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# Recent advances in amino acid *N*-carboxyanhydrides and synthetic polypeptides: chemistry, self-assembly and biological applications

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Polypeptides are fascinating materials with unique properties for various biological materials. We highlight here recent advances in amino acid *N*-carboxyanhydrides (NCAs) and synthetic polypeptides from the aspects of chemistry, self-assembly and biological applications. New synthetic methodologies, mechanistic studies and optimization of polymerization conditions for the preparation of well-defined novel polypeptides are comprehensively reviewed and evaluated. Functional polypeptides, mostly prepared from novel NCA monomers, with ultra-stable helical conformation, stimuli-sensitive properties, or glycoprotein mimetics are summarized. We also highlight a number of interesting self-assembled structures of polypeptides in solid state and solution, with particular emphasis on those structures other than amphiphilic self-assembly. The biological applications of polypeptides in drug and gene delivery are also reviewed. Future directions and perspectives are discussed in the conclusion.

## 1. Introduction

Polypeptides prepared by the ring-opening polymerization (ROP) of α-amino acid *N*-carboxyanhydrides (NCAs) are emerging biomaterials that have received increasing attention due to their various biomedical applications including drug delivery, gene therapy, antibiotics and tissue engineering.[1,2] One attractive feature of polypeptides is that they offer considerable chemical diversity just by the

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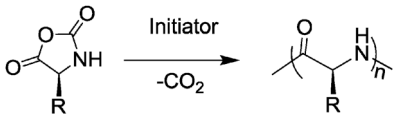
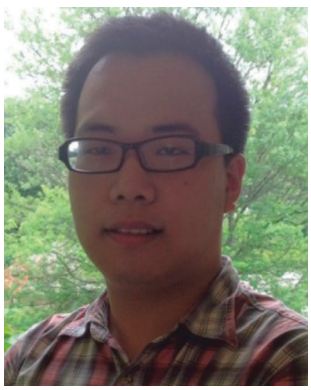
twenty canonical amino acids alone, not even considering numerous non-canonical amino acids that are readily accessible from commercial sources. Moreover, polypeptides are fascinating and unique in their ability to self-assemble into ordered structures,[3] which is especially valuable when hierarchical architectures or complex functions are required for special applications. Significant progress has been made in the NCA/polypeptide field in the past decade with new synthetic tools being developed;[4–9] plenty of novel polypeptide-based materials were synthesized and new applications were explored.[10–13] A few excellent review articles have summarized the previous important advances and milestones in this field.[2,14–20] Here, we highlight the most recent progress in the NCA/polypeptide in the past ten years. Progress in oligopeptides prepared from the solid phase peptide synthesis (SPPS) is not covered in this feature article.

## 2. Controlled polymerization of NCA for the synthesis of polypeptides

NCAs are usually prepared by phosgenation of side-chain protected α-amino acids. In the most classical NCA polymerization mechanism, ring opening of NCA results in the release of one molecule of carbon dioxide and exposure of a primary amine, which behaves as the active species to open the next NCA monomer and the repetition of this step finally generates the polypeptides (Scheme 1). Typical initiators for ROP of NCAs are nucleophiles (primary amines) or bases (tertiary amines and alkyl oxides). Controlled NCA polymerization technologies include pioneering works by Deming using cobalt and nickel organometallic catalysts,[4] Hadjichristidis using a high vacuum setup,[5] Schlaad using ammonium salts,[6] and Giani using low temperature to regulate polymerization.[7] In addition, investigations by Heise[21,22] and Messman[23] provided special and informative insights into how parameters such as temperature, pressure and solvent purity impact the controlled polymerization.




Scheme 1 ROP of NCA for the preparation of polypeptides.



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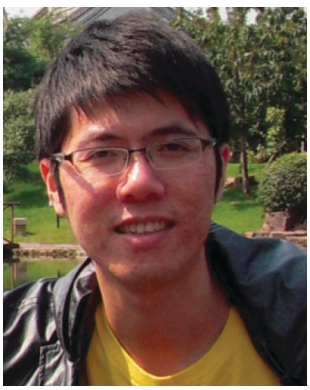

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### 2.1 Organosilicon amines

Since 2007, Lu and Cheng developed a simple and convenient approach for the controlled NCA polymerization using organosilicon amines.[8,13,24–27] They first discovered that hexamethyldisilazane (HMDS),[8] an inexpensive and commonly used silylation reagent, gave unexpected excellent control over the polymerization (Scheme 2a) of various NCA monomers, including γ-benzyl-L-glutamate NCA (Bn-Glu-NCA) and ε-carbobenzyloxy-L-lysine NCA (Z-Lys-NCA). Using HMDS, the molecular weights (MW) of the resulting poly(γ-benzyl-L-glutamate) (PBLG) and poly(ε-carbobenzyloxy-L-lysine) (PZLL) are excellently controlled and the molecular-weight distribution (MWD = $M_w/M_n$) of the polypeptides is generally less than 1.2. Block copolypeptides such as PBLG-*b*-PZLL and PZLL-*b*-poly(L-leucine) can be readily prepared by sequential addition of NCA monomers. They later on expanded this system to various organosilicon amines, with attention on providing a simple approach for C-terminus functionalization of polypeptides (Scheme 2b).[24] The facile introduction of new functional groups (*e.g.* alkyne, alkene, azide or norbornene, *etc.*) provides extra "chemical handles" facilitating potential post-polymerization modifications of the materials. Because organosilicon amines in many situations resemble protected amines, they are therefore especially useful in circumstances where free amines are not tolerated (see Section 2.3).

The most intriguing part of the organosilicon amines mediated NCA polymerization is that it introduced a brand new mechanism that differs from the well documented conventional "amine mechanism" or "activated monomer mechanism". By conducting mechanistic studies using FT-IR, mass spectrometry (MS), NMR and kinetic monitoring, the authors discovered that a unique end-group, trimethylsilyl carbamate (TMS-CBM), exists in both chain initiation (intermediate **1**, Scheme 3) and chain propagation steps.

Lu and Cheng proposed a tentative mechanism for the HMDS-mediated NCA polymerization, which to some extent resembles the mechanism of group-transfer polymerization (GTP) of acrylic monomers mediated by similar organosilicon compounds.[28] Because of the steric hindrance of HMDS, it is very difficult for HMDS to directly nucleophilically attack the NCA *via* a classical $S_N2$ mechanism. To generate the identified intermediate **1** with TMS-CBM structure, it was initially proposed that a TMS group from HMDS firstly transferred to a carbonyl of NCA (Scheme 3a) and the anhydride ring was then opened by the *in situ* generated TMS-amine. The chain propagation might involve a six-membered ring transition state between the TMS-CBM group of the growing polypeptide and the incoming NCA monomer, by which the TMS group was transferred to the reactive center to regenerate the TMS-CBM structure. However, new evidence such as that HMDS can polymerize $^N$methyl-glycine-NCA yielding a polypeptoid by the same TMS-CBM end-group (Scheme 3b, unpublished results) indicates that there might be other approaches to generate the intermediate **1**, simply because the *N*-substituted NCA cannot undergo the tautomerization as proposed in the initial route (Scheme 3a). Moreover, it was shown that succinic anhydride whose ring structure resembles NCA very much can also react with an equal amount of HMDS and give product **2** in very high yield (Scheme 3c, unpublished results), suggesting that HMDS can indeed open anhydride rings without involving the nitrogen on the NCA ring. It was proposed that both reactions (Scheme 3b and c) probably

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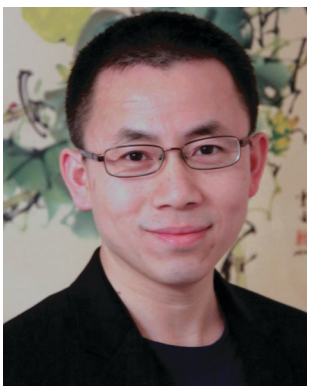

