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Recent Advances in Drug Delivery Systems based on Polypeptides Prepared from *N*-Carboxyanhydrides

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Abstract

Recent years have seen a dramatic increase in interest in the use of ring-opening polymerization of *N*-carboxyanhydride (NCA) monomers as a method to prepare well-defined polypeptides and peptide-hybrid materials. The resulting molecules are often capable of assembling into a variety of different structures, including micelles, vesicles, nanoparticles and hydrogels, and therefore have been explored as novel drug delivery systems. Peptides are attractive in this regard due to their rich chemical functionality and ability to assemble through the formation of secondary structures (for example α -helices and β -sheets). In addition, they are inherently biodegradable and biocompatible. This review describes recent advances in the field, covering aspects such as improved methods with which to prepare better-defined polypeptides, crosslinking of assemblies to enhance biostability, the preparation of materials that respond to a variety of stimuli (including light and intra- or extracellular redox conditions), functionalization with targeting ligands to enhance cellular uptake, assemblies for siRNA delivery and approaches to theranostic systems.

Introduction

Even after decades of research, efficient drug delivery to diseased tissues still represents a significant challenge. The small molecule nature of most potent drugs is largely responsible as they frequently possess poor solubility, undergo rapid metabolism and can freely diffuse in both healthy and diseased cells resulting in non-specific distribution in the body. The use of macromolecular drug delivery systems has proven very successful in increasing the efficacy of delivery of such therapeutics due to their increased circulation time and enhanced ability to enter and be retained within tumors.

Polypeptide-based materials are ideal for drug delivery systems due to their biocompatibility, biodegradability, tunable structural architecture and ability to form secondary structures. Amphiphilic polypeptide materials can self-assemble into a plethora of different architectures through non-covalent interactions, possessing the ability to encapsulate and deliver drugs. By adopting ordered conformations such as α -helices and β -sheets, the use of polypeptide-based materials allows better control over nanoscale structure formation. The self-assembly of amphiphilic polymers can be directed by controlling chemical and physical parameters, such as hydrophobic-hydrophilic ratio, molecular weight and chemical nature.^{1,2} A straightforward route to synthetic polypeptide materials is through ring opening polymerization (ROP) of *N*-carboxyanhydrides (NCAs).³⁻⁵ NCAs are versatile precursors to homo-polypeptides and block copolypeptides. The polymerization technique is particularly useful in the design of peptide-hybrid materials, which combine peptide segments with non-peptidic macromolecular architectures. These hybrids hold great potential due to the combination of the desirable features of non-peptidic synthetic polymers, such as solubility and processability, with those of polypeptides *i.e.* secondary structure and biocompatibility.^{6,7}

Recently, an abundance of research has been carried out into the use of polypeptide-based vehicles as drug delivery systems. Assemblies such as micelles, vesicles, hydrogels, nanogels, capsules, fibres, dendritic and macromolecular drug conjugates, nucleic acid complexes and polyplexes have all been reported.⁸⁻¹⁵ Some of the systems are well established and investigated, and are currently in clinical trials.^{9,16} Most of the formulations in clinical use or trials at the moment are simple monofunctional micellar systems. The next phase of drug delivery research, already well underway, is the introduction of multifunctionality to the systems to achieve highly efficient delivery, with minimal side effects for the patient. The ultimate drug delivery system should have a number of important additional characteristics as well as biocompatibility; it should be stable to dilution in biological media, have stealth properties to avoid uptake by the reticuloendothelial system and only release drug at the required site in a controlled manner. Methodology to achieve these goals is under intense investigation. The biostability of delivery systems can be enhanced by modification of the chemical building blocks for crosslinking. Stealth properties can be introduced into carriers through incorporation of synthetic polymers with inherent stealth properties such as poly(ethyleneglycol) (PEG). A number of approaches can aid release of drugs specifically in the required tissues, including introduction of stimuli-responsiveness to systems and creation of systems that actively target certain cells or certain cell characteristics. Recently, a number of thorough reviews have been published on the subject of polypeptides, poly(amino acids) and stimuli responsive drug delivery systems.^{5,7,10,12,16-18}

This review will focus on the latest advances in multifunctional polypeptide-based drug delivery systems created solely from ROP of NCAs. Initially an overview of the synthetic strategy, *i.e.* ROP of NCAs, will be given. In the remaining sections block copolypeptides and peptide-hybrid block copolymer systems which form nanoparticles (micelles and vesicles primarily), will be

discussed in terms of (I) increasing the biostability of the systems, (II) stimuli responsiveness, (with particular focus on redox- and photo-responsive systems) and (III) recent advances in active targeting and additional functionalities. There will be some overlap in the sections especially with regards to stimuli-responsiveness, as the majority of the systems discussed are multifunctional.

Synthetic Strategy: ROP of NCAs

Reports of ROP of NCAs have been widespread in the literature for over 100 years since their first introduction by Leuchs *et al.*^{4,19,20} The polymerization is most commonly initiated by primary amines, however, the existence of two possible mechanisms, namely the normal amine mechanism (NAM) and activated monomer mechanism (AMM) has always rendered it problematic to gain precise control of the reaction (Figure 1). Nonetheless, in the past number of years there have been major advances in this area making ROP of NCAs a convenient and efficient method to generate synthetic polypeptides and peptide-based block copolymers on a multi-gram scale. The key to gaining control is to prevent or at least limit the AMM route. This can be achieved in a number of ways. **Deming *et al.* employed transition metal catalysts which undergo oxidative addition with NCA monomers thus preventing deprotonation of the NCA N-H**^{21,22}. **Lu *et al.* used trimethylsilylated primary amines which produce NCA ROP via a group transfer mechanism.**^{23,24} Both types of initiator significantly reduce the polymerization time, as well as allowing precise control and resulting in well-defined polymers. Schlaad and co-workers utilized simple amine hydrochloride salts as initiators resulting in good control but with longer reaction times (e.g. three days).²⁵ The use of highly purified NCA monomers (e.g. *via* flash column chromatography), the optimization of experimental technique (e.g. high vacuum) and investigation of reaction conditions (e.g. temperature, pressure) can also result in good control.²⁶⁻

²⁹ In work comparable to that of Schlaad, Conejos-Sánchez *et al.* have reported using ammonium salts with non-nucleophilic tetrafluoroborate anions as initiators for the ROP.³⁰ Most recently, Zou *et al.* have detailed a facile method for well-controlled ROP of NCAs involving simply flowing nitrogen through the polymerization mixture.³¹

Non-peptidic synthetic polymers with terminal amines can also be used as initiators for the ROP resulting in peptide-hybrid copolymers, where one block is a homo-poly(amino acid) and the other is a synthetic polymer with advantageous properties. These peptide hybrid block copolymers have potential inherent biocompatibility and stealth properties owing to the properties of the non-peptidic polymers used such as PEG. PEG is widely used as the hydrophilic portion of amphiphilic block copolymers as it facilitates solubility in aqueous solution through steric stabilization, prevents protein adsorption and can protect the peptide block from enzymatic degradation. This results in decreased uptake by the liver, increased circulation time in the bloodstream and thus enhanced bioavailability. A number of excellent reviews have previously been published on this topic.^{5,6}

