles for adequate delivery. Recognition of foreign entities is implemented by a family of proteins known as opsonins which absorb to the surface of the nanosystem and promote phagocytosis. While the exact mechanisms behind opsonization have yet to be fully delineated, certain physico-chemical and surface properties which affect the level of opsonization have been ascertained.[67–69] Furthermore, as macromolecules, some conjugates will trigger the activation of the complement system, the innate immune response, limiting the effectiveness of the treatment besides the subsequent development of hypersensitivity reactions or allergies.[70]

(c) Level 3 (tissue-specific barriers and tumor stroma). Some organs are provided with highly specific blood-tissue barriers (blood-brain, blood-ocular, blood-retinal, blood-testis, blood-thymus and blood-air). Crossing the blood-brain barrier (BBB) using polypeptide conjugates has only recently gained much attention following the publication of the so-called "Trojan Horse" strategy.[71] However, to the best of our knowledge, there is currently no reported evidence for polypeptide conjugates crossing any other of the above-mentioned barriers apart from our own recently patented work on the BBB.[72] However, solid tumors develop a deficient lymphatic drainage and an abnormal architecture of neovasculature with wide fenestration within the stroma, allowing permeation of macromolecules. In this scenario, passive targeting by the so-called enhanced permeability and retention (EPR) effect[73] has stimulated the development of anti-cancer polypeptide conjugate therapies.[35,74,75] However, for future developments, it is important to take into account that the EPR effect is a rapidly evolving concept.[76]

(d) Level 4 (cellular barriers). The passive diffusion of molecules with a molecular mass above 1 kDa is efficiently blocked by the cell membrane. Conjugates can enter the cell through endocytosis (including clathrin-mediated, caveolin-mediated and clathrin-and-caveolin-independent), phagocytosis, or macropinocytosis.[77,78] To enhance endocytic cell internalization, different ligands can be conjugated to the surface of the polypeptides (e.g., folic acid, cholesterol, or RGD based peptides).[79,80]

(e) Level 5 (subcellular barriers). Targeting of specific organelles (e.g., the nucleus or mitochondrion) represents the least researched area within drug delivery. Endocytic processes define the trafficking of a given agent to different subcellular vesicles or organelles. Most polypeptide-drug conjugates are designed to enter the cell by clathrin-mediated endocytosis to be actively transported to lysosomes for further degradation, a process which is advantageous for bioresponsive-drug release.[78,81] In clathrin-mediated endocytosis, endosomal escape is the only way to ensure access of the carrier to targeted organelle. In this case, the incorporation of bioresponsive elements in the conjugate backbone or specific targeting sequences are required.

3. Key Design Features of Polypeptide Conjugates

In general, polypeptide conjugates used in drug delivery consists of four parts: the biodegradable polymer matrix, the active agent (e.g., small molecules), the linker, and targeting (and/or imaging) moieties. However, the linking and targeting moieties are not always essential conjugate design features.

Conjugation of active agents to the polypeptidic carrier can have a prominent effect on the final physico-chemical properties. However, conjugates do tend to replicate the biodistribution and pharmacokinetic behavior of the original polymer matrix if no major conformational changes are induced by the conjugated drug(s) (importance of drug(s) nature and loading).[82] This topic is thoroughly discussed in Section 4.

Conversely, the polypeptide matrix itself can affect the activity of the conjugated agent, especially when functional groups determining activity are blocked or utilized for conjugation. The chemotherapeutic agent paclitaxel (PTX), for example, is connected to the polypeptide through the 2′-hydroxyl position which is essential for tubulin binding.[83] Thus, PTX conjugated to P(L-Glu) is inactive and needs to be degraded within the cell to provide its therapeutic effect.[84] Polymer conjugates of Doxorubicin (Dox), also require in-cell degradation, as the anti-tumorigenic effect requires Dox interaction with the DNA and the polymer conjugate cannot penetrate the nuclear membrane.[85] However, polymer conjugation prevents Dox-related cardiotoxic effects.[86] Nevertheless, polymer conjugation does not always alter the activity of the conjugated agent and, in some cases (e.g., phloridzin (Prz),[87,88] doxycycline[88]), drug activity is increased.

The versatility of PAAs is demonstrated by the diversity of developed systems to date; ranging from linear or branched homopolypeptides to block (and random) copolypeptides capable of generating hierarchically assembled nanostructures from synthesized polypeptide conjugates (e.g., micelles and core cross-linked micelles, vesicles, cross-linked networks and hydrogels).[18,89–91] In each case amino acid building block choice is driven by the required functionality. Additionally, ROP of NCAs renders polypeptides with conserved N-terminal ends and can be used to introduce functionalities at the C-terminal site for latter conjugation strategies. Synthetic aspects of polypeptide production are outside of the scope of this review and can be found elsewhere.[14,16,20,91,92]

In the context of Polypeptide Therapeutics, amino acids can be classified from two different points of view.

(1) Functional classification: According to the chemical reactivity of the associated side chains, amino acids can be classified as functional (where side chains contain functional groups suitable for active molecule conjugation) and non-functional (where side chain modification is unpractical or inaccessible).
(2) Structural classification: According to the structural or architectural properties brought to the scaffold, amino acids can be grouped as hydrophobic (e.g., Ala, Val, Leu, Ile, Phe, Tyr, Trp), α-helix forming (e.g., Leu, Phe), β-sheet forming (e.g., Ala, Val), random coil promoters (mainly ionizable side chains in their charged state), polar (or ionizable). [89]

Although the conjugation of active agents to the polypeptide backbone does not necessarily need to occur through side-chain functionalities, most of the examples in the literature operate within this strategy. This fact is attributed to the multivalency of PAA backbones and high cargo loading attainable. There are a number of amino acids with functional side groups that have yet to be thoroughly explored for polypeptide therapeutic design, including methionine, serine, threonine, and cysteine.[21] In practice, glutamic acid, aspartic acid, and lysine are the most exploited amino acids in polypeptide conjugate synthesis.

4. Physico-Chemical Properties Driving the Bio-Nano Interface

Interaction of a polypeptide conjugate with the bio-nano interface is driven by a complex system of simultaneous interactions which requires the step-wise design of experimental conditions in relevant physiological environments to completely reveal the material's performance in a biological milieu.[52] Polypeptide conjugates are discussed in the following section in relation to the influence of active agent incorporation on the final physico-chemical properties and the impact on their biological output.

4.1. Effect of Size

The size of the conjugate is an important parameter that determines its fate in the bloodstream, its targeting ability, and mechanisms of cellular uptake. In the bloodstream, nanosystems smaller than 5 nm rapidly penetrate capillary fenestrae, equilibrate with the extracellular matrix, and become rapidly cleared by renal glomerular capillaries. Larger particles exhibit prolonged circulation (except in cases when clearance is guided by factors other than size), although the RES eliminates nanosystems larger than 200 nm from the circulation. Thus, the diameter of the nanosized therapeutics suitable for in vivo application should lie in the range from 5 to 200 nm. It is important to note that even in this size range, nanomedicines display non-uniform size-behavior, unique for each material.[93] This fact is often disregarded and few studies have investigated size optimization for the nanosystems.

A serious problem with passive size-dependent accumulation of nanoconstructs is similar capillary porosity in different tissues. Studies into the anti-cancer efficiency of amphiphilic block copolymer PEG-P(L-Asp)-Dox found that micelles demonstrated tissue cytotoxicity that was probably related to accumulation specificity and retention time in the specific tissue.[94,95] As shown in Figure 3,

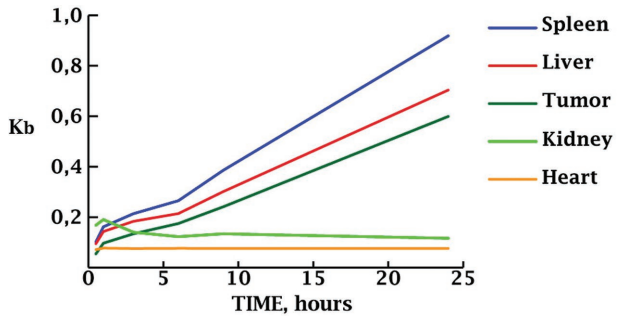


Figure 3. Tissue-to-blood concentration ratio ($K_b = C_{tissue}/C_{blood}$) of PEG-PAsp-Dox. Adapted from.[95]

the conjugate concentrated in the spleen, liver, and tumor because of the corresponding size of fenestrae (approx. 100–200 nm).[69] Thus, highly selective tissue targeting with polypeptide conjugates (and nanomedicines in general) remains a great challenge.

4.1.1. Effect of Conjugation on Size

Self-assembly of nanoconstructs is highly dependent on the nature of the assembling polymers and conjugated drug(s) and many different driving forces specific for each system are involved in its regulation. Self-assembly is regulated by the minimization of the interfacial energy directed by hydrophobic/hydrophilic balance, π–π stacking, dipole interactions, and hydrogen bonding among different domains. The balanced forces determine the compactness, conformation, and size of the conjugate.

Of note, many hydrophilic polypeptides and related copolymers do not aggregate in water and self-assembly of such systems is often induced by simple conjugation with a hydrophobic drug. For example, both PEG-P(L-Asp) and PEG-P(L-Glu) form nanosized systems after conjugation with Dox, while non-conjugated polymers do not aggregate in aqueous solution.[96]

When highly hydrophobic drugs are conjugated to self-assembled PAA (co)polymers, or when the content of the hydrophobic drug increases, the size of the resulting polymer-drug conjugate usually rises significantly.[97] The size increase is in direct non-linear proportion to the drug conjugation ratio. The assessment of a series of CPT conjugated PEG_{5000}-P(L-Glu) graft-copolymers demonstrated that an increased CPT content led to an increase in final nanosystem size from 30 nm (15.2 wt% of CPT) to 65 nm (25.1 wt% of CPT).[98] Zhou et al. obtained similar results for a synthesized series of PEG_{45}-$P(L\text{-}Lys)_{25}$-SS-CPT_x (x = 3, 6, and 8), where all of the conjugates formed spherical particles with diameters of 85, 118, and 152 nm, respectively.[99] Analogous trends were observed for other hydrophobic drugs such as Dox.[100] For PEG-P(L-Asp) copolymer conjugates with the anti-tuberculosis drugs Pyrazinamide and Isoniazid, conjugation with Pyrazinamide (86% conjugation) resulted in the formation of micelles with an average diameter of 78.2 nm[39] while Isoniazid conjugation (65% modification) led to the formation of significantly larger micelles (266 ± 27 nm).[101]

Post-synthetic modification with hydrophobic moieties through coordination bonds leads to nanosystem compaction, as was shown for many metal-containing drugs. For example, spherical nanoconjugates of γ-P(L-Glu)-(citric acid)-Cisplatin (CDDP) displayed an average size of 107 ± 6.3 nm while free γ-P(L-Glu) was larger, at 212 ± 8.6 nm.[102] The same trend is typical for physical encapsulation of hydrophobic drugs.

Some drugs have changeable effects on the size of the conjugate. Assessment of γ-P(L-Glu) series with different anti-diabetes drug Prz content, demonstrated that all conjugates formed spherical nanoconstructs with average sizes of 149 ± 23 (P(L-Glu)-Prz-7%), 187 ± 4.0 (P(L-Glu)-Prz-15%), and 170 ± 19 (P(L-Glu)-Prz-25%) nm.[87] However, some drugs have a less pronounced effect on size. This includes P(L-Glu)-Dox conjugates with 15% and 30% loading, which demonstrated even less pronounced size dependence with nanosystem sizes of 6.2 ± 1.3 nm and 7.5 ± 0.7 nm, respectively.[88]

Molecules for active targeting are conjugated either at the polymer side-chains[67] or at the terminal ends of the polymer and only a few targeting molecules per chain are present. Nevertheless, the effect of even a low conjugation level on conjugate size can be pronounced depending on residue nature. Targeting moieties are usually hydrophilic and, in most cases, conjugation results in an increase in nanosystem size independently of the polymer matrix nature, mainly if terminal conjugation is used. For terminal end modification of PEG-poly(γ-benzyl-L-Glutamate) (PEG-P(L-Glu-Bz)) with glycyrrhetinic acid (GA), the size of the modified micelles was higher than for the non-modified copolymer (181.1 and 175.4 nm, respectively).[103] An increase in size also occurred for dendrimeric P(L-Lys) nanosystems modified with folic acid[104] or galactose[105] and for PEG-P(L-Asp) nanosystems modified with biotin.[106] In general, terminal modification resulted in a size increase from 3% to 70%.

