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Biodegradable stimuli-responsive polypeptide materials prepared by ring opening polymerization

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Abstract

The stimuli-responsive polypeptides have drawn extensive attention for their promising applications in biotechnology considering their biocompatibility, biodegradability, and bioactivity. In this tutorial review, we summarize the most recent progress in this area, including thermo-, redox-, photo-, and biomolecule responsive polypeptides over the past decade. The design and synthesis of stimuli-responsive polypeptides will be briefly introduced. The correlation between structure and property, particularly the effects of polypeptide conformation will be emphasized here. In addition, the applications of stimuli-responsive polypeptides in controlled drug release and tissue engineering are briefly discussed.

Introduction

Stimuli-responsive polymers, also named as "smart" polymers, can undergo chemical structure change or physical property variation in response to environmental stimuli, such as temperature, pH values, ionic strength, light, biomolecules, etc.[1] Given these unique properties, stimuli-responsive polymers have been used in nanotechnology and biotechnology. Among all the stimuli-responsive polymers, polypeptides are particularly attractive due to their biocompatibility and biodegradability.[2-4] Another attractive feature of polypeptides is their diverse functionality considering their abundant monomer sources from more than twenty natural amino acids and their synthetic derivatives.[3, 4] Moreover, compared to conventional polymers, polypeptides can form higher ordered secondary structures such as α-helix and β-sheet, and the conformational transition among different secondary structures can be triggered by external stimuli.[5, 6] Such feature provides an additional pathway to modulate the stimuli-responsive properties of polypeptides.

Generally, polypeptides can be prepared by several synthetic methods, and each of them has unique advantages. Here, we focus on polypeptides prepared by ring opening polymerization (ROP) of α-amino acid N-carboxyanhydrides (NCA).[7, 8] Recent progress in living/controlled ROP of synthetic functional NCA monomers combined with the employment of orthogonal conjugation reactions, *i.e.*, "Click" chemistry, greatly promoted the development of stimuli-responsive polypeptides. A variety of stimuli-responsive polypeptides have been synthesized and their applications in biotechnology were also explored in the past decade.

In this tutorial review, we highlight the recent progress on stimuli-responsive synthetic polypeptides including thermo-, redox-, photo- and biomolecule responsive polypeptides over the past decade. We will focus on the relationship between structure and properties, especially the effects of

polypeptide conformation. PH responsive polypeptides, as the most extensively investigated polypeptides, will not be discussed here.[9]

Synthetic strategy

Polypeptides can be synthesized by ROP of NCAs initiated by nucleophiles (primary amine) or bases (tertiary amine or alkyl oxide), transition metal catalysts and recently developed organosilicon amines. The mechanism of ROP of NCAs has been thoroughly discussed by several excellent reviews regarding the synthetic polypeptides.[3, 7, 10] It is worth pointing out that the recently developed NCA monomer purification techniques of using flash chromatography[11] and the employment of "Click" chemistry greatly promoted the preparation of stimuli-responsive polypeptides with well-defined composition and molecular structures as well as functionalities.

Herein, we want to emphasize two general strategies used to prepare stimuli-responsive polypeptides. The first one is based on the stimuli-responsive monomers synthesized by incorporating stimuli-responsive groups to the side chain of canonical amino acids. The subsequent conversion to corresponding NCAs followed by controlled ROP directly result in the formation of stimuli-responsive polypeptides. This strategy is quite straightforward and gives polypeptides with known end group, controllable molecular weight, and narrow molecular weight distribution. **Table 1** summarizes the polypeptides made by this strategy as well as their general properties discussed in the following sections. However, this strategy suffers from multiple-step synthesis and tedious purification of new NCA monomers. On the other hand, the post modification strategy provides a more facile pathway to make stimuli-responsive polypeptides. Based on easily available polypeptide precursors, stimuli-responsive groups can be incorporated via conjugation with the pendent functional groups. The major disadvantage of post modification strategy is the incomplete conversion

of functional groups. Meanwhile, with employing the high efficient “Click” chemistry, the grafting efficiency can be greatly enhanced and the undesired byproducts can be suppressed. A library of novel polypeptides with pendent clickable groups, such as azide, alkyne, allyl groups, has been reported recently as summarized in **Scheme 1**.

INSERT TABLE 1 HERE

INSERT SCHEME 1 HERE

Thermo-responsive polypeptides

Temperature is one of the most frequently used stimuli because of its great control in space and time. A great deal of efforts has been devoted to preparing thermo-responsive polymers due to their promising applications in biotechnology in the past decades. At a critical temperature including lower critical solution temperature (LCST) and upper critical solution temperature (UCST), thermo-responsive polymers can undergo a reversible phase transition from soluble status to precipitation, or *vice versa*. Typically, the thermo-responsive property originates from a delicate hydrophilic/hydrophobic balance among different subunits within polymer chains. For an LCST-type polymer, upon temperature increase, the hydrophilic moieties undergo a hydration-to-dehydration transition, which causes the corresponding decrease of polymer solubility in water. While the most extensively investigated thermo-responsive polymer is poly(N-isopropyl acrylamide) (PNIPAM), polymers derived from oligo(ethylene glycol) (OEG) (meth)acrylate (OEGA or OEGMA) have been developed as alternative thermo-responsive materials with tunable LCST.[12] By conjugating OEG units to the polypeptide side chain, a series of thermo-responsive polypeptides was obtained with variable LCSTs.

As illustrated in **Figure 1**, we developed a new synthetic method to prepare thermo-responsive

polypeptide by direct ROP of OEGylated L-glutamic acid (L-EG_xGlu) NCAs, in which x represented the repeating number of ethylene glycol units.[13] Depending on the chain length of OEG, these OEGylated poly(L-glutamate) (poly(L-EG_xGlu)) showed distinct solubility in water. For example, poly(L-EG_1Glu) was insoluble in both water and most common organic solvents, while poly(L-EG_2Glu) and poly(L-EG_3Glu) displayed reversible LCST behaviors in water. With longer OEG side chain, poly(L-EG_3Glu) had higher LCST than poly(L-EG_2Glu).

