Perspective Article

Polypeptide-based materials prepared by ring-opening polymerisation of anionic-based $\alpha$-amino acid N-carboxyanhydrides: A platform for delivery of bioactive-compounds

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ABSTRACT

Polymisation of $\alpha$-amino acid N-carboxyanhydrides (NCAs) is one of the most common techniques to prepare synthetic polypeptides. Of special interest are the NCAs derived from $\alpha$-amino acids, L-aspartic acid and L-glutamic acid, since most investigations have been focused on their use to synthesise multiblock copolypeptides or hybrid synthetic polypeptide-polymers to design excipients suitable for delivery of bioactive compounds. This perspective highlights advantages of using L-aspartic acid and L-glutamic over other natural $\alpha$-amino acids in that their pendant carboxyl group serves as a reactive handle for coupling a variety of reactive groups, and because the resulting polypeptides have the ability to adopt secondary structures. In addition, recent progress in the ring-opening polymerisation of NCAs will be discussed. Throughout, we provide representative examples that shed light on the NCAs polymerisation process, and we finally share our perspectives concerning the practical use of anionic $\alpha$-amino acids as building blocks for future investigations.

1. Introduction

Natural proteins, which are ubiquitous and essential for the structure and function of cells, tissues and organs, the transport of molecules, and the catalysis of biochemical reactions that are needed to sustain cellular processes, are constituted from 20 naturally occurring amino acids [1]. These materials are polypeptide copolymers that derive their properties from precisely controlled sequences and compositions of their constituent amino acid monomers [2]. Researchers have long been fascinated by mimicking these natural polypeptides and combining them with synthetic polymers to obtain complex architectures that can exhibit superior features for potential applications in biomedicine and biotechnology such as tissue engineering, drug delivery, and as therapeutics [3–5]. Currently, three main techniques are employed for the synthesis of polypeptides: ring-opening polymerisation (ROP) of $\alpha$-amino acid N-carboxyanhydrides (NCAs) [6], solid phase peptide synthesis (SPPS) [7] and protein biosynthesis [8]; the former enables the production of high molecular weight (MW) polypeptides with narrow dispersities in a facile manner [6].

The $\alpha$-amino acids L-aspartic acid (Asp) and L-glutamic acid (Glu), which present a carboxyl group at the $\beta$ and $\gamma$ carbons, respectively (Fig. 1), are useful precursors for constructing synthetic polypeptides. For simplicity, we invariably refer to them as anionic $\alpha$-amino acids using the AAA acronym. Nonetheless, these amino acids must be protected prior to NCA formation in order to prevent the activation of the side chain carboxylic groups during polypeptide synthesis, otherwise it would lead to undesired branched polypeptides [9]. Different types of side chain-protecting groups are available for polypeptide synthesis. For instance, to produce poly(L-aspartic acid) (PAA) or poly(L-glutamic acid) (PGA), the $\beta/\gamma$-carboxyl groups in Asp and Glu, respectively, are typically protected as benzyl esters (Scheme 1) using benzyl alcohol or benzyl halide [10–12], which will lead to the corresponding protected polypeptides. Afterwards, benzyl groups are easily removed by hydrogenation, or by an acidic or basic treatment to release the carboxyl groups [13,14]. Other protecting groups such as, tert-butyl (tBu) or trityl...
AAA lend themselves to engineer synthetic polypeptide-based copolymers (PBCs), where the copolymers consist of either a copolypeptide or a polypeptide–polymer conjugate that can be produced in several different architectures, e.g. block, graft, star, etc. Amphiphilic block PBCs with appropriate hydrophobic-hydrophilic balance can spontaneously self-assemble in an aqueous media, thus leading to a library of stable nanostructures, such as micelles, vesicles, nanogels, nanofibers and solid nanoparticles \cite{16,17}. Therefore, NCAs from AAA derivatives stand out as ideal monomers to prepare PBCs. Additionally, AAA provide β-benzyl-acid ester NCAs like the following advantages: i) the feasibility to polymerise some amino and solid nanoparticles \cite{16,17}. Therefore, NCAs from AAA derivatives serve as a reactive handle for coupling a variety of reactive groups or even pre-formed polymers that are orthogonal to NCA polymerisation, thus leading to a library of side-chain-modified NCAs (SCM NCAs) \cite{20}, iv) synthetic polypeptides of AAA derivatives exhibit transitions between α-helical and random coil conformations. This phenomenon occurs because their secondary structures are sensitive to temperature and especially to pH variations. The control of this unique ability can offer a wide range of applications in materials science \cite{21,22}, and v) AAA are exceptional building blocks for imparting hydrophilicity to amphiphilic copolymers, which leads to their self-assembling in aqueous media \cite{23}.

In this perspective, we wish to highlight significant progress achieved in the last five years in the polymerisation of AAA NCAs derivatives. We also focus on recent advances and applications concerning the design and synthesis of PBCs that are created solely by ROP of NCAs from derivatives of AAA. Throughout, we provide an overview of these achievements discussing selected and representative examples. We finally discuss the principal advantages of using AAA as building blocks in the design of PBCs to build novel excipients for the delivery of bioactive compounds, and we share our perspectives for their use in future investigations.