The effect of conjugation on size when more than one drug or a drug and a targeting moiety are used is often more significant than that observed for single component conjugation, possibly due to both intra- and inter-molecular interactions. P(L-Glu) dendrimers with oligomeric silsesquioxane core with a size of 3 nm grew to 135 nm after conjugation with Dox and Biotin. The conjugated nanoassembly displayed an unusual morphology, as each nanoassembly was an aggregate of smaller nanoobjects of 2–3 nm (the size of single dendrimers). This suggests that hydrophobic interactions within the conjugates, due to the presence of Dox molecules on the periphery of the nanosystem, drove the self-assembly process.[107] Dox and

Wortmannin conjugation to PEG-P(L-Asp-hydrazide) block copolymers generated nanosystems of less than 70 nm in diameter, although when the amount of Wortmannin increased, nanoassembly size also increased gradually. The only exception was observed for micelles with a 50:50 ratio which displayed a size of 91 nm.[108]

In some reports, molecular weight (M_w), is used to explain biological performance and can affect biodistribution in a similar way to size. For tumor targeting, the EPR effect is most pronounced for agents with an M_w above 50 kDa (either single molecule or self-assembled nanosystem).[109] For P(L-Glu)-CPT conjugates, an increase in M_w from 33 to 50 kDa enhanced anti-tumor efficacy against B16 melanoma cancer cells without significant changes in the maximal tolerated dose.[110] Similar results were also obtained for P(L-Glu)-CDDP conjugates with different M_w.[111]

The M_w of the matrix is a crucial parameter for polypeptide conjugates suitable for MRI as it affects T1 relaxivity, water exchange rate, accumulation in target tissues, and rotational correlation lifetime of Gd(III) species (so far only Gd-containing polypeptide conjugates have been investigated).[112] Polymeric P(L-Glu)-diethylenetriaminepentaacetic acid (DTPA)-Gd conjugates displayed sustained signal enhancement from necrotic tissues, while oligomeric P(L-Glu)-DTPA-Gd and low M_w conjugates showed a much shorter signal.[113] The M_w of each conjugate also affected the speed of signal disappearance—the quicker clearance of lower M_w conjugates resulted in quicker signal disappearance. The 50 and 87 kDa conjugates demonstrated prolonged signal increase duration in the tumor periphery and interstitium with no significant difference when compared to each other.[114]

4.2. Effect of Charge

Nanosystem surface charge has a prominent effect on the in vivo fate of conjugates, determining the recognition in and clearance from the circulation, interactions with cell membranes, cellular uptake, and toxicity. Surface charge also determines the physical stability of the system and aggregation tendency after exposure to biological media.[115] In the blood, zeta (ξ) potential determines the pharmacokinetic properties of the conjugate and the possibility of phagocytosis. Conjugates with a positive zeta potential such as (P(L-Glu-hydrazide)-*b*-poly(*N*,*N*-dimethylaminopropyl methacrylamide))3-g-PEG-Dox (ξ = +46.3 mV) are not directly applicable in vivo, because of charge-associated toxicity, but can be transformed to negatively charged nanoconstructs by conjugation or complexation with anionic molecules (e.g., siRNA).[100] It is generally accepted that polypeptide conjugate suitable for in vivo application should carry a small negative or neutral charge in order to inhibit opsonization and RES elimination.

4.2.1. Effect of Zeta Potential on Cellular Uptake

Nanosystem binding to the lipid cell membrane is the first step in cellular uptake and is greatly affected by nanosystem surface charge. Variations in zeta potential can be used to control binding to the cell both in vitro and in vivo. Cellular surface charge is usually negative due to sulfated proteoglycans (core proteins anchored in the lipid bilayer and glycosaminoglycan side chains of dermatan, keratin, or chondroitin sulfate).[116] Nanosystems with higher surface charges tend to bind strongly to the membrane, and this increases cellular uptake. However, moderate cationic polymers have shown high insertion efficiency in negatively charged films, whereas highly cationic and anionic polymer displayed reduced interaction with such monolayer films.[117]

Even though cellular uptake is higher for polycations, negatively charged conjugates can also penetrate cellular membranes and are preferential for biomedical use due to lower toxicity profiles. For anionic polypeptides, uptake is also dependent on the absolute value of the zeta potential. For a p(X-Ala$_m$-Lys) series, where X is either free, acetylated, succinylated, or maleilated Glu, polypeptides (Figure 4) with a larger negative charge density (Suc-Glu) displayed greater uptake by murine macrophages when compared to the less negatively charged acetylated and maleilated conjugates since C—C double bond in maleic acid residue is conjugated with C=O, and this influences the state of the terminal carboxylic group.[118]

4.2.2. Zeta Potential in Polymer Conjugate Design

During conjugation, the zeta potential value of the system changes according to the charge of the modifying moiety. For example, CDDP conjugates of PEG$_{114}$-*b*-P(L-Glu)$_{12}$ block copolymer (10% CDDP) displayed a relatively low zeta potential of −3.93 ± 0.5 mV due to the conjugation of the carboxylate anion with platinum.[116] The drug loading

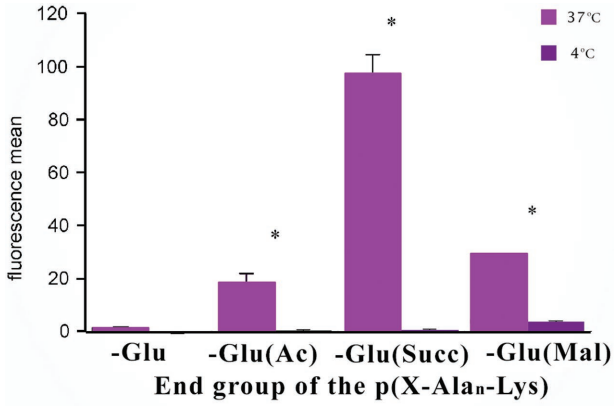


Figure 4. Effect of the terminal group of p(X-Ala$_m$-Lys) on the cellular uptake. Adapted from.[118]

influences the charge density of the nanosystem in direct non-linear proportion. For example, zeta potentials for two samples of P(L-Glu)$_{160}$-PEG$_{5000}$ with 10% and 20% loadings of CDDP was of –17.7 ± 1.0 mV and –8.5 ± 1.3 mV, respectively.[111] This trend seems logical from an electrostatic point of view, although its mathematical expression is often complicated and rarely reported.

In some cases, zeta potential behavior does not follow expected trends. Tsai et al. demonstrated unusual changes in zeta potential for γ-P(L-Glu) conjugated with Dox and galactose. When conjugated with Dox, zeta potential of the system increased from –37 to –27 mV and after conjugation with galactose zeta-potential slightly decreased (–32 mV). This was expected because Dox bears a slightly positive charge while the hydroxyl groups of galactose are slightly negative. However, conjugation of Dox and galactose significantly decreased the zeta potential to –75 mV with no obvious reason.[119] Thus, prediction of synergistic effect on zeta potential remains a serious challenge in the preliminary design of polypeptide conjugates.

Zeta potential can also be controlled by changing the ratio of monomers of opposite charge. For a series of random P(L-Glu)-P(L-Lys) polymers by varying lysine/glutamate content it was possible to change the value of zeta potential from –30.3 to +4.9 mV.[120] Further CDDP loading increased the zeta potential of all nanosystems. For example, a study found that the zeta potentials of aggregates for three compounds (Glutamate/Lysine molar ratio of 4/1, 1.5/1, and 1/1) to be –30.3, –21.8, and –15.5 mV before and –21.0, –19.8, and –10.8 mV after CDDP conjugation, respectively. However, each conjugate displayed different zeta potential changes at different pH. By careful manipulation of drug loading and monomer ratio, the authors managed to tune pH-dependent surface charge reversion at high level of precision.[120]

4.3. Effect of Conformation

The conformation of the polymer chain is not usually considered as a key parameter determining applicability of a conjugate as a drug delivery system. Compared to size and charge, chain conformation has minimal influence on recognition and elimination from the bloodstream, However, conformation strongly affects nanosystem stability, interaction with cellular membranes, and biological activity.[121] For example, PEG-*b*-P(L-Glu)-CDDP and PEG-*b*-P(D-Glu)-CDDP, which adopt helical conformations with different handedness, displayed no differences in biodistribution or anti-cancer activity. However, micelles with random P(D,L-Glu) copolymer matrix, which are unable to form helical conformation, were less active and cleared faster, even though the nanosystem size was similar. The authors concluded that an α-helical structure additionally stabilized micelles and extended their bioavailability.[122] Additionally, polypeptides containing amino acids that stimulate α-helical conformation (Ala, Phe, Leu) are generally much stronger membrane disrupting agents than amino acids stimulating β-sheet conformation (Ile, Val).[12]

The conformation of polypeptides and polypeptide-based conjugates is very sensitive to pH of the solution and to conjugation or, more generally, to the electrostatically equilibrated structure of the nanosystem. Upon pH change, functional groups in the amino acid residues become ionized/neutralized and polymers undergo conformational transitions.[123]

In some cases, conformation is concentration dependent. For example, PEG-P(L-Glu) complexes with polylysines at concentrations of 0.02, 0.05, and higher than 0.1 mg mL^{-1}, micelles existed as random coil, mixed and β-sheet conformation, respectively. Most probably, PEG chains suppress the collapse of the nanosystems into a random coil due to increased surface area/volume ratio.[124]

4.3.1. Effect of Conjugation on Conformation

Conjugation has a complex effect on conformation because, in addition to utilizing the functional groups of the polypeptide, conjugated moieties introduce new spatial and charged elements that change the electrostatic equilibrium. Conjugated molecules can be thus classified as stabilizing or destabilizing. Serine, for example, has tendency to stabilize ordered structures (either α-helix or β-sheet) as was shown for a series of branched P(L-Lys). The incorporation of terminal Ser and Leu in the side-chains of branched P(L-Lys) led to the acquisition of a more ordered (α-helical) conformation in comparison with Ala (Figure 5).[125] Glutamate replacement of leucine in P[L-Lys(Leu$_{1.0}$-Ser$_{0.9}$-DL-Ala$_{7.0}$)] changed the tendency to form helical structure, with a dependence on the amino acid position in the sequence as shown in the Figure 6. Under acidic conditions glutamate-containing polymers tended to stabilize or destabilize helical structures depending on the surroundings. Under appropriate conditions, polymers with both serine and glutamate formed β-sheet structures.[126]

Conjugated moieties allows stabilization of the conjugate conformation in the organism that is especially important for polypeptides with narrow pH range of conformational stability such as P(L-Glu) that tends to form α-helix at pH < 4.5. Conjugation with the hydrophilic anti-cancer agent D-penicillamine stabilized the helix conformation even in a completely water soluble state at pH 7.4 (normal pH of blood plasma).[127,128] The secondary structure of P(L-Glu) can also be stabilized as a helix when functionalized with amine/guanidine as shown in Figure 5.[129] Remarkably, one study achieved stable helicity of up to 90%–95% following γ-4-((2-(piperidin-1-yl)-ethyl)aminomethyl)benzyl incorporation into the P(L-Glu)

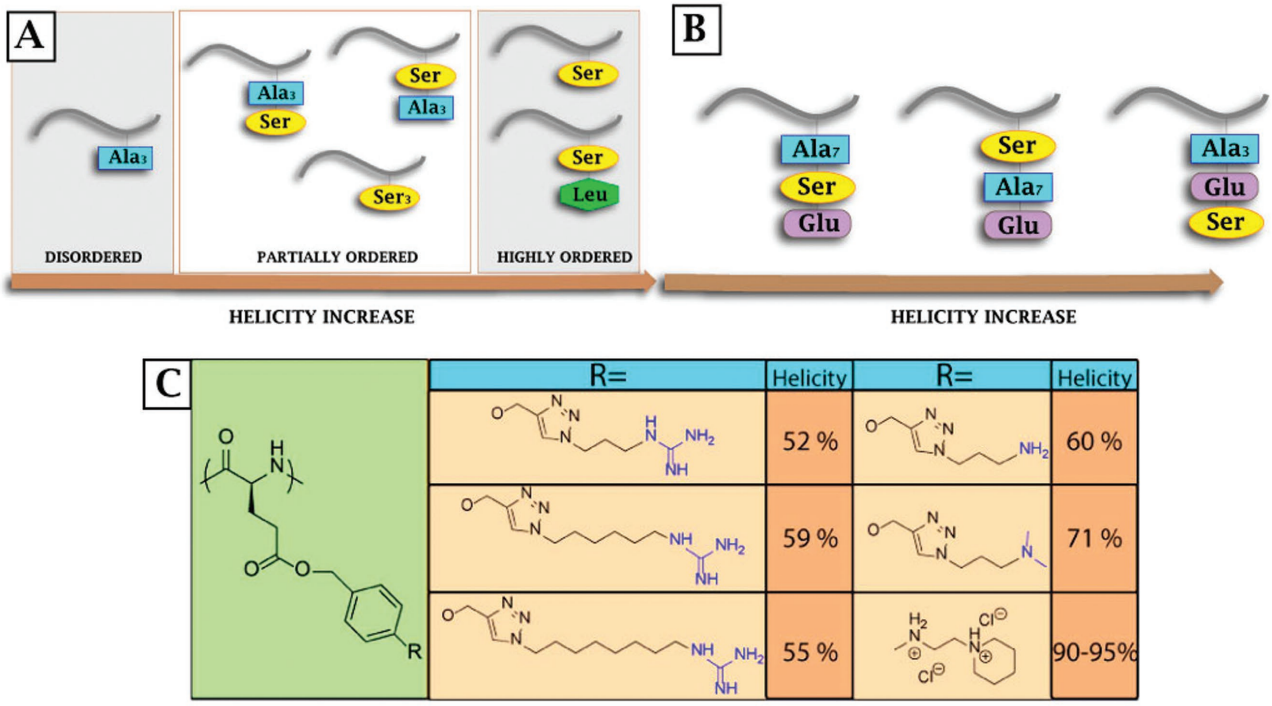


Figure 5. A,B) Effect of the terminal amino acid sequence on the helicity of the PLys adapted from,[125, 126]; C) effect of terminal group on helicity of γ-4-((2-(piperidin-1-yl)ethyl)aminomethyl)benzyl-PGA. Adapted from[129] and[130].

structure. Interestingly, the helical structure remained even after around 40% addition of the other functionalized moieties.[130]

Many active agents have minimal effect on conformation change after conjugation and nanosystem can display behavior similar to a non-modified polypeptide. An illustrative example of such systems is P(L-Glu)-Dox which is negatively charged at pH 7.6 and displays a random coil conformation. At acidic pHs, the charge is neutralized and the conjugate adopts an α-helical conformation, similar to that observed for control P(L-Glu).[131] However, upon Dox conjugation, such conformational change occurs at slightly less acidic environments (pH 5.7 vs 4.5 for P(L-Glu)-Dox and P(L-Glu), respectively).