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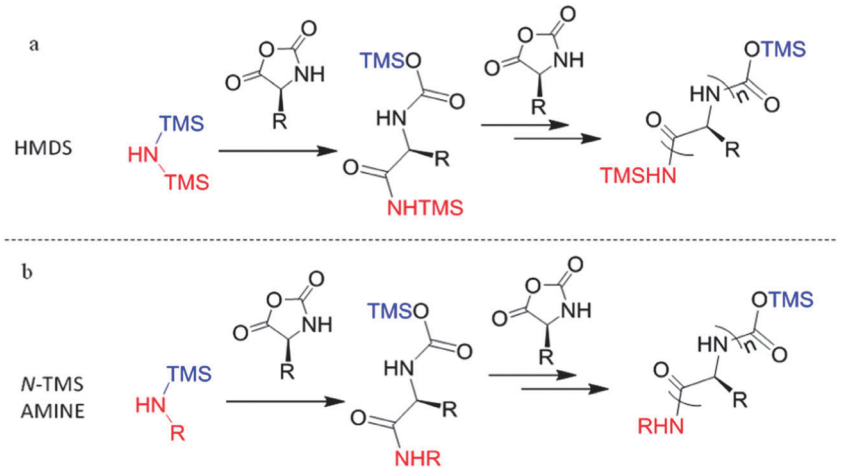
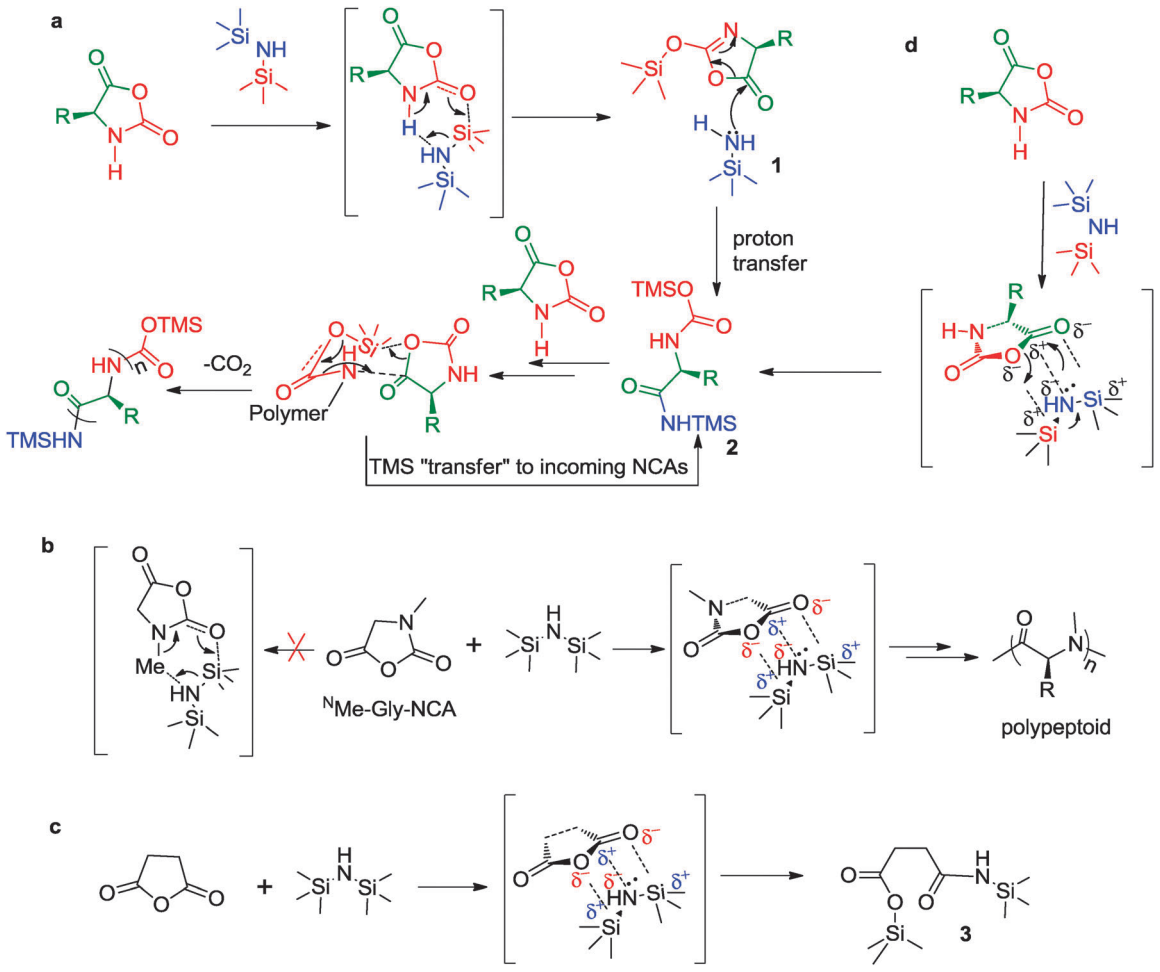



Scheme 2 Organosilicon amines mediated NCA polymerization.[8,24]




Scheme 3 Proposed mechanism for the HMDS mediated NCA polymerization.

proceed *via* the simultaneous formation of an amide bond and TMS transfer to the carboxylate in a cooperative manner. Therefore, the cooperative mechanism of opening the NCA ring and TMS transfer to generate intermediate **1** (Scheme 3d) might appear to be a feasible route for the chain initiation step of the HMDS-mediate NCA polymerization.

Despite their simplicity and effectiveness, one of the most significant drawbacks of those organosilicon amines is their lower reactivity compared with the organometallic catalysts and the vacuum NCA polymerization in which carbon dioxide was actively removed to force the equilibrium to move towards the ring-opening polymerization. This low reactivity makes HMDS and related

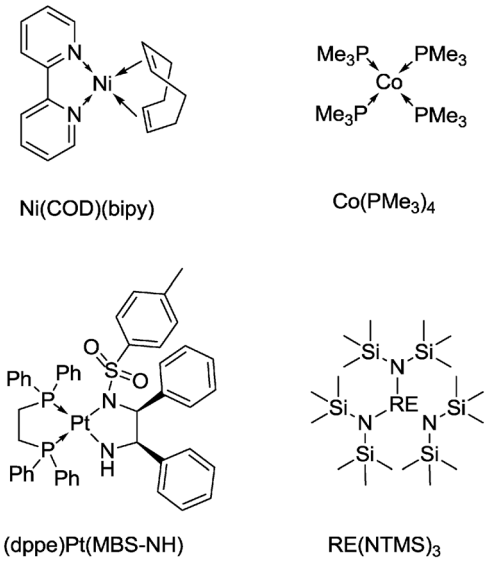
 

organosilicon amines difficult to polymerize less-reactive-monomers (*e.g.* L-proline NCA), resulting in low monomer conversion, low MW, and slow kinetics. For example, the highest MW of PBLG prepared by HMDS mediated polymerization of Bn-Glu-NCA is *ca.* 100 kDa, which is at a 500/1 monomer/initiator (M/I) ratio. In another scenario, when HMDS was used to polymerize a less reactive monomer, γ-(4-vinylbenzyl)-L-glutamate NCA (VB-Glu-NCA) for instance, the polymerization was so slow that only 50–60% monomer conversion was obtained even at M/I as low as 200/1 and the MW of the resulting poly(γ-(4-vinylbenzyl)-L-glutamate) (PVBLG) was barely beyond 30 kDa. To accelerate the polymerization of VB-Glu-NCA by HMDS, Cheng's group developed a dual-catalyst system to balance controlled polymerization and reactivity.[27] In a screen of several acylation nucleophiles, they observed that a trace amount of 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD) dramatically boosted the HMDS-mediated VB-Glu-NCA polymerization with only a slight loss of overall control of the reaction. TBD has been previously used as an effective organocatalyst for lactide (LA) polymerization.[29] The hypothesis here is that TBD might accelerate the polymerization due to its strong nucleophilicity by opening the NCA ring and forming certain active intermediates to react with the chain propagation center.[30] This phenomenon has also been confirmed for a few other nucleophiles such as 1,3-bis(2,4,6-trimethylphenyl)imidazol-2-ylidene (NHC) and 4-(dimethylamino)pyridine (DMAP) (unpublished results). They also reported similar results for another less reactive monomer, γ-(4-allyloxylbenzyl)-L-glutamate *N*-carboxyanhydride (AOB-Glu-NCA), in a separate study.[31]

### 2.2 Other polymerization systems and mechanistic studies

A handful of metal based catalysts have been explored and reported by Deming for controlled NCA polymerization since 1997, among which the Ni(COD)(bipy) and $Co(PMe_3)_4$ were the best two catalysts (Scheme 4).[4] Lin *et al.* have shown that a platinum-based organometallic catalyst, (dppe)Pt(MBS-NH), gave well-controlled NCA polymerization (Scheme 4).[32] Shen and coworkers reported a screening on rare earth (RE) catalysts for NCA polymerization.[33] A few catalysts with various metals and ligands were studied and the underlying mechanism was analyzed. Interestingly, they identified a strong base $RE(NTMS)_3$ (Scheme 4), which is normally a poor catalyst for NCA polymerization, that gave a reasonably well controlled ROP of NCAs.