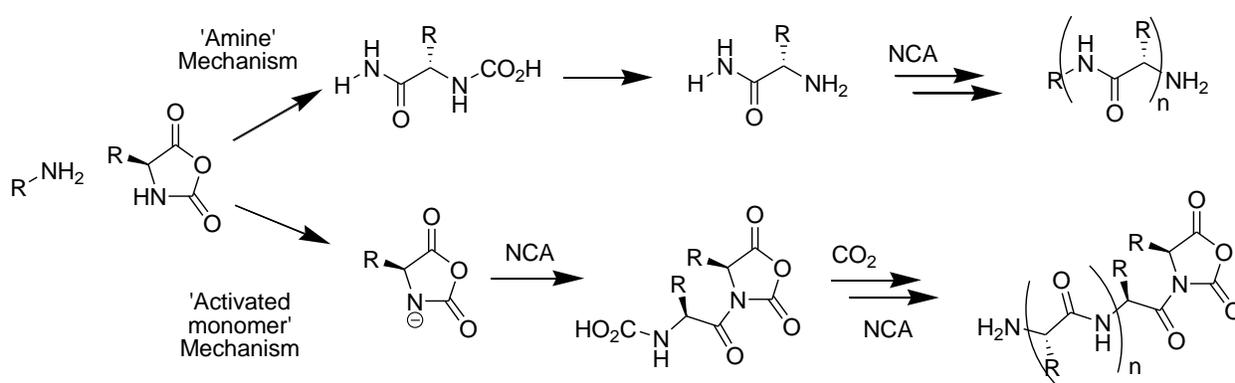


Figure 1 Mechanisms for ROP of NCAs when using primary amine initiation

1. Crosslinking for Increased Biostability

After biocompatibility, one of the most basic requirements of a drug delivery system is stability in the body. If a system is not stable in biological fluids this could result in burst release of the payload in non-targeted areas. Increased stability results in increased blood circulation time and increased bioavailability. The most commonly used method for increasing the stability of systems is crosslinking. Table 1 depicts a summary of the crosslinked nanoparticles that are discussed in this section. Nanoparticles can be shell-crosslinked or core-crosslinked or both. Shell-crosslinked particles can obstruct burst release of the encapsulated drug, while crosslinking the particle core can enhance the stability of the systems by preventing disintegration on dilution. Crosslinking can be carried out using small molecule crosslinking agents or by stimulating the self-crosslinking ability of certain systems for example with UV light. There are occasionally some disadvantages associated with crosslinking including complicated crosslinking routes and low reaction efficiency, extensive purification and expensive crosslinkers. Systems with the ability to self-crosslink bypass such problems and usually involve mild conditions thereby eliminating inter-particle crosslinking and avoiding aggregation. Additionally, in some circumstances it is not desirable to decrease the biodegradability of the particle and slow the release of the payload. Some recent advances have been made to avoid potential problems in this area, such as the introduction of unimolecular micelles and tri-domain particle systems wherein neither the shell nor the core is the crosslinked region, thereby not interfering with drug action or release.

Table 1 Summary of crosslinked particles discussed and their responsiveness

Polymer	Architecture	Crosslinker	Responsiveness	Reference
PEG-PAsp-P(Leu-co-Tyr)	Micelle	Iron (II) Chloride	pH	31
PEG-PAsp-PPhe	Micelle	Ketal	pH	32
PEG-PAsp	Micelle	1,6-hexanediamine	pH	33
PGA-PLys-r-DOPA	Vesicle	Oxygen	pH	39
PEG-PCys-PPhe	Micelle	Hydrogen peroxide	Redox	41
PEG-PCys-PGA	Nanogel	Cys NCA	Redox	43
PEG-Cys-Phe (Star)	Nanogel (Unimolecular micelle)	Cys	Redox	44
PLys-ss-PEG	Micelle	Cystamine	Redox	46
PEG-P(propargyl-Glu)	Micelle	Bis(Z-azidoethyl) disulfide (BADDS)	Redox	47
P(diethyleneglycol-L-Glu)-tetramine (Star)	Micelle (unimolecular)	Disulfide tetramine	Redox	48
PEG-P(Glu-co-cinnamyl-Glu)	Micelle	UV light	Photo	37
PEG-P(cinnamyl-Glu)	Micelle	UV light	Photo	38

1.1 Crosslinking with External Factors (small molecule agents, self- and photo-crosslinking)

1.1.1 Micelles

Tri-domain micellar systems with core-shell-corona morphology have recently been reported by Rios-Doria *et al.*³² The system is pH-responsive and based on PEG-*b*-poly(aspartic acid)-*b*-poly(D-leucine-co-tyrosine). The poly(D-leucine-co-tyrosine) comprises the core (drug reservoir) and PEG forms the corona with a middle layer or shell of poly(aspartic acid). Using iron (II) chloride, crosslinks are formed between the metal and the carboxylic acid groups of the poly(aspartic acid) layer, which allows release of drug at low pH such as that found in a tumour microenvironment. The system is versatile and capable of encapsulating a range of hydrophobic anticancer drugs with high loading efficiency, due to the use of both D- and L- stereoisomers of amino acids in the core of the micelles. This disrupts the secondary helical structure of the polypeptide introducing a random coil structure thereby allowing for greater drug loading. The crosslinked systems displayed enhanced stability *in vivo*. A similar approach has been taken by

Lee *et al.* who have reported core-shell-corona micelles consisting of PEG-poly(L-aspartic acid)-poly(L-phenylalanine) on this occasion with pH hydrolysable ketal crosslinks (Figure 2).³³ Later, Bae and coworkers crosslinked the poly(aspartate) portion of diblock PEG-poly(aspartate) nanoassemblies with 1,6-hexanediamine using carbodiimide coupling.³⁴ Grafting to synthesize hyperbranched polymers is also a method of potentially increasing the stability of assemblies. By preparing grafted nanoassemblies, where PEG-poly(benzylaspartate) grafts were conjugated to PEG-poly(aspartic acid), a comparison of the stability of both grafted and crosslinked systems was possible. The crosslinked systems proved to be much more stable and were shown to encapsulate doxorubicin and release it in a controlled manner in response to pH.

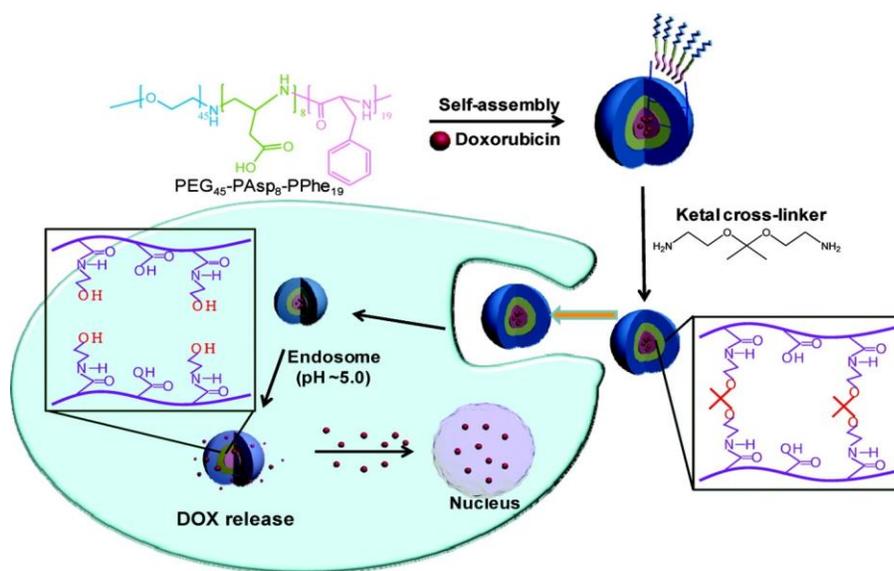


Figure 2 Illustration of shell cross-linking of doxorubicin-loaded polymer micelles with a pH-labile ketal cross-linker and intracellular release triggered by endosomal pH³³. Reprinted with permission from Lee *et al.*, *Biomacromolecules*, **2011**, *12*, 1224-1233. Copyright 2011 American Chemical Society.