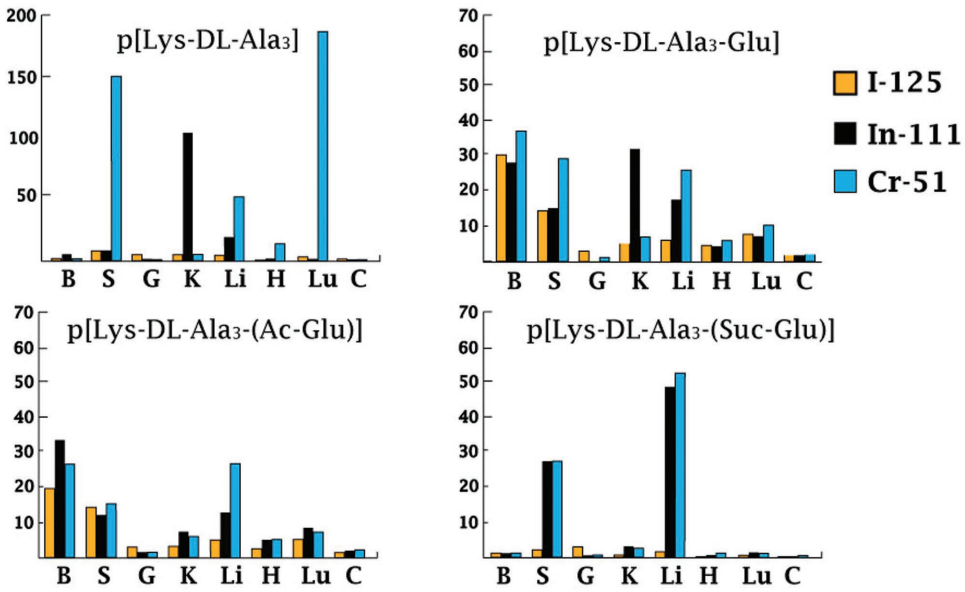


Figure 6. Effect of the terminal group and radioisotope nature on biodistribution. Adapted from[82].

When a conjugated drug destabilizes secondary structure, additional moieties may be incorporated in order to achieve the necessary conformation. Methotrexate (MTX), for example, has a tendency to destabilize the secondary structure of P(Lys-DL-Ala$_x$) but the conjugate can be further stabilized with helix-stabilizing amino acids (such as Leu) or destabilized with Glu.[132] The study also found that the incorporation of D-amino acids into polypeptide polymers with L-chirality mediates the destabilization of secondary structure.

Even small changes to a polypeptide backbone can destabilize polymer conjugate conformation. For example, polycysteine molecules modified with galactose or glucose adopt predominantly α-helical conformation. The oxidation of the thiol group to sulfone destabilized the α-helix and causes a transition to a disordered conformation, perhaps due to the strong interaction of sulfone groups with water molecules. This results in the disruption of peptide hydrophobic packing and the increase of steric crowding that consequently results in the destabilization of the helix. Interestingly, separation of the thioester group by an additional methylene group led the conjugate to retain an α-helical conformation even after oxidation to sulfone.[133]

Chain topology also influences the amount of secondary structure defects. For example, linear and 3-arm star P(ε-carbobenzyloxy-L-Lys) and P(L-Glu-Bz) both displayed an α-helix conformation. However, smaller star-copolymers displayed smaller helix persistence length which may affect the binding sites.[134] Due to increased steric effects and charge repulsion, the helicity of star-copolypeptides is usually lower than that of linear analogues.[135]

4.4. Effect of Geometry

While geometry is an important characteristic to consider during nanomedicine design, there have been few extensive studies on this topic until recent times. However, it is now evident that many nanoconjugate properties, including transport, degradation and release profile, are shape-dependent.[136]

Conjugate diameter affects nanosystem velocity, diffusion, and adhesion to the blood vessels, airways, and intestine. However, shape has more complex effect on these characteristics because non-spherical nanosystems demonstrate flow-dependent alignment. Shape can also affect the targeting ability, as local curvature affects ligand and opsonin adsorption and the degree to which nanosystems fit to the contours of cellular membrane. Conjugate shape changes may lead to increased cell attachment, resistivity to detachment, as well as increased cell internalization.[136] However, nanodisks, as compared to nanospheres, have a tendency to localize within phospholipid bilayers, but not to penetrate the membrane.[137] Shape may even be the main driving attribute in some biological scenarios.[138] Several studies have demonstrated that shape, and in particular local particle shape, and not size, has a dominant influence on phagocytosis when alveolar macrophages where exposed to non-spherical particles of different sizes and shapes.[139,140] This was due to the influence of local shape on macrophage actin structure[140–142] and is an important issue to take into account in the rational design of a given immunotherapy.

4.4.1. Geometry of Conjugates

It has been long understood in polymer chemistry that altering synthetic conditions can generate polypeptide-based polymers with multiple different morphologies.[143] However, there are only a few reports on polypeptide conjugates with a shape other than spherical. Many researchers have synthesized conjugates with ellipsoidal geometry based on P(L-Glu-Bz). This includes the synthesis of nanosystems with ellipsoidal morphology from different γ-P(L-Glu-Bz)-PEG-60 and γ-P(L-Glu-Bz)-Bz-50 copolymers in different ratios.[144] The formation of such mixed nanosystems occurs without conformation change of the initial polymer molecules, as shown for γ-P(L-Glu-Bz)-Bz-50 and P(L-Glu-Bz)-β-CD-50 (cyclodextrin).[145] Both polymers remained in an α-helical conformation before and after nanosystem formation.

For P(L-Glu-Bz)-P(L-Glu) conjugates with CDDP, the polymer and the conjugate adopted ellipsoidal shapes (aspect ratio of 1.3, 37 ± 7 nm $\times$ 27 ± 6 nm) with a hydrodynamic diameter of 49–58 nm. The authors observed no significant changes in the morphology with CDDP loading up to 8.3% .[146]

However, terminal modifications with small targeting molecules do not affect the shape of polymer nanoassemblies. When comparing P(L-Glu-Bz) with benzyl, MeO-PEG, or PEG-biotin as a terminal groups or with benzyl and fluorescein isothiocyanate (FITC isomer I) as terminal groups, nanosystem morphology was dependent on polymer nature. Constructs containing PEG formed spherical species while other nanosystems displayed an ellipsoidal morphology.[147]

Finally, one recent study found that P(L-Glu)-Dox conjugates formed fibril aggregates of approximately 40 nm in diameter and two to tens of micrometres in length.[131]

4.5. Effect of Composition

4.5.1. Influence of Stereoisomery

Stereoisomery of amino acids affects the conformation, biodegradability, and activity of conjugates on the cellular

Macromol. Biosci. **2016**, DOI: 10.1002/mabi.201600316

level. For branched P[L-Lys-DL-Ala-X-cADj] (cAD = cisaconityl daunomycin), conjugates with D- and L-Leu had similar cytotoxicity, while conjugates with D-Glu were seven times less toxic and conjugates with L-Glu were four times more toxic than Leu-containing nanosystems. Stereochemically controlled toxicity is thus dependent on amino acid type and is specific for each system.[148] For corresponding MTX conjugates, replacement of L-Leu and L-Glu with D-analogues produced a four to five fold decrease in activity.[132]

Polypeptides conjugated with D-amino acid sequences behave identically to the L-analogues due to identical physico-chemical properties (except conformation handedness). Poly-D-amino acids, however, are not in-cell degradable as was shown for P(L-Glu) and P(D-Glu) conjugates with the NIR-813 fluorescent dye.[149] Another study found no differences in P(L-Glu)-DTPA-Gd using D- or L-PGlu, indicating that affinity to necrotic tissues is not mediated by specific processes whether an enzymatic or a receptor-mediated transport mechanism is taking place.[113]

4.5.2. Effect of Active Agent Characteristics

The biodistribution of micelles is considerably more affected by size, charge, and surface properties than by the nature of the drug to be delivered, as the drug is usually concentrated in the core.[125] However, upon conjugation, the nature of the drug can determine chain topology leading to different biodistribution and pharmacokinetics when compared to the non-modified polypeptide.

One interesting study constructed a series of conjugated radioactive metals (^{125}I, ^{51}Cr and ^{111}In) and branched P(L-Lys) with short side chains of $(DL\text{-}Ala)_3$, amphoteric $(DL\text{-}Ala)_3$-Glu, polyanionic $(DL\text{-}Ala)_3$-(Ac-Glu), and highly polyanionic $(DL\text{-}Ala)_3$-(Suc-Glu). Similar elution profiles suggested structural similarity of the conjugates independent of the radiolabel used. Interestingly, polymers labeled with different metals demonstrated similar blood levels but different organ distribution as shown on Figure 6.[150] Unfortunately, the reason for these differences in behavior remains undetermined. Despite the fact that radiometals are retained at a higher level at the clearance sites of the matrix polypeptide, the degree of retention depended on both the nature of the polymer and the radiometal used.[150] This study revealed another challenging problem for polypeptide conjugate design, namely understanding how the nature of the drug affects biodistribution of the whole conjugate.

A further study assessed two other conjugates: P[L-Lys-Ala-SucGlu]-Dau with and without terminally conjugated 5(6)-carboxyfluorescein. Remarkably, the conjugate with Dau and 5(6)-carboxyfluorescein was more effective than the Dau conjugate, suggesting that even negligible modification (one to two molecules per conjugate) can play crucial role in cellular uptake.[148]

In the case of the previously discussed γ-P(L-Glu)-Prz conjugates, the polymer matrix did not interfere with the activity of the drug or its binding to the Prz target, the SGLT1 sodium-glucose linked transporter.[62,87]

4.5.3. Effect of Topology

Although scarcely reported, conjugate topology can affect conjugate cellular uptake. In a recent study, star-shape polyglutamates St-P(L-Glu) were directly compared with their linear analogues P(L-Glu) with regards to cell trafficking as well as in vivo biodistribution and pharmacokinetics.[13] Importantly, a 3-fold cell uptake enhancement for the St-P(L-Glu) was demonstrated when compared to a linear counterpart. Both polyglutamates showed similar biodistribution profiles with renal excretion and no specific accumulation in any organ. However, the star-shaped polymer displayed longer retention times and greater distribution volume.[13] Investigations into the cytotoxicity of synthesized poly(γ-4-((2-(piperidin-1-yl)ethyl)aminomethyl)benzyl-L-glutamate) (P(PA-L-Glu-Bz))-containing polymers demonstrated the highest FITC uptake for star copolymer 8-arm PEG_{113}-*b*-$P(PA\text{-}L\text{-}Glu\text{-}Bz)_{91}$ and the lowest for a graft-copolymer. These findings correlated to polypeptide capability in triggering membrane pore formation and suggests that the cytotoxicity of polymer/DNA complexes is cell-dependent and signifies that topology of the polymer affects cytotoxicity.[135]

4.6. Effect of the Linker

In drug design, the scrutiny of the linker role is rarely extended above the cleavage mechanism and stimuli responsibility for controlled release applications. Nevertheless, in addition to its direct function, the linker influences physico-chemical parameters, toxicity, drug loading, and conjugate solubility. For example, incorporation of glutamyl linkers into the P(L-Glu)-PTX conjugate to yield poly(L-γ-glutamyl-glutamine)-paclitaxel nanoconjugate (PGG-PTX) drastically increased conjugate water solubility and showed a better therapeutic index in preclinical models. Maximum tolerated dose for PGG-PTX versus P(L-Glu)-PTX was found to be 350 versus 160 mg PTX kg^{-1}, respectively.[97]

For P(L-Lys citramide) conjugates with Norfloxacin, studies demonstrated spacer-dependent effects on aggregation behavior. Of the three linking strategies used (lysine linker, carbamate linkers, or direct linking to polymer), conjugates with the lysine linker tended to form aggregates. This may be due to electrostatic interactions between the residual primary amine group and the carboxyl groups present as pendent groups along the

polymer chain or covalent amide bonds between amine and carboxylic acid groups. The carbamate space did not lead to detectable levels of aggregation.[151]

Linkers also affect the hydrodynamic radius and zeta potential of the nanosystems. An increase in the number of carbons in the acid motif of the backbone results in a decrease on the hydrodynamic radius and an increase in zeta-potential in the following order Ami > Asp > Glu.[152]

The structure of the linker can also affect conjugate cytotoxicity. When analyzed using the MCF-7 breast cancer cell line, PEG_{45}-P(L-Lys), PEG_{45}-$P(L\text{-}Lys)_{25}$-CPT_6, and PEG_{45}-$P(L\text{-}Lys)_{25}$-SS-CPT_6 (additionally incorporated disulfide bond) displayed IC_{50} values of 500.0, 6.8, and 0.88 μg mL^{-1}, respectively. In general, conjugates were more active against OVCAR-3 cell line and less active against MCF-7, SKOV-3, and MDA-MB-468 cells when compared with free CPT.[99]

Additionally, a γ-P(D-Glu)-vancomycin (Van) conjugate demonstrated linker-dependent anti-bacterial behavior. P(D-Glu)-Van, P(D-Glu)-*N*-succinylethane 1.2-diamine-Van, and free Van displayed an anti-bacterial activity against *Bacillus subtilis* equal to an 8, 9, and 14 mm zone of inhibition at 1 mg mL^{-1}, respectively, using a filter disk assay. Similar results were obtained against Van-resistant strains of *Lactobacillus casei* with inhibition area of 5 and 3 mm for conjugated and free Van, respectively.[153]

4.7. Effect of Ligand Patterning

Simultaneous binding to multiple ligands often occurs in biological systems. The application of polyvalent interactions may be beneficial in drug delivery system design as it allows strong binding from low surface area ligands, increased binding specificity, the creation of conformational contact between large biological surfaces, macroscopic reorganization, and redistribution of molecules.[154] Typical drug conjugate design includes the synthesis of constructs with random surface distribution of ligands that limit or completely restrict manipulations on ligand grouping, cluster size, and spacing. Even though many studies devoted to ligand patterning of small oligolysine with mannose and diglycoside clusters have been published,[155,156] this topic remains scarcely investigated for polypeptide conjugates. Optimal cluster arrangement results in better targeting properties due to longer binding time on the cell surface and binding of more conjugates, all of which increase the probability of endocytosis. In cells overexpressing folate receptors, these receptors exist in the membrane as clusters of three or more molecules. Quite recently, Poon et al. prepared mixed micelles from non-functionalized and functionalized linear dendritic polymer $(P(L\text{-}Asp)\text{-}Bz)_{12}$-PED (polyester Dendron)-PEG_{600}-FAx, where $x = 1$–16 with 0%–100% folate functionalization providing micelles with different cluster arrangement. Maximum