In conjunction with the development of new organometallic catalysts, efforts have also been made to improve the traditional primary amine initiated NCA polymerization. Messman *et al.* did a mechanistic study by analyzing the end-group structure of an ROP of *O*-benzyl tyrosine NCA. Their results indicated that polypeptides prepared by high vacuum techniques exclusively proceeded by an amine mechanism, in contrast to the mixed mechanisms for polypeptides prepared in the glove box.[23] Heise also performed careful and comprehensive studies examining how the factors such as pressure and temperature affect the polymerization of various NCA monomers.[21,22] Their results pointed out that the γ-benzylester cleavage by terminal amine (backbiting) is the major impurity for Bn-Glu-NCA polymerization and in the case of Bn-Asp-NCA, a more complicated scenario was discovered at elevated temperature, which showed impurities that include side-chain

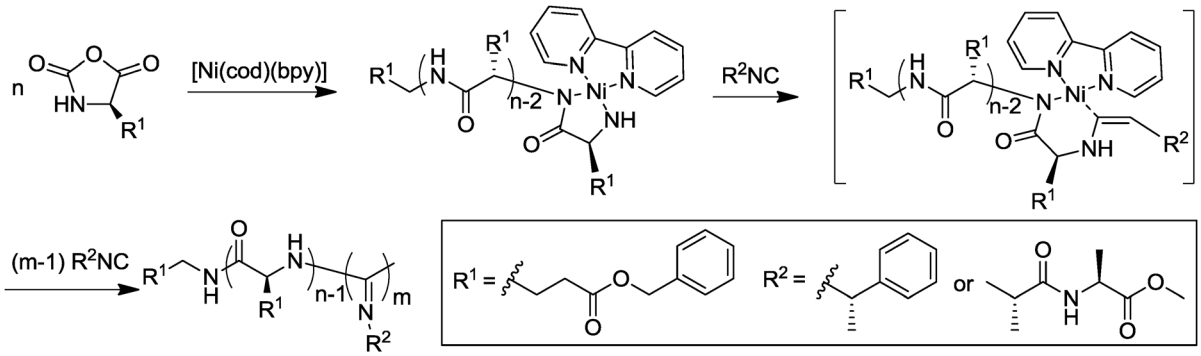



Scheme 4 The structures of selected organometallic catalysts for NCA polymerization.

ester cleavage and formamide end-capping from DMF. These studies are very informative and are useful for optimizing conditions of polymerizations of Glu- and Asp-based NCAs.

### 2.3 Hybrid polypeptide materials

Synergistic incorporation of polypeptide blocks with other classes of polymers that contain segments with distinct structures and properties may create hybrid materials with combined advantages that are useful for unique applications.[34,35] To generate hybrid polypeptides, conjugation of polypeptides with other polymers *via* highly efficient and orthogonal coupling reactions is a common strategy. For example, Lecommandoux *et al.* reported the novel syntheses of well-defined block copolymers composed of a PBLG sequence and a poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) block by "click" coupling alkynyl- and azide-terminated PBLG and PDMAEMA precursors.[36] One particularly attractive approach for synthesizing hybrid polypeptides is sequentially integrating the ROP of NCAs with other polymerization technologies, such as ROP of cyclic ester monomers,[37] atom transfer radical polymerization (ATRP),[38–41] nitroxide-mediated polymerization (NMP),[42,43] or reversible addition–fragmentation chain-transfer (RAFT) polymerization.[44,45] Kros *et al.* reported that synthesis of rod-rod block hybrid poly-(γ-benzyl-L-glutamate)-*block*-polyisocyanide copolymers by the direct combination of ROP of NCA and isocyanide polymerization using the Ni(bpy)(cod) complex as the only catalyst (Scheme 5).[46] The ability of the Ni(bpy)(COD) to subsequently control two completely different types of polymerizations in one pot without any intermediate separation illustrated the powerfulness of this catalyst and can potentially generate a vast amount of hybrid materials with similar structures. Cheng's group reported the integration of ROP of NCAs and ring-opening metathesis polymerization (ROMP) to prepare polypeptide-grafted brush-like polymers in a one-pot fashion.[25] The strategy involves ROMP of




Scheme 5 Synthesis of poly(γ-benzyl-L-glutamate)-*block*-polyisocyanide copolymers by a combination of ROP of NCA and isocyanide polymerization.[46]

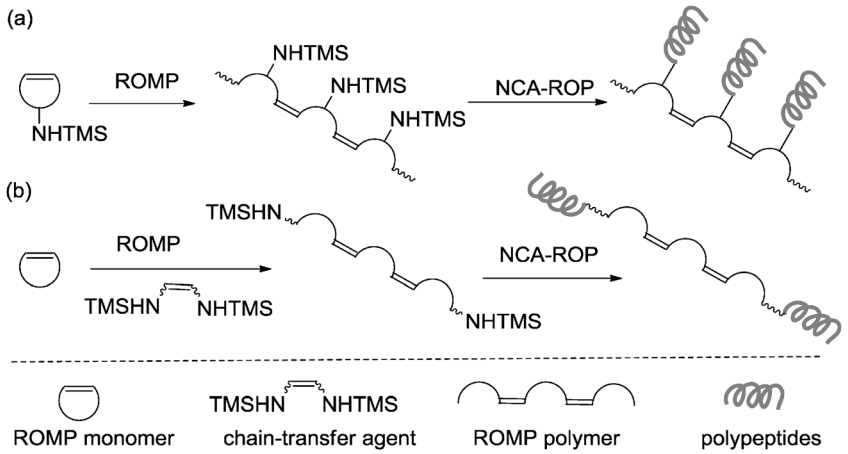



Fig. 1 (a) Synthesis of polypeptide-containing brush-like copolymers using a ROMP monomer bearing a tethered *N*-TMS amine;[25] (b) synthesis of linear hybrid block copolymers *via* a *N*-TMS amine functionalized CTA.[26]

a norbornene monomer containing *N*-TMS amine followed by controlled ROP of NCAs mediated by the pendent *N*-TMS amine group. The excellent compatibility between *N*-TMS amines and ROMP catalysts could also be utilized to prepare linear hybrid block polymers by using an *N*-TMS amine functionalized *cis*-alkene as chain transfer agent (CTA) (Fig. 1).[26] Recently, Li *et al.* reported a new type of molecular bottlebrush with poly-L-lysine (PLL) as the backbone using $N^{\varepsilon}$-bromoisobutyryl-L-lysine NCA, which contains an initiation group for subsequent ATRP (Section 3.1).[41] The same group also reported a DNA grafted polypeptide molecular bottlebrush.[47] Hybrid bottlebrushes with gold nanoparticles and hydrogels can be readily prepared using the resulting DNA grafted polypeptides *via* DNA hybridization.

# 3. New monomers and functional polypeptides

To expand the scope of polypeptides from the twenty canonical amino acids, there is a strong motivation to generate novel NCA monomers from non-canonical amino acids or to modify side-chains of canonical amino acids. Indeed, this approach has recently emerged as one of the most productive topics in the NCA and polypeptide field.[27,31,41,48–67] In this section, we summarize recent efforts in developing various functional NCA monomers. Three select topics, ionic helical polypeptides, stimuli-sensitive polypeptides, and glycopolypeptides, will be highlighted to show the application of new NCA monomers in the synthesis and design of various functional polypeptides.

## 3.1 Overview of new NCA monomers

Table 1 summarizes a number of novel NCAs reported during the past 5–6 years. In 2009, Hammond and co-workers[52] reported the first example of a clickable NCA monomer bearing a propargyl group. This monomer can undergo the copper-mediated [2+3] alkyne–azide 1,3-dipolar cycloaddition reaction or thiol–yne reaction to introduce side-chain PEGylation or glycosylation as reported by a few groups.[55,56,68] Since then, examples of other functional groups introduced to NCA monomers include chloro-,[49] azide-,[58] allyl-,[31,50,53,62] vinyl benzyl-groups,[13,27] and an ATRP initiator.[41] Functional polypeptides were prepared by polymerization of these NCA monomers followed by post-polymerization modifications (*e.g.*, thiol–ene reaction, azide–alkyne click reaction, ozonolysis and reductive amination) to derivatize the functionality of the polypeptides. Li introduced the ATRP initiator functionality into the Lys-NCA to combine the ROP of NCA with the well-developed controlled radical polymerizations.[41] Deming and co-workers very recently developed allyloxycarbonyl