Notably, these thermo-responsive polyglutamates displayed distinguished features compared to traditional polymers. The corresponding thermo-responsive properties strongly depended on the samples' secondary structures. For instance, copolymerization of different monomers was a general strategy to tune the LCST of samples. For these OEGylated glutamate NCAs, random copolymerization of L-EG_1Glu NCA with either L-EG_2Glu NCA or L-EG_3Glu NCA did not produce thermo-responsive polypeptide except the copolypeptides composed of L-EG_2Glu and L-EG_3Glu. In this case, the LCST of the (co)polyglutamates can be continuously tuned between 32 and 57 °C. The reason was that random copolypeptides of poly(L-EG_1Glu-co-EG_2Glu) and poly(L-EG_1Glu-co-EG_3Glu) formed predominating β-sheet structures while copolypeptides of poly(L-EG_2Glu-co-EG_3Glu) adopted helical conformation. Furthermore, racemic poly(rac-EG_3Glu) homopolypeptide was prepared by copolymerization of L-EG_3Glu and D-EG_3Glu NCAs, which adopted a random coil conformation, did not show any LCST behaviors up to 70 °C in contrary to the α-helical counterpart. The underlying reason was attributed to the nature of hydrogen bonding interaction of amide bonds, *i.e.*, intramolecular interactions for helical conformation versus intermolecular interactions for random coil. In the case of poly(L-EG_2Glu) and poly(L-EG_3Glu), the amide bonds formed intramolecular hydrogen bonding to stabilize α-helical conformation, and the solubility mainly arose from the

hydrophilic OEG side chain. Any disruption of such regularity in poly(rac-EG_3Glu), however, promoted the formation of intermolecular hydrogen bonding between water and amide bonds, which in return significantly increased the polypeptide solubility. As a consequence, poly(rac-EG_3Glu) lost its thermo-responsive property in water. Although transition from rigid helical conformation to random coil will increase samples' entropy, we believe the extra intermolecular hydrogen bonding will be the dominant factor for the increase of samples' hydrophilicity. Besides the monomers' chiral purity, the molecular weight of polypeptides also affected their thermo-responsive property since the stability of helical structure was associated with chain length. For both poly(L-EG_2Glu) and poly(L-EG_3Glu), their helical content increased with molecular weights, so the corresponding LCST had obvious decrease with increase of molecular weight (**Figure 2**).[14]

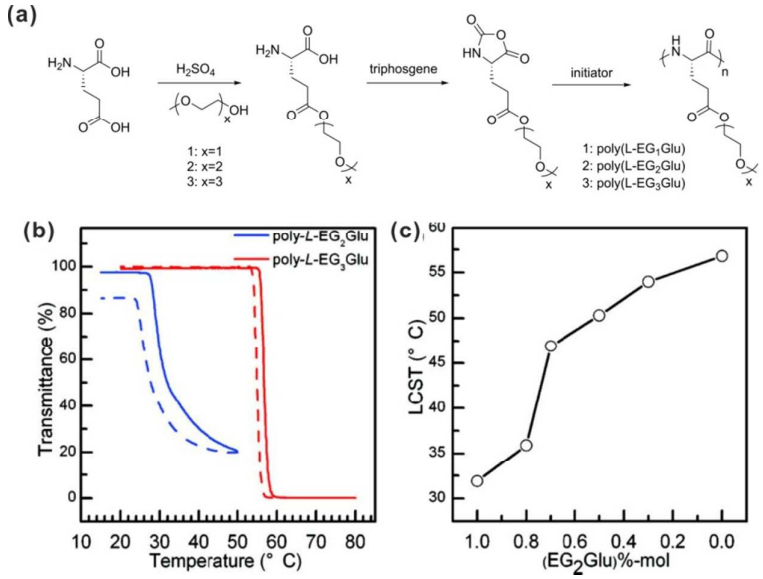

Figure 1. (a) Synthetic routes to poly(L-EG_xGlu) homopolypeptides. (b) Plots of transmittance as a function of temperature for aqueous solutions (2 mg/mL) of poly(L-EG_2Glu) and poly(L-EG_3Glu). Solid line: heating; dashed line: cooling. (c) LCST of poly(L-EG_2Glu-co-EG_3Glu) copolypeptides as a function of sample composition.[13] Adapted with permission from Ref. 13. Copyright (2011) American Chemical Society.

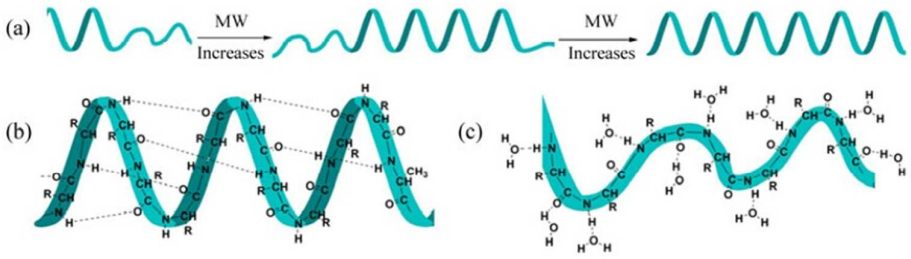

Figure 2. (a) Schematic illustration of helicity increase with molecular weight, (b) intramolecular hydrogen bonding illustration of helical polypeptide, and (c) intermolecular hydrogen bonding for random coil polypeptide.[14] Reproduced from Ref. 14 with permission from Springer Science and Business Media.

In addition to direct polymerization of functional NCA monomers, post modification provides an alternative way to construct thermo-responsive polypeptides. "Click" chemistry, which has the characteristics of high efficiency, mild reaction conditions, rapid reaction rate and minimal byproducts, greatly improves the grafting efficiency of functional groups. Chen et al. prepared a series of thermo-responsive polypeptides by ROP of γ-propargyl-L-glutamate NCA followed by "Click" reaction between pendant alkyne groups and 1-(2-methoxyethoxy)-2-azidoethane (MEO_2-N_3) and 1-(2-(2-methoxyethoxy)ethoxy)-2-azidoethane (MEO_3-N_3).[15] These polypeptides showed sharp LCST type transitions in water, and their LCST can be tuned by varying molecular weight, side chain length, and concentration of both polypeptides and salt. Very recently, Hammond and coworkers reported preparation of a dual pH and thermo-responsive polypeptide by conjugation of OEG and diisopropylamine groups to the side chain of poly(γ-propargyl-L-glutamate) using "Click" chemistry.[16]