2. Ring-opening polymerisation of N-carboxyanhydrides

Mechanistic aspects of the NCA polymerisation have been explained in detail in the literature elsewhere and will be only briefly mentioned in this review. For a broader insight on mechanisms, we refer the interested reader to some recently published and detailed reviews on the different mechanisms of NCA polymerisation \cite{6,24}. The most relevant pathway of NCA polymerisation for polypeptide synthesis is the so-called ‘normal amine mechanism’ (NAM); the carbonyl group (CS) is highly electrophilic and can be easily attacked by a nucleophile, typically a primary amine, which induces ROP initiation (Scheme 2). The main advantage of unhindered primary amines as NCA initiators is that they facilitate a controlled polymerisation with MW control via the monomer to initiator feed ratio of polypeptides with low dispersities.

A wide range of primary amine compounds can be used to initiate the NCA polymerisation: for instance, functional amines, alkyne and azido \cite{25}, vinyl \cite{26,27} and halide \cite{28} amines have been used, allowing straightforward functionalisation of the polypeptide C-terminus and access to complex polypeptide architectures. Dendritic primary amine initiators produce multiarm star polypeptides, and even dopamine has also been utilised to produce a therapeutic polypeptide material \cite{29,30}. Additionally, pre-formed polymers (i.e., polymers with functional primary amines) have acted as macroinitiators to prepare hybrid materials, for example, polymacroactones amino terminated to yield block copolymers \cite{31}, copolymacroactones amino functionalised to render graft copolymers \cite{32,33}, star-shaped polymers \cite{29}, and for grafting of synthetic polypeptides from inorganic solid surfaces \cite{34,35}.

Nonetheless, the level of control in NCA polymerisation can encounter some issues when carried out at room temperature (and with primary amine initiator); for instance, the presence of side reactions (end-group termination and chain transfer) can limit the production of high MW polymers with narrow molecular weight distributions (MWDs) \cite{36}. Performing the ROP of NCAs at low temperatures has indeed helped to avoid such issues, and the robustness of Glu derivatives has been instrumental to fine-tune the polymerisation process. Some research groups have efficiently performed the NCA polymerisation at reduced temperatures for the synthesis of polypeptides. For instance, the Heise group has carried out the ROP of several NCAs at 0 °C, and end-group termination was suppressed especially for the synthesis of helix-forming polypeptides \cite{37}. More recently, Li et al. performed the ROP of BLG NCA in DMSO or 1,4-dioxane under frozen conditions at −18 °C; the cryo-NCA polymerisation showed narrower MWD \( (D = 1.06–1.11) \) than regular ROP at 25 °C \( (D = 1.13–1.16) \); good control over the \( M_n \) was obtained in both systems \cite{38}.

![Fig. 1](image_url)

**Fig. 1.** Chemical structures of Asp and Glu as well as their ionic forms: L-aspartate and L-glutamate, respectively. Throughout this paper we interchangeably refer to them as AAA.
2.1. Influence of secondary structure in polypeptide synthesis

In contrast to conventional synthetic polymers, synthetic polypeptides can fold into different secondary structures such as α-helix or β-sheets in the same way as do proteins [21,39]. The secondary structure adopted by these polypeptides can play a crucial role in their own synthesis. For instance, it has been recently reported that the formation of α-helical structures in synthetic polypeptides (i.e., PBLG) can catalyse their own growth through cooperative interactions that accelerate the polymerisation process [40]. In contrast, β-sheet forming synthetic polypeptides reduces the propagation of the ROP process due to steric hindrance of the chain end groups [19]. This phenomenon will be discussed in more detail later in the review. Furthermore, secondary conformation can affect the physicochemical properties of the final polymer (i.e., solubility, mechanical properties, degradation), which has a large effect on the applications of these polypeptides, such as drug delivery, gelation formation [21].

It is well known that PGA exhibits random coil-to helix transition with decreasing pH, and poly(ℓ-lysine) (PLys) undergoes random coil-to helix transition with increasing pH [22]. Lecommandoux and colleagues exploited this unique transition of these synthetic polypeptides to design unilamellar vesicles [41]. They prepared a zwitterionic diblock copolymer poly(ℓ-lysine)_{15}-b-poly(L-glutamic acid)_{15} (PGA_{15}-b-PLys_{15}) with positive charges of the protonated lysine and negative charges of the deprotonated glutamic acid at neutral pH, respectively. The team studied the self-assembly behavior in acid and basic conditions. At acidic pH, the PGA block is neutralised, and its secondary conformation changes from a charged coil to a neutral and more compact α-helical structure. The structure variation comes with a decrease in solubility, and the gained hydrophobicity is the driving force for self-assembly, and insoluble PGA forms the core of the aggregates while PLys blocks form the shell. Under basic conditions the opposite happened; the protonated PLys block (∼NH₃⁺) is transformed into neutral and insoluble polylysine blocks, which forms the core of these aggregates. This system demonstrates how the secondary structure can be modulated to engineer “smart” nanodevices that respond to external stimuli, such as pH, for potential application as bioactive delivery systems.

In hybrid copolymers, the secondary structure adopted by the synthetic polypeptide segment is also significant. The hydrodynamic radius in nanostructures (e.g. micelles or vesicles) can be controlled. For example, micelles from poly(butadiene)-b-poly(L-glutamic acid) (PB-b-PGA) were demonstrated to respond to pH or ionic strength with a change in hydrodynamic radius. The observed size variations were attributed to the neutralisation of the polypeptide block that changed from a random coil conformation (charged form) into a neutral and compact α-helical structure (“rod”). Interestingly, the size of these nanostructures can be reversibly controlled if the pH and ionic strength vary [42]. Therefore, PBCs that involve the robustness of AAA as building blocks leverage their secondary conformation to self-assemble in solution. The ability to utilise the secondary structure of the synthetic polypeptides leads to “smart” nanostructures that can meet appealing applications for the delivery of bioactive compounds.