Table 1 The molecular structures of functional NCAs

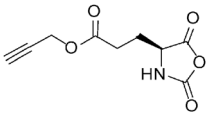
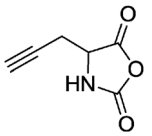
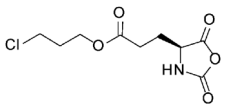
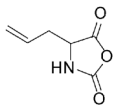
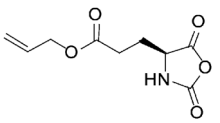
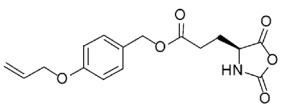
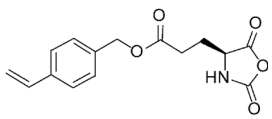
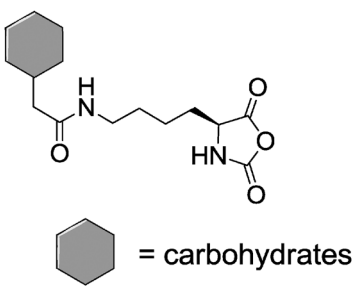
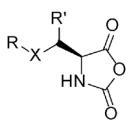
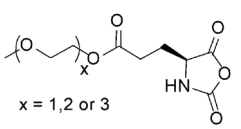
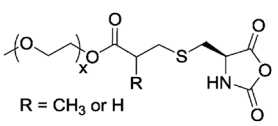
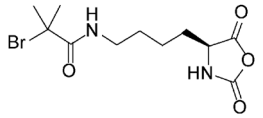
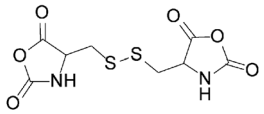
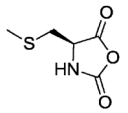
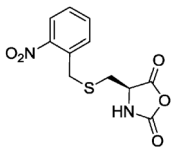
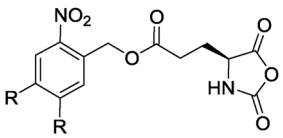
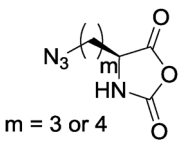
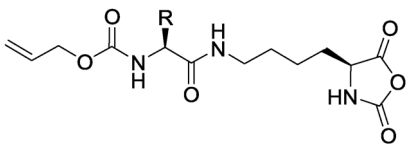
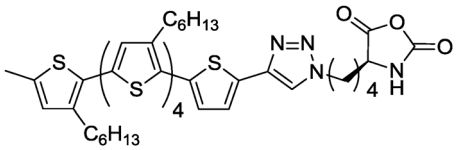
| NCA | Molecular structure | Post modification reaction or function | Ref. |
|---|---|---|---|
| PG-NCA |  | Alkyne–azide [2+3] Huisgen cycloaddition (*e.g.*, peglation and glycosylation); thiol–ynereactions | 52, 55, 56, 69 |
| PG-Gly-NCA |  | Alkyne–azide [2+3] Huisgen cycloaddition (*e.g.*, peglation and glycosylation); thiol–ynereactions | 70, 71 |
| CP-NCA |  | Alkyne–azide [2+3] Huisgen cycloaddition after post-polymerization nucleophilic substitution (*e.g.*, glycosylation and molecular bottle brushes) | 49 |
| DL-Allylglycine |  | Thiol–ene reactions | 50, 62 |
| AL-NCA |  | Thiol–ene reactions | 53 |
| AOB-Glu-NCA |  | Thiol–ene reactions | 31 |
| VB-Glu-NCA |  | Ozonolysis, Suzuki-coupling, olefin metathesis | 13, 27 |
| α-Glyco-Lys-NCAs |   | Mimics of glycosylated peptides and proteins | 51, 66, 72 |
| Glyco NCAs (R = $Ac_4$Gal, $Ac_4$Glu or $Ac_7$Lac; X = O or S; R′ = Me or H) |  | Mimics of glycosylated peptides and proteins; oxidative sensitive | 57 |
| L-$EG_x$Glu-NCA |   | Thermo-responsive polypeptides | 48 |
| $EG_x$MA-Cys-NCA (R = $CH_3$) and $EG_x$A-Cys-NCA (R = H) |   | Thermo-responsive polypeptides | 63 |

Table 1 (*continued*)

| NCA | Molecular structure | Post modification reaction or function | Ref. |
|---|---|---|---|
| Br-Lys-NCA |  | ATRP to prepare molecular bottlebrushes | 41 |
| Cys-NCA |  | Redox sensitive; star polymers and nanogels | 54 |
| Met-NCA |  | Alkylation | 60 |
| NBC-NCA |  | Photo-responsive | 64 |
| DMNB-Glu-NCA (R = OMe) |  | Photo-responsive | 65 |
| Anl-NCA<br>Anv-NCA |  | Alkyne–azide [2+3] Huisgen cycloaddition | 58 |
| $K^{AM}$ NCA (R = $CH_2CH_2SCH_3$ or $CH(CH_3)CH_2CH_3$) |  | Cylindrical polypeptide brushes | 59 |
| Hexithiophene-Lys-NCA |  | Organic semiconductor unit; organic photovoltaic and organic field effect transistor | 67 |

(alloc)-α-aminoamide substituted NCAs, the alloc-α-aminoamide groups served as masked initiators for tandem catalysis to prepare cylindrical brushes based entirely on polypeptides.[59] They also demonstrated in an earlier example that the thioether group in poly(L-methionine) could be directly functionalized by alkylation with various alkylation reagents under mild conditions, allowing facile preparation of water-soluble polypeptides.[60] A few other NCAs monomers were prepared to bear the functional groups needed directly without post-polymerization modifications. For example, synthetic glycopolypeptides[51,57] and pegylated[48,61,63] polypeptides with thermo responsive properties were prepared from direct polymerization of glycosylated and pegylated NCAs. Cys-NCA has been reported as a crosslinker to prepare nanogels for drug delivery applications.[54] Impressively, Holmes synthesized a hexithiophene functionalized Lys-NCA and polymerized it by HMDS. The hierarchical self-assembly of these organic semiconductor functionalized polypeptide helices leads to interesting properties when used in organic photovoltaic and organic field transistor devices.[67]

### 3.2 Ionic helical polypeptides

Water soluble peptides that adopt stable helical conformations are attractive motifs because of their importance in basic science and their broad utility in medicine and biotechnology. There is often a dilemma in the design of water-soluble and bioactive helical polypeptides: polypeptides composed entirely of charged amino acids have excellent aqueous solubility but limited helicity due to side-chain charge repulsion; increasing the proportion of hydrophobic amino acids tends to increase helicity by the contribution of side-chain hydrophobic interactions, but the resulting polypeptides often show poor water solubility. Helix-stabilization strategies used for oligopeptides,

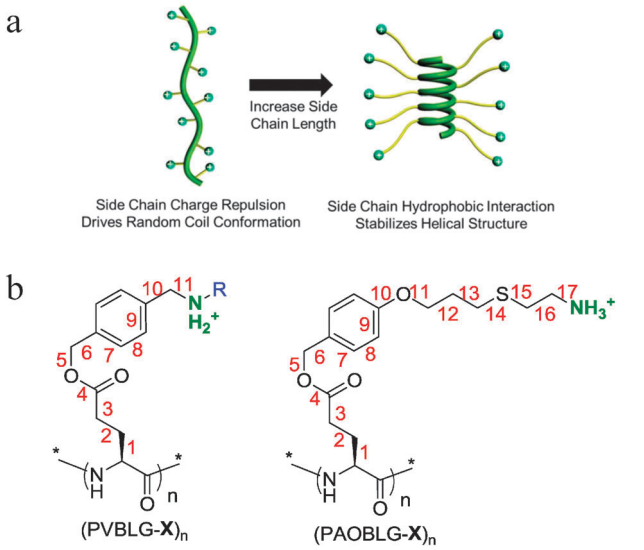



Fig. 2 (a) Schematic illustration of the elongated hydrophobic side-chains leading to formation of polypeptide helixes with unprecedented stability.[13] (b) Structure of the polypeptides with 11 and 17 σ-bonds of side chain charge-backbone distances.[13,31]

such as salt bridges and side-chain tethering, however, generally require the design of peptides with specific sequences. For polypeptides prepared by polymerization, such helix-stabilization strategies cannot be simply applied.

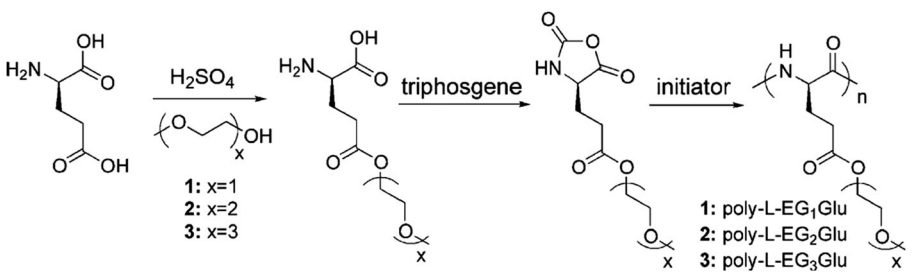
Recently, Cheng and coworkers circumvented this problem and generated a class of water-soluble, ultra-stable α-helical polypeptides by elongating charge-containing amino-acid side chains to position the charges distally from the polypeptide backbone (charge-backbone distance ≥11 σ-bonds) (Fig. 2a).[13,31] A series of polypeptides denoted as PVBLG-X were facilely prepared *via* ROP of VB-Glu-NCA, followed by highly efficient post-polymerization modifications to introduce ammonium cations on the side-chain. These polypeptide electrolytes contain sufficiently long hydrophobic side chains that contribute to their remarkable helical stability against changes in pH, temperature, and various strong denaturing reagents (Fig. 2b). Their follow-up work showed that by further increasing the charge-backbone distance to 17 σ-bonds, very short polypeptides (DP = 10) that conventionally cannot adopt a helix showed unusually high helicity and stability (Fig. 2b). These ionic helical polypeptides are useful materials in nucleic acid delivery applications (Section 5.2).