Photo-crosslinkable systems are desirable as the process is usually fast, effective, can be well-controlled and achieved in mild conditions. Systems that exploit similar photo-responsiveness for

triggered drug delivery functions will be discussed in detail later. One of the most frequently used photo-crosslinkable groups is the cinnamic group due to its excellent UV responsiveness.³⁵⁻
³⁷ Ding *et al.* reported photo-crosslinkable micelles based on diblock and triblock copolymers of PEG-poly(L-glutamic acid-co- γ -cinnamyl-L-glutamate) prepared by post-ROP modification of the glutamic acid block with cinnamyl alcohol.³⁸ Upon UV exposure the micelles were crosslinked in water and were shown to be able to release the drug Rifampin. In comparable work, Yan *et al.* have reported photo-crosslinked micelles based on PEG-poly(γ -cinnamyl-L-glutamate) this time from polymerization of a cinnamyl-modified NCA monomer.³⁹ The latter method resulted in better control over functionalities and architectures. It was possible to load and release Paclitaxel from the micelles, with the crosslinked systems displaying a slower release rate than the non-crosslinked.

1.1.2 Vesicles

Micelles are the forerunners of drug delivery systems and have been extensively studied.⁷ However, polymer vesicles or polymersomes are emerging as more versatile systems since they can be used to deliver both hydrophobic and hydrophilic drugs simultaneously. While numerous reports of micelle crosslinking exist, there are relatively few dealing with the crosslinking of vesicles. Deming and coworkers prepared DOPA-containing diblock copolypeptides that self-assembled into vesicles. The DOPA residues were crosslinked under oxidative conditions which produced a marked increase in membrane stability.⁴⁰ Another example was reported by Sulistio and co-workers, where the inner layer of the shell of a polypeptide vesicle was crosslinked *via* an oxygen-mediated process.⁴¹ The vesicles were based on poly(glutamic acid)-*block*-poly(lysine)-*ran*-(3,4-dihydroxyphenylalanine) (DOPA) formed by hexamethyldisilazane-initiated ROP of the corresponding NCAs. Crosslinking the vesicles was carried out at pH 12 where the poly(lysine)-

ran-poly(DOPA) block forms the core of the particle. *In situ* crosslinking with oxygen resulted in the oxidation of the phenolic groups of the DOPA residues to DOPA-quinone species, which then underwent dimerization or attack by the pendent amines of the lysine residues. The crosslinked vesicles were shown to be more stable than the non-crosslinked counterparts, maintaining their structure when the pH was lowered to 3, whereas the non-crosslinked ones changed their configuration (Figure 3).

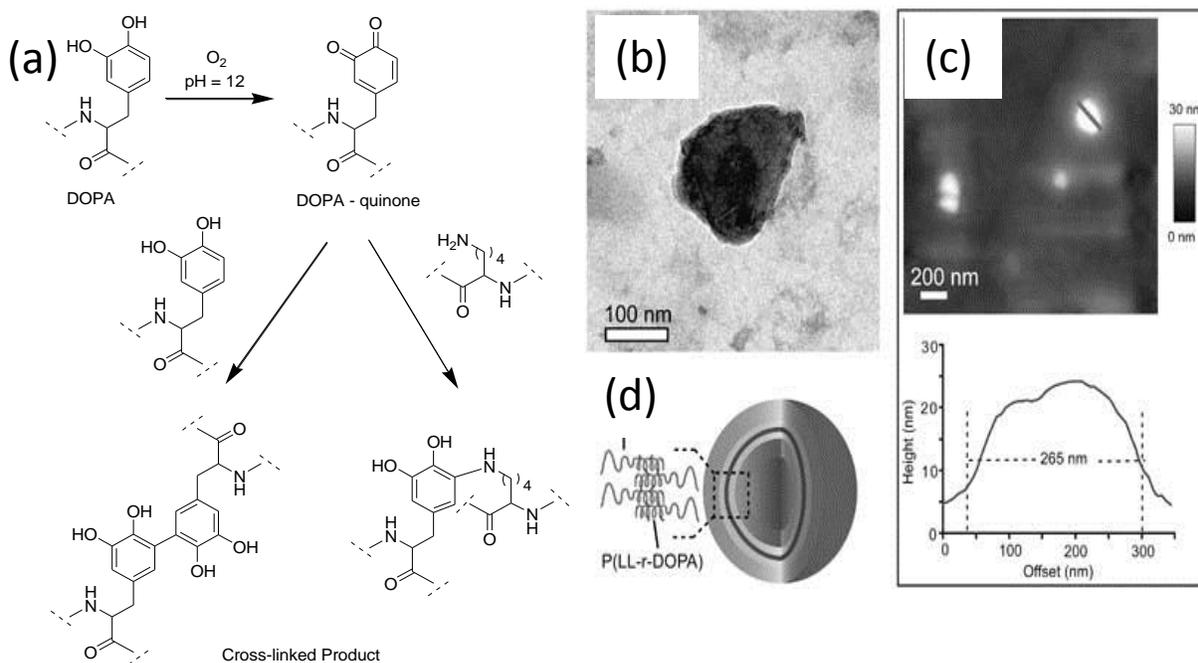


Figure 3 (a) Crosslinking of DOPA at pH 12 *via* generation of DOPA-quinonic intermediates (b) TEM image and (c) AFM image with z-profile analysis of the cross-linked vesicle from PLGA-*b*-P(LL-*r*-DOPA) block copolymers self-assembly at pH = 12. (d) The proposed structure of the cross-linked vesicle at pH = 12. Reproduced with permission (John Wiley & Sons) from ⁴¹.

1.2 Crosslinking with disulfide bonds: Redox-responsive polymers

A popular method of introducing crosslinking into systems is through the use of disulfide bonds, which can result in redox-responsive polymers.⁴² The development of polymers, which respond differently to intra- and extracellular redox conditions is a promising area in tailored drug delivery. Redox-sensitive nanoparticles are selectively cleaved in the cytoplasm by glutathione (GSH), a tripeptide found in cells at millimolar concentrations which degrades disulfide bonds to the corresponding thiols. Disulfide bonds can be introduced into block copolymers by using disulfide-containing monomers or disulfide-containing crosslinkers/initiators.

1.2.1 Nanoparticles from Disulfide-containing Monomers

In order to create redox-responsive polypeptides, monomers containing cysteine are the most commonly used. Wang *et al.* prepared redox-sensitive shell cross-linked PEG-polypeptide hybrid micelles with core-shell-corona morphology based on PEG, cysteine and phenylalanine.⁴³ The micelles were self-crosslinkable through the oxidation of the thiol groups on the poly(cysteine) portion, using dilute hydrogen peroxide. *In vitro* drug release studies show that the micelles could rapidly release the drug payload in response to the intracellular GSH level, and reduce drug loss in the extracellular environment.

Disulfide-containing nanoparticles can also be core-crosslinked and when this is the case they are sometimes termed nanogels.^{41,44} A core-crosslinked nanogel with potential drug delivery applications has been reported by Xing *et al.*⁴⁵ The nanogel is prepared by the ROP of two NCAs, L-cysteine and γ -benzylglutamate initiated by an amine-terminated PEG to form a peptide-hybrid copolymer with stealth properties. The cysteine NCA is a bifunctional monomer in that it can act as both a monomer and crosslinker *via* the disulfide linkages. The resultant nanogel had a core-shell structure as evidenced by DLS, TEM and AFM with a size of 250 nm. It

disassembled in the presence of dithiothreitol (DTT) indicating crosslinking of the core by disulfide bonds. An investigation into the loading and release of the anti-cancer drug indomethacin was carried out showing efficient loading and release behaviour.