2.2. Recent advances in NCA polymerisation

Of great interest for researchers is the ability to prepare NCA-derived synthetic polypeptides in the presence of water and impurities, which are known to cause NCA degradation and also chain termination [6]. The interest arises from four main drawbacks that complicate the production of synthetic polypeptides. First, the purity of the NCA is a limiting factor, because some of the electroophilic impurities can catalyse side reactions during the NCA ROP. Repeated precipitations/crystallisations of NCAs under anhydrous conditions is the most common applied purification method on the laboratory scale, but it is time-consuming and produces organic waste. Second, NCAs are known to be hydrolytically unstable in the presence of moisture, with some of the more hydrophilic NCAs being particularly sensitive and their handling typically requires an air-free environment that can be achieved using inert conditions, such as a glovebox or Schlenk line techniques [43]. Some of these techniques can be unaffordable, and are more difficult to use than standard chemical apparatus and they can also reduce the scale of NCA production. Thirdly, during the polymerisation, the presence of nucleophilic species other than those deliberately added as an initiator can trigger polymerisation producing undesired polymer chains and result in wide MWDs [44]. Fourthly, some impurities generated during the phosgenation of α-amino acids (i.e., hydrogen chloride (HCl) or acyl chloride) even present in trace amounts can react with the growing polymer chain-end in the course of the polymerisation. The result is a limited control of the polymerisation and low yields [45].

Nature overcomes these drawbacks by synthesising...
biomacromolecules in cells with precision, even though the cellular environment contains numerous molecules and species other than the building blocks. Over the last five years, taking inspiration from how nature produces proteins, progress has been made in the synthesis of well-defined NCA-derived polypeptides, in the presence of other species, such as water and impurities [46]. NCAs from AAA derivatives have been the first choice to perform the polymerisations. For example, $\gamma$-benzyl-L-glutamate NCA (BLG-NCA) or $\beta$-benzyl-L-aspartate NCA (BLA-NCA), have been widely used due to their convenient synthetic and purification processes and good solubility in various solvents as well as their distinct tendency to adopt an $\alpha$-helical conformation under various conditions [47,48]. Chen and colleagues [46] demonstrated controlled polypeptide synthesis by non-purified NCA monomers in an oil/water biphasic system that mediates impurity segregation and enables very fast polymerisation that outpaces competing side reactions. The method was named SIMPLE (Segregation-Induced-Monomer-Purification and initiator-Localisation promoted rate-Enhancement). For example, the polymerisation of BLG-NCA using a monomer/initiator feed ratio of 200 using this method proved to reduce the polymerisation rate significantly to 19 min with a conversion of 98%. Conventional polymerisation of BLG-NCA by primary amines, stronger nucleophiles than basic initiators, has been conducted in solvents like DMF, which usually takes hours or even days to finish [43]. In 2020, they improved this method and reported the synthesis of polypeptides directly from unpurified NCAs by adding a small-molecular amine scavenger (AS) in-situ to eliminate the remaining organic impurities in the emulsion polymerisation system (Fig. 2a) [49]. When the polymerisation of purified BLG-NCA took place with and without an AS, very similar MW of the resulting polymers were produced, with identical dispersities (Fig. 2b). Accordingly, the addition of the AS did not interfere with the NCA polymerisation. Interestingly,
the ROP of unpurified BLG-NCA was studied, and the addition of AS greatly improved the SIMPLE polymerisation, resulting in polypeptides with a monomodal GPC peak, expected MW, and greatly reduced dispersity (Fig. 2c). This strategy showed great potential for the synthesis of PEG-containing homo-, block and random polypeptides with controlled MW and low dispersities in open-air conditions at any scale.

In 2020, Lv et al. reported the preparation of star polypeptides with exceptionally high MW (up to 85 MDa) and low dispersity (D < 1.05) through dendritic polynime-initiated ultrafast ROP of NCAs [50]. The obtained polymers exhibited low dispersity could form uniform spherical micelles with diameters between 23 and 238 nm (Fig. 3a). They used polyamidoamine (PAMAM) dendrimers acting as macro-initiators, and the primary amines anchored on the surface served to initiate the NCA polymerisation (Fig. 3b). The BLG-NCA was chosen to be polymerised because the resulting PBLG adopts α-helical conformation under various conditions, which accelerates the polymerisation through interhelix cooperative macrodipole interactions. This technique takes advantage of the helix-macrodipole interaction, in which the macroinitiator constrains the helices in an approximately parallel array where the dipoles of neighboring α-helices enhance one another, leading to a faster rate of growth in the dendritic systems [40].

The polymerisation was performed in DCM with a low dielectric constant, which was crucial because this solvent minimised the disruption of the macrodipole interactions between α-helices. Interestingly, using different generations of PAMAM containing between 16 and 128 surface amines (G2 to G5) to initiates the NCA polymerisation, the conversion was >99% within 8 min (Fig. 3c). Such a high conversion is due to the fact that the macroinitiator polymerisation follows a cooperative mode to the proximity of α-helices. They monitored the progress of the polymerisation by in situ FT-IR spectroscopy as well as by circular dichroism. Both techniques clearly confirmed the formation of α-helical structures. The strategy was employed to polymerise other NCAs with reactive handles (e.g. alkynyl and alkenyl groups) from Glu and lysine derivatives, and it demonstrated the feasibility to extend the polymer chain since the end group remains intact, enabling block copolymerisation. The simplicity of this approach for producing polypeptides within minutes is remarkable compared to conventional ROP of NCAs, which may take several hours or even days to target high MWs.