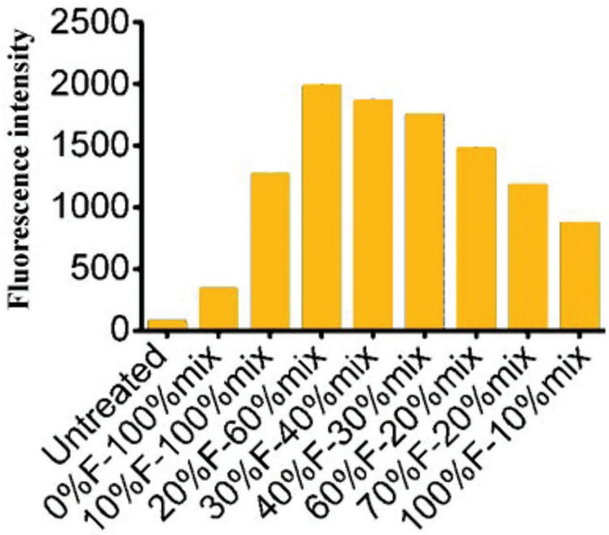


Figure 7. Effect of ligand pattern on the cellular uptake obtained by usage of polymer mixtures. Reprinted with permission from[157].

activity corresponded to the 20%F-60% mix micelles (Figure 7). The binding energy increased with an increase in folate modification, from 10% to 40% (1.5 ± 0.4 to 6.9 ± 0.9 folates/Dendron) and then starts to decrease due to steric binding interference due to an excess of ligands clustered in a small binding area.[157]

Another study demonstrated that P(L-Lys)-catechin conjugates displayed significant enhancement of concentration-dependent inhibition against collagenase from *Clostridium histolyticum* (ChC) compared to free catechin. This may occur due to efficient multivalent interactions between collagenase and catechin from P(L-Lys)-catechin. Catechin conjugates also inhibit hyaluronidase in contrast with non-conjugated catechin.[158]

5. Stimuli-Triggered Drug Release

Smart stimuli responsive materials have greatly evolved over the last few decades. The potential for molecular programming of nanomaterials to respond to small changes in the environment has led to the synthesis of materials in different fields including materials science[159] and biomedicine.[89]

The human body represents a multitude of intrinsic microenvironments appropriate for the spatio-temporal control of drug release. A multitude of systems have so far been described which have the ability to deliver a cargo or to activate the therapeutic effect, in response to an endogenous environmental factor[160] (e.g., pH, redox potential, enzyme availability, or reactive oxygen species) or external stimuli (e.g., magnetic field, temperature, light).[89] Relevant examples of polypeptide conjugates

described so far as drug delivery vehicles with inbuilt endogenously stimulated triggered release mechanisms are discussed within this section.[89,160,161]

5.1. pH-Responsive Drug Release

5.1.1. pH in Relevant Physiological Environments

pH-responsive macromolecules are developed taking into account the pH gradients experienced depending on their pharmacokinetics and pharmacodynamics following the route of administration and arriving to the site of action. In addition, their design should also consider the differential physiological pH environments comparing normal against pathological conditions. The design of active nanosystems responding to pH changes at the tissue level plays a special role for the treatment of solid tumors (and inflammation related pathologies)[162] where many regions are transiently or chronically acidic, playing an important role in tumor progression and metastasis, and therefore highlighting the need of non-invasive techniques for in vivo imaging and measurement of pH.[161,163] At the cellular level, a mildly acidic pH gradient is encountered following endocytic uptake pathway.[77,78]

5.1.2. Polypeptide-Based pH-Responsive Drug Release Systems

Considering that the vast majority of amino acids employed to design polypeptide conjugates are limited to Glu, Asp, and Lys, the chemical toolbox for pH-responsive systems for direct drug conjugation to polypeptide backbone employed up to date is reduced to compatible chemistries with amines and carboxylic acids (e.g., esters, carbamates). However, rationally designed linkers offer the opportunity to expand the toolbox to different pH-labile groups such as hydrazone, acetal, cis-acotinyl, Schiff-base or β-thiopropionate among others, which have all been introduced into pH-responsive polymer–drug conjugates to date.[164]

Pioneering the development of amphiphilic block copolypeptide-drug conjugate micelles, Prof. Kataoka designed a series of PEG-P(L-Asp) block copolymer drug conjugates with the ability to assemble into micelles after conjugation to anthracycline derivatives through pH labile hydrazine bonds (Dox and Epirubicin (Epi)).[95,165] In one remarkable example, Bae et al. modified the surface of PEG-P(L-Asp-hyd-Dox) block copolymer micelles, with an already proven outstanding pH-control on drug release profile (Figure 8A),[95] with piloting residues of folate

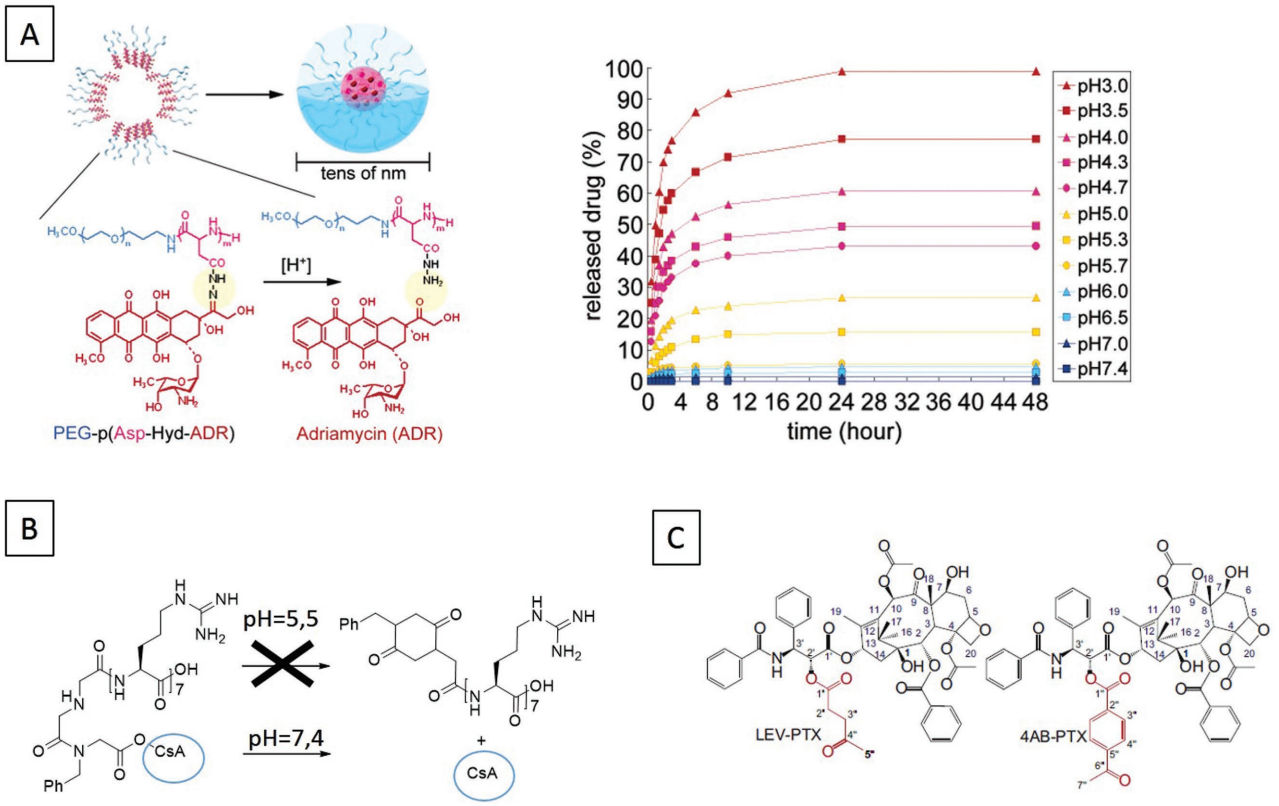


Figure 8. A) Schematic representation of PEG-P(L-Asp-hyd-Dox) block copolymer micelles showing the chemical structure and drug release profile. Reprinted with permission from[95]; B) Chemical structures of PEG-p(Asp-Hyd-LEV-PTX) and PEG-p(Asp-Hyd-4AB-PTX), adapted from[169]; C) Chemical structure of P(L-Arg-CsA) conjugated through a pH-responsive self-immolative linker adapted from[60].

for cancer cell-targeting. In vitro evaluation in human pharyngeal cancer cells proved the advantage of active targeting and an outstanding control of the release profile as a function of pH.[166] Of note, the NC-6300 system, which relies on a similar design, but incorporating Epi without active targeting residues, displayed an improved anti-tumor activity against hepatocellular carcinoma compared to the native Epi with significantly reduced cardiotoxicity.[32] Further proof-of-concept studies also validated NC-6300 against triple negative breast and liver tumors[165] and so granted the enrolment of this candidate into phase I clinical trials for advanced or metastatic tumors.[167] Phase I trials are currently ongoing in Japan with NC-6300.[168]

Modulation of the pH-lability, and as a consequence, the responsiveness to pH, depends on the bond's chemical environment. Following this approach, different PTX prodrugs with modulated release characteristics have been recently published by Alani et al. (Figure 8B).[169] They synthesized two micelle-forming conjugates of PTX: PEG-p(Asp-Hyd-LEV-PTX) and PEG-p(Asp-Hyd-4AB-PTX) using two different modifications of the drug. One in which the hydrazone moiety is modulated through the use of alkylic (LEV) and another using aromatic (4AB) ketones. Micelles displayed a notable release rate of LEV-PTX under acidic pH, although they observed no 4AB-PTX release after 24 h. The authors took advantage of such behavior to modulate the release profile of LEV-PTX by co-assembling mixed micelles of PEG-p(Asp-Hyd-4AB-PTX) and PEG-p(Asp-Hyd-LEV-PTX), so illustrating an alternative pharmacokinetic tuning solution for PTX-prodrug strategies.

Satchi-Fainaro and co-workers recently reported a polyglutamate-based combination conjugate bearing Dox through a hydrazone linker (EMCH) and PTX through direct conjugation (ester bond) as drugs in a synergistic ratio. The combination conjugate was highly effective in inhibiting the growth of mammary tumors compared to a combination of free drugs and drugs conjugated to polymers separately.[170] In an additional study, a comparative evaluation of linear P(L-Glu) vs. dendritic PEG-Polyglycerol bearing the abovementioned combination therapy revealed that activity against mammary tumors for the combination P(L-Glu) conjugate was superior to the dendritic polymer carrier which showed no differences in activity compared to the combination of drugs conjugated to the dendrimer separately.[171]

Aside from hydrazone as a pH-labile linker, other chemical entities such as cis-aconityl moiety have been incorporated into polypeptidic carriers such as P(D-Lys). Wei-Chiang et al. prepared the first model of pH-sensitive linkage for Dau release from a lysosomotropic P(D-Lys) conjugate.[172]

In spite of a lack of polypeptidic carriers designed for topical administration, Rothbard et al. have very elegantly conjugated the immunosuppressor drug cyclosporine A (CsA) to P(L-Arg) oligomers through a pH-labile linker for use in the topical delivery in inflammatory skin diseases.[60] In contrast with the typical release upon acidification, this self-immolative linker (Figure 8C) is stable under acidic pH (skin barrier) and takes advantage of an intramolecular nucleophilic attack of the secondary amine in the neighboring carbonyl group. This is enabled at neutral pH through the deprotonation of the amine. They proved how, in contrast to free CsA which cannot penetrate the skin, R7-CsA could reach dermal T lymphocytes and inhibit cutaneous inflammation in mouse.

5.2. Redox Responsive Drug Release

5.2.1. Redox Characteristics of Relevant Physiological Environments

In general, redox microenvironments at different extracellular and intracellular regions are caused by the presence of reducing/oxidizing agents, as well as redox proteins, providing an opportunity for the controlled delivery of different active agents. At the extracellular level, cysteine and cystine constitute the major source of thiol/disulfide pairs in mammalian plasma (8×10^{-3} M). This is one of the main redox control nodes for protein signaling that, together with glutathione (GSH) marginal levels (2×10^{-6} M), represents the main pathways for disulfide bond reduction in a mildly oxidizing extracellular environment.[173,174] Although systemic circulation of polymeric vehicles are thought to progress under relative oxidizing environment, the above mentioned conditions might promote the inactivation of disulfide-based drug delivery systems with prolonged blood half-life.[175]

The intracellular environment, in contrast, provides a highly reductive environment, mediated by the action of multiple proteins (e.g., thioredoxin, glutaredoxin, protein disulfide isomerase) and small reductant molecules, mainly represented by GSH.[176] Noticeably, the redox environment in endosomes has been reported to be mildly oxidizing when compared to other organelles and cellular environments, suggesting the need for late lysosomal digestion or escape from endosomes in order to efficiently deliver the cargo for reductive dependent strategies.[177] In addition, the tumor microenvironment has been reported to be a highly reductive environment and so represents an attractive target for the development of redox-responsive anti-cancer nanomedicines.[178]

5.2.2. Polypeptide-Based Redox-Triggered Drug Release Systems

Several examples of different redox-sensitive linkers for targeted controlled drug delivery are present in the

literature,[179] but the field is dominated by the employment of disulfide bonds. The fact that disulfide-containing carriers can take advantage of the large intra/ extracellular redox gradient, renders them very attractive for the design of drug and gene-delivery systems.