Besides poly(L-glutamate) derivatives, OEG functionalized poly(L-cysteine) [poly(L-EG_xMA-C) or poly(L-EG_xA-C)] was also designed towards thermo-responsive polypeptides via thiol-ene Michael addition between L-cysteine and oligo(ethylene glycol) monomethyl ether (meth)acrylates [EG_x(M)A).[17] In contrast to the poly(L-glutamate) derivatives, which adopted α-helical conformation in aqueous solution, these OEGylated poly(L-cysteine) homopolypeptides adopted a mixed

conformation. Their secondary structure and LCST behaviors were strongly affected by the length of OEG side chain. Only poly(L-EG_xMA-C) or poly(L-EG_xA-C) with 3 to 5 OEG units displayed LCST behavior in water. In addition to thioether bond, disulfide linkage was also used in the construction of OEGylated poly(L-cysteine). Very recently, we prepared an OEGylated disulfide bond containing poly(L-cysteine) derivative, *i.e.*, poly(L-EG_x-SS-Cys), by reacting L-cysteine with OEGylated sulfenyl chloride. Surprisingly, these poly(L-cysteine) derivatives with 3 and 4 OEG repeating units showed irreversible thermo-responsive behavior in water, and this behavior was probably ascribed to chemical crosslinking caused by disulfide bonds exchange. Meanwhile, a diblock copolymer comprising of PEG and poly(L-EG_4-SS-Cys) can form thermogel in water, which can be used as reduction responsive injectable hydrogel.[18]

However, not all polypeptides conjugated with OEG units in the side chain can display thermo-responsive behaviors. Even subtle variation in the molecular structure can disrupt the delicate hydrophilic/hydrophobic balance and cause the loss of thermo-responsive property. For example, Deming reported the preparation of OEGlyated poly(L-lysine), which was nonionic helical polypeptide and did not show thermo-responsive behaviors.[19] Also, poly(L-EG_xAsp) (x=1, 2, 3), which were prepared from OEGylated aspartic NCAs, did not show any LCST behavior in water. Compared to poly(L-EG_xGlu), poly(L-EG_xAsp) with one less methylene group in the side chain cannot keep stable α-helical conformation. Actually, poly(L-EG_xAsp) (x=2, 3) adopted random coil conformation and had good water solubility.[20]

One important application of thermo-responsive polypeptides is to construct responsive hydrogels, which can be used as minimally invasive injectable biomaterials in drug delivery and tissue engineering. For example, the thermogelling polypeptide can undergo a sol-gel transition with

temperature increase from room temperature or below to physiological temperature. At low temperature, polypeptide system remains as low viscous solution, which can be easily mixed with drugs or cells. After simple injection of the mixture to subcutaneous layer or muscle, the increase of temperature causes rapid *in situ* sol-gel transition to form hydrogel network, which is an ideal candidate as a sustained drug release system or a three dimensional matrix for cell growth in tissue repair. Great efforts have been devoted to designing thermogelling polypeptides considering their great advantages compared to other non-degradable systems. Firstly, the gel modulus can be easily adjusted by controlling the composition and the chain length of hydrophilic and hydrophobic blocks through selection of natural or synthetic amino acids. Also, the gel properties can be further modulated by the higher ordered structure of polypeptides, such as α-helix and β-sheet. Secondly, the polypeptide materials are quite stable *in vitro* and can be stored for rather long time, but the degradation of the polypeptide materials can easily occur in the existence of enzymes. It is worth noting that the degradation products are zwitterionic amino acids, which can minimize the change of local pH values and will not deactivate the pharmaceutical species nor denature the cells.

Jeong and coworkers have made great contribution to this area. They prepared a series of thermogelling block copolypeptides including poly(alanine)-b-polyoxamer-b-poly(alanine) (PA-b-PLX-b-PA),[21] PEG-b-poly(alanine) (PEG-b-PA),[22-24] PEG-b-poly(alanine-co-phenylalanine) (PEG-b-PAF),[25, 26] and investigated their potential applications in drug delivery[25, 26] and cell therapy.[24] In the case of PA-b-PLX-b-PA, the copolypeptide formed thermogel at a typical concentration of 5.0 to 10.0 wt %,[21] and the sol-gel transition temperature decreased from 39 to 13 °C with increase of copolypeptide concentration. The sol-gel transition can be ascribed to the increase of β-sheet content within PA block as well as the dehydration of PLX block upon temperature increase. The

secondary structure played a critical role in the thermogel formation. With increase of the racemic alanine ratio in the PA block, a decrease of β-sheet content in the copolypeptide resulted in a higher sol-gel transition temperature. The effect of chiral purity on thermogel formation was further investigated by comparing PEG-b-poly(L-alanine) (PEG-b-L-PA) and PEG-b-poly(racemic alanine) (PEG-b-rac-PA).[22] The PEG-b-L-PA with a mainly β-sheet conformation underwent a sol-gel transition at a concentration of 6 to 12 wt % at around 10 to 37 °C. In contrast, PEG-b-rac-PA, in which the polypeptide block adopted a random coil conformation, cannot form gel at the physiological temperature. In addition to effects of chirality, the chain length of polypeptide block can also affect the secondary structure and the properties of the formed thermogel.[23] For PEG-b-L-PA diblocks, the secondary structure of PA block underwent a transition from antiparallel β-sheet to α-helix with increase of both PA block length and PEG block length. In the mean time, the corresponding assembly nanostructures changed from fibrous structures to spherical micelles. Interestingly, the samples' critical gelation concentration decreased with increase the α-helical content of PA block. They also demonstrated that incorporation of a more hydrophobic phenylalanine into polypeptide block can lower the thermogelling temperature and critical gelation concentration.[25] These thermogel had good stability. The PEG-b-L-PA formed hydrogel remained intact over half a month in phosphate buffered saline (PBS) while it almost completely degraded *in vivo* in the presence of enzymes. Given these advantages, an insulin delivery system based on PEG-b-L-PA was developed, and the hypoglycemic effect more than 18 days was observed.

Chen et al. designed a thermogelling system made by ROP of L-glutamate NCAs containing different hydrophobic side groups using PEG-NH_2 as macroinitiator.[27] They found that the secondary structure of poly(L-alkyl-glutamate) transformed from α-helix to β-sheet with increase of side chain length.