In early 2021, Xia et al. found that crown ether (CE) can catalyse the polymerisation of BLG-NCA, γ-benzyl-D-glutamate NCA (BDG-NCA) and γ-benzyl-DL-glutamate NCA (BDLG-NCA) initiated by conventional primary amines initiators in solvents with low polarity and low hydrogen-bonding ability (i.e., DCM and chloroform) (Fig. 4a) [51]. Interestingly, they hypothesised that a catalyst promoting amine/NCA interactions would further accelerate the polymerisation, enabling the rapid and efficient synthesis of polypeptides. Among the CE tested, 18-crown-6 enabled the fastest polymerisation kinetics. For instance, in the ROP of BLG-NCA, a 95% conversion was reached after 18 min for the preparation of α-helical PBLG when adding 18-crown-6 as catalyst, which was indeed faster than the polymerisation in the absence of CE under identical conditions (75% conversion after 12 h) (Fig. 4b). It is remarkable that this strategy showed great importance of ordered α-helical structure for the accelerated polymerisation since the ROP of racemic BDLG-NCA was much slower than that of BLG-NCA, with 50% monomer conversion after 3 h (Fig. 4c). The lower conversion is because polymerisation of racemic DL monomer prevents the formation of the α-helix. This fact results in the elimination of the macrodipole of the α-helix, a key element for this polymerisation phenomenon [40]. Interestingly, the method was also instrumental in synthesising versatile NCA-derived polypeptide materials with functionalised C-terminus (Fig. 4d). The authors proposed a likely mechanism of the CE-catalysed rate acceleration (Fig. 4c) with the aid of NMR and diffusion-ordered spectroscopy (DOSY) experiments. In parallel experiments, DOSY analysis demonstrated that there exist molecular interactions between an amino-terminated initiator (R-NH2) and 18-C-6 in DCM (complex 1). In addition, the analysis also confirmed the binding interactions between CE and NCA (complex 2). To further understand the intermolecular interactions of the CE/R-NH2/NCA complex, they collected DOSY spectra at low temperature, and the experiments proved that CE is able to bind with both propagating polypeptide chain end and NCA (complex 3). They concluded that CE participates in the reaction and promotes the polypeptide/NCA binding, facilitating the ring-opening of NCAs in DCM, to then continue the growth of the polypeptide chain through the NAM.

Researchers have been intensively testing other catalysts to fine-tune the ROP of NCAs to produce well-defined polypeptides. There have been a number of different organocatalysts developed over the last 6 years, namely, N-heterocyclic carbenes (NHCs) [52] which can produce linear synthetic polypeptides (in the presence of primary amines) or cyclic polypeptides (without the presence of primary amines), N,N′bis[3,5-bis (trifluoromethyl)phenyl]-thiourea (TU-S) and a fluorinated alcohol catalysts, 1,3-bis(2-hydroxyhexafluoropropyl)benzene (1,3-Bis-HFAB) which have been shown to initiate BLG-NCA with an aminoalcohol initiator, to produce well-controlled polymers (D ≤ 1.07) at room temperature in minutes [53,54]. In another study, Liang et al., have used effectively the (S)-1,1′-binaphthyl-based phosphoric acid organocatalytic system that mediated the ROP of BLC-NCA with primary amine initiators [55]. Although this organocatalyst facilitated the synthesis of PBLG with expected MW and narrow dispersities, its utilisation in the ROP of other NCAs like alanine-NCA, phenylalanine-NCA or β-benzyl-L-aspartic acid-NCA failed because of the poor solubility of these monomers or the resulting polymers in the solvent used.

3. Copolyptide and polypeptide-polymer bioconjugates by ROP of NCAs

3.1. Copolyptides from SCM NCAs

In order to produce PLA and PGA, the side chain-protected polymers must be formed initially (i.e., PBLA and PBLG). Then, a deprotection process is performed to regenerate the carboxyl groups of the polymers. Orthogonally reactive amino acids in NCA polymerisation have received
considerable attention because functional amino acids whose reactivity is compatible with the polymerisation of NCAs lead to complex architectures since the resulting polymers can be easily modified in one controlled post-polymerisation step \[56\]. As far as we know, the use of orthogonally reactive NCAs from Asp has been undescribed apart from an early study of Hashimoto in the 1960s who prepared γ-4-chlorobenzyl-L-aspartate NCA \[57\], and more recently Zhang et al. reported mono-, di- and triethylene glycol modified aspartate NCAs \[58\]. On the contrary, significant efforts have been devoted for engineering PBCs using SCM NCAs from Glu (Fig. 5). The range of functional groups that has been incorporated into L-glutamic acid NCA has increased notably: alkyne \[59\], alkene \[60\], halogen \[61,62\], oligoethylene
glycol [63], mesogen [64], photoresponsive [65], azido [66], among others [67], and this diversity is expected to increase. For broader insight into other SCM NCAs, the reader is referred to reviews by Deming and Huesmann et al. [20,56].