In the context of polypeptidic conjugates, an interesting study based on P(D-Lys) structures has been developed by Shen and co-workers. They have conjugated MTX to the P(D-Lys) using two different types of spacers and have evaluated its cytotoxic activity in Chinese Hamster Ovary (CHO) cells.[180] In the P(D-Lys)-SS-MTX conjugate, MTX was conjugated to the polymer using a disulfide spacer as a probe to study the reducing cytoplasmic environment. The fact that disulfide reduction did not demonstrate any dependence on GSH led the authors to hypothesize that the conjugate experienced an enzymatic degradation in a prelysosomal compartment. Based on these studies, Feener et al, synthesized an analogue conjugate in which [^{125}I]iodotyramine, instead of MTX, was again linked to P(D-Lys) through a disulfide linker.[181] The resultant P(D-Lys)-SS-[^{125}I]tyn conjugate was used as a probe to detect disulfide cleavage in the endocytic pathway. Several analyses of subcellular fractionations allowed the authors to hypothesize that in spite of the cytosol, as would be expected, the redox environment of the Golgi apparatus was the most probable place of disulfide cleavage.

Using a different approach, Zhou and collaborators recently designed a complex redox-responsive high drug-loaded, tumor targeted nanosystem.[99] This nanoconstruct is based on PEG-P(L-Lys) in which the folate is covalently attached to P(L-Lys) amines through the PEG moiety. The system was FITC-labeled and conjugated CPT to the amine groups of block-PLys via disulfide bonds. Carrying all these different moieties, the CPT-conjugate formed stable nanoparticulate systems in the blood stream with uniform sizes and experienced enhanced accumulation and tumor uptake both passive and actively. These self-assembled conjugates could also be loaded with a secondary active agent (Dox) physically encapsulated inside the core. Following FA-receptor mediated endocytosis, the conjugate was disassembled in the reductive environment of the cytosol via the cleavage of the disulfide linkers releasing CPT and Dox and leading to excellent in vitro anti-tumor activity in various cancer cell lines.

Looking for efficient gene silencing, Takemoto et al. grafted siRNA to the side chains of a PAsp (PAsp-SS-siRNA) polymer through disulfide bonding.[182] This released conjugated siRNA into the cytosolic milieu due to the reductive conditions. Subsequent P(Asp-SS-siRNA) complexation with a poly-L-aspartamide containing 1,2-diaminoethane side chains aimed to enhance biodegradability. Using this strategy, they achieved strong target-specific gene silencing in vitro employing the mouse melanoma B16F10-Luc cell line.

Another interesting example is the polymer-protein conjugate based on P(L-Glu) reported by Talelli and Vicent (Figure 9A).[183] In this system, P(L-Glu) is covalently bounded to lysozyme, used as a model protein, through disulfide bonds and was used for the design of a reduction sensitive PUMPT (Polymer Masked–Unmasked Protein Therapy) system. The P(L-Glu) cover protected and masked the protein efficiently, but disulfide reduction and therefore protein unmasking, restored lysozyme activity. Very recently, this strategy has been validated via the use of a PEG-P(L-Glu) block copolymer for the intraperoxisomal delivery of engineered human alanine:glyoxylate aminotransferase liver peroxisomal enzyme.[184] This can be used to treat primary hyperoxaluria type I, a rare genetic disease characterized by an abnormally high concentration of urinary oxalate that can progress to end-stage-renal-disease and to a potentially fatal condition called systemic oxalosis.

The self-immolative disulfide carbonate (and carbamate) represents another interesting linker for polypeptide conjugate design (Figure 9B).[185] This linker has been employed for the development of a luciferin-releasable system based on a cell penetrating motif, octaarginine. The study conjugated this probe system through the self-immolative disulfide carbonate linker designed for quantification of carrier uptake. Following disulfide cleavage, intramolecular nucleophilic attack of thiol on carbonyl atom at the intermediate specie yields a cyclic product releasing the native probe; a very interesting approach to consider for future designs.

5.3. Enzyme Responsive Release

Enzymes have long been identified as powerful biological targets for site-specific design of drug delivery systems.[186] Many disorders are associated with an enzyme imbalance, mainly overexpression of proteases, including cancer[187] or inflammatory diseases,[188] amongst many notable others. For example, following polypeptide conjugates pathway throughout the body, extracellular proteases such as matrix metalloproteases (MMPs) play an important role in the degradation of extracellular matrix components and cell membrane proteins and are overexpressed in damaged tissues.[189,190] Intracellularly, cathepsins represent the major family of proteases involved in the degradation and turnover of intracellular proteins. Due to their higher activity under acidic pH, endosomal and lysosomal locations are particularly relevant for triggering polypeptide conjugates degradation.[191] Therefore, proteases have constituted a powerful alternative for the design of specific stimuli responsive polypeptide conjugates and their

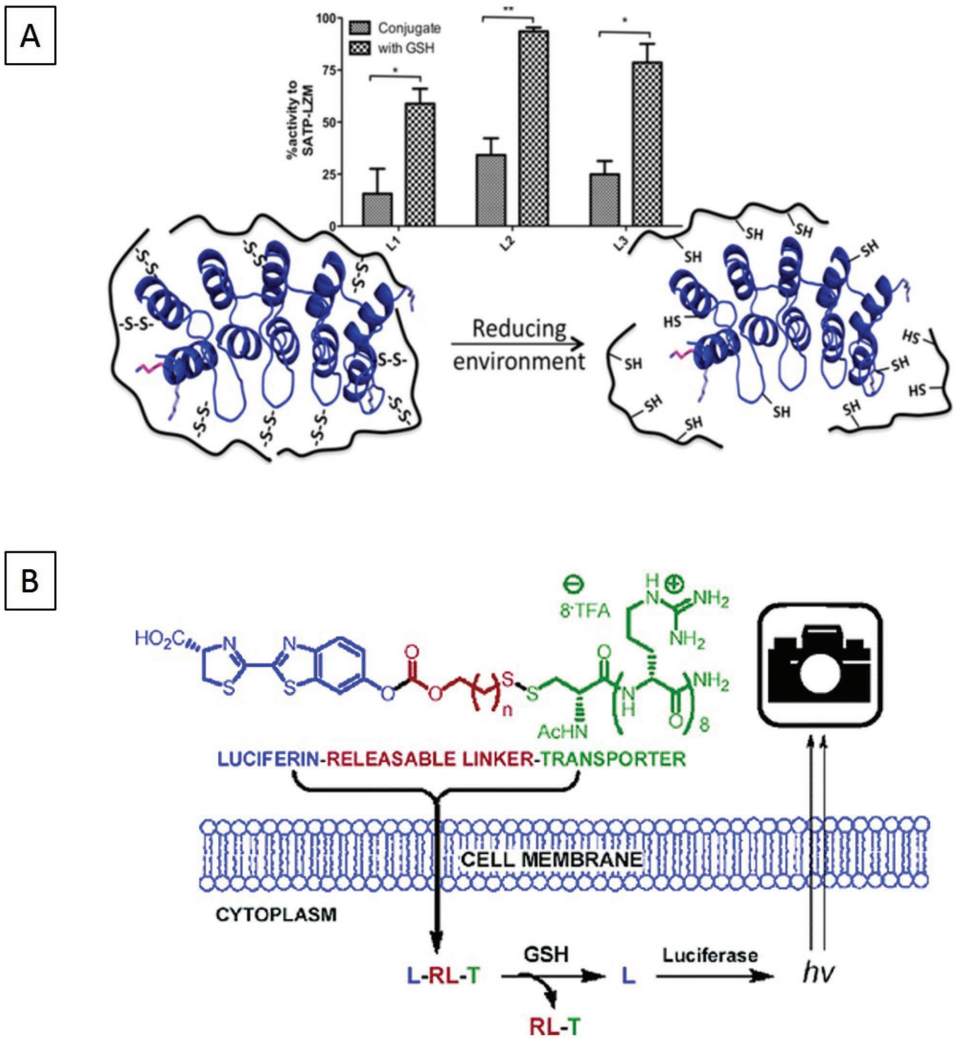


Figure 9. A) Schematic representation of the PUMPT strategy developed with P(L-Glu)-lysozyme polypeptide-protein conjugate sensitive to intracellular GSH levels. Reprinted with permission from[183]; B) Chemical structure and representation of the Luciferin releasable probe conjugated through a self-immolative disulfide-carbonate. Reprinted with permission from[185].

use as a trigger for drug delivery relies on their ability to promote polypeptide matrix degradation and hence facilitation of drug release in a site-specific manner.

The anti-cancer polypeptide-drug P(L-Glu)-PTX (Opaxio) in Phase III clinical trials represents one of the best examples in this class as, although PTX is covalently bound through an ester bond, its 37 wt% loading makes its release highly depending on cathepsin B levels.[33,192] A correlation between estrogen levels and cathepsin B activity has now been reported[149] and therefore only patients with baseline estradiol levels >25 pg mL^{-1} would benefit of this conjugate.[193] P(L-Glu)-CPT is another example of a P(L-Glu)-drug conjugate to reach Phase I clinical trials for ovarian and colorectal carcinomas.[194] CPT was also conjugated via an ester bond but in this case through a Gly linker in order to stabilize the CPT lactone ring.

6. Perspective and Outlook

The physico-chemical parameters of the conjugate, and hence the biological performance, are defined by an intricate interplay of multiple structural factors. This highlights the need for detailed structure-activity relationship studies to develop the hierarchical strategies of polypeptide conjugate design. However, structural complexity also represents a unique opportunity, since small changes at the structural level might endow nanomedicines with outstanding and unexpected biological performance. Future development of polypeptide-drug conjugates may proceed toward the determination of dependencies between physico-chemical parameters and biological activity and to the elaboration of careful synthetic strategies for the design of conjugates with preliminary designated characteristics. Extensive research in the adjacent areas of nanomedicine

Macromol. Biosci. 2016, DOI: 10.1002/mabi.201600316

and polypeptide chemistry has allowed us to delineate possible future trends and opportunities to fully exploit the potential of polypeptide therapeutics:

(1) Different polarity of amino acids and enormous possibilities of side chain modification allow for the synthesis of a variety of architectures. A recent study reports that a combination of soft and rigid helix turns in γ-P(L-Glu-Bz)-block-poly(O-benzyl-L-hydroxyproline) results in the formation of lamellar structures with a "zig-zag" morphology.[195] Additionally, several interesting aggregation modes have been described for polypeptide-based polymers. These include helical rods and rings,[196] disk-like micelles with cylindrical pores,[197] bilamellar vesicles, and bicells.[198] The transfer of different geometries achieved for polypeptide-based polymers to the area of conjugates will allow the expansion of conjugate applicability and, in theory, reveal unknown effects of the geometry on the in vivo response in various pathological conditions. Many polyamino acids display tunable secondary conformation transformation from α-helix to β-sheet[199] and studies show that this conformation can be controlled via multitude of factors as discussed in Section 4.[200] Future development directed toward the synthesis of stimuli-tunable conformation drug conjugates together with full exploitation of amino acids chemical diversity might allow for a step-wise adaption of the polypeptidic vehicle upon interface with biological media allowing for highly specific targeting site and release of the cargo within the body. For example, recent reports have shown the ability of short amphiphilic polyproline fragments to target mitochondria probably due to the specific characteristics of the polyproline helix conformation.[12,201–203] Another interesting approach toward organelle specific delivery relies on an adaptive response through the different stimuli gradients along the body of a P(L-Lys)-block–poly(L-Leu) diblock copolymer with β-carboxylic amidated lysine residues using 2,3-dimethylmaleic anhydride, rendering anionic micelles at neutral solution.[204] As a result, the system demonstrated step-wise hydrolysis following the endocytic uptake pathway resulting in an adaptive charge-reversal of the nanosized micelles optimal for systemic circulation and endocytic uptake, followed by endosomal escape and nuclei internalization.

(2) The shift to increased conjugate biodegradability is not a new paradigm in nanomedicine, but improvements in the in vivo degradability are not always possible. PEGylation remains the main strategy in the synthesis of stealth polypeptide conjugates even though PEG is not biodegradable and could present serious problems associated and hypersensitive reactions (e.g., anaphylactic reactions).[205] Several alternatives to PEG, such as polysarcosine, have recently been proposed,[16,22,206] although their use in the field of polymer conjugates has yet to be reported.