Moreover, the subtle variation in the side group further affected the sol-gel transition of copolypeptide. On the other hand, Heise and coworkers prepared a diblock copolymer comprising of PEG and oligo(tyrosine), which underwent sol-gel transition at an extremely low concentration, *i.e.,* 0.25 to 3.0 wt %, within the physiological temperature range.[28] It was suggested that the delicate amphiphilic balance between PEG and oligo(tyrosine) blocks, close packing of β-sheet tyrosine block at elevated temperature and the polar phenolic groups were responsible for the sol-gel transition at such a low concentration.

Although poly(L-EG_2Glu) with relative large molecular weight can display reversible thermo-responsive behaviors, this sample did have unmatched characteristics, which were not observed in previous reported systems. In particular, poly(L-EG_2Glu) itself had inherent amphiphilic feature. It cannot hold long-term stability as helical structure because its diethylene glycol side chain was not hydrophilic enough to stabilize the helical conformation. Although fresh prepared poly(L-EG_2Glu) adopted helical secondary structures, the conformation of poly(L-EG_2Glu) can slowly evolve from predominate helical structure to partial β-sheet conformation. Such changes accompanied a significant percentage transition from intramolecular interactions to intermolecular interactions. As a result, the molecular level interaction variation caused macroscopic physical properties change for poly(L-EG_2Glu) from soluble helical polymer to loosely associated aggregates due to β-sheet formation. Moreover, the conformational transition rate can be modulated by external stimuli such as thermal annealing, ionic strength, etc. This also explained the reason of its partially reversible LCST transition. Above all, the nature of hydrogen bonding and stimuli-responsive properties of poly(L-EG_2Glu) polypeptide were strongly associated with molecular weight and sample history. To the best of our knowledge, such properties have not been observed in synthetic polymers.

Using such properties, we constructed several functional materials.

For example, we prepared a series of nonionic alkyl-peptide amphiphiles, namely alkyl-poly(L-EG_2Glu), via ROP of L-EG_2Glu NCA using hexyl/dodecyl/hexadecyl amine as initiators. These alkyl-peptide amphiphiles spontaneously formed transparent hydrogel in water at a relative low critical gelation concentration, *ca.* 2 wt %. The partial β-sheet conformation of poly(L-EG_2Glu) segment accounted for the formation of nanoribbons, which further formed fibril network of hydrogel (**Figure 3**).[29] These physically crosslinked hydrogels showed excellent shearing thinning and rapid recovery features, which endowed them promising candidates as injectable hydrogels for controlled delivery or cell therapy applications. Using PEG_{44}-NH_2 as macroinitiator, we prepared a series of PEG_{44}-b-poly(L-EG_2Glu)$_x$ diblock copolymers, which displayed distinct properties in water for poly(L-EG_2Glu) with different molecular weight. When the average polymerization degree (x) of poly(L-EG_2Glu) block was lower than 30, PEG_{44}-b-poly(L-EG_2Glu)$_{25}$ self-assembled into nanoribbons and ultimately formed hydrogel in water. Though these copolypeptides did not show a sol-gel transition with increasing temperature, a thermo-induced increase in storage modulus was observed in the range of 20 to 45 °C.[30] On the other hand, PEG_{44}-b-poly(L-EG_2Glu)$_x$ with x larger than 40 formed micelles and the poly(L-EG_2Glu) adopted mainly helical structure. However, thermal annealing can induce that the assembled morphology changed from wormlike micelles to nanotubes. The reason was that thermal annealing as stimuli can promote the transformation of helical structure to β-sheet conformation for thermo-responsive poly(L-EG_2Glu) block (**Figure 4**).[31]

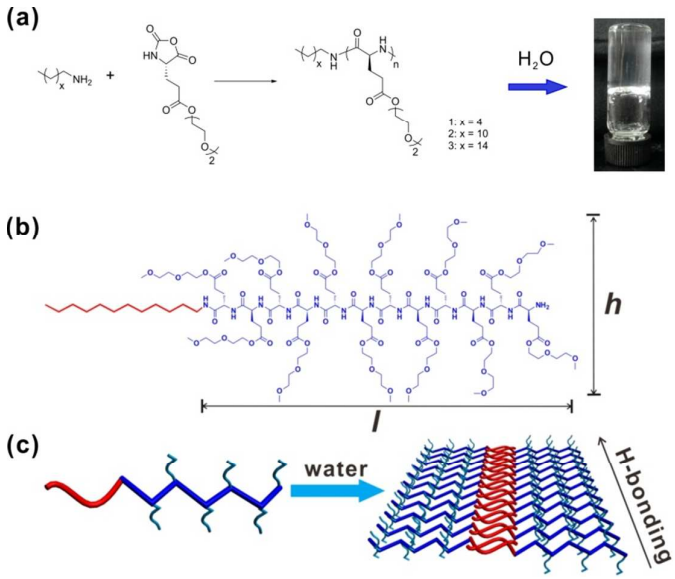

Figure 3. (a) Synthetic routes to alkyl-poly(L-EG_2Glu) amphiphiles. (b) Molecular illustration of dodecyl-poly(L-EG_2Glu)$_{12}$ amphiphile. (c) Self-assembly mechanism of alkyl-peptide amphiphiles into nanoribbons.[29] Adapted with permission from Ref. 29. Copyright (2013) American Chemical Society.

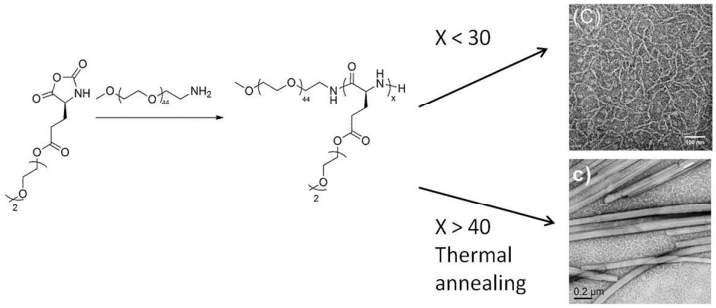

Figure 4. Synthetic route to PEG_{44}-b-poly(L-EG_2Glu)$_x$ and the distinct self-assembled morphology with different polymerization degree of poly(L-EG_2Glu) block.