Among PBCs, those consisting just of polypeptides stand out because of the versatility of the polymerisation reaction (i.e., ROP of NCAs) in particular the ability to incorporate a large variety of amino acid monomers. These copolypeptides offer the flexibility of biomolecular design, which allows the addition of different functionalities. Moreover, copolypeptides may adopt secondary structures analogous to those of natural proteins. Monomers are formed by converting α-amino acids into α-amino acid NCAs. Then, sequential addition of NCA monomers into the polymerisation reaction (i.e., one-pot strategy) renders multi-block architectures that exhibit well-defined block length and low dispersity [68–70]. To produce copolypeptides the ROP of NCAs is the preferred technique. Alternative living polymerisation techniques, such as reversible addition-fragmentation chain transfer polymerisation (RAFT), allow the synthesis of polymer architectures and functional

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**Fig. 5.** Side-Chain-Modified NCAs from L-glutamic acid.

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**Fig. 6.** (a) Scheme showing the designed block sequence of the ABCDE pentablock copolypeptide. (b) Normalised GPC-LS traces of intermediate polypeptide after synthesis of each block during the synthesis of the copolypeptide. Adapted with permission from Ref. [73]. Copyright (2019) American Chemical Society.
materials because it is compatible with several monomers. This technique also produces polymers that exhibit predictable MW, low dispersities, and above all, retention of the terminal functional group that permits extending the polymer chain [71]. In fact, the combination of RAFT and NCA ROP enables the synthesis of different polymer architectures, like hybrid copolymers [72].

This strategy was recently exploited using several SCM NCAs for the controlled synthesis of multiblock copolypeptides; Wang et al. synthesised block copolypeptides with up to 20 blocks using BLG-NCA enabled by ultrafast polypeptide chain propagation in a water/chloroform emulsion system. Also, a pentablock copolypeptide bearing five different side-chain functionalities with a sequence of ABCDE was prepared using BLG-NCA, ε-carboxybenzyl-l-lysine NCA (ZLL-NCA), γ-(4,5-dimethoxy-2-nitrobenzyl)-L-glutamate NCA (DMNB-NCA), γ-(4-propargyloxybenzyl)-L-glutamate NCA (POB-NCA) and L-leucine NCA (Leu-NCA) using an amino-terminated poly(ethylene glycol)-block-poly(ε-benzyl-L-glutamate) (PEG-b-PBLG) macroinitiator (Fig. 6a). The good control over the NCA polymerisation is reflected by the shift toward higher MW after each NCA is sequentially added (Fig. 6b). The team did not report the selective deprotection of the PBLG and PZLL blocks to regenerate the COOH and NH₂ functional groups, respectively. Ideally, it would be expected that under a proper treatment for a selective deprotection, neither the 4,5-dimethoxy-2-nitrobenzyl-2-Nitrobenzyl (DMNB) nor 4-propargyloxybenzyl (POB) pendant groups were altered, leading to those functional groups being in predetermined positions. Nonetheless, the purpose of the investigation was to demonstrate the versatility of the polymerisation method. This approach paved the way to develop complex architectures by incorporating side-chains that can be exploited for post-polymerisation modifications of multiblock copolypeptides [73].

### 3.2. Polypeptide-polymer conjugates

Synthetic polypeptide-polymer conjugates make up a new class of functional biomaterials comprising natural and synthetic building blocks. The co-existence of synthetic polypeptides and synthetic polymers in a single structure has the advantage to combining the functionality and structure properties of polypeptides with the processability and economy of polymers to generate hybrid copolymers [44,74]. These polymers present exceptional stability and can exhibit stimuli responsiveness. Amphiphilic copolymers can self-assemble in different nanostructures that show features of biological materials [75]. The variety of hybrid copolymers that can be designed is immense because there is flexibility in the length and complexity of the polypeptide sequence, the chemical nature, the length and architecture of the polymer, and mainly the architecture of the conjugate. In addition, the toolbox of synthetic routes to generate polypeptide-polymer conjugates is well established. The reader is directed to detailed reviews elsewhere [6,22,24,25].

Advances in ROP of NCAs along with other polymerisations techniques, such as, atom transfer radical polymerisation (ATRP), reversible addition-fragmentation chain transfer polymerisation (RAFT), nitroxide mediated polymerisation (NMP) ring-opening metathesis polymerisation (ROMP) over recent years has provided easy access to prepare synthetic polypeptide-polymer hybrid materials. There are two main strategies for the synthesis of NCA-derived polypeptide-polymers, convergent and divergent synthesis [76]. The convergent synthesis involves the coupling of pre-synthesised polypeptide and synthetic polymer building blocks typically utilising click chemistry (see Fig. 7). For divergent synthesis, AB or ABA block copolymers can be produced from the ROP of NCAs from synthetic polymer macroinitiators containing primary amine groups (see Fig. 7). For the interested reader there are some recent reviews that discuss these in more detail than we will cover here [77,78]. Typically, the polypeptide block is made from γ-benzyl L-glutamate, β-benzyl L-aspartate, or N'-benzoxycarbonyl L-lysine because the polymerisation of these NCAs produces controlled polymers [44].