As aforementioned, crosslinking can have some limitations and unimolecular micelles, such as those formed from multi-arm star amphiphilic polymers, can be used as alternatives since they commonly display enhanced stability without hindering drug release. Ding and co-workers reported star copolymers created from ROP of L-cysteine and L-phenylalanine initiated by amine-terminated PEG.⁴⁶ The polymers were shown to form redox-responsive nanogels in solution and accelerated release of doxorubicin was observed in GSH pretreated HeLa cells as opposed to non-pretreated cells.

1.2.2 Nanoparticles from Disulfide-containing Linkers/Initiators

Use of disulfide-functionalized initiators to carry out the polymerization is an efficient method of introducing redox-responsiveness. Ding *et al.* have recently shown the increased efficacy of redox-responsive micelles based on poly(lysine) created by ROP of the lysine NCA, initiated by disulfide-functionalized PEG.⁴⁷ Doxorubicin was loaded into the micelles and the biocompatibility (hemolytic, cell and tissue compatibilities) were proven. Ren and co-workers have reported sheddable micelles which can detach their shells upon reduction stimulus, prepared in a similar manner using a cystamine-conjugated PEG to initiate the ROP of leucine NCA.⁴⁸ The micelles showed accelerated doxorubicin release in the presence of DTT; 40% of the drug was released within 12 hours in the presence of DTT whereas it took 100 hours to release only 25% in the absence.

Redox-responsive crosslinked micelles can also be achieved using click chemistry. Cheng *et al.* prepared micelles from the self-assembly of PEG-*b*-poly(γ -propargyl-L-glutamate) generated from NCA polymerization.⁴⁹ The micelles were subsequently crosslinked by click chemistry between bis(2-azidoethyl)disulfide (BADs) and the pendent alkynyl groups. Crosslinking was found to increase the stability of the micelles as well as increasing the drug-loading capacity due to the compacted crosslinked core. The micelles showed an accelerated release profile of doxorubicin in reductive conditions and higher cell proliferation inhibition towards HeLa cells pre-treated with glutathione.

A more inherently stable unimolecular star system was recently introduced by Liu and co-workers.⁵⁰ These dual-responsive redox-sensitive and thermo-responsive micelles were prepared by ROP of diethyleneglycol-L-glutamate initiated by a tetra(amine) with a disulfide-bond core. An attractive feature of these systems is that they also form hydrogels at certain concentrations (<4 wt%). They were shown to be redox- and thermo-responsive and to have the mechanical properties that can be controlled by the disulfide bond core and star shape (Figure 4).

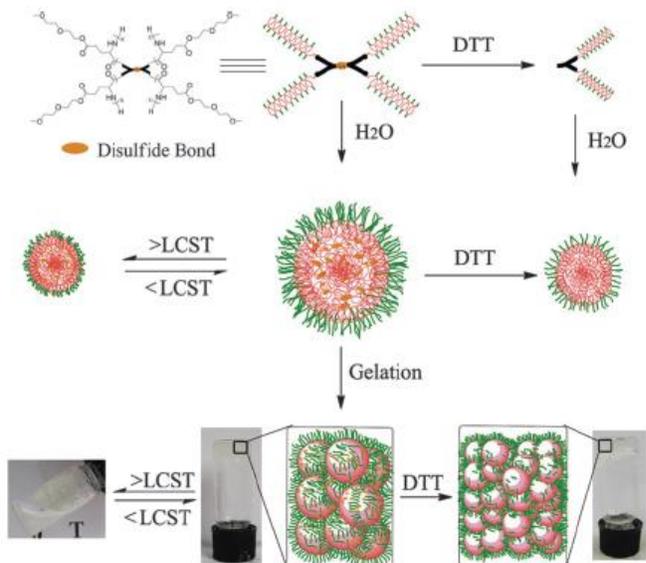


Figure 4 Illustration of dual stimuli-sensitive micellization and hydrogelation process of star polypeptide. Reproduced from Ref. ⁵⁰ with permission from The Royal Society of Chemistry.

2. Advances in Stimuli-Responsive/Triggered Release

As demonstrated by the preceding discussion on redox-responsive examples, one of the most exciting areas of growth in drug delivery is the development and advancement of stimuli-responsive polypeptide delivery systems. Very intricate and elegant systems have been reported over the past two decades.^{51,52} Multifunctional stimuli-responsive or ‘smart’ polypeptide drug delivery materials allow increasing control over the release of payloads, combining the possibility of efficient delivery to the required sites with decreased distribution of payloads in unwanted areas of the body. There are many other external stimuli that can be harnessed in a similar manner to redox, including pH, temperature, light, ultrasound, electronic and magnetic fields, biomolecules, enzymes and chemicals. A number of excellent reviews on stimuli responsive polymer systems have been published previously.^{17,53,54} This part of the review will

focus on recent developments in light responsive polypeptide particle systems in particular and their use in drug delivery.

2.1 Photoresponsive Systems

Recently, photoresponsive drug delivery vehicles have come to the forefront of polymer research owing to the precise spatial and temporal control that such systems allow.⁵⁵ Light-responsive carriers possess non-invasive stimuli responsiveness, biocompatibility and remote activation and also offer the possibility of quick release of payload as compared to other stimuli-sensitive carriers e.g. pH-responsive. A number of photoresponsive moieties are regularly used in this area including nitrobenzyl, azobenzene and spiropyran groups. Light-responsive systems can be prepared by homo-/copolymerization of photoresponsive monomers, by post-polymerization functionalization or by initiation of the polymerization with the photoresponsive moiety.

Most recently Liu *et al.* have reported the first synthesis of a photoresponsive polypeptide-hybrid polymer from ROP of a photoresponsive *S*-(*o*-nitrobenzyl)-L-cysteine *N*-carboxyanhydride, initiated by amino-terminated PEG.⁵⁶ The polymers were shown to self-assemble into spherical nanoparticles in aqueous solution and displayed a reduction in size after irradiation, indicating their responsiveness to light. Controlled release of doxorubicin was achieved by changing the light irradiation times which induced the gradual photocleaving of the poly(nitrobenzylcysteine) core of the particles and reduction in micelle diameter (Figure 5).

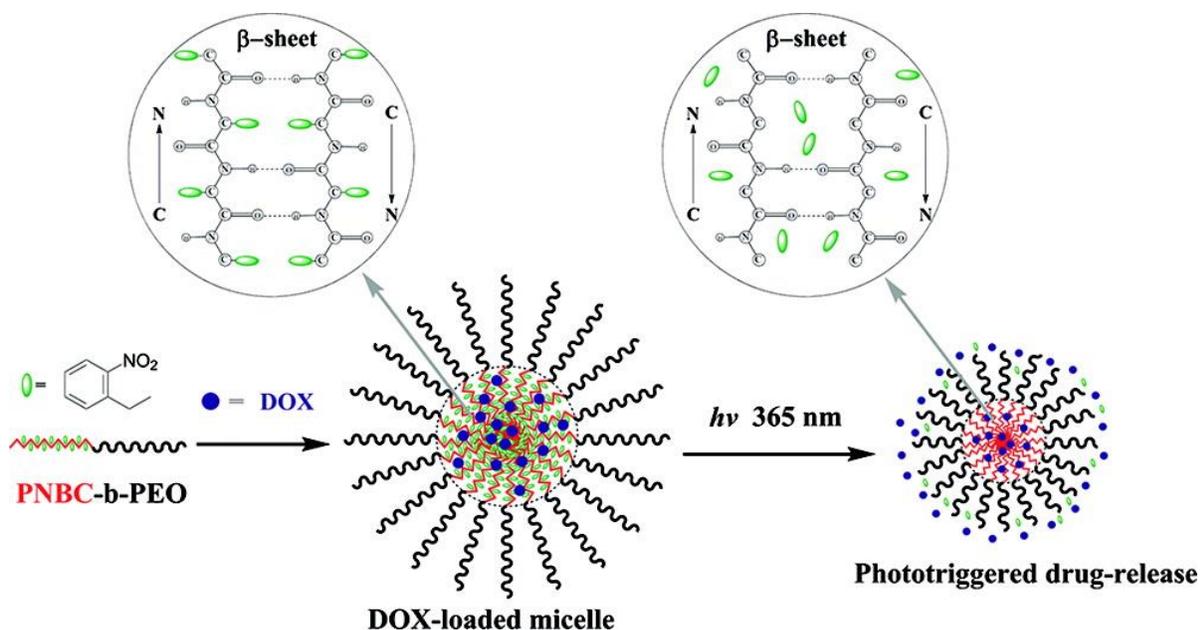


Figure 5 Schematic representation of self-assembly and photo-triggered drug release from poly(*S*-(*o*-nitrobenzyl)-*L*-cysteine)-*b*-PEG⁵⁶. Reprinted with permission from Liu & Dong, *Biomacromolecules* **2012**, *13*, 1573-1583. Copyright 2012 American Chemical Society