### 3.3 Stimuli-sensitive polypeptides

Stimuli-sensitive polymers capable of responding to internal or external stimuli are especially useful materials in drug delivery.[73] In the presence of specific triggers such as pH change, temperature variation, photon, enzyme, or redox environment, these polymers were designed in a manner that their chemical and/or physical structures can undergo rapid and dramatic rearrangement eventually resulting in new properties and functions. Many polypeptides have demonstrated their ability in responding to various external/internal triggers.[74,75] For example, pH-sensitive PLG, poly(L-histidine), PLL as well as modified poly(L-aspartic acid) and poly(L-serine) were extensively studied; various pH-responsive nano-/micro-structures (vesicles, micelles, and nanoparticles) were obtained from those smart materials and used as delivery vehicles (Section 5.2).[10,76–80]

Li *et al.* reported the first thermo-responsive polypeptide in 2011.[48,81] They developed a new pegylated-L-glutamate NCA with different oligo(ethylene glycol) (OEG) lengths (Scheme 6). The solubility, helicity and the lower critical solution temperature (LCST) of the resulting polypeptides strongly depend on the length of the OEG side-chain. Interestingly, they showed that the secondary structures of the polypeptide are critical for the thermo-responsive property because racemic polypeptides with the same OEG side-chain, which adopt a random coil confirmation, do not exhibit LCST behavior (Fig. 3). By copolymerizing pegylated-L-glutamate NCAs with different OEG length, the LCST can be modulated to a broad range by manipulating the feeding ratio of NCA monomers. Cysteine-based thermo-responsive polypeptides were reported by the same group using a similar strategy.[63] Thermo-responsive polypeptides can also be prepared by conjugating azide functionalized OEG groups on alkyne functionalized polypeptides, which also showed tunable temperature-dependent solubilities under biologically relevant conditions.[82]

Redox-responsive polypeptide based material has been reported recently. Zhang and co-workers have synthesized a block copolymer poly(ethylene glycol)-*b*-poly(L-cysteine)-*b*-poly(L-phenylalanine) (PEG-PCys-PPhe) and self-assembled it to give micelles with a core–shell–corona structure. The thiol groups on the PCys block can undergo reversible crosslinking and breaking under oxidative and reductive conditions.[83] Deming reported an oxidation triggered helix-to-coil transition of glycopolypeptides and their results of transition mechanism studies suggest that the polarity and steric effects play an important role in the transition.[57]




Scheme 6 Synthesis of the LCST polypeptides.[48]

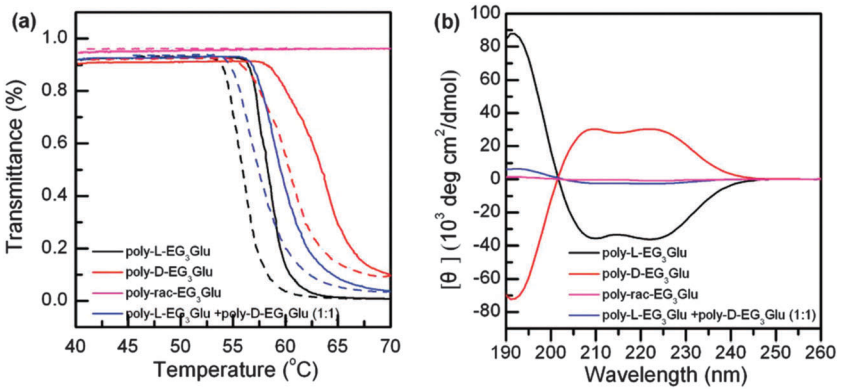



Fig. 3 Plots of transmittance as a function of (a) temperature and (b) CD spectra for poly-L-$EG_3$Glu and poly-D-$EG_3$Glu and poly-*rac*-$EG_3$Glu homopolypeptide. Solid line: heating; dashed line: cooling. Reprinted with permission from ref. 48. Copyright (2011) American Chemical Society.

Among all the triggering mechanisms being developed and studied, light stimulus is particularly attractive because of its non-invasive nature and precise spatial and temporal control. Dong's group demonstrated for the first time that light responsive polypeptides can be made by the ROP of a novel NCA monomer, namely *S*-(*o*-nitrobenzyl)-L-cysteine *N*-carboxyanhydride (NBC-NCA).[64] Separately, Cheng's group recently developed a light-sensitive NCA monomer based on glutamate, DMNB-Glu-NCA (Table 1), which contains a photo labile (UV and two-photon sensitive) moiety 3,4-dimethyoxy-2-nitrobenzyl. Photocontrolled gene plasmid release using polymers prepared from this monomer has been demonstrated (Section 5.2).[65]

### 3.4 Glycopolypeptides

Carbohydrates play a critical role in a large number of biological processes like metabolism, signal transduction, protein post-translational modification, adhesion, and recognition.[84,85] In particular, the recognition of carbohydrates with carbohydrate-binding proteins known as lectins has triggered research interests in glycopolymers as synthetic analogues to study the carbohydrate–lectin interaction, as well as their use as scaffolds for tissue engineering and as drug carriers. Glycopolypeptides are particularly interesting biomaterials for this purpose as they are the closest mimics of natural glycoproteins. Deming and Gupta pioneered the synthesis of glycopolypeptides using carbohydrate functionalized NCAs. Several synthetically-challenging NCA monomers were elegantly synthesized and polymerized to yield fully water-soluble glycopolypeptides bearing various carbohydrates.[51,57,66,72] Heise synthesized glycopolypeptides[70,71] *via* a grafting approach using alkyne-functionalized polypeptides and azide-derivatized carbohydrates. Selective glycopolypeptides–lectin interactions were studied by Gupta and Heise aiming to study the multivalency ligand effect on lectin binding stoichiometry and affinity, the binding with lectins in the self-assembled glycopolypeptides (*e.g.*, nanorod[86] and polymersome[70]), and the effect of the helical/random coil conformations of a polypeptide backbone on the lectin binding.

## 4. Self-assembly of polypeptides

Synthetic polypeptides form specific secondary structures with well-defined interactions, both in solution and in solid state. By incorporating polypeptides into macromolecules and controlling their intermolecular interactions,[87] a variety of self-assembled nanostructures[35,88–96] (*e.g.* micelle-like aggregates, hollow tubules, sheets and vesicles) have been made and these assemblies can further organize into hierarchical structures. Since polypeptides offer both the protein-like functionality and the polymer processability, the self-assembled polypeptide materials hold great promise in the medical and biotechnology applications.[18,97] The past two decades have witnessed growing interest in self-assembled structures from synthetic polypeptides and polypeptide-containing hybrid macromolecules. Many of these polypeptide or hybrid systems are amphiphilic[35,88,93–95,98–100] in design and their aggregation behaviors follow the classic theory of surfactant micellation.[101,102] A few excellent reviews have been recently published and offer a comprehensive overview of the self-assembly of amphiphilic polypeptides.[1,35,92,94,95,103,104] Herein, we highlight a few interesting examples that approached self-assembly which are attributed to the specific secondary structures from polypeptides.

### 4.1 Helical assembly of polypeptides

Boden and coworkers presented a statistical mechanical model for the self-assembly of rod-like, β-sheet-forming peptides into helical tapes, which further associate into twisted ribbons, fibrils or fibers at higher concentration (Fig. 4).[105–107] The model accounts for the balance between the elastic energy cost of twisting ribbons and the energy gains from the supramolecular associations to predict the morphology of the assemblies. Numerous experimental works have qualitatively supported the prediction and demonstrated how the morphology and properties of the self-assembling structures can depend on the molecular parameters of the peptide building blocks.[108,109] The helical assembly process demonstrated in these systems follows a cooperative supramolecular polymerization mechanism,[110] which was originally proposed by Oosawa in 1962 to describe the helical aggregation of

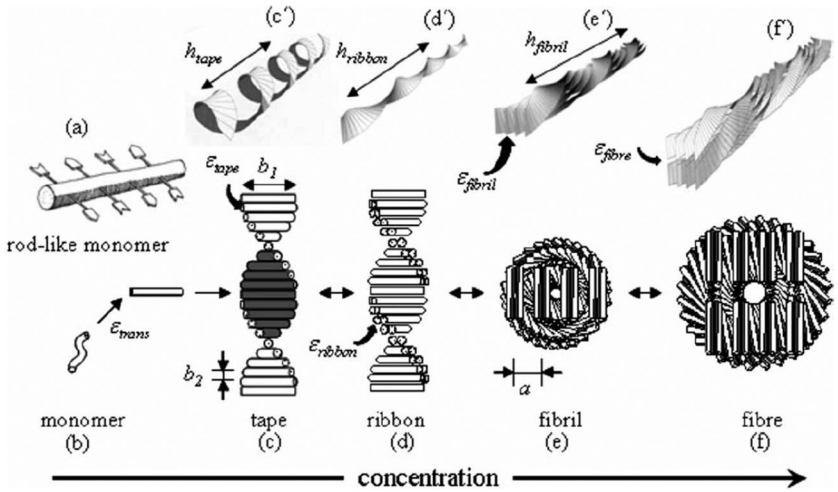



Fig. 4 Model of hierarchical self-assembly of chiral rod-like units. Reprinted with permission from ref. 105. Copyright (2001) National Academy of Sciences, U.S.A.