Furthermore, the use of SCM NCAs in orthogonal reactions has widened the library of synthetic polypeptide-polymer conjugates for specific applications. The synthetic polymer, on the other hand, has been much more varied, for instance: hydrophobic polymers, e.g. poly(styrene), poly(macrolactones), or hydrophilic polymers like poly(ethylene glycol) or poly(vinylalcohol) among many others. Table 1 and Table 2 gather a selection of representative systems where AAA were utilised as building blocks. The library of copolymers presented is not meant to be exhaustive but rather represents a selection that brings together the work generated in the last five years, which in turn will hopefully encourage further work in the field.

### 4. PBCs as bioactive-compound delivery systems

PBCs are attractive for their use in drug delivery because of their biocompatibility, biodegradability and tuneable secondary structures [6]. The amount of different side chain functionalities available for

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**Fig. 7.** The two synthetic routes to build polypeptide/synthetic-polymer conjugates, convergent and divergent.
Table 1
Polypeptide-based copolymers engineered from L-aspartic acid through NCA ROP.

| Copolymer Architecture | Diameter (nm) | Bioactive cargo | Ref. |
|-------------------------|---------------|-----------------|------|
| Poly(L-phenylalanine-b-poly(L-aspartic acid) (PPA-PAA) | Nanoparticles 226 | Placlitaxel | [79] |
| Cat-poly(L-aspartic acid)-poly(L-phenylalanine) (cat-PAsp-Phe) | Nanoparticles 50.8 | BMP-2, IGF-1 | [80] |
| Hybrid copolymers | Micelles 90 | Methotrexane | [81] |
| Methoxypoly(ethylene glycol)-b-poly(aspartic acid-g-imidazole) (mPEG-PAsp-g-im) | Micelles 100 | Chlorin e6, Lutein | [84,85] |
| Methoxypoly(ethylene glycol)-b-poly(aspartic acid-imidazole) (mPEG-PAsp-IM) | Micelles 65 | DOX | [86] |
| Poly(ethylene glycol)-b-poly(aspartic acid) hydrazine functionalised (PEG-PASP-NH₂) | Nanoparticles 68.3 | siRNA | [87] |

(continued on next page)
synthetic polypeptides not only provide sites for the covalent conjugation of bioactive cargoes and facile crosslinking for enhanced structural stability, but also enables manipulation of the physical properties of the resulting nano-assemblies through the design of smart, trigger-responsive chemistry [20]. We define a bioactive cargo as a compound of natural origin (i.e., plant, microorganism or animal) used as a therapeutic agent in biomedicine. An essential aspect of drug delivery systems is the nanoparticle size because it significantly influences their therapeutic and diagnostic applications, such as, blood circulation half-life, targeting, cellular uptake and tumour penetration. Nanoparticles (NPs) must be larger than 10 nm to avoid renal filtration barrier, but they must be smaller than 200 nm to avoid activation of the complement system and be quickly removed from the blood stream. Preferably, the optimal NPs design will be disease-specific [109].

AAA lend themselves for designing amphiphilic PBCs because they are water-soluble polypeptides bearing long, charged side chains according to different mechanisms. For instance, micelles that contain icosahedral side groups can interact electrostatically with oppositely charged drugs [119,120] and bioactive macromolecules, such as DNAs, RNAs and proteins, when functional moieties can be incorporated into the side chains of the polypeptides [121–124]. There is a synergistic interaction between the bioactive compound and the AAA motifs in these excipients, but they have limited (if any) pharmacological activity. In other words, micelles do not interfere with the active pharmaceutical ingredient [119,125]. Likewise, in micellar structures, the hydrophobic core could be used to embed hydrophobic drugs [79], or the pendant carboxyl groups can be used to complex drugs via electrostatic interactions [118]. When AAA are present as excipients, the association or encapsulation of bioactive compounds may happen according to different mechanisms. For instance, micelles that contain ionisable side groups can interact electrostatically with oppositely charged drugs [119,120] and bioactive macromolecules, such as DNAs, RNAs and proteins, when functional moieties can be incorporated into the side chains of the polypeptides [121–124]. There is a synergistic interaction between the bioactive compound and the AAA motifs in these excipients, but they have limited (if any) pharmacological activity. In other words, micelles do not interfere with the active pharmaceutical ingredient [119,125]. Likewise, in micellar structures, the hydrophobic core could be used to embed hydrophobic drugs [79], or the pendant carboxyl groups can be used to complex drugs via electrostatic interactions [126].

Polymer vesicles, on the other hand, are very attractive and have large demand as excipients because they offer the possibility to host hydrophilic and hydrophobic cargo in the aqueous core and hydrophobic part of the membrane, respectively [127]. Synthetic polypeptides from AAA can enhance the formation of PICosomes (polypeyn complex vesicles) because of two main characteristics: the charge and the adopted helical conformation. Accordingly, two mechanisms pave the way to form PICosomes: i) if one polypeptide is hydrophobic and α-helical, e.g. PBLG, the anisotropic packing of the α-helices facilitate the formation of vesicles, and ii) if one polypeptide block is charged, e.g. PGA, PAA or PLL, the charge complexion with other charged polypeptide or small molecules enable the vesicular assembly. Indeed, both Glu and Asp fulfil these requirements and stand out as ideal building blocks to construct PICosomes [110,128].