(3) There is a generalized trend to explore novel nanosized delivery systems with increasing complexity and chemical functionality. This is rationally motivated by various factors: the complexity of human body, the underlying biochemical mechanisms in life threatening pathologies, which requires engineered nanomedicines capable of adaptation to a multitude of environments, and the bio-nano interface interactions which exist along the voyage to the site of action. However, there is now a consensus to reach a compromise between complexity, required to achieve highly specific and efficient activity against the target disease, and chemical simplicity, required for translation into real therapies. This necessitates that a given conjugate can be produced in a robust, reproducible, and scalable manner to ensure industrial feasibility and avoid premature failure at clinical trials.[207]

(4) Incorporation of novel chemical functionalities have proven their value in different contexts, and beyond the increase in manufacturing complexity, potential opportunities to improve and complement polypeptide therapeutics toolbox are conceptualized in the following scientific examples with other polymeric drug delivery systems:

a. Despite strong evidence for specific protease-sensitive linkers as powerful biological targets for drug release, this concept has not been widely exploited in polypeptide conjugates. However some polymer-drug conjugates can serve as interesting examples to illustrate this approach and might be adapted through the proper chemistry to polypeptide conjugate design. As relevant examples, Cathepsins B and D cleave specific tetrapeptide sequences,[208] in particular Gly-Phe-Leu-Gly (GFLG) and Gly-Phe-Ala-Leu (GFAL), in tumor cells.[209–213] Other studies have exploited the presence of enzymes in the extracellular environment, such as short peptide sequences (MMP2 cleavable linker = H_2N-GPLGIAGQ-COOH), cleavable by MMPs.[214] While these representative examples highlight the potential of enzyme triggered drug delivery, further studies are still required to obtain precise information of the target enzyme levels at the desired site to fine-control cell uptake and to demonstrate that in vivo drug release is correlated to enzymatic activity.

b. Although poorly explored in polymer (and polypeptide) conjugate design, reactive oxygen species (ROS), mainly generated in the mitochondria through diverse endogenous sources, represent an important trigger for stimulated release.[215,216] A number of existing responsive functionalities have proven the great potential for ROS-mediated drug release via appropriate

linker design (or model compounds) as well as ROS-mediated degradation or response of polymeric materials. These include phenylboronic acids and esters,[217] thioketals,[218,219] poly-L-methionine[220] and poly-L-proline[221,222] among others. As an example, proline is the only natural-occurring amino acid capable of forming a tertiary amide bond. This bond is more easily oxidized as compared to secondary amide bonds and, therefore, could be applied for ROS triggered release of active molecules within polypeptidic conjugates. Although there are still no examples with conjugates, its benefits have been already demonstrated by Sung and co-workers who synthesized different types of ROS cleavable scaffolds based on poly(L-proline) and investigated their applications in tissue engineering.[222] They prepared porous polymeric scaffolds via crosslinking a PEG-poly(ε-caprolactone)-poly(carboxyl-ε-caprolactone) (PEG-PCL-cPCL (4%-86%–10%)) block copolymer with biaminated PEG-oligo(proline)-PEG. Treatment with hydrogen peroxide cleaved all proline residues within 6 d and addition of the ROS generator SIN-1 accelerated this further. This proof of concept study suggests that it may be feasible to adapt synthetic strategies for the incorporation of such responsive linkers toward the development of ROS mediated polypeptide conjugates delivery platforms.

c. Molecular recognition of specific molecules in biological media represents a highly challenging task, mainly due to the complex composition of the physiological environment which requires highly specific and efficient interactions. In this context, the intrinsic ability of phenyl boronic acids and derivatives to interact selectively with diols is of significant note.[223] This approach is highly attractive for the development of glucose sensitive systems toward an on-demand insulin release in response to changes in blood glucose levels. To this end, Zhao et al. have developed a polypeptidic nanogel based on PEG-block-γ-P(L-Glu-Bz)-co-(γ-propargyl-L-Glu-graft-glucose).[224] This system can release physically entrapped insulin in response to specific levels of glucose in phosphate buffered saline. The construct displayed appropriate cytocompatibility and hematocompatibility allowing us to envisage the potential and feasibility of this approach for the chronic treatment of diabetes with on-demand insulin release systems following a polypeptidic conjugate design.

As shown in this review, the multivalency of the different polypeptides together with their biodegradability, the tunable architectural properties including size, shape, zeta potential, conformation, and rationally designed polymer-drug(s) linkers, have already been used in many different clinical applications upon conjugation of drugs or imaging agents as single agents or in combination therapy ranging from i.v. to topical administration. However, an even better understanding of polypeptide chemistry together with the clinical knowledge of the pathological environments to target (enzyme type and concentration, pH, ROS, GSH level, etc.), would allow us to improve the already available medicine armory and more importantly, to identify novel therapeutic approaches for unmet clinical needs. However, we must remember to take into account the robustness and industrial feasibility of polypeptide conjugate production in order to facilitate the transfer from bench to bedside.

Acknowledgements: O.Z. and J.J.A.-C. contributed equally to this work. The authors acknowledge Stuart Atkinson for English editing and the Spanish Ministry of Economy and competitiveness (Grants SAF2013-44848-R, CTQ2010-18195 and SPTQ1300×006465XV0) and the European Research Council (Grants ERC-CoG-2014-648831 MyNano) for financial support.

Received: July 25, 2016; Revised: September 2, 2016; Published online: ; DOI: 10.1002/mabi.201600316

Keywords: drug conjugates; drug delivery systems; nanomedicine; polymer therapeutics; polypeptide

[1] R. Duncan, *J. Controlled Release* **2014**, *190*, 371.
[2] R. Duncan, M. J. Vicent, *Adv. Drug Delivery Rev.* **2013**, *65*, 60.
[3] X. Pang, X. Yang, G. Zhai, *Expert Opin. Drug Delivery* **2014**, *11*, 1075.
[4] Y. Wu, D. Y. W. Ng, S. L. Kuan, T. Weil, *Biomater. Sci.* **2015**, *3*, 214.
[5] Y. Qi, A. Chilkoti, *Curr. Opin. Chem. Biol.* **2015**, *28*, 181.
[6] M. Talelli, M. Barz, C. J. F. Rijcken, F. Kiessling, W. E. Hennink, T. Lammers, *Nano Today* **2015**, *10*, 93.
[7] D. S. He, E. Wagner, *Macromol. Biosci.* **2015**, *15*, 600.
[8] H. Cabral, K. Kataoka, *Sci. Technol. Adv. Mater.* **2010**, *11*, 014109.
[9] H. Jatzkewitz, *Z. Naturforsch, B J. Chem. Sci.* **1955**, *10*, 27.
[10] H. Ringsdorf, *J. Polym. Sci. C—Polym. Symp.* **1975**, 135.
[11] M. S. H. Akash, K. Rehman, S. Chen, *Polym. Rev.* **2015**, *55*, 371.
[12] H. Lu, J. Wang, Z. Song, L. Yin, Y. Zhang, H. Tang, C. Tu, Y. Lin, J. Cheng, *Chem. Commun.* **2014**, *50*, 139.
[13] A. Duro-Castano, J. Movellan, M. J. Vicent, *Biomater. Sci.* **2015**, *3*, 1321.
[14] A. Duro-Castano, R. M. England, D. Razola, E. Romero, M. Oteo-Vives, M. Angel Morcillo, M. J. Vicent, *Mol. Pharmacol.* **2015**, *12*, 3639.
[15] M. L. Amin, J. Y. Joo, D. K. Yi, S. S. A. An, *J. Controlled Release* **2015**, *207*, 131.
[16] M. Barz, R. Luxenhofer, R. Zentel, M. J. Vicent, *Polym. Chem.* **2011**, *2*, 1900.
[17] T. J. Deming, *Prog. Polym. Sci.* **2007**, *32*, 858.
[18] A. Duro-Castano, I. Conejos-Sanchez, M. J. Vicent, *Polymers* **2014**, *6*, 515.
[19] S. Hehir, N. R. Cameron, *Polym. Int.* **2014**, *63*, 943.
[20] N. Hadjichristidis, H. Iatrou, M. Pitsikalis, G. Sakellariou, *Chem. Rev.* **2009**, *109*, 5528.
[21] T. J. Deming, *Chem. Rev.* **2016**, *116*, 786.
[22] N. Gangloff, J. Ulbricht, T. Lorson, H. Schlaad, R. Luxenhofer, *Chem. Rev.* **2016**, *116*, 1753.

[23] R. Luxenhofer, Y. C. Han, A. Schulz, J. Tong, Z. J. He, A. V. Kabanov, R. Jordan, *Macromol. Rapid Commun.* **2012**, *33*, 1613.
[24] A. C. Engler, A. Shukla, S. Puranam, H. G. Buss, N. Jreige, P. T. Hammond, *Biomacromolecules* **2011**, *12*, 1666.
[25] C. R. Cohen, J. Brown, A.-B. Moscicki, E. A. Bukusi, J. R. A. Paull, C. F. Price, S. Shiboski, *PLoS One* **2011**, *6*.
[26] I. McGowan, K. Gomez, K. Bruder, I. Febo, B. Chen, B. Richardson, M. Husnik, E. Livant, C. Price, C. Jacobson, M. T. N. P. Team, *AIDS* **2011**, *25*, 1057.
[27] S. P. Ltd., "VivaGel®-Prevention of recurrence in BV-Phase 3-NCT02236156", **2014**.
[28] C. F. Price, D. Tyssen, S. Sonza, A. Davie, S. Evans, G. R. Lewis, S. Xia, T. Spelman, P. Hodsman, T. R. Moench, A. Humberstone, J. R. A. Paull, G. Tachedjian, *PLoS One* **2011**, *6*.
[29] Z. Feng, Y. Lai, H. Ye, J. Huang, X. G. Xi, Z. Wu, *Cancer Sci.* **2010**, *101*, 2476.
[30] M. E. Fox, S. Guillaudeu, J. M. J. Fre, K. Jerger, N. Macaraeg, F. C. Szoka, *Mol. Pharmacol.* **2009**, *6*, 1562.
[31] J. Nemunaitis, N. Senzer, B. Cooper, M. Nemunaitis, C. Bedell, J. W. Singer, F. B. Oldham, *Int. J. Gen. Med.* **2011**, *4*, 5.
[32] L. V. Kiew, S. K. Cheong, K. Sidik, L. Y. Chung, *Int. J. Pharm.* **2010**, *391*, 212.
[33] C. J. Langer, K. J. O'Byrne, M. A. Socinski, S. M. Mikhailov, K. Lesniewski-Kmak, M. Smakal, T. E. Ciuleanu, S. V. Orlov, M. Dediu, D. Heigener, A. J. Eisenfeld, L. Sandalic, F. B. Oldham, J. W. Singer, H. J. Ross, *J. Thorac. Oncol.* **2008**, *3*, 623.
[34] M. E. R. O'Brien, M. A. Socinski, A. Y. Popovich, I. N. Bondarenko, A. Tomova, B. T. Bilynskyi, Y. S. Hotko, V. L. Ganul, I. Y. Kostinsky, A. J. Eisenfeld, L. Sandalic, F. B. Oldham, B. Bandstra, A. B. Sandler, J. W. Singer, *J. Thorac. Oncol.* **2008**, *3*, 728.
[35] L. Paz-Ares, H. Ross, M. O'Brien, a. Riviere, U. Gatzemeier, J. Von Pawel, E. Kaukel, L. Freitag, W. Digel, H. Bischoff, R. García-Campelo, N. Iannotti, P. Reiterer, I. Bover, J. Prendiville, a. J. Eisenfeld, F. B. Oldham, B. Bandstra, J. W. Singer, P. Bonomi, *Br. J. Cancer* **2008**, *98*, 1608.
[36] S. P. Ltd., "DEP™ docetaxel in patients with advanced cancer. Phase 1 - ", **2014**.
[37] S. Karmaker, T. K. Saha, Y. Yoshikawa, H. Yasui, H. Sakurai, *J. Inorg. Biochem.* **2006**, *100*, 1535.
[38] M. J. Vicent, E. Pérez-Payá, *J. Med. Chem.* **2006**, *49*, 3763.
[39] M. Silva, N. L. Ricelli, O. E. Seoud, C. S. Valentim, A. n. G. Ferreira, D. N. Sato, C. Q. F. Leite, E. I. Ferreira, *Arch. Pharm.* **2006**, *339*, 283.
[40] T. Ke, E.-k. Jeong, D. L. Parker, Z.-r. Lu, *Int. J. Nanomed.* **2007**, *2*, 191.
[41] T. Xing, X. Yang, F. Wang, B. Lai, L. Yan, *J. Mater. Chem.* **2012**, *22*, 22290.
[42] C. Zou, M. Brewer, X. Cao, R. Zang, J. Lin, Y. Deng, C. Li, *Gynecol. Oncol.* **2007**, *107*, 441.
[43] J. W. Singer, R. Bhatt, J. Tulinsky, K. R. Buhler, E. Heasley, P. Klein, P. De Vries, *J. Controlled Release* **2001**, *74*, 243.
[44] S. C. Ghosh, E. Auzenne, M. Khodadadian, D. Farquhar, J. Klostergaard, *Bioorg. Med. Chem. Lett.* **2009**, *19*, 1012.
[45] C. Fante, A. Eldar-Boock, R. Satchi-Fainaro, H. M. I. Osborn, F. Greco, *J. Med. Chem.* **2011**, *54*, 5255.
[46] M. J. Vicent, *Aaps J.* **2007**, *9*, E200.
[47] A. Vaidya, Y. Sun, Y. Feng, L. Emerson, E. K. Jeong, Z. R. Lu, *Pharm. Res.* **2008**, *25*, 2002.
[48] R. Hu, C. He, J. Liu, Y. Wu, J. Li, Z. Feng, J. Huang, H. Xi, Z. Wu, *J. Pharm. Sci.* **2010**, *99*, 3041.
[49] S. Karmaker, T. K. Saha, H. Sakurai, *Macromol. Biosci.* **2007**, *7*, 456.
[50] J. Cui, R. DeRose, J. P. Best, A. P. R. Johnston, S. Alcantara, K. Liang, G. K. Such, S. J. Kent, F. Caruso, *Adv. Mater.* **2013**, *25*, 3468.
[51] A. Takahashi, Y. Yamamoto, M. Yasunaga, Y. Koga, J.-i. Kuroda, M. Takigahira, M. Harada, H. Saito, T. Hayashi, Y. Kato, T. Kinoshita, N. Ohkohchi, I. Hyodo, Y. Matsumura, *Cancer Sci.* **2013**, *104*, 920.
[52] A. Nino-Pariente, V. J. Nebot, M. J. Vicent, *Curr. Pharm. Des.* **2016**, *22*, 1274.
[53] C. Cai, L. Wang, J. Lin, *Chem. Commun.* **2011**, *47*, 11189.
[54] R. Duncan, R. Gaspar, *Mol. Pharmacol.* **2011**, *8*, 2101.
[55] C. J. Cheng, G. T. Tietjen, J. K. Saucier-Sawyer, W. M. Saltzman, *Nat. Rev. Drug Discovery* **2015**, *14*, 239.
[56] S. Mitragotri, J. Lahann, *Nat. Mater.* **2009**, *8*, 15.
[57] M. J. Alonso, N. S. Csaba, *Nanostructured Biomaterials for Overcoming Biological Barriers*, The Royal Society of Chemistry, Cambridge, CB4 0WF, UK **2012**.
[58] R. Duncan, L. Izzo, *Adv. Drug Delivery Rev.* **2005**, *57*, 2215.
[59] M. A. Dobrovolskaia, *J. Controlled Release* **2015**, *220*, 571.
[60] J. B. Rothbard, S. Garlington, Q. Lin, T. Kirschberg, E. Kreider, P. L. McGrane, P. A. Wender, P. A. Khavari, *Nat. Med.* **2000**, *6*, 1253.
[61] K. Sahu, M. Sharma, H. Bansal, A. Dube, P. K. Gupta, *Laser Med. Sci.* **2013**, *28*, 465.
[62] Y. Ikumi, T. Kida, S. Sakuma, S. Yamashita, M. Akashi, *J. Controlled Release* **2008**, *125*, 42.
[63] Y. Y. Zou, H. Fu, S. Ghosh, D. Farquhar, J. Klostergaard, *Clin. Cancer Res.* **2004**, *10*, 7382.
[64] M. A. Islam, P. Bajracharya, S.-K. Kang, C.-H. Yun, E.-M. Kim, H.-J. Jeong, Y.-J. Choi, E.-B. Kim, C.-S. Cho, *J. Nanosci. Nanotechnol.* **2011**, *11*, 7091.
[65] S. Mignani, S. El Kazzouli, M. Bousmina, J.-P. Majoral, *Adv. Drug Delivery Rev.* **2013**, *65*, 1316.
[66] H. Yang, C. T. Leffler, *J. Ocul. Pharmacol. Ther.* **2013**, *29*, 166.
[67] S. D. Li, L. Huang, *J. Controlled Release* **2010**, *145*, 178.
[68] D. E. Owens, N. A. Peppas, *Int. J. Pharm.* **2006**, *307*, 93.
[69] *Targeting of Drugs 6. Strategies for stealth therapeutic systems*, Springer Science Business Media, LLC, London **1998**, p. 294.
[70] S. M. Moghimi, *J. Controlled Release* **2014**, *190*, 556.
[71] I. Tamai, A. Tsuji, *J. Pharm. Sci.* **2000**, *89*, 1371.
[72] A. Duro-Castaño, V. J. Nebot, M. J. Vicent, Eur PCT/EP 2016/067554, **2016**; Eur EP15382422. 2, **2015**.
[73] H. Maeda, J. Wu, T. Sawa, Y. Matsumura, K. Hori, *J. Controlled Release* **2000**, *65*, 271.
[74] F. Canal, J. Sanchis, M. J. Vicent, *Curr. Opin. Biotechnol.* **2011**, *22*, 894.
[75] P. Bonomi, *Exp. Rev. Anticancer Ther.* **2007**, *7*, 415.
[76] A. Z. Wang, *Sci. Trans. Med.* **2015**, *7*, 294ec112.
[77] I. Canton, G. Battaglia, *Chem. Soc. Rev.* **2012**, *41*, 2718.
[78] R. Duncan, S. C. W. Richardson, *Mol. Pharmacol.* **2012**, *9*, 2380.
[79] L. A. Bareford, P. W. Swaan, *Adv. Drug Delivery Rev.* **2007**, *59*, 748.
[80] A. Eldar-Boock, K. Miller, J. Sanchis, R. Lupu, M. J. Vicent, R. Satchi-Fainaro, *Biomaterials* **2011**, *32*, 3862.
[81] S. Biswas, V. P. Torchilin, *Adv. Drug Delivery Rev.* **2014**, *66*, 26.
[82] M. V. Pimm, S. J. Gribben, G. Mezo, F. Hudecg, *J. Labelled Compd. Radiopharm.* **1995**, *36*, 157.