Redox-responsive polypeptides

The redox-responsive polypeptides have drawn considerable attention recently because of their potential applications in drug and gene delivery, especially for the cancer therapy. The most extensively investigated redox-responsive groups are based on thiol chemistry, such as disulfide bond and thioether group. While the thioether group can be oxidized to sulfoxide and sulfone, the disulfide bond can be reversibly reduced to thiol group in the presence of dithiothreitol (DTT) or glutathione

(GSH).

Disulfide bond can be rapidly and selectively cleaved under intracellular condition while it displays high stability under extracellular physiological conditions due to the large GSH concentration difference between intracellular and extracellular environments. Utilizing this feature, several redox-responsive polypeptides containing disulfide bond have been synthesized. Two synthetic strategies were applied to synthesize disulfide bond containing polypeptides, either by using a disulfide containing PEG as macroinitiator or using poly(cysteine) as a component. For example, a PEG detachable diblock copolypeptide, PEG-SS-P[Asp(DET)], was synthesized by ROP of β-benzyl L-aspartate NCA using disulfide bond containing PEG-NH_2 as macroinitiator followed by aminolysis in the presence of excess diethylenetriamine (DET).[32] Compared to PEG-P[Asp(DET)], PEG-SS-P[Asp(DET)] was a more effective gene carrier. The cleavage of PEG chain in the endosome promoted the efficient endosomal escape of PEG-SS-P[Asp(DET)]/DNA polyplex micelles, which probably accounted for the high transfection efficiency. In addition to the disulfide linkage, ROP of cysteine or cystine NCAs was also used to incorporate disulfide to give redox-responsive polypeptides. For example, Qiao et al. prepared a core crosslinked star (CCS) shaped polypeptide using sequential monomer addition and arm-first approach.[33] Cystine NCAs were used as crosslinker to form the core of CCS polypeptide. Then, the core can be further modified using different primary amine or cleaved in the presence of excess DTT. Using PEG-NH_2 as macroinitiator, Chen and coworkers synthesized a dual pH and reduction responsive diblock copolypeptide through copolymerization of γ-benzyl-L-glutamate (BLG) NCA or ε-benzyloxycarbonyl-L-lysine (ZLL) NCA with L-cystine NCA. After deprotection, the copolypeptides formed nanogels crosslinked by disulfide bonds from cystine moieties in PBS solution. These nanogels was demonstrated a promising delivery system for doxorubicin (DOX) because the

DOX release from these nanogels can be greatly accelerated in intracellular reductive and acidic conditions.[34] Recently, a shell crosslinked (SCL) polymeric micelles was prepared by self-assembly of PEG-b-poly(L-cysteine)-b-poly(L-phenylalanine) (PEG-b-PCys-b-PPhe).[35] The *in situ* shell crosslinking of the micelles due to the oxidation of thiol groups of PCys block can greatly depress the drug release in the extracellular environment. Under the intracellular condition, the cleavage of disulfide bonds in the presence of high concentration of GSH caused the shell disruption, which promoted the drug release.

In contrast to the reduction responsive disulfide bond, the thioether bond can be used to construct oxidation responsive polypeptides. Deming and Kramer synthesized a glycosylated L-cysteine (glycol-C) monomer by coupling of alkene terminated C-linked glycosides of D-galactose or D-glucose to L-cysteine using thiol-ene "Click" Chemistry.[5] After ROP of the corresponding NCA monomers, they obtained an oxidation responsive glycopolypeptide with switchable conformation under different oxidation status. The oxidation of thioether bond to more polar sulfone group caused conformational transition of these glycopolypeptides from α-helix to random coil but without losing their solubility in water. On the other hand, the analogous glycopolypeptide based on L-homocysteine remained as α-helix upon oxidation to sulfone. Similar conformation switchable property was further demonstrated by the OEGylated poly(L-cysteine) derivatives [poly(L-EG_xMA-C) or poly(L-EG_xA-C)].[36] Poly(L-EG_xMA-C) and poly(L-EG_xA-C) underwent β-sheet dominant conformation to random coil conformation transition upon oxidation accompanying with increased water solubility and LCST. Moreover, PEG-b-poly(L-EG_xMA-C) diblock formed spherical micelles in aqueous solution, and the micelles can be disrupted upon oxidation due to the conformational switch of poly(L-EG_xMA-C) block. These micelles could be used as drug delivery system for inflamed tissues, which exhibit more

oxidative environment than normal tissues.

While oxidation of thioether group to sulfone is irreversible, partial oxidation of thioether group to sulfoxide group can be reversibly reduced in the presence of appropriate reducing agents or reductase enzymes. Deming et al. designed an enzyme responsive copolypeptide by incorporation of oxidable methionine units into hydrophobic copolypeptide, poly(L-methionine)$_{65}$-b-poly(L-leucine$_{0.5}$-*stat*-L-phenylalanine$_{0.5}$)$_{20}$ ($M_{65}(L_{0.5}/F_{0.5})_{20}$). Upon mild oxidation, poly(L-methionine) block underwent α-helix to random coil conformational transition accompanying with enhanced hydrophilicity. The resultant amphiphilic copolypeptide, $M^{O}_{65}(L_{0.5}/F_{0.5})_{20}$ can self-assemble into vesicles in aqueous solution (**Figure 5**). In the presence of DTT and methionine sulfoxide reductase (MSR) enzyme, the vesicles can be disrupted and release the encapsulated drug due to the reduction of methionine sulfoxide to methionine.[37] In addition to the enzymes, the sulfoxide group can be reduced by thioglycolic acid as well. Similar with poly(L-methionine), poly(α-D-galactose-L-homocysteine) (poly(α-gal-C^{H})) and poly(S-OEGylated-L-homocysteine) (poly(EG_x-C^{H})), which share the same core structure as poly(L-methionine), were able to undergo the α-helix to random coil conformational transition upon oxidation to sulfoxide. Particularly, poly(EG_4-C^{H}) displayed an LCST behavior while the oxidized poly(EG_4-C^{O}) with sulfoxide groups lost the thermo-responsive property.[6]

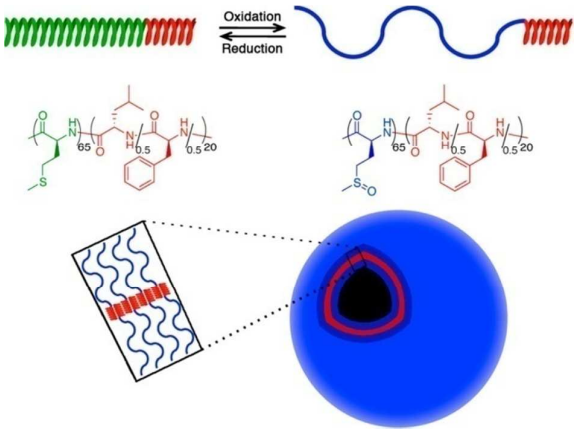

Figure 5. Schematic showing structure, redox properties, and proposed self-assembly of $M^{O}_{65}(L_{0.5}/F_{0.5})_{20}$

copolypeptides into vesicles.[37] Reprinted with permission from Ref. 37. Copyright (2013) American Chemical Society.