Among the nanostructures obtained from PBCs, polypeptide-based
Table 2
Polypeptide-based-block copolymers engineered from L-glutamic acid through NCA ROP.

| Copolymer Architecture | Diameter (nm) | Bioactive cargo | Ref. |
|------------------------|--------------|----------------|------|
| Micelles               |              |                |      |
| Poly(L-glutamic acid-b-L-phenylalanine) (PGA<sub>x</sub>-PPA<sub>y</sub>) | Micelles 173-368 | Amphotericin B | [90] |
| Poly(L-leucine)-b-poly(L-glutamic acid) (PLeu-b-PGA) | Hydrogel – | Calcein | [92] |
| Bz-Poly(L-alanine)-b-(L-glutamic acid)-b-(L-alanine)-Bz (Bz-A<sub>5</sub>E<sub>11</sub>A<sub>5</sub>-Bz) Bz = benzaldehyde | Micelles 28 | DNA | [93] |
| Poly(y-benzyl-L-glutamate-b-y-propargyl-L-glutamate) (PBLG-b-PPLG<sub>Nu</sub>) Nu = nucleobase (zidovudine) | Vesicles 216 | DNA | [93] |
| Poly(y-propargyl-L-glutamate-b-y-benzyl-L-glutamate-b-y-propargyl-L-glutamate) (PPLG<sub>Nu</sub>-b-PBLG-b-PPLG<sub>Nu</sub>) Nu = nucleobase (zidovudine) | Polymersomes 60-350 | Bromophenol/rhodamine | [94] |
| Poly(L-glutamic acid)-b-poly(L-phenylalanine) (Pglu-b-Pphe) | Hydrogel – | DOX | [95] |
| 32-Poly(L-glutamic acid)-b-oligo(L-valine) (32-PLGA<sub>35</sub>-b-OLV<sub>5</sub>) | (continued on next page) |
### Table 2 (continued)

| Copolymer                                                                 | Architecture       | Diameter (nm) | Bioactive cargo | Ref.   |
|---------------------------------------------------------------------------|--------------------|---------------|-----------------|--------|
| Hybrid copolymers                                                        |                    |               |                 |        |
| Poly[(globalide-r-o-pentadecalactone)-g-L-glutamic acid] (P[(Gl-r-PDLr)-g-LGlu]) | Micelles           | 200–600       | DOX             | [32]   |
| Poly(2-ethyl 2-oxazoline)-b-poly(L-glutamic acid) (PEtOX-b-PGlu)         | Nanoparticles/micelles | 91.2          | SN-38           | [96]   |
| Poly(ImBF₄-L-glutamic acid)-b-poly(propylene glycol)-b-poly(ImBF₄-L-glutamic acid) (Pl₄BF₄-b-PPG-b-Pl₄BF₄) | Hydrogel           | –             | Ciprofloxacin   | [97]   |
| ImBF₄ = 1-butylimidazolium tetrafluoroborate                               |                    |               |                 |        |
| Poly[N-isopropylacrylamide-co-allyl poly(ethylene glycol)]-b-poly(y-benzyl-L-glutamate)] [P (NIPAM-co-APEG)-b-PBLG] | Nanoparticles/micelles | 180–260      | Paclitaxel      | [99]   |
| Poly(2-deoxy-2-methacrylamido-D-glucose)-b-poly(y-benzyl-L-glutamate) [PMAG-b-PGlu (OBzl)] | Polypexes         | 249.8         | pDNA            | [100]  |
| Poly(ethylene imine)-poly(l-lysine)-poly(L-glutamic acid) (PEI₈₈-PLys-PGlu) | Unimolecular nanomedicine | 47            | Camptothecin    | [101]  |
| Poly(norbornene)-g-poly(L-glutamic acid) (PNBₑ₀-g-PGAₙₐₜ)                | Nanoparticles/Micelles | 50–100        | 20(S)-ginsenoside (Rg₃) / Sorafenib | [102,103] |
| Methoxypoly(ethylene glycol)-b-poly(L-glutamic acid-co-phenylalanine) [mPEG-b-PGlu-co-Phe)] | Micelles           | 105–121       | Paclitaxel      | [91]   |
| (continued on next page)                                                  |                    |               |                 |        |
hydrogels distinguish from other types of nanocarriers because they have 3D networks that can encapsulate large biologics. Synthetic polypeptide-based hydrogels can be engineered to exhibit stimuli-triggered and drug-controlled release capacities. Particularly, pH-responsive hydrogels are capable of responding to perturbations in the environmental pH. For instance, several locations in the body exhibit substantial pH changes during normal function or as a part of a disease state. pH-variations exist within sites like the gastrointestinal tract, vagina, blood vessels, inflamed tissue/wounds or the extracellular tumour environment that can trigger a pH response [129].

Table 2 (continued)