There are a number of reports of photoresponsive amphiphilic block copolymers with spiropyran (SP) and azobenzene groups attached, which are also activated by UV-visible light. Mezzenga *et al.* reported the preparation of photoresponsive polypeptide-hybrid diblock copolymers based on a SP-modified poly(glutamic acid) by post-polymerization modification, from amine-terminated PEG-initiated ROP (Figure 6).⁵⁷ The glutamic acid was functionalized with SP groups *via* esterification of the free carboxylic acid groups after deprotection of the benzyl moieties. The amphiphilic block copolymers self-assembled into flower-like micelles and micellar aggregates. Dissolution and assembly of micelles was shown to be controllable by light, although no drug loading or release data was reported.

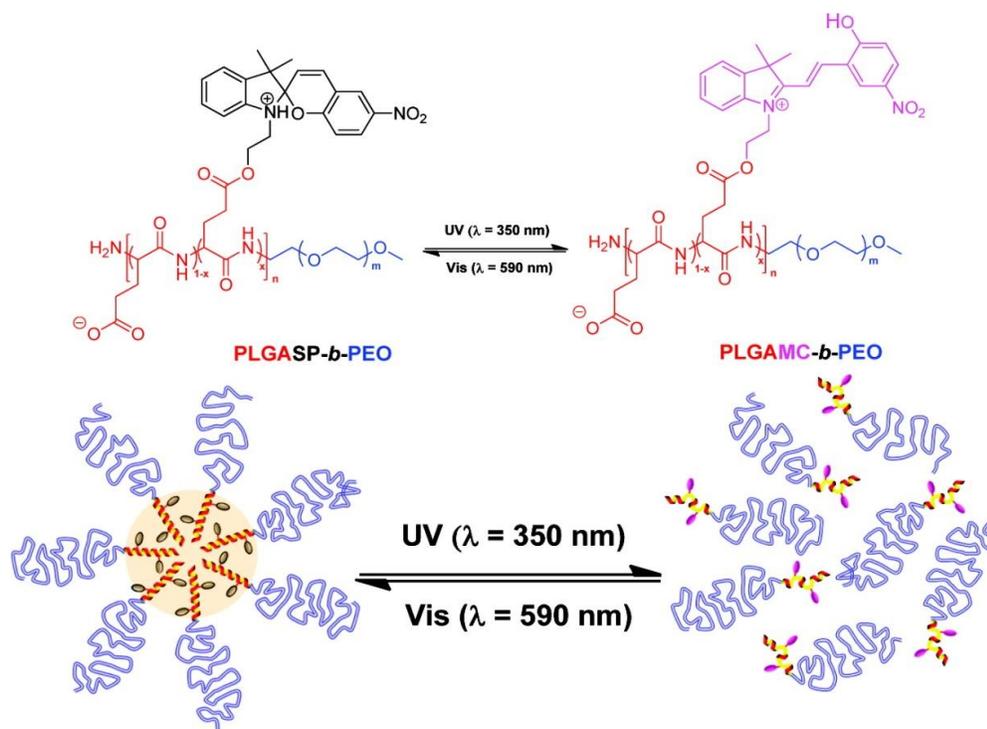


Figure 6 Schematic representation of photoresponsive micellization-dissolution process for spiropyran-poly(glutamic acid)-*b*-PEG⁵⁷. Reprinted with permission from Kotharangannagari *et al.*, *Macromolecules* **2011**, *44*, 4569-4573. Copyright 2011 American Chemical Society.

One of the drawbacks, however, of using UV light is the lack of penetration into tissues and the possibility of tissue damage. As an alternative, near-infrared light (NIR), can penetrate deep into tissues with less damage than UV or visible light and as such would be a highly desirable method for control of drug release. Kumar *et al.* have reported the development of a NIR light-sensitive polypeptide block copolymer by functionalizing poly(glutamic acid)-PEG hybrids with a number of NIR two-photon-absorbing chromophores.⁵⁸ Upon exposure to NIR light the chromophore is photocleaved, destabilizing the micelle due to a shift in the hydrophobic-hydrophilic balance. NIR-responsive release of the antibacterial drug Rifampicin (RIF) and the anti-cancer agent Paclitaxel (PCT) were investigated separately.

There are a number of systems which combine different types of stimuli sensitivity displaying multi-stimuli responsiveness.⁵³ Light-sensitive and temperature-sensitive triblock copolymers based on two PEG-poly(alanine) diblocks coupled by an azobenzene were reported by Jeong *et al.*⁵⁹ Exposure to UV light switches the azobenzene to its *trans* form and larger micelles are formed in aqueous solution as opposed to the *cis* form. The system is also temperature-responsive due to the PEG-poly(alanine) blocks and as such shows good potential as a drug delivery vehicle although no drug studies have been reported.

2.2 Other Systems

Chemical substances are also being explored as stimuli for drug delivery.⁶⁰ An advantage of this method of stimulation is that the activity and concentration of chemical substances can be easily controlled by external modification, which is not the case with other stimuli. Recently, Wang *et al.* introduced the possibility of using β -cyclodextrin as a trigger molecule by incorporating adamantane into micelles and thereby making use of host-guest chemistry. The *N*-hydroxysuccinimide ester of adamantanamine was coupled to Z-L-lysine and subsequently converted to the NCA (lys(Ad) NCA) using α,α -dichloromethylmethyl ether. ROP of this NCA was carried out using a tetra-amino-modified PEG (TAPEG) resulting in a four-armed block copolymer capable of forming micelles in solution (PLys(Ad)₂-*b*-PEG-*b*-PLys(Ad)₂). The diameter of the micelles in response to β -cyclodextrin was measured, and the assemblies were shown to be dissociated after 24 hour exposure. In the absence of β -cyclodextrin the micelles are very stable with no change in diameter over two weeks. The release of doxorubicin from the micelles was found to be accelerated in the presence of β -cyclodextrin (Figure 7).