Photo-responsive polypeptides

Among all the stimuli-responsive polypeptides, photo-responsive polypeptides are of particular interest since the light allows remote control of the drug release process in both spatial and temporal controlled manner. The research of photo-responsive polypeptides is relatively limited compared to other stimuli-responsive polypeptides. Photo-responsive moieties that have been incorporated into polypeptides include coumarin, 2-nitrobenzyl, cinnamyl, and spiropyran. Photo-responsive polypeptides have been used to construct photo-crosslinked polypeptide nanogels, photo-triggered drug delivery system, and photo-regulated self-assembly.

Using PEG-NH_2 as macroinitiator, Chen and coworkers prepared photo-crosslinkable and pH responsive copolypeptides, *i.e.*, PEG-b-poly(L-glutamic acid-co-γ-cinnamyl-L-glutamate) (PEG-b-P(LGA/CLG)) diblock and (P(LGA/CLG)-b-PEG-b-P(LGA/CLG)) triblock copolypeptides by ROP of BLG-Glu NCA followed by deprotection of benzyl groups and subsequent conjugation with cinnamyl alcohol. Both copolypeptides can self-assemble into micelles in aqueous solution with P(LGA/CLG) as the core. Upon UV irradiation, the micelle cores were crosslinked to produce nanogels due to the photodimerization of cinnamyloxy groups.[38] The nanogels can be used as drug delivery system and the drug release can be controlled by varying the compositions of block copolymers and solution pH. In addition to post modification, cinnamyl moieties can also be introduced into NCA monomer. Yan et al. synthesized a cinnamyl modified amino acids, γ-cinnamyl-L-glutamate, by selective conjugation of L-glutamic acid with cinnamyl alcohol. The subsequent conversion to NCAs and ROP resulted in the

formation of polypeptide containing photo-crosslinkable units.[39]

On the other hand, photochromic groups that can be cleaved by light were also incorporated into polypeptide to fabricate photo-triggered drug release system. Dong and coworkers designed a photo-responsive copolypeptide, *i.e.*, PEG-b-poly(S-(*o*-nitrobenzyl)-L-cysteine) (PEG-b-PNBC), from an S-(*o*-nitrobenzyl)-L-cysteine NCA (NBC-NCA) monomer. The PEG-b-PNBC spontaneously formed micelles in aqueous solution with PNBC as the core. These micelles were demonstrated the ability to form photo-triggered drug release system. Upon UV irradiation, the progressively decreased hydrophobic interaction between DOX and PNBC block promoted the release of DOX due to the photo-cleavage of pendant *o*-nitrobenzyl groups.[40] Among all the photo-responsive polypeptides, near-infrared (NIR) light responsive polypeptides are particularly attractive considering the deeper penetration and less harmful to tissues in contrast to UV or visible light. A NIR light responsive polypeptide was recently synthesized by incorporation of 6-bromo-7-hydroxycoumarin- 4-ylmethyl groups into PEG-poly(L-glutamic acid).[41] The micelles formed by the copolypeptide can be disrupted by the shifted hydrophilic/hydrophobic balance caused by the NIR induced cleavage of coumarin groups.

While the above introduced photo-responsive polypeptides undergo light induced irreversible structural change, spiropyran group provides the possibility to fabricate photo-responsive polypeptides with reversible structural change or phase transition. Spiropyran group undergoes spiropyran (SP) to merocyanine (MC) isomerization upon UV light and reverts back to the SP form upon exposure to visible light or heat. By taking advantages of this reversible behavior, Mezzenga et al. prepared a photo-responsive polypeptide containing spiropyran group, which can undergo a reversible aggregation-dissolution-aggregation transition upon exposure to UV light or visible light

(**Figure 6**).[42] This transition was speculated to be caused by the hydrophobic to hydrophilic switch as well as the decrease of helical content of copolypeptide arising from the conversion of SP form to MC form.

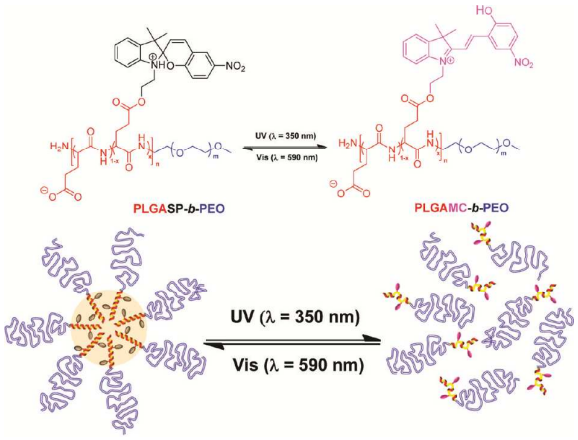

Figure 6. Schematic illustration of photo-responsive micellization/dissolution process for the PLGASP-b-PEO block copolymer.[42] Reprinted with permission from Ref. 42. Copyright (2011) American Chemical Society.

Biomolecule responsive polypeptides

Biomolecule responsive polypeptides can be fabricated by integrating biomolecules, such as sugars, into the side chain of polypeptides. In this section, we will briefly summarize the recent progress of lectin responsive glycopolypeptides and glucose sensitive polypeptides.

Glycopolypeptides have drawn considerable attention recently since they can mimic the natural glycoprotein and the related biomolecular recognition process with lectins. Lectins are carbohydrate binding proteins, which perform recognition on the cellular and molecular level and are highly specific for sugar moieties. The most common used lectins in the investigation of glycopolypeptides are concanavalin A (Con A) and *Ricinus communis agglutinin* (RCA). While Con A binds with mannosyl

and glucosyl groups, RCA specially and selectively binds with galactosyl groups. Sugar moieties can be incorporated into the polypeptide by either a native O-linkage or a C-linkage. Similar with the other stimuli-responsive polypeptides mentioned above, two strategies were employed to prepare glycopolypeptides, either via ROP of glycosylated NCA monomer or by post modification of the synthetic polypeptide precursors.