[83] C. Jamieson, E. M. Moir, Z. Rankovic, G. Wishart, *J. Med. Chem.* **2006**, *49*, 7253.
[84] S. A. Shaffer, C. Baker-Lee, J. Kennedy, M. S. Lai, P. De Vries, K. Buhler, J. W. Singer, *Cancer Chemoth. Pharmacol.* **2007**, *59*, 537.
[85] T. Xing, X. Z. Yang, F. Wang, B. Lai, L. F. Yan, *J. Mater. Chem.* **2012**, *22*, 22290.
[86] K. S. Gada, V. Patil, R. Panwar, A. Hatefi, B.-A. Khaw, *Drug Delivery Transl. Res.* **2012**, *2*, 65.
[87] S. Sakuma, T. Sagawa, Y. Masaoka, M. Kataoka, S. Yamashita, Y. Shirasaka, I. Tamai, Y. Ikumi, T. Kida, M. Akashi, *J. Controlled Release* **2009**, *133*, 125.
[88] I. Conejos-Sanchez, I. Cardoso, M. Oteo-Vives, E. Romero-Sanz, A. Paul, A. R. Sauri, M. A. Morcillo, M. J. Saraiva, M. a. J. Vicent, *J. Controlled Release* **2015**, *198*, 80.
[89] Y. Shen, X. Fu, W. Fu, Z. Li, *Chem. Soc. Rev.* **2015**, *44*, 612.
[90] T. J. Deming, *Wiley Interdiscip. Rev.: Nanomed. Nanobiotechnol.* **2014**, *6*, 283.
[91] C. Deng, J. Wu, R. Cheng, F. Meng, H.-A. Klok, Z. Zhong, *Prog. Polym. Sci.* **2014**, *39*, 330.
[92] W. Zhao, Y. Gnanou, N. Hadjichristidis, *Chem. Commun.* **2015**, *51*, 3663.
[93] H. Cabral, Y. Matsumoto, K. Mizuno, Q. Chen, M. Murakami, M. Kimura, Y. Terada, M. R. Kano, K. Miyazono, M. Uesaka, N. Nishiyama, K. Kataoka, *Nat. Nanotechnol.* **2011**, *6*, 815.
[94] Y. Bae, S. Fukushima, A. Harada, K. Kataoka, *Angew. Chem. Int. Ed.* **2003**, *42*, 4640.
[95] Y. Bae, N. Nishiyama, S. Fukushima, H. Koyama, M. Yasuhiro, K. Kataoka, *Bioconjug. Chem.* **2005**, *16*, 122.
[96] J. Vega, S. Ke, Z. Fan, S. Wallace, C. Charsangavej, C. Li, *Pharm. Res.* **2003**, *20*, 826.
[97] S. Van, S. K. Das, X. Wang, Z. Feng, Y. Jin, Z. Hou, F. Chen, A. Pham, N. Jiang, S. B. Howell, L. Yu, *Int. J. Nanomed.* **2010**, *5*, 825.
[98] W. Tai, R. Mo, Y. Lu, T. Jiang, Z. Gu, *Biomaterials* **2014**, *35*, 7194.
[99] Z. Zhou, J. Tang, Q. Sun, W. J. Murdoch, Y. Shen, *J. Mater. Chem. B* **2015**, *3*, 7594.
[100] J. Qian, M. Xu, A. Suo, W. Xu, T. Liu, X. Liu, *Acta Biomater.* **2014**.
[101] M. Silva, A. S. Lara, C. Q. F. Leite, E. I. Ferreira, *Arch. Pharm.* **2001**, *334*, 189.
[102] Y. Xiong, W. Jiang, Y. Shen, H. Li, C. Sun, A. Ouahab, J. Tu, *Biomaterials* **2012**, *33*, 7182.
[103] W. Huang, W. Wang, P. Wang, Q. Tian, C. Zhang, C. Wang, Z. Yuan, M. Liu, H. Wan, H. Tang, *Acta Biomater.* **2010**, *6*, 3927.
[104] K. Jain, U. Gupta, N. K. Jain, *Eur. J. Pharm. Biopharm.* **2014**, *87*, 500.
[105] P. Agrawal, U. Gupta, N. K. Jain, *Biomaterials* **2007**, *28*, 3349.
[106] M. Lee, J. Jeong, D. Kim, *Biomacromolecules* **2015**, *16*, 136.
[107] Y. Pu, S. Chang, H. Yuan, G. Wang, B. He, Z. Gu, *Biomaterials* **2013**, *34*, 3658.
[108] Y. Bae, T. A. Diezi, A. Zhao, G. S. Kwon, *J. Controlled Release* **2007**, *122*, 324.
[109] J. W. Singer, *J. Controlled Release* **2005**, *109*, 120.
[110] J. W. Singer, R. Bhatt, J. Tulinsky, K. R. Buhler, E. Heasley, P. Klein, P. de Vries, *J. Controlled Release* **2001**, *74*, 243.
[111] H. Yu, Z. Tang, D. Zhang, W. Song, Y. Zhang, Y. Yang, Z. Ahmad, X. Chen, *J. Controlled Release* **2015**, *205*, 89.
[112] Z. Chen, D. Yu, C. Liu, X. Yang, N. Zhang, C. Ma, J. Song, Z. Lu, *J. Drug. Targeting* **2011**, *19*, 657.
[113] E. F. Jackson, E. Esparza-Coss, X. Wen, C. Ng, S. L. Daniel, R. E. Price, B. Rivera, C. Charnsangavej, J. G. Gelovani, C. Li, *Int. J. Radiat. Oncol. Biol. Phys.* **2007**, *68*, 830.
[114] F. Ye, T. Ke, E.-K. Jeong, X. Wang, Y. Sung, M. Johnson, Z.-R. Lu, *Mol. Pharmacol.* **2006**, *3*, 507.
[115] N. N. Mahmoud, K. M. Al-Qaoud, A. G. Al-Bakri, A. M. Alkilany, E. A. Khalil, *Int. J. Biochem. Cell B.* **2016**, *75*, 223.
[116] N. J. Klein, G. I. Shennan, R. S. Heyderman, M. Levin, *J. Cell Sci.* **1992**, *102*, 821.
[117] F. Hudecz, M. A. Alsina, F. Reig, *Biopolymers* **2003**, *70*, 323.
[118] R. Szab, L. Peiser, A. Plddemann, S. Bsze, S. Heinsbroek, S. Gordon, F. Hudecz, R. Szabo, *Bioconj. Chem.* **2005**, *16*, 1442.
[119] W. B. Tsai, H. Y. Lai, J. L. Lee, C. W. Lo, W. S. Chen, *Langmuir* **2014**, *30*, 5510.
[120] Y. Huang, Z. Tang, X. Zhang, H. Yu, H. Sun, X. Pang, X. Chen, *Biomacromolecules* **2013**, *14*, 2023.
[121] V. Gimenez, C. James, A. Arminan, R. Schweins, A. Paul, M. J. Vicent, *J. Controlled Release* **2012**, *159*, 290.
[122] Y. Mochida, H. Cabral, Y. Miura, F. Albertini, S. Fukushima, K. Osada, N. Nishiyama, K. Kataoka, *ACS Nano* **2014**, *8*, 6724.
[123] S. C. Shukla, A. Singh, A. K. Pandey, A. Mishra, *Biochem. Eng. J.* **2012**, *65*, 70.
[124] O. F. Mutaf, A. Kishimura, Y. Mochida, A. Kim, K. Kataoka, *Macromol. Rapid Commun.* **2015**, *36*, 1958.
[125] F. Hudecz, M. V. Pimm, v. Rajnavolgyi, G. b. Mezo, A. Fabra, D. Gaal, A. L. Kovacs, A. Horvath, M. r. Szekerke, *Bioconjug. Chem.* **1999**, *10*, 781.
[126] G. Mezo, J. Remenyi, J. Kajtar, K. Barna, D. Gaal, F. Hudecz, *J. Controlled Release* **2000**, *63*, 81.
[127] S. Wadhwa, R. J. Mumper, *J. Controlled Release* **2012**, *158*, 215.
[128] S. Wadhwa, R. J. Mumper, *Mol. Pharmacol.* **2010**, *7*, 854.
[129] R. Zhang, N. Zheng, Z. Song, L. Yin, J. Cheng, *Biomaterials* **2014**, *35*, 3443.
[130] L. Yin, H. Tang, K. H. Kim, N. Zheng, Z. Song, N. P. Gabrielson, H. Lu, J. Cheng, *Angew. Chem. Int. Ed.* **2013**, *52*, 9182.
[131] J. Lai, Y. Huang, *RSC Adv.* **2015**, *5*, 48856.
[132] F. Hudecz, J. A. Clegg, J. Kajtar, M. J. Embleton, M. V. Pimm, M. Szekerke, R. W. Baldwin, *Bioconjug.Chem.* **1993**, *4*, 25.
[133] J. R. Kramer, T. J. Deming, *J. Am. Chem. Soc.* **2012**, *134*, 4112.
[134] A. Gitsas, G. Floudas, M. Mondeshki, H. J. Butt, H. W. Spiess, H. Iatrou, N. Hadjichristidis, *Biomacromolecules* **2008**, *9*, 1959.
[135] L. Yin, Z. Song, K. H. Kim, N. Zheng, H. Tang, H. Lu, N. Gabrielson, J. Cheng, *Biomaterials* **2013**, *34*, 2340.
[136] J. A. Champion, Y. K. Katare, S. Mitragotri, *J. Controlled Release* **2007**, *121*, 3.
[137] Y. Zhang, S. Tekobo, Y. Tu, Q. Zhou, X. Jin, S. A. Dergunov, E. Pinkhassik, B. Yan, *ACS App. Mater. Int.* **2012**, *4*, 4099.
[138] N. P. Truong, M. R. Whittaker, C. W. Mak, T. P. Davis, *Expert Opin. Drug Delivery* **2015**, *12*, 129.
[139] J. A. Champion, S. Mitragotri, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 4930.
[140] D. Paul, S. Achouri, Y.-Z. Yoon, J. Herre, C. E. Bryant, P. Cicuta, *Biophys. J.* **2013**, *105*, 1143.
[141] D. V. Koester, K. Husain, E. Iljazi, A. Bhat, P. Bieling, R. D. Mullins, M. Rao, S. Mayor, *Proc. Natl. Acad. Sci. USA* **2016**, *113*, E1645.
[142] T. A. Masters, B. Pontes, V. Viasnoff, Y. Li, N. C. Gauthier, *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 11875.
[143] L. Zhang, A. Eisenberg, *Science* **1995**, *268*, 1728.
[144] M. E. Martinez-Barbosa, S. Cammas-Marion, L. Bouteiller, C. Vauthier, G. Ponchel, *Bioconjug. Chem.* **2009**, *20*, 1490.