| Copolymer                                                      | Architecture           | Diameter (nm) | Bioactive cargo | Ref.     |
|----------------------------------------------------------------|------------------------|---------------|----------------|---------|
| Methoxypoly(ethylene glycol)-b-poly(L-glutamic acid)-b-poly(L-leucine) (mPEG-PGA-PLeu) | Micelles               | 55            | DOX            | [104]   |
| Methoxypoly(ethylene glycol)-b-poly(L-glutamic acid-metrodinazole) (PEG-b-P(GL-g-MN)) | Micelles               | 14.9–24.5     | Cisplatin      | [105]   |
| Poly(L-glutamic acid)-g-methoxy poly(ethylene glycol) (PLG-g-mPEG) | Nanodisk/nanosheet     | 14.6          |                | [106]   |
| Methoxypoly(ethyl glycol)-b-poly(L-glutamic acid)-b-poly(N-octylglycine) (PEG_x-b-PGA_y-b-PNOG_z) | Hydrogel               | –             | Bovine serum albumin | [107]   |
| Poly(L-glutamic acid)-b-poly(ethyl glycol)-b-poly(L-glutamic acid) / poly(L-lysine)-b-poly (ethyl glycol)-b- poly(L-lysine) [(PGA-b-PEG-b-PGA) / PLL-b-PEG-b-LLL)] | Hydrogel               | –             | Insulin       | [108]   |
| 4arm-Poly(ethylene glycol)-b-poly(L-glutamic acid) sodium salt (4a-PEG-PLGs) |                        |               |                |         |
The pH-responsive behavior of the hydrogel network is imparted by the presence of ionisable pendant groups in the polymer backbone. In particular, PAA or PGA contains a large number of carboxyl groups on their side chains and can be ionised in solutions at a pH greater than their acid dissociation constant, or $pK_a$. Therefore, the hydrogel swells at $pH > pK_a$ because of the large osmotic pressure generated by the presence of the ions [130,131]. pH-responsive hydrogel systems have been widely used for the controlled drug delivery of various therapeutics like proteins, small drug molecules, chemotherapeutics and genetic material. In addition, the functional pendant groups COOH in AAA have been exploited because they provide excellent sites for the attachment of crosslinking functionalities that favor the hydrogel formation [132]. These outstanding features make AAA promising candidates to construct novel hydrogels that could exhibit superior characteristics for selective delivery of bioactive agents [130,133,134].

**5. Summary and future outlook**

Synthetic polypeptide-based copolymers from natural α-amino acids have met important applications as bioactive delivery systems since the use of polypeptides offers a broad spectrum of functional groups inherent to α-amino acids. Negative-charged amino acids are particularly attractive because their acid functionality (COOH) can serve as a handle to incorporate functional groups or preformed polymers, or it can be exploited in its free form to interact with positive-charged molecules.

NCAs from AAA derivatives, particularly γ-benzyl-L-glutamate NCA or β-benzyl-γ-aspartate NCAs, have been investigated extensively more than others due to their relative ease of synthesis, manipulation and purification in the laboratory. Nonetheless, due to the instability of the NCAs, the use of stringent methods to avoid moisture and the use of glovebox or Schlenk techniques have been used for their polymerisation. In the past five years, significant efforts have been attempted to overcome those issues; accordingly, we highlight the following conclusions:

i) the ROP of NCAs has progressed from the use of stringent methods in synthesis to the polymerisation of unpurified NCAs in open vessels. The use of novel initiators and/or (organo)catalysts that favor initiator/NCAs interactions and growing polymer chain and NCA interactions has resulted in a great increase in rate of polymerisation, that even outpaces side reactions.

ii) the use of solvents with low dielectric constants (e.g. chlorinated solvents like DCM or chloroform) to prepare helix-forming polypeptide (for example, PBLG with high-MW and monodisperse polymers) can be instrumental in carrying out the NCA polymerisation, shortening the reaction time. Therefore, the polymerisation may follow a cooperative manner that is driven by interaction of neighboring α-helices.

iii) The use of an emulsion system with the ROP at the interface, called SIMPLE, which can produce well-controlled multiblock hybrid polypeptide-polymers is a big step forward in the field, as it allows the use of crude NCAs thereby solving some of the issues mentioned above.

We firmly believe that the abovementioned achievements pave the way for laboratories worldwide to perform NCA polymerisations in a more efficient and affordable way since access to complex techniques or expensive tools (i.e., high vacuum, Schlenk glassware or glovebox) may sometimes restrict the synthesis of polypeptides. Such advances have facilitated the synthesis of not only synthetic copolypeptides but also synthetic polypeptide-polymer conjugates that exhibit diverse architectures (e.g. block, graft, star) with predictable MW and narrow MWDs.

The use of different SCM NCAs in orthogonal reactions has generated special interest in the synthesis of copolypeptide or hybrid copolymers. Innovative polymers bearing specific functionalities with well-defined block sequences, narrow dispersities and tunable $M_n$'s have been successfully prepared. Particularly, the use of Glu derivatives is very attractive because helical, water-soluble synthetic polypeptides bearing various moieties can be attained, which can find several biological and...
biomedical applications. A difficulty has been the engineering of PBCs using SCM NCAs from Asp derivatives. Accordingly, new progress in NCA polymerisation can provide an opportunity to build SCM NCAs from Asp derivatives. Accordingly, new progress in their use in orthogonal reactions could lead to the construction innovative excipients.

Albeit the engineering of PBCs has moved forward significantly, it is noteworthy that the resulting nanocarriers have thrived mainly for drug or nucleic acid delivery. However, there is a potential niche for them in their use in areas like food science, where this nanotechnology can address issues relevant to food or nutrition. Thus, it is imperative to design edible-novel excipients capable of encapsulating, protecting and releasing functional compounds (e.g. nutraceuticals or phytochemicals) for innovative biomaterials, for instance, printable biomaterials and bioinks releasing functional compounds (e.g. nutraceuticals or phytochemicals) or edible-novel excipients capable of encapsulating, protecting and releasing functional compounds (e.g. nutraceuticals or phytochemicals) or issues relevant to food or nutrition. Thus, it is imperative to design innovative biomaterials, for instance, printable biomaterials and bioinks releasing functional compounds (e.g. nutraceuticals or phytochemicals) or edible-novel excipients capable of encapsulating, protecting and releasing functional compounds (e.g. nutraceuticals or phytochemicals).

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