Gupta et al. synthesized per-acetylated-O-glycosylated lysine NCA monomers by reacting their corresponding propargyl 1, 2-orthoester of per-O-acetylated carbohydrates with lysine derivatives followed by conversion to corresponding NCA monomers. Subsequent ROP of these glycosylated NCA monomers and removal of the acetyl groups resulted in formation of water soluble O-glycopolypeptide.[43] The binding of glycopolypeptide, *i.e.*, poly(α-manno-O-lys), with Con A was investigated using precipitation, hemagglutination assays, and isothermal titration calorimetry. The racemic glycopolypeptides displayed a slightly higher binding stoichiometry compared to their α-helical counterparts. Wenz and coworkers designed a O-linked glycopolypeptide via thiourea linker,[44] and they found the galactosylated polypeptide showed special interaction with T lymphocytes and enabled to be internalized into the cytoplasm of T cells.

The synthesis of C-linked glycopeptides was pioneered by Deming and coworkers.[45] By employment of C-linked sugars and amide linkages to lysine, a new glycosylated-L-lysine monomer was developed. Compared to the O-linked glycopolypeptides, the C-linked glycopolypeptides have much higher molecular weight and better mimics of natural glycoproteins. Recently, the same group investigated the effects of secondary structure of hydrophilic block on the self-assembled morphology using glycosylated amphiphilic polypeptides. The hydrophilic glycopolypeptide with random coil conformation preferred forming vesicles while the analogous glycopolypeptide with α-helical

conformation formed irregular aggregates. Moreover, the formed galactosyl vesicle can specifically bind with RCA_{120}.[46]

The post modification strategy, on the other hand, provides a facile way to prepare glycosylated polypeptides, especially by utilizing high efficient "Click" chemistry. Chen et al. prepared a series of glycopolypeptides by combining the pendant alkyne groups of poly(γ-propargyl-L-glutamate) with azido sugars using Cu(I) catalyzed cycloaddition "Click" reaction.[47] Huang et al. recently synthesized diblock amphiphilic glycopolypeptides comprising of poly(γ-benzyl-L-glutamate) (PBLG) block and galactosylated poly(L-propargylglycine) block using similar method. These amphiphilic glycopolypeptides can self-assemble into polymersomes and selectively bind with RCA_{120}.[48] In addition to Cu(I) catalyzed alkyne/azide cycloaddition reaction, the metal free thiol-ene and thiol-yne photochemistry were also applied in the preparation of glycopolypeptides. Krannig and Schlaad prepared a statistical copolypeptide consisting of poly(L-glutamic acid) and poly(allylglycine) or poly(propargylglycine). The copolypeptide was further modified with thiol functionalized glucose using thiol-ene or thiol-yne "Click" reaction.[49] The incorporation of glucose moieties was found to enhance both the helical stability and water solubility of the copolypeptide, and the selective binding of the glucosylated copolypeptide with Con A was demonstrated.

Glucose sensitive polypeptide, presented as another biomolecule responsive polypeptide, can be used to fabricate promising insulin delivery system for the treatment of diabetes. Glucose responsive polypeptide can be designed by incorporation of phenylboronic acid (PBA) moieties into the polypeptide side chain. PBA can reversibly bind with glucose by forming a six member boronic cyclic ester accompanying with the increase in hydrophilicity. Recently, Chen and coworkers developed a PBA functionalized block copolypeptide, *i.e.*, PEG-b-P(GA-co-GPBA), by reacting PEG-b-

poly(L-glutamic acid) with 3-aminophenylboronic acid.[50] These copolypeptides can self-assemble into micelles in phosphate buffer at physiological pH. The insulin encapsulated micelles can accelerate release of insulin at a higher glucose concentration. More importantly, the alternant insulin release ability of the micelles triggered by glucose demonstrated their promising application as self-regulated drug delivery system (**Figure 7**).

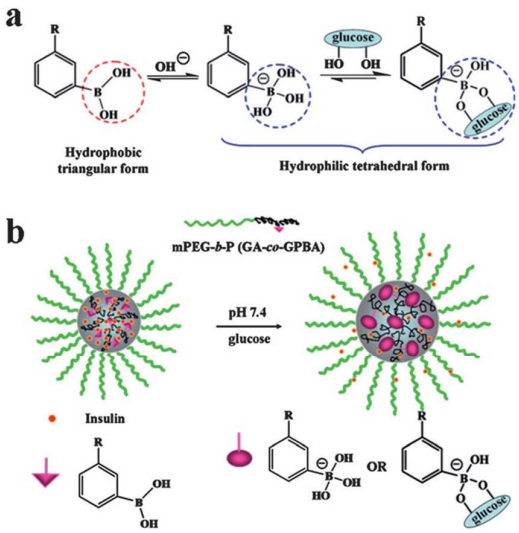

Figure 7. Complexation equilibrium between PBA derivative and glucose (a) and glucose sensitive behavior of insulin-loaded micelle in PB at pH 7.4 (b).[50] Reproduced from Ref. 50 with permission from The Royal Society of Chemistry.

Other responsive polypeptides

In addition to the above mentioned stimuli responsive polypeptides, chemical responsive polypeptides were also synthesized by Deming and coworkers. The poly(methionine) and poly(homocysteine) derivatives can be reversibly alkylated to polysulfonium in a near quantitative yield.[51] The increased polarity and electronic repulsion arising from side chain will induce a conformational transition. Specially, poly(α-gal-C^H) and poly(EG_4-C^H) were demonstrated to undergo a conformational transition from α-helix to random coil upon alkylation.[6]