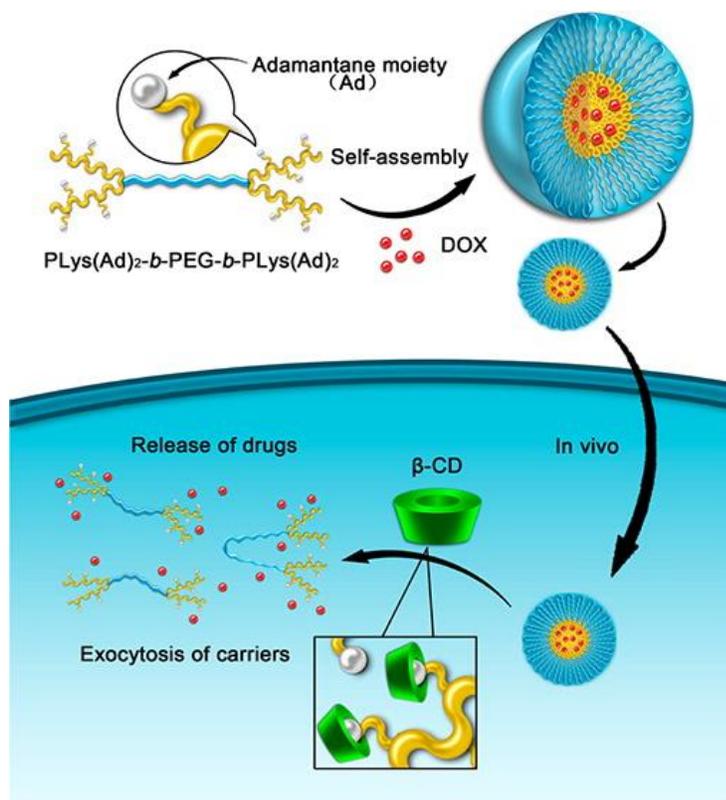


Figure 7 Schematic illustration of the formation of micelles and triggered drug release mechanism⁶⁰. Reprinted with permission from Wang *et al.*, *Macromolecules* **2013**, 2, 201-205. Copyright 2013 American Chemical Society.

3.0 Functionalization for Active Targeting and Promotion of Cell Uptake

The majority of the drug delivery systems discussed thus far act *via* passive targeting, namely the enhanced permeation and retention (EPR) effect which results in accumulation of the particles in tumours. The ability to actively target specific cells is desirable for increased efficiency of delivery and reduced side effects. A number of micelles with active targeting capabilities are in clinical trials.⁹ There are several of methods of functionalizing polypeptides with targeting ligands including post-polymerization modification, for example conjugation using click

chemistry,^{29,61} or polymerization of NCA monomers with attached functional moieties.^{62,63} Many targeting ligands have been incorporated into various polypeptide systems. The nature of the ligand usually exploits the fact that its receptor is over-expressed on the surface of the diseased cells. This section will present recent developments in targeting in terms of the ligands used for targeting functionalization, namely sugars, folic acid and transferrin. In addition, polypeptides with functionality required for siRNA delivery and for use as theranostic systems will briefly be discussed.

3.1 Functionalization with Sugars

The use of sugars for targeting is widespread in the literature as glycopeptides have many therapeutic and biotechnological applications.^{64,65} Stöhr *et al.* detailed the preparation of glyco-NCAs through the attachment of peracetylated sugars (glucose, mannose, galactose) *via* a thiourea linker to the ϵ -amino group of α -Boc-lysine and α -Z-lysine. The corresponding NCAs were copolymerized with PEGylated lysine NCA and ϵ -TFA-Lysine-NCA and both the sugar residues and lysine units were subsequently deprotected. The peptide-hybrids were shown to be specifically taken up by human T lymphocytes at 37 °C demonstrating good potential as drug delivery systems.⁶⁶

Previously, Huang *et al.* reported saccharide-decorated amphiphilic block copolymers *via* post-polymerization functionalization.⁶⁷ The poly(L-lysine)-*block*-poly(L-tyrosine) copolymers were capable of forming both micelles and vesicles, which could be crosslinked by UV irradiation *via* dimerization of the tyrosine residues thus increasing the biostability. The disaccharide lactobionolactone, was coupled to the free amino groups on the lysine segment post-polymerization, in order to introduce galactose residues that act as a targeting group to

hepatocytic cells and also to help combat the innate cytotoxicity of poly(lysine). These glycopeptide nanoparticles, Lac-g-P(Lys)-b-P(Tyr), were investigated for release of fluorouracil and doxorubicin and were shown to do so rapidly upon enzymatic degradation of the assemblies (Figure 8). This system is multifunctional, and combines a number of the desired aspects of an efficient delivery system such as targeting *via* the saccharide ligand and enhanced stability through crosslinking.

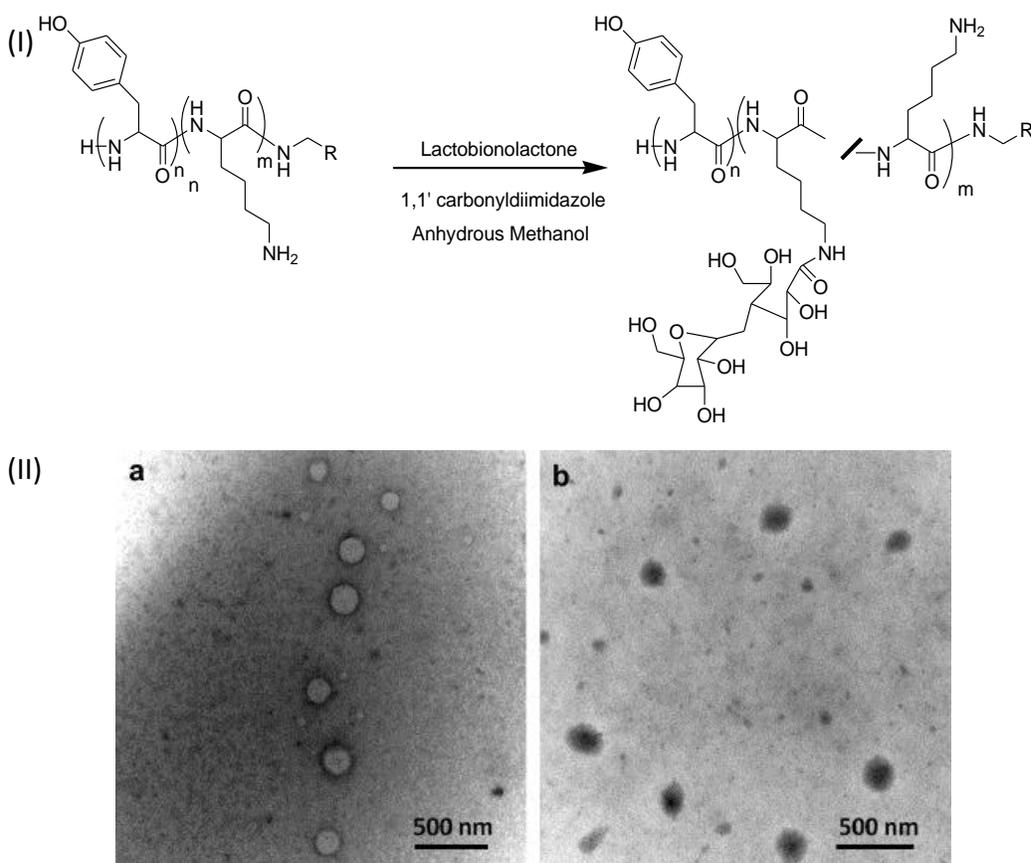


Figure 8 (I) Synthesis of Lac-g-P(Lys)-b-P(Tyr) block copolypeptides; (II) TEM images of (a) self-assembled aggregates from Lac-g-P(Lys)₆₆P(Tyr)₂₂ and (b) the Dox-loaded Lac-g-P(Lys)₆₆P(Tyr)₂₂ particles. Reprinted with permission (Elsevier) from ⁶⁷.

More recently, this group have detailed the preparation of similar multifunctional vesicles based on saccharide- and hexanoyl- modified poly(L-lysine). The amphiphilic nature of the polymer and thus the size of the vesicles, is dictated by the saccharide and hexanoyl substitution. The vesicles can actively target HepG2 cells through the saccharide ligand, they are pH responsive and display increased stability *via* crosslinking with genipin. A high level of drug loading was achieved through sonication and the application of a pH gradient between the outside and inside of the vesicles. The vesicles showed accelerated drug release at acidic pH and the rate could be tuned by the degree of crosslinking, resulting in a very promising drug delivery system.