[145] M. E. Martínez-Barbosa, L. Bouteiller, S. Cammas-Marion, V. Montembault, L. Fontaine, G. Ponchel, *J. Mol. Recognit.* **2008**, *21*, 169.
[146] L. De Miguel, I. Popa, M. Noiray, E. Caudron, L. Arpinati, D. Desmaele, G. Cebrian-Torrejon, A. Domenech-Carbo, G. Ponchel, *Pharm. Res.* **2014**, *32*, 1794.
[147] F. Segura-Sanchez, V. Montembault, L. Fontaine, M. E. Martinez-Barbosa, K. Bouchemal, G. Ponchel, *Int. J. Pharm.* **2010**, *387*, 244.
[148] F. Hudecz, J. A. Clegg, J. Kajtar, M. J. Embleton, M. Szekerke, R. W. Baldwin, **1992**, *3*, 49.
[149] M. P. Melancon, W. Wang, Y. Wang, R. Shao, X. Ji, G. J. Gelovani, *Pharm. Res.* **2007**, *24*, 1217.
[150] M. V. Pimm, S. J. Gribben, K. Bogdan, F. Hudecz, *J. Controlled Release* **1995**, *37*, 161.
[151] S. Gac-Breton, J. Coudane, M. Boustta, M. Vert, *J. Drug. Targeting* **2004**, *12*, 297.
[152] Y. Xue, X. Tang, J. Huang, X. Zhang, J. Yu, Y. Zhang, S. Gui, *Colloid Surf. B.* **2011**, *85*, 280.
[153] E. J. F. Prodhomme, A. L. Tutt, M. J. Glennie, T. D. H. Bugg, **2003**, 1148.
[154] M. Mammen, S.-K. Choi, G. M. Whitesides, *Angew. Chem. Int. Ed.* **1998**, *37*, 2754.
[155] E. A. L. Biessen, F. Noorman, M. E. Van Teijlingen, J. Kuiper, M. Barrett-Bergshoeff, M. K. Bijsterbosch, D. G. Rijken, T. J. G. Van Berkel, *J. Biol. Chem.* **1996**, *271*, 28024.
[156] N. Frisont, M. E. Taylor, E. Soilleux, M. T. Bousser, R. Mayer, M. Monsigny, K. Drickamer, A. C. Roche, *J. Biol. Chem.* **2003**, *278*, 23922.
[157] Z. Poon, S. Chen, A. C. Engler, H. I. Lee, E. Atas, G. Von Maltzahn, S. N. Bhatia, P. T. Hammond, *Angew. Chem. Int. Ed.* **2010**, *49*, 7266.
[158] N. Ihara, S. Schmitz, M. Kurisawa, J. E. Chung, H. Uyama, S. Kobayashi, *Biomacromolecules* **2004**, *5*, 1633.
[159] T. Aida, E. W. Meijer, S. I. Stupp, *Science* **2012**, *335*, 813.
[160] H. Chen, D. Liu, Z. Guo, *Chem. Lett.* **2016**, *45*, 242.
[161] S. Bhatnagar, V. V. K. Venuganti, *J. Nanosci. Nanotechnol.* **2015**, *15*, 1925.
[162] E. S. Lee, Z. Gao, Y. H. Bae, *J. Controlled Release* **2008**, *132*, 164.
[163] A. I. Hashim, X. Zhang, J. W. Wojtkowiak, G. V. Martinez, R. J. Gillies, *NMR Biomed.* **2011**, *24*, 582.
[164] X. Pang, Y. Jiang, Q. Xiao, A. W. Leung, H. Hua, C. Xu, *J. Controlled Release* **2016**, *222*, 116.
[165] M. Harada, I. Bobe, H. Saito, N. Shibata, R. Tanaka, T. Hayashi, Y. Kato, *Cancer Sci.* **2011**, *102*, 192.
[166] Y. Bae, W. D. Jang, N. Nishiyama, S. Fukushima, K. Kataoka, *Mol. BioSyst.* **2005**, *1*, 242.
[167] N. Kowa Company, IND Application for NC-6300 (K-912) Epirubicin Micelle in Japan **2013**.
[168] M. Yasuhiro, in *Nanomedicines Design, Delivery and Detection*, 1st ed. (Ed: B. Martin), The Royal Society of Chemistry, Cambridge **2016**.
[169] A. W. G. Alani, Y. Bae, D. A. Rao, G. S. Kwon, *Biomaterials* **2010**, *31*, 1765.
[170] E. Markovsky, H. Baabur-Cohen, R. Satchi-Fainaro, *J. Controlled Release* **2014**, *187*, 145.
[171] H. Baabur-Cohen, L. I. Vossen, H. R. Krüger, A. Eldar-Boock, E. Yeini, N. Landa-Rouben, G. Tiram, S. Wedepohl, E. Markovsky, J. Leor, M. Calderón, R. Satchi-Fainaro, *J Controlled Release* **2016**.
[172] W. C. Shen, H. J. P. Ryser, *Biochem. Biophys. Res. Commun.* **1981**, *102*, 1048.
[173] P. S. Samiec, C. Drews-Botsch, E. W. Flagg, J. C. Kurtz, P. Sternberg, R. L. Reed, D. P. Jones, *Free Radical Biol. Med.* **1998**, *24*, 699.
[174] D. P. Jones, J. L. Carlson, V. C. Mody, J. Y. Cai, M. J. Lynn, P. Sternberg, *Free Radical Biol. Med.* **2000**, *28*, 625.
[175] T. Ishida, M. J. Kirchmeier, E. H. Moase, S. Zalipsky, T. M. Allen, *Biochim. Biophys. Acta Biomembr.* **2001**, *1515*, 144.
[176] K. M. Cai, J. Yen, Q. Yin, Y. Liu, Z. Y. Song, S. Lezmi, Y. F. Zhang, X. J. Yang, W. G. Helferich, J. J. Cheng, *Biomater. Sci.* **2015**, *3*, 1061.
[177] C. D. Austin, X. H. Wen, L. Gazzard, C. Nelson, R. H. Scheller, S. J. Scales, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 17987.
[178] L. L. Policastro, I. L. Ibanez, C. Notcovich, H. A. Duran, O. L. Podhajcer, *Antioxid. Redox Signaling* **2013**, *19*, 854.
[179] L. Liu, P. Liu, *Front. Mater. Sci.* **2015**, *9*, 211.
[180] W. C. Shen, H. J. P. Ryser, L. Lamanna, *J. Biol. Chem.* **1985**, *260*, 905.
[181] E. P. Feener, W. C. Shen, H. J. P. Ryser, *J. Biol. Chem.* **1990**, *265*, 18780.
[182] H. Takemoto, A. Ishii, K. Miyata, M. Nakanishi, M. Oba, T. Ishii, Y. Yamasaki, N. Nishiyama, K. Kataoka, *Biomaterials* **2010**, *31*, 8097.
[183] M. Talelli, M. J. Vicent, *Biomacromolecules* **2014**, *15*, 4168.
[184] A. Roncador, E. Oppici, M. Talelli, A. Niño-Pariente, C. Borri Voltattorni, M. J. Vicent, B. Cellini, *Nanomedicine: Nanotechnol. Biol. Med.* **2016**.
[185] L. R. Jones, E. A. Goun, R. Shinde, J. B. Rothbard, C. H. Contag, P. A. Wender, *J. Am. Chem. Soc.* **2006**, *128*, 6526.
[186] X. S. Puente, L. M. Sanchez, C. M. Overall, C. Lopez-Otin, *Nat. Rev. Genet.* **2003**, *4*, 544.
[187] A. J. Littlewood, I. Evans, G. Bilbe, W. B. Bowler, D. Farley, B. Wlodarski, T. Kokubo, T. Inaoka, J. Sloane, D. B. Evans, J. A. Gallagher, *Cancer Res.* **1997**, *57*, 5386.
[188] J. A. Liu, G. K. Sukhova, J. S. Sun, W. H. Xu, P. Libby, G. P. Shi, *Arterioscler. Thromb., Vasc. Biol.* **2004**, *24*, 1359.
[189] M. Egeblad, Z. Werb, *Nat. Rev. Cancer* **2002**, *2*, 161.
[190] R. P. Iyer, N. L. Patterson, G. B. Fields, M. L. Lindsey, *Am. J. Physiol. Heart Circ. Physiol.* **2012**, *303*, H919.
[191] B. Turk, D. Turk, V. Turk, *Biochim. Biophys. Acta Prot. Struct. Mol. Enzymol. y* **2000**, *1477*, 98.
[192] S. A. Shaffer, C. Baker Lee, A. Kumar, J. W. Singer, *Eur. J. Cancer* **2002**, *38*, S129.
[193] T. M. Beer, C. Ryan, J. Alumkal, C. W. Ryan, J. Sun, K. M. Eilers, *Anticancer Drugs* **2010**, *21*, 433.
[194] G. M. Springett, C. Takimoto, M. McNamara, J. H. Doroshow, S. Syed, E. Eastham, D. Spriggs, S. Pezzulli, G. Michelson, J. Dupont, *J. Clin. Oncol.* **2004**, *22*, 226S.
[195] M. Gkikas, J. S. Haataja, J. Seitsonen, J. Ruokolainen, O. Ikkala, H. Iatrou, N. Houbenov, *Biomacromolecules* **2014**, *15*, 3923.
[196] C. Cai, J. Lin, T. Chen, X.-S. Wang, S. Lin, *Chem. Commun.* **2009**, 2709.
[197] X. Lin, X. He, C. q. Hu, Y. Chen, Y. Mai, S. Lin, *Polym. Chem.* **2016**.
[198] H. R. Marsden, J.-W. Handgraaf, F. Nudelman, N. A. J. M. Sommerdijk, A. Kros, N. Rod, R. Peptosomes, *J. Am. Chem. Soc.* **2010**, *132*, 2370.
[199] C. Chen, D. Wu, W. Fu, Z. Li, *Biomacromolecules* **2013**, *14*, 2494.
[200] S. Zhang, C. Chen, Z. Li, *Chin. J. Polym. Sci. Eng. Ed.* **2013**, *31*, 201.
[201] D. Kalafut, T. N. Anderson, J. Chmielewski, *Bioorg. Med. Chem. Lett.* **2012**, *22*, 561.

[202] I. M. Geisler, J. Chmielewski, *Pharm. Res.* **2011**, *28*, 2797.
[203] L. Li, I. Geisler, J. Chmielewski, J.-X. Cheng, *J. Controlled Release* **2010**, *142*, 259.
[204] S. S. Han, Z. Y. Li, J. Y. Zhu, K. Han, Z. Y. Zeng, W. Hong, W. X. Li, H. Z. Jia, Y. Liu, R. X. Zhuo, X. Z. Zhang, *Small* **2015**, *11*, 2543.
[205] U. M. Sahiner, S. T. Yavuz, M. Gokce, B. Buyuktiryaki, I. Altan, S. Aytac, M. Tuncer, A. Tuncer, C. Sackesen, *Pediatr. Int.* **2013**, *55*, 531.
[206] K. Klinker, M. Barz, *Macromol. Rapid Commun.* **2015**, *36*, 1943.
[207] O. C. Farokhzad, R. Langer, *ACS Nano* **2009**, *3*, 16.
[208] Y. Choe, F. Leonetti, D. C. Greenbaum, F. Lecaille, M. Bogyo, D. Bromme, J. A. Ellman, C. S. Craik, *J. Biol. Chem.* **2006**, *281*, 12824.
[209] H. Maeda, Y. Matsumura, *Crit. Rev. Ther. Drug Carrier Syst.* **1989**, *6*, 193.
[210] H. Soyez, E. Schacht, S. Van der kerken, *Adv. Drug Delivery Rev.* **1996**, *21*, 81.
[211] T. Minko, P. Kopeckova, J. Kopecek, *Pharm. Res.* **1999**, *16*, 986.
[212] J. Kopecek, P. Kopeckova, *Adv. Drug Delivery Rev.* **2010**, *62*, 122.
[213] F. M. Veronese, O. Schiavon, G. Pasut, R. Mendichi, L. Andersson, A. Tsirk, J. Ford, G. F. Wu, S. Kneller, J. Davies, R. Duncan, *Bioconjug. Chem.* **2005**, *16*, 775.
[214] L. Zhu, P. Kate, V. P. Torchilin, *ACS Nano* **2012**, *6*, 3491.
[215] C. C. Song, F. S. Du, Z. C. Li, *J. Mater. Chem. B* **2014**, *2*, 3413.
[216] Q. H. Xu, C. L. He, C. S. Xiao, X. S. Chen, *Macromol. Biosci.* **2016**, *16*, 635.
[217] Y. F. Zhang, Q. Yin, L. C. Yin, L. Ma, L. Tang, J. J. Cheng, *Angew. Chem. Int. Ed.* **2013**, *52*, 6435.
[218] D. S. Wilson, G. Dalmasso, L. X. Wang, S. V. Sitaraman, D. Merlin, N. Murthy, *Nat. Mater.* **2010**, *9*, 923.
[219] M. S. Shim, Y. N. Xia, *Angew. Chem. Int. Ed.* **2013**, *52*, 6926.
[220] A. R. Rodriguez, J. R. Kramer, T. J. Deming, *Biomacromolecules* **2013**, *14*, 3610.
[221] S. S. Yu, R. L. Koblin, A. L. Zachman, D. S. Perrien, L. H. Hofmeister, T. D. Giorgio, H. J. Sung, *Biomacromolecules* **2011**, *12*, 4357.
[222] S. H. Lee, T. C. Boire, J. B. Lee, M. K. Gupta, A. L. Zachman, R. Rath, H. J. Sung, *J. Mater. Chem. B* **2014**, *2*, 7109.
[223] R. J. Ma, L. Q. Shi, *Polym. Chem.* **2014**, *5*, 1503.
[224] L. Zhao, C. S. Xiao, J. X. Ding, X. L. Zhuang, G. Q. Gai, L. Y. Wang, X. S. Chen, *Polym. Chem.* **2015**, *6*, 3807.

Macromol. Biosci. **2016**, DOI: 10.1002/mabi.201600316