Conclusions and perspectives

The recent progress in the living ROP of NCAs combined with the employment of orthogonal conjugation chemistry greatly promoted the development of stimuli-responsive polypeptides in the past decade. A large number of novel functional NCA monomers have been synthesized, and their corresponding (co)polypeptides show intriguing responsive properties triggered by external stimuli, such as temperature, redox, light, and biomolecules, etc. In contrast to conventional polymers, not only chemical compositions but also secondary structures of polypeptides play a critical role on their properties. For example, poly(L-EG$_x$Glu), which adopts α-helical conformation, displays a LCST behavior in aqueous solution while its disordered analogous poly(rac-EG$_x$Glu) loses the thermo-responsive property. Furthermore, external stimuli can induce the conformational transition of polypeptides and further change their macroscopic properties, for instance, the redox-induced α-helix to random coil transition discussed above. However, not all polypeptides can change their secondary structure upon external stimuli. The sophisticated designs of their chemical structures combined with appropriate stimuli-responsive groups are both responsible for their conformational transition behavior. The research focused on the relationship between secondary structure of polypeptides and their properties is still limited so far. More efforts should be devoted for better understanding of the effects of side chain functionality and secondary structure. Various applications of stimuli responsive polypeptides have been explored in biotechnology, including drug delivery and tissue engineering by taking advantages of their biocompatibility and biodegradability. There are still plenty rooms for the further development of stimuli-responsive polypeptides, including potential applications in tissue scaffold, cell growth matrix, target drug delivery and chiral separation of biomolecules, etc.

Acknowledgements

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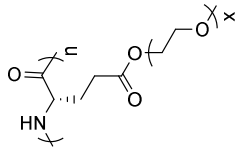
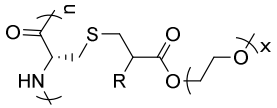
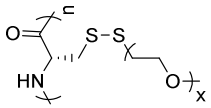
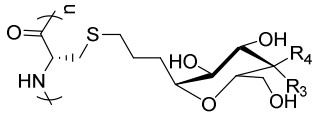
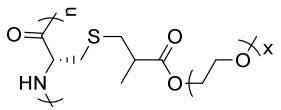
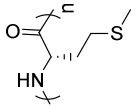
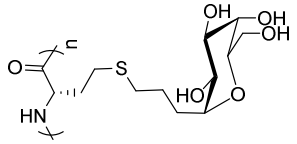
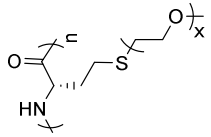
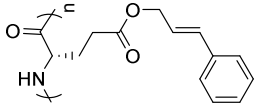
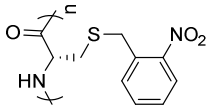
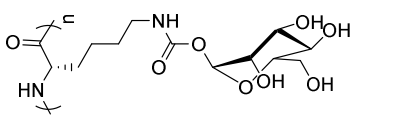
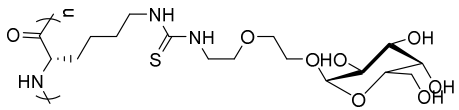
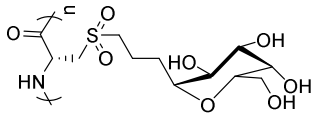
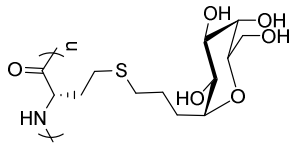
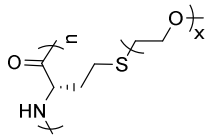
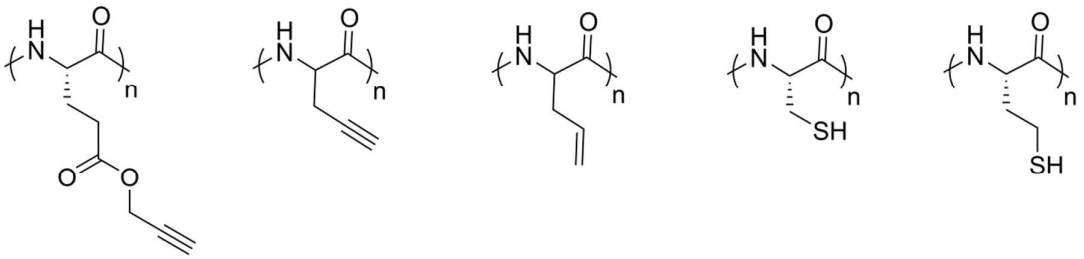
Table 1. Stimuli-responsive polypeptides synthesized by direct ROP of novel functional NCA monomers discussed in this review

stimuli	Chemical structure of polypeptide	structural change	conformational change	Macroscopic property change	Ref
Temperature	 X= 2, 3	dehydration of OEG units	remain α-helical conformation	reversible LCST transition	13
	 R = H, CH_3 X = 3, 4/5	dehydration of OEG units	remain mixed conformation	reversible LCST transition	17
	 X=3, 4	dehydration of OEG units and disulfide bonds exchange	remain mixed conformation	Irreversible LCST transition	18
Redox	 R_3 = OH, R_4 = H or R_3 = H, R_4 = OH	thioether bonds oxidize to sulfoxide	remain α-helical conformation	remain good water solubility	5
		thioether bonds oxidize to sulfone group	transition from α-helix to random coil	remain good water solubility	

	 X = 3, 4/5	thioether bonds oxidize to sulfoxide group	remain mixed conformation	enhanced water solubility and LCST	36
		thioether bonds oxidize to sulfone group	transition from β-sheet to random coil	loss of LCST behavior	
		thioether bonds oxidize to sulfoxide group	transition from α-helix to random coil	transition from hydrophobic state to hydrophilic state	37
		thioether bonds oxidize to sulfone group	remain α-helical conformation	transition from hydrophobic state to hydrophilic state	
		thioether bonds oxidize to sulfoxide group	transition from α-helix to random coil	remain good water solubility	6
	 X= 2, 4	thioether bonds oxidize to sulfoxide group	transition from α-helix to random coil	enhanced water solubility and loss of LCST behavior	6
Light		photo-dimerization of cinnamyl groups	not investigate	crosslinking	39

		photo-cleavage of *o*-nitrobenzyl groups	remain β-sheet conformation	promote release of cargo	40
biomolecule		bind with Con A	not investigate	transition from soluble state to precipitation	43
		not investigate	not investigate	endocytosis by T lymphocytes	44
		bind with RCA_{120}	not investigate	transition from soluble state to precipitation	46
chemistry		alkylation with methyl or benzyl group	transition from α-helix to random coil	remain good water solubility	6

 X= 2, 4	alkylation with methyl or benzyl group	transition from α-helix to random coil	enhanced water solubility and loss of LCST behavior	6



Scheme 1. Polypeptides with pendent clickable groups discussed in this review.