3.2 Functionalization with Folic acid

Folate-decorated systems have been investigated for many types of polymers including polypeptide-based, as folate receptors are over-expressed on the surface of certain types of cancer cells. Previously, a targeted polypeptide-based multifunctional micellar system was reported by Bae *et al.*⁶⁸ The micelles were composed of PEG-poly(aspartate), decorated with folate and conjugated to a drug, adriamycin, *via* a hydrazone linkage. This system incorporates targeting, stealth properties and an already conjugated drug. The efficacy of folate-PEG-(polyaspartate-hydrazone-adriamycin) micelles was compared with that of non-targeted micelles and the effective dose of the targeted system improved.

More recently, core cross-linked star polymers composed of a poly(L-cysteine) core and poly(L-lysine) arms were functionalized with a PEG-folic acid conjugate *via* thiol-ene click chemistry resulting in poly(PEG-*b*-L-Lysine)_{arm}-poly(L-cysteine)_{core} stars.⁴⁴ Uptake of the folate-conjugated and non-folate-conjugated version of the stars was investigated with the breast cancer cell line MDA-MB-231. The folate-conjugated star polymer was shown to display cell association and

internalization for 55% of cells after 3 hours of incubation. In the case of the star polymers lacking the folate ligand, only 13% of cells displayed a low level of non-specific attachment, indicating the polymers are promising as cancer targeting drug delivery vehicles.

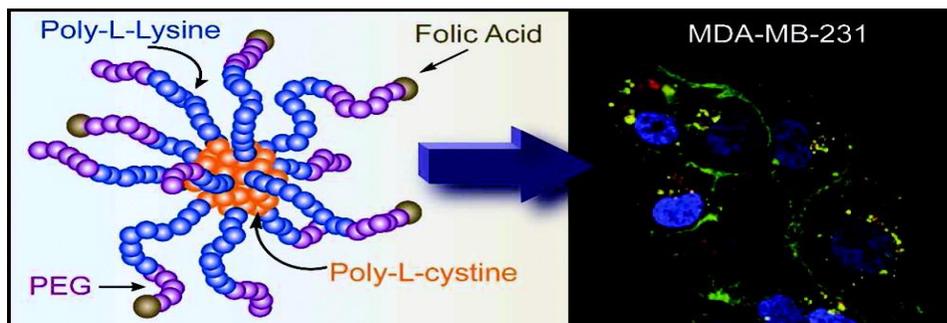


Figure 9 Schematic illustration of folic acid decorated poly(PEG-*b*- L-Lysine)_{arm}-poly(L-cysteine)_{core} stars and an image of their internalization in breast cancer cell line MDA-MB-231.⁴⁴ Reprinted with permission from Sulistio *et al.*, *Biomacromolecules* **2011**, *12*, 3469-3477. Copyright 2011 American Chemical Society.

3.3 Functionalization with Transferrin

Deming and coworkers previously reported block co-polypeptide vesicles based on poly(arginine) and poly(leucine).^{69,70} In order to overcome the cytotoxicity associated with the positive charge due to the arginine, the less cytotoxic poly(L-glutamate) was used as the hydrophobic block. This alteration however, means that the vesicles are unable to enter cells unaided and in order to enhance the internalization an iron-binding glycoprotein, transferrin was conjugated. An increased uptake of the vesicles in cells was observed, however, it was found that the vesicles traffic to early endosomes but not lysosomes, indicating the possibility they are recycled back to the cell surface. This observation may create an opportunity for the design of more advanced

vesicles equipped with an endosome-disrupting function to allow increased localization of the assemblies in the cytosol, where it would be required to release the payload.

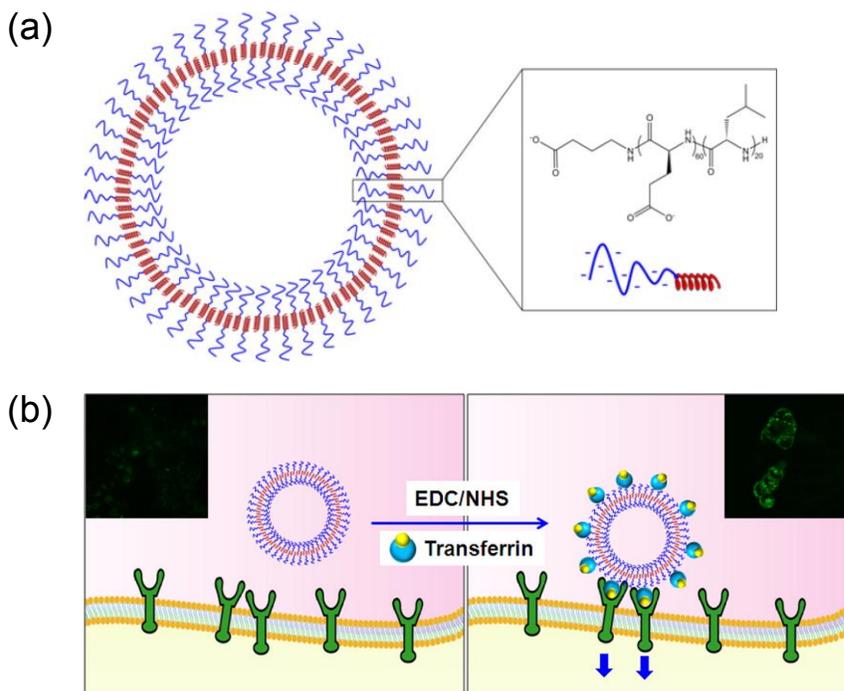


Figure 10 (a) Schematic representation of poly(glutamic acid)-poly(leucine) vesicles (b) Schematic illustration of transferrin-receptor binding and the resultant difference in internalization in cells⁷⁰. Reprinted with permission from Choe *et al.*, *Biomacromolecules* **2013**, *14*, 1458-1464. Copyright 2013 American Chemical Society.

Interestingly, the functional moiety does not necessarily need to be conjugated to the particle to have an effect on its targeting ability as evidenced by Song *et al.*, who have shown that simply co-administering the tumor-penetrating peptide *internalizing(Arg-Gly-Asp-D-Phe-Cys* (iRGD) was sufficient to enhance the targetability of their cisplatin-loaded PEG-poly(glutamic acid) micelles.¹⁷

3.4 DNA and siRNA Delivery

One of the most promising areas of therapy for the future is delivery of small interfering RNAs (siRNA). A number of problems are associated with siRNA delivery, such as a short serum half-life and poor cellular internalization ability, which can possibly be overcome through the use of polypeptide-based delivery vehicles. A recent paper by Zheng *et al.* combines the approaches of drug and gene delivery by preparation of a nanoparticle capable of co-delivering a chemotherapeutic drug and an siRNA.⁷¹ The system is comprised of a triblock copolymer of PEG-*b*-poly(L-lysine)-*b*-poly(L-leucine) and was loaded with docetaxel and siRNA-Bcl-2.