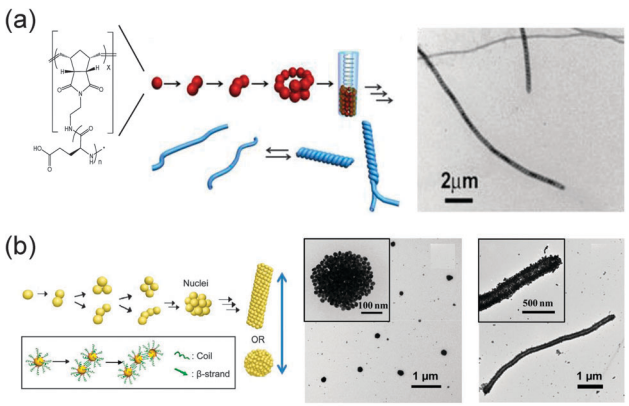



Fig. 5 (a) Supramolecular polymerization of polypeptide-grafted comb-like polymers into tubular superstructures. Reprinted with permission from ref. 112. Copyright (2011) American Chemical Society (b) Nucleation-controlled supramolecular polymerization of polypeptide-grafted gold nanoparticles. Reprinted with permission from ref. 113. Copyright (2013) American Chemical Society.

proteins in solution.[111] To expand the concept beyond the typical rod-like building blocks, Lin and Cheng reported the synthesis of polypeptide-grafted comb polymers and the use of their tunable secondary interactions in solution to achieve controlled supramolecular polymerization (Fig. 5a).[112] The formation of an anti-parallel β-sheet from grafted PLGs of neighboring macromolecular units upon association, as a thermodynamically more favorable configuration than extended coils, provided the driving force for supramolecular polymerization. The resulting tubular supramolecular structures, with external diameters of hundreds of nanometers and lengths of tens of micrometers, are stable and resemble, to some extent, biological superstructures assembled from proteins (*e.g.* tubulins). This study shows that highly specific polypeptide interactions can guide the self-assembly of large macromolecules monomers into giant supramolecular polymers. The general applicability of this strategy was further demonstrated by carrying out helical assembly from gold nanoparticles (NPs) grafted with the same polypeptides on the surface. Besides, nucleation-controlled supramolecular polymerization of PLG-grafted gold NPs was also reported very recently.[113] The supramolecular structures depend on the grafting density of the polypeptides on the NPs and the sizes of the NPs (Fig. 5b).

### 4.2 Self-assembly of block copolypeptides in the solid state and the effects of chain topology

Block copolymers can self-assemble into specific, ordered nanostructures upon microphase separation. As polypeptides can adopt ordered conformations, such as α-helices or β-strands, hybrid block copolymers composed of a peptide segment and a synthetic block may possess either rod-coil character (in the case of helical peptides), or have the capability to undergo intermolecular hydrogen bonding (*e.g.* for β-sheet forming peptides). Klok and Lecommandoux reported the solid-state structure, organization and properties of peptide-synthetic hybrid block copolymers. Unlike many other synthetic polymers that form flexible, coil-like structures, synthetic polypeptides can adopt rigid rod-like, α-helical structures with intra hydrogen bonding, or β-pleated sheet-like structures with inter hydrogen bonding with the adjacent neighbors. This offers opportunities to direct nanoscale structure formation, such as lamellar microphase-separated structures.[95] Floudas and Spiess examined the effect of chain topology on the nanophase self-assembly in a series of model diblock and star co-polypeptides of PBLG and PZLL.[103] They found that diblock copolypeptides are nanophase separated with PBLG and PZLL domains comprising α-helices packed in a hexagonal lattice. In contrast, star copolypeptides are only weakly phase separated (Fig. 6), and the PBLG and PZLL have reduced helix persistence under the confinement. The study indicates the strong effect of chain topology on the copolypeptide miscibility and the persistence of helical peptides, and sheds some light on how to design polypeptide nanomaterials with complex architectures.

### 4.3 Self-assembly of block copolypeptides in solution

On the other hand, a number of block copolypeptides have been reported to self-assemble into nanostructures in solutions, such as

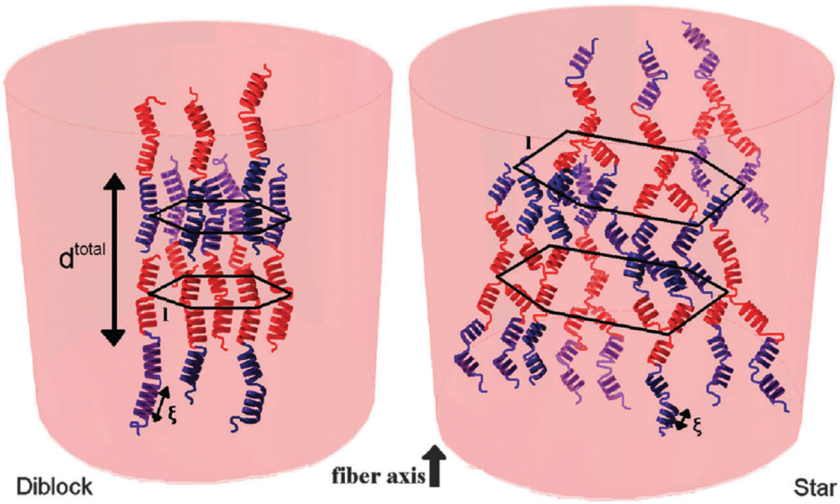



Fig. 6 Schematic representation of the self-assembly in diblock (left) and star (right) polypeptides. Reprinted with permission from ref. 103. Copyright (2008) American Chemical Society.

micelles, vesicles, fibers and other morphologies.[35,70,88,90,91,99,114] Typically one of the blocks conveys the initial aggregation characteristics of the copolypeptides, which can be an α-helical segment or a β-sheet structure. The second block is more flexible that provides solubility. For example, Lecommandoux reported the formation of vesicles from dextran-*b*-PBLG in water, where the PBLG helices are parallel-packed inside and stabilized by dextran chains as the inner and outer shell.[115] Furthermore, the functionality can also be incorporated into these blocks to be susceptible to environmental stimuli. Deming and co-workers reported vesicular assemblies whose size and structure were sensitive to pH and salt concentration (Fig. 7).[10,116] Cargo can be loaded and released controllably. The self-assembly of polypeptides is more complex than that of general polymers due to the existence of different secondary structures, besides the phase separation in self-assembly.

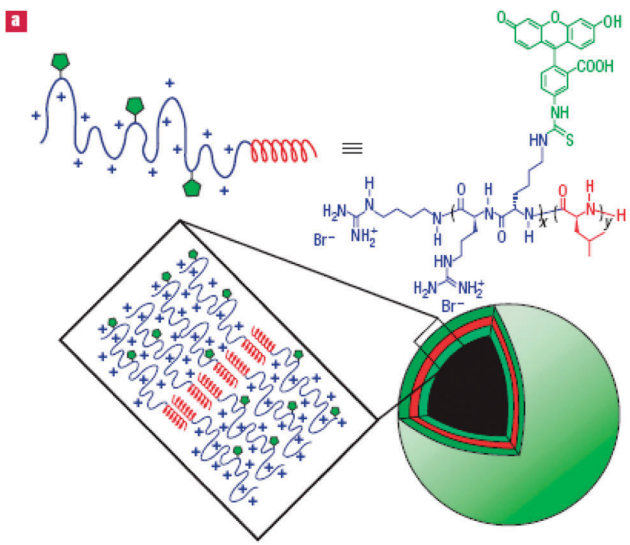



Fig. 7 Schematic diagram of proposed self-assembly of polypeptide vesicles. Reprinted with permission from Macmillan Publishers Ltd: [*Nature Materials*] ref. 116. Copyright (2007).

In another report, by tuning the peptide sequence and pH of solution, a variety of architectures, such as β-sheet-plates, β-sheet-fibers and α-helix-particles could be formed spontaneously by one molecular system.[117] Functional small molecules with designed structure can also attribute to form the hierarchical structure from polypeptides. A supramolecular framework was reported by Houbenov's group in 2011, which was simply formed by mixing PBLG-*b*-PLL and 2′-deoxyguanosine 5′-monophosphate (dGMP) in the solution. It was found that π-stacked dGMPs are inserted between the fully extended β-strand of PLL blocks in the lamellar structure *via* ionic interaction with the side-chain ammonium of PLL. The supramolecular framework is cage-like and possesses pores in the dGMP-PLL domain.[118]

## 5. Biomedical applications of polypeptides

The biomedical applications of polypeptides have been pursued for many years. One of the most successful examples is Glatiramer Acetate, a commercial drug to treat multiple sclerosis. This FDA-approved drug is a random polypeptide (average $M_W$ 6.4 kDa) composed of glutamic acid, lysine, tyrosine and alanine prepared from a random ROP of NCAs. Moreover, polypeptides have also been actively exploited as antibiotics, carriers for drug and gene delivery, and scaffolds for regenerative medicines.