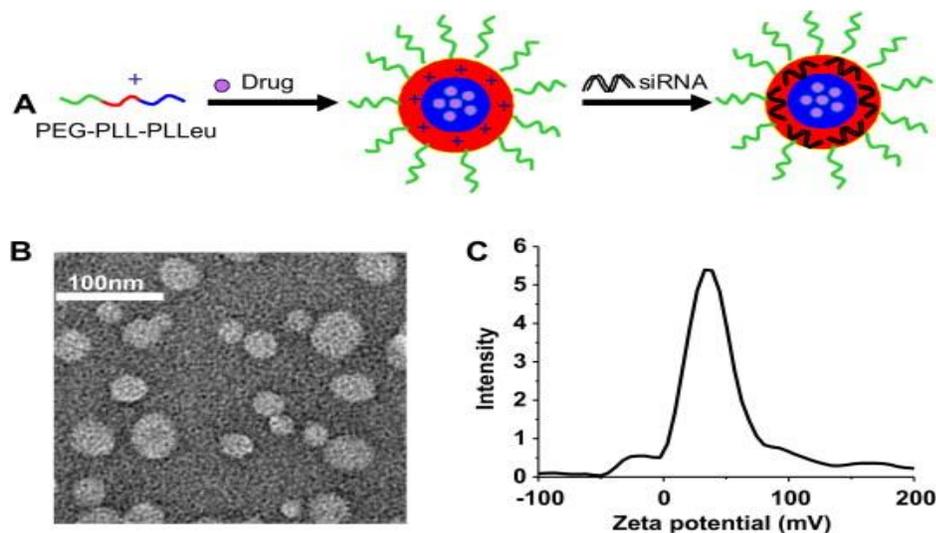


Figure 11 (A) Schematic illustration of self-assembly of PEG-poly(lysine)-poly(leucine) vesicles and siRNA loading; (B) TEM of vesicles; (C) zeta potential of vesicles. Reprinted with permission (Elsevier) from ⁷¹.

Gabrielson *et al.* have designed a library of cationic, helical polypeptides based on the architectural characteristics of cell penetrating peptides. A number of the polymers have been shown to effectively mediate gene delivery while at the same time maintaining the membrane

destabilization properties associated with helicity which facilitate cell penetration.⁷² Polypropyleneimine-poly(L-lysine) (PPI-PLL) dendritic core star polymers were also investigated for plasmid DNA and siRNA complexation and compared to linear PLL⁷³. A 300-fold increase in DNA transfection ability was observed in the case of the star polymers, which was attributed to their ability to form well-defined spherical polyplexes of around 100nm diameter.

3.5 Theranostic Systems

Another growing area is incorporating imaging ability into the delivery systems *i.e.* multifunctional theranostics. An interesting example of this was reported by Sanson and co-workers who developed multifunctional vesicles for combined therapy and imaging.⁷⁴ Block copolymer vesicles based on poly(trimethylene carbonate) (PTMC) and poly(glutamic acid) were loaded with both hydrophobically-modified magnetic nanoparticles, maghemite (γ -Fe₂O₃) and the anti-cancer drug doxorubicin. Synthesis of the block copolymer was carried out by ROP of an amine-terminated PTMC. The magnetic particle-loaded vesicles demonstrated the ability to be guided by a permanent magnetic field gradient and proved useful for magnetic resonance imaging (MRI), showing enhanced contrast. Release of doxorubicin from the dually-loaded vesicles was enhanced by the application of an RF oscillating magnetic field.

Recently, Xiao *et al.* reported multifunctional, unimolecular micelles with enhanced stability, combining active targeting and stimulus-responsive drug delivery with positron emission tomography imaging ability.⁷⁵ The polymers were composed of Boltorn H40-poly(ϵ -glutamate-hydrazone-doxorubicin)-*b*-poly(ethylene glycol) conjugated with *cyclo*(Arg-Gly-Asp-D-Phe-Cys)(cRGD) for targeting and macrocyclic chelators (1,4,7-triazacyclononane-N, N', N''-

triacetic acid) [NOTA] for ^{64}Cu -labeling and PET imaging (H40-DOX-cRGD). Boltorn H40 is a hyperbranched polyester and synthesis of H40-DOX-cRGD involves ROP of benzyl glutamate NCA with PEG macroinitiators. Doxorubicin was conjugated to the poly(glutamic acid) portion of the micelle *via* a pH-sensitive hydrazone linkage after conversion of the benzyl groups to hydrazide groups through an ester-amide exchange reaction. The intricate synthesis of the multifunctional system is shown in figure 12.

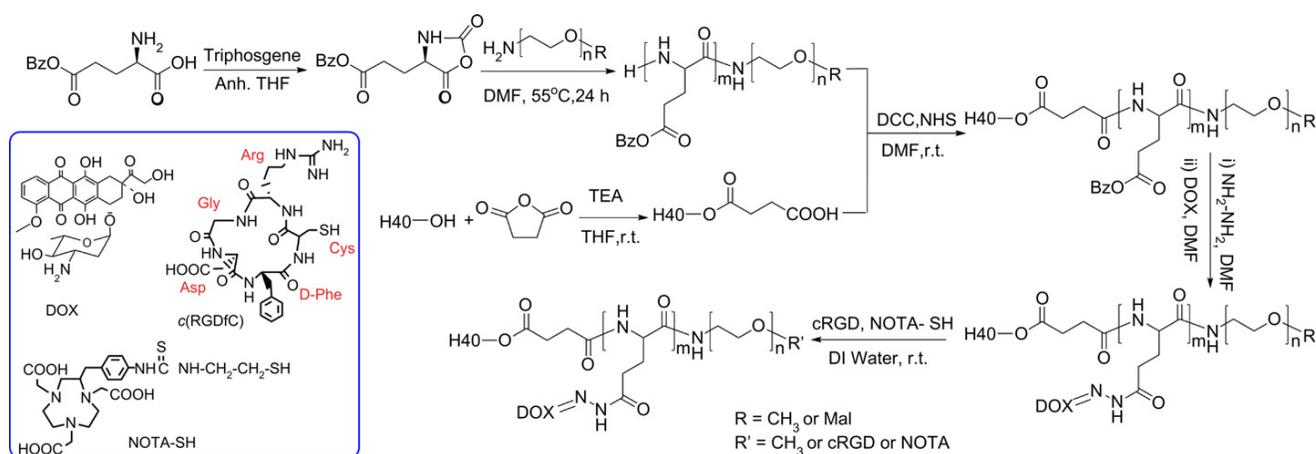


Figure 12 Synthetic scheme for multifunctional micelles Boltorn H40-poly(L-glutamate-hydrazone-doxorubicin)-b-PEG decorated with cRGD and NOTA.⁷⁵

This micellar system showed accelerated release of the conjugated doxorubicin at lower pHs. cRGD conjugation resulted in increased accumulation of the assemblies in human glioblastoma cells *via* $\alpha_v\beta_3$ -integrin endocytosis, compared to the control micelles lacking the cell penetrating peptide sequence. PET imaging using the ^{64}Cu -labelled micelles also showed greater accumulation of the targeted assemblies in the tumor cells which was further corroborated by biodistribution and *ex-vivo* fluorescence imaging. This example shows the potential power of multifunctional drug delivery systems.

Summary and Future Perspective

Throughout this review recent advances in the design and preparation of polypeptide-based assemblies with the potential for drug delivery use have been discussed. The developments in the efficiency and control of the ROP of NCAs have made the explosion in polypeptide-based drug delivery systems possible. A large volume of foundation work on self-assembling peptide-based materials has been carried out over the past few decades and now the task of improving and increasing the efficacy of the existing systems through multifunctionality, is underway. Biocompatibility and biostability are essential considerations when it comes to the design of vehicles and introduction of stealth characteristics and crosslinking, are key to achieving these goals. Release of the payload in the desired area of the body is the other significant challenge, however developments in stimuli-responsiveness and active targeting are proving very successful in the realization of this objective.

It has been shown that there are many features in the design of new multifunctional nanoparticle drug delivery systems that can be varied; different combinations of amino acid building blocks and non-peptidic architectures, choice of targeting ligands, stimuli-responsive moieties and methods of stability enhancement, resulting in the opportunity to create a diverse library of nanodevices for all therapeutic eventualities.

Ultimately, superior drug delivery systems will be created which will be smart, superfunctionalized nanodevices incorporating stealth properties, optimized biostability, stimuli-sensitivity, active targeting and imagibility, as well as the function of the therapeutic payload. The potential impact of these superfunctionalized systems on the area of drug delivery is profound.

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