### 5.1 Membrane active and antimicrobial polypeptides

Peptides have been used in the transmembrane domain in delivery vehicles and as antimicrobial drugs for many years. Often, those peptides contain approximately 1/3 of positively charged (*e.g.* Lys) and 2/3 of hydrophobic amino acid sequences that favor the α-helix. It has been generally accepted that the activities of these peptides are strongly related to their amphiphatic helical conformations.[119–121] Polymeric peptide analogues such as poly(β-peptides)[122,123] and peptoids[124,125] have been commonly used as substitutes for cell-penetrating peptides

(CPPs) and antimicrobial peptides (AMPs), with only a few reports of polypeptides prepared from ROP of NCA. Deming *et al.* initiated an early study in 2001 to screen membrane active polypeptides using a parallel and combinatory approach.[126] In this study, they randomly co-polymerized Z-Lys-NCA with a few hydrophobic amino acid NCAs and created a library of polypeptides. By employing an optical assay, they screened these polymers and studied how the parameters such as the side chain structure of the amino acid, hydrophobicity and MW influenced their membrane disruption results. They concluded that polypeptides containing amino acids favoring α-helical structures (*e.g.* Ala, Phe, and Leu) are generally much stronger membrane disruption agents compared to those containing amino acids favoring β structures (*e.g.* Ile and Val). In a later study from the same group, they synthesized a block copolypeptide, poly(L-homoarginine)$_{60}$-*b*-poly(L-leucine)$_{20}$ ($R_{60}L_{20}$) and self-assembled the material to vesicles by extrusion. These vesicles are about 1 μm in size and demonstrated superior cell penetrating properties compared with the poly(L-lysine)$_{60}$-*b*-poly(L-leucine)$_{20}$ ($K_{60}L_{20}$). A mechanistic study revealed that these vesicles are internalized through macropinocytosis, which is not surprising considering its large size and lack of receptor-specific ligands. The majority of the internalized vesicles were trapped in the endosome.[127] Leong and Park utilized the same parallel and combinatory approaches as Deming to explore polypeptides for antimicrobial activities.[128] They identified a few polymers (*e.g.* $P(K_{10}F_{7.5}L_{7.5})$ and $P(K_{10}F_{15})$) with promising minimum inhibitory concentration (MIC) values that are comparable or superior to natural AMPs. However, those polymers also showed very strong hemolytic ability, hampering their real application. Hammond and co-workers synthesized AMPs from the ROP of γ-propargyl-Glu-NCA followed by grafting different amine-containing side-chains through alkyne–azide click chemistry.[129] Their study focused on understanding how primary, secondary, tertiary and quaternary amines impact the antimicrobial activities. Those polypeptides are non-hemolytic with moderate antimicrobial activities towards both gram-positive and gram-negative pathogens tested. The same group recently developed a new class of cationic peptidopolysaccharides as AMPs by mimicking the bacterial peptidoglycan structures.[130] Having various cationic polypeptides grafted to chitosan, these novel materials showed outstanding broad-spectrum antimicrobial activities against a number of clinically significant bacteria and fungi. It is believed that the structural affinity with the microbial cell-wall of these peptidopolysacharides gives rise to their excellent activity and selectivity.

### 5.2 Gene and siRNA delivery

Due to their desired biocompatibility and biodegradability, cationic polypeptides have been routinely used as non-viral gene delivery vectors. PLL is the first set of polypeptides used for gene delivery, which can ideally condense anionic DNA *via* electrostatic interactions at molecular weights higher than 3000 Da.[131,132] Nevertheless, because of its MW-dependent toxicity and inability to induce endosomal escape, PLL displays unsatisfactory transfection efficiencies when used stand-alone.[133] In order to address this problem, PLL is modified with oligo-histidine which helps facilitating the endosomal escape *via* the "proton-sponge" effect of the imidazole groups.[134–136] Poly(L-histidine) itself, however, shows weak DNA binding capacities and its application as a gene transfection vector is restricted. As an alternative, poly(L-arginine), which exhibits strong DNA binding and cell penetrating capacities, has aroused more interest. The p$K_a$ of the guanidinium group on the side chain is ~12.5, and it tends to form multiple hydrogen bonds with lipid bilayers.[137] Therefore, poly(L-arginine) with a minimum of 4–8 arginine repeating units could effectively condense DNA and trigger gene transfection. A further increase in the repeating units leads to unappreciable increase in the transfection efficiency, which is ascribed to the excessive binding with DNA that restricts intracellular DNA unpackaging.[138]

Kataoka's group developed a series of *N*-substituted poly(L-aspartamide), which showed desirable gene transfection efficiencies with minimal cytotoxicity.[79,139,140] They revealed minimal membrane destabilization at physiological pH, yet there was a significant enhancement in the membrane destabilization at the acidic pH mimicking the late endosomal compartment (pH similar to 5).[79] They further revealed an odd–even effect of repeating aminoethylene units in the side-chain of *N*-substituted poly(aspartamides) on gene transfection profiles (Fig. 8).[140] Briefly, it was found that polymers with an even number of repeating aminoethylene units outperformed their odd-number counterparts by an order of magnitude, which was closely related to the buffering capacity of the polymers as well

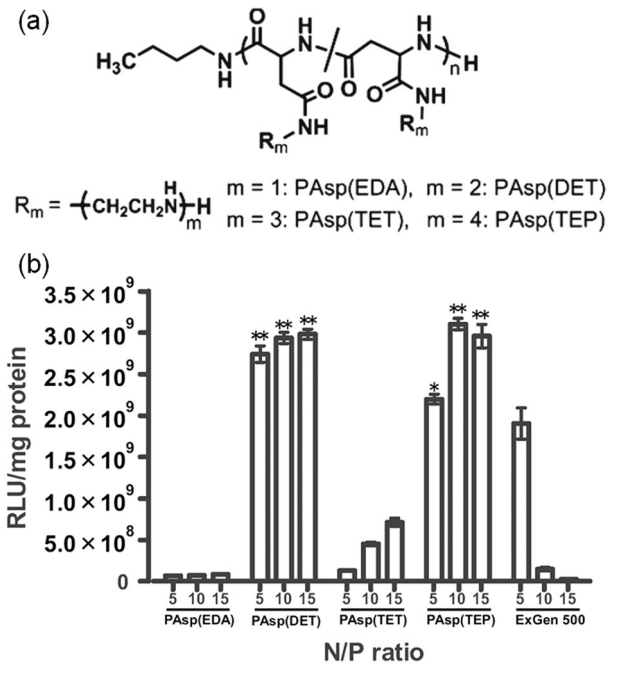



Fig. 8 Odd-even effect of repeating aminoethylene units in the side-chain of *N*-substituted poly(aspartamides) on gene transfection.[140] (a) Chemical structures of poly(aspartamides) with different repeating aminoethylene units; (b) *in vitro* transfection efficiency of poly(aspartamides) at varying N/P ratios with Huh-7 cells determined by luciferase assay, Commercial transfection reagent ExGen 500 was used as a control. Reprinted with permission from ref. 140. Copyright (2011) American Chemical Society.

as their capability in selectively disrupting membrane integrity at endosomal pH. Based on the poly(aspartamides), they also developed a promising siRNA delivery system by covalently attaching siRNAs. Specifically, a comb-like polymer PAsp(-SS-siRNA) was generated by grafting siRNAs to the side-chain of the poly(L-aspartamides) through disulfide linkage. Using this PAsp(-SS-siRNA), they observed better stability of the formed polyion complex and a potent gene silencing compared with using conventional monomeric siRNAs. They reasoned that this PAsp(-SS-siRNA) forms stable PICs due to its larger size and higher anionic density, giving rise to more efficient internalization.[141] In a more recent study, they expanded the system by switching the disulfide linker to an acid-labile maleic acid amide linker for the siRNA grafting, which resulted in accelerated endosomal escape and reduced immune response.[142]

In an attempt to improve the polypeptide-mediated gene transfection efficiency, a variety of strategies have been adopted. Shim *et al.*[78] developed acid-transforming PEG-conjugated poly(ketalized serine) [PEG-poly(kSer)] as non-viral gene vectors, which may undergo conversion of poly(kSer) to naturally occurring poly(serine) upon acid-hydrolysis of ketal linkages, thus facilitating intracellular DNA unpackaging. Sanjoh *et al.*[143] developed PLL complexes functionalized with a pH-sensitive charge-conversional poly(aspartamide) derivative for the controlled gene delivery to human endothelial cells, which displayed charge conversion in the endosomal/lysosomal compartments to promote intracellular DNA release. Additionally, Kang *et al.*[144] synthesized poly(L-lysine)-*g*-sulfonylurea derivatives, which targeted genes to insulin secreting cells (RINm5F) that express sulfonylurea receptors to allow elevated gene delivery efficacy.

Gabrielson and Cheng recently developed high MW, cationic, α-helical polypeptides, termed PVBLG-8, for non-viral gene delivery.[145] The helical structure of PVBLG-8 is stabilized by increased hydrophobic interaction of the side-chains and reduced side-chain charge repulsion, which is achieved by maintaining a minimum separation distance of 11 σ-bonds between the polypeptide backbone and the side chain charge (Section 3.2).[13] PVBLG-8 showed CPP-like membrane activity correlated to its helical conformation, and because of its high charge density and higher MW as compared to CPPs, PVBLG-8 was also able to condense and deliver DNA and siRNA to mammalian cells, making it a better material for gene delivery than traditional CPPs.[146] They also demonstrated that the helical structure of PVBLG-8 is essential for triggering effective cell penetration and gene transfection, which lead to pore formation on cell/endosomal membranes (Fig. 9). In addition, trigger-responsive domains were incorporated and different molecular architectures were designed to further improve the transfection efficiency of PVBLG-8.[65,147] In the former case, photo-responsive groups can be introduced into PVBLG-8 polymer chains *via* copolymerization with DMNB-Glu-NCA (Section 3.1). Upon light irradiation, the resulting negatively charged PLG led to charge density and conformation change of the polypeptides, which facilitated intracellular DNA release and showed improved transfection efficiency with lower cytotoxicity.[65] In the latter case, diblock, ABA triblock, graft, and star (8-arm) PEG-PVBLG-8 copolymers were synthesized and evaluated for *in vitro* transfection compared with the PVBLG-8 homopolymer. The star copolymers, resembling the ''multivalent cationic sphere'', gave the highest transfection efficiency with relatively low cytotoxicity.[147] Moreover, PVBLG-8 was included in supramolecular self-assembled nanoparticles (SSNPs) to promote cellular internalization and endosomal escape. The SSNPs were used for oral delivery of therapeutic TNF-α siRNA and *in vivo* DNA delivery.[148,149] Because of the excellent helicity stability of PVBLG-8 against pH changes, the helicity-dependent permeability was maintained under different

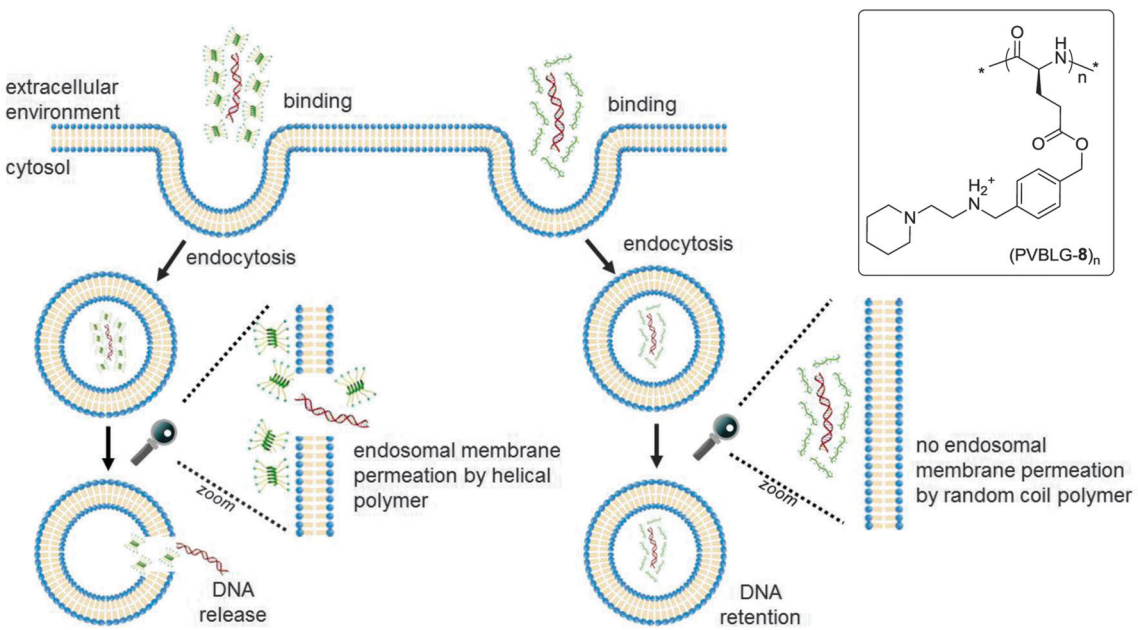



Fig. 9 Cartoon illustration of PVBLG-8 mediated transmembrane activity and gene delivery.

physiological conditions (*e.g.*, acidic stomach and weakly basic intestinal environments).

### 5.3 Drug delivery

Besides their applications in non-viral gene delivery, polypeptides also demonstrated enormous potential in drug delivery and drug derivatization. Since most anti-cancer chemotherapy drugs are hydrophobic and cytotoxic small molecules, delivery systems that can improve solubility, pharmacokinetics, and enhanced selective distribution to target cells are generally required. Polypeptides demonstrated remarkable superiorities partially due to their excellent biodegradability and biocompatibility. For instance, poly(L-glutamate) and poly(L-aspartate) have been extensively exploited for camptothecin (CT-2106), paclitaxel (NK105 and CT-2103) and Cisplatin (NC-6004) delivery in preclinical and clinical settings.[16,150–156]

Amphiphilic polypeptide based micelles/vesicles are commonly used as drug carriers. Hua *et al.*[157] and Wang *et al.*[158] developed a triblock polymer of PLLeu-PEG-PLLeu and a four-arm polymer of $PZLL_2$-PEG-$PZLL_2$, which formed micelles and vesicles in water, respectively. They showed high encapsulation efficiencies for hydrophobic paclitaxel and hydrophilic DOX·HCl, respectively, and controlled drug release profiles were observed. In addition, PEG-PLL-PLLeu based micelles were recently reported to co-deliver docetaxel and siRNA-Bcl-2; this highly stable and non-cytotoxic co-delivery system showed synergistic tumor suppression *in vivo*.[159] Kataoka reported the Y-shape two-armed PEG-*b*-PLG-cholesterol based metallosome for the co-delivery of a platinum complex (through coordination with carboxylic acid) and other encapsulated agents, another sibling of their famous PLG-Cisplatin system.[160] Based on the non-covalent interaction within amphiphilic peptides, supramolecular peptide hydrogels were also prepared for drug embedment.[161] After antibiotics loading and injection into rabbit eyes, the hydrogels effectively prevented scar formation post glaucoma filtering operation, and demonstrated sustained therapeutic efficacy against glaucoma.

Tumor microenvironments often display different properties to normal tissues, including lower pH ($<6.0$), higher temperature (39–42 °C), and over-expression of some enzymes, which have been exploited as triggers for on-demand release of therapeutic or diagnostic agents. Stimuli-responsive polypeptides are thus another important category for drug delivery. A well-known example is poly(L-histidine), which promotes drug release at the tumor site for its p$K_a$ of 6.0, making it hydrophobic at physiological pH while hydrophilic at the pH of the tumor site.[162] Chen's group investigated a series of random poly(L-glutamic acid-*co*-L-lysine)s [P(Glu-*co*-Lys)s] copolymers as pH-triggered charge-reversal polypeptides for Cisplatin delivery. By manipulating the relative ratio of Glu to Lys, the surface charge of the Cisplatin/P(Glu-*co*-Lys) nanoparticles could be reversed to positive from negative at tumor extracellular pH (pH 6.5–7.2) to enhance the drug uptake and inhibition of cancer cell proliferation.[163] Zhong's group reported a redox and pH dual-sensitive micelles based on lipoic acid (LA) and *cis*-1,2-cyclohexanedicarboxylic acid (CCA) decorated poly(ethylene glycol)-*b*-poly(L-lysine) (PEG-P(LL-CCA/LA)) block copolymers.[164] Stimuli-responsive blocks can also be introduced into polypeptides as spacers or crosslinkers to trigger release of loaded drugs in certain environments.[165–168] Moreover, temperature-responsive polypeptides, including elastin-, silk-, and collagen-like polypeptides provide a "hear-targeting" strategy for tumor-specific drug delivery.[169] They usually share the phase-transition temperature of about 37–42 °C. At physiological body temperature, the polypeptides were hydrophilic and allowed circulation of the conjugated drugs; when reaching the tumor regions, the high temperature caused conformation alteration of the polypeptide and they became hydrophobic, which led to drug precipitation and localization at the tumor site.[170,171]

## 6. Conclusions and perspectives

There was indeed a blossom of the NCA and polypeptide field in the past decade. The ongoing advancing synthetic technologies for the ROP of NCAs and the development of various interesting functional NCA monomers will give convenient access to novel homo- and hybrid-polypeptide materials with well-defined topological structures and high-performance characteristics for various mechanical, environmental and biological applications. Despite the promising perspectives, the functions of synthetic polypeptides are still in their infancy compared with their natural biological counterparts (proteins) in terms of activity, complexity and delicacy. It is still impossible to rival those delicate evolved proteins and substantial efforts are needed dedicating to both fundamental and applied research. To this end, there are several exciting, important and challenging research topics which include the design of new NCA monomers that are potentially moisture resistant to allow facile NCA preparation and purification, design of functional NCA and polypeptides by exploring new structures using noncanonical amino acids or learning from interesting post-translational modifications in proteins (*e.g.*, acylation, methylation, glycosylation, and phosphorylation), exploration of properties and functions of hybrid polypeptides integrating other biological macromolecules such as nucleic acids and polysaccharides, construction of complex hierarchical nano-/micro-architectures and frameworks beyond simple amphiphilic self-assembly and phase separation by taking advantage of the unique secondary structures of polypeptides to generate synthetic polypeptides with protein-like biological functions, such as catalysis and recognition. With the continuing endeavor in this field, synthetic polypeptides are expected to play a more important role in the design of functional materials.

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