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*Review*

# Peptide-Based Polymer Therapeutics

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**Abstract:** Polypeptides are envisaged to achieve a major impact on a number of different relevant areas such as biomedicine and biotechnology. Acquired knowledge and the increasing interest on amino acids, peptides and proteins is establishing a large panel of these biopolymers whose physical, chemical and biological properties are ruled by their controlled sequences and composition. Polymer therapeutics has helped to establish these polypeptide-based constructs as polymeric nanomedicines for different applications, such as disease treatment and diagnostics. Herein, we provide an overview of the advantages of these systems and the main methodologies for their synthesis, highlighting the different polypeptide architectures and the current research towards clinical applications.

**Keywords:** polymer therapeutics; polypeptides; drug delivery; nanomedicine

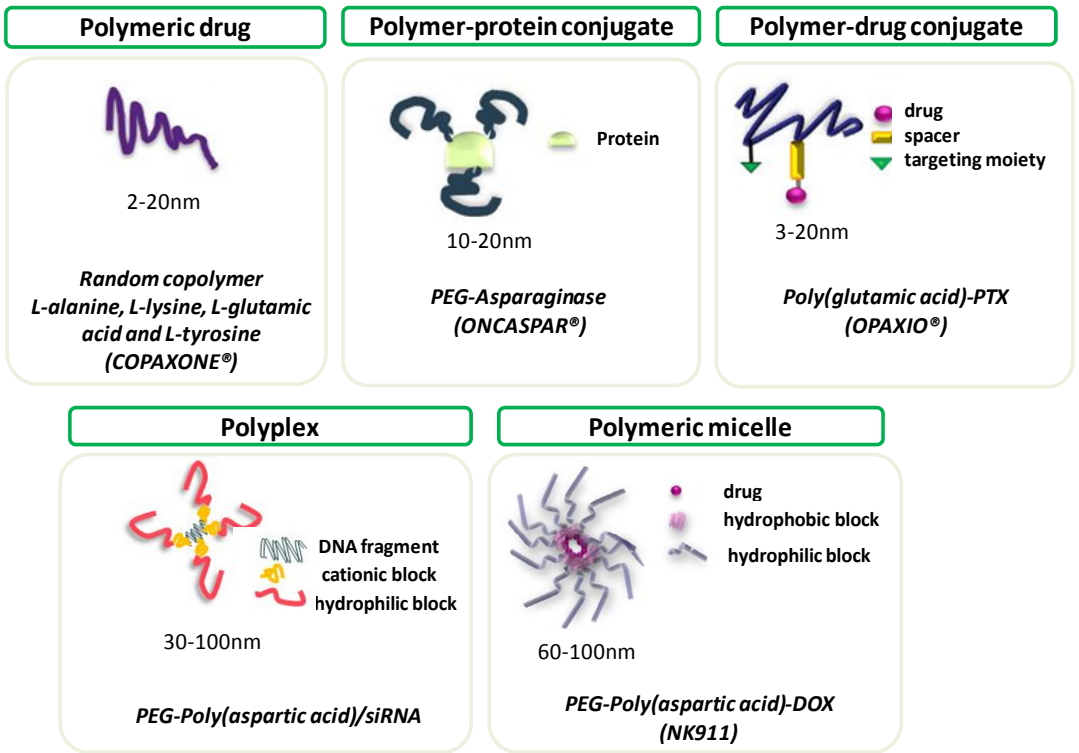
## 1. Introduction

Nanomedicine, as it is currently known, is a relatively young field in exponential growth addressing unmet medical and pharmaceutical needs in cancer and other diseases [1]. Areas such as controlled drug/gene delivery, tissue engineering, molecular imaging or diagnostics have made important progress through nanotechnology [2]. Within the 40 nano-products in routine use for healthcare, Polymer Therapeutics is considered one of the most revolutionary systems. Coined by Professor Ruth Duncan, Polymer Therapeutics defines a family of new chemical entities (NCEs) considered the first

polymeric nanomedicines [3]. This term covers a variety of complex macromolecular systems, their common feature being the presence of a rationally designed covalent chemical bond between a water-soluble polymeric carrier (with or without inherent activity) and the bioactive molecule(s). Although a tripartite design has been typically described for these systems: polymer, linker and the bioactive molecule, the use of additional targeting moieties and/or imaging agents are also covered [4].

Polymer Therapeutics includes five different groups: *polymeric drugs*, polymers with inherent activity [5,6] *polymer-protein conjugates* [3,7,8]; polyplexes which are multi-component systems developed as non-viral vectors for gene/small interfering ribonucleic acid (siRNA) delivery [9,10], *polymeric micelles* where the bioactive agent is covalently bound [11,12] and *polymer-drug conjugates* [3] (Figure 1). This field has been exhaustively revised and the following background references are recommended [4,13–15].

**Figure 1.** Schematic representation of polymer therapeutic families. Adapted from [3].




Covalent linkage to a polymer confers to the bioactive molecule enhanced solubility and stability, a different pharmacokinetic profile at the whole organism, cellular and even subcellular level, enhancing the drug therapeutic index [16]. With a rational selection of the polymer carrier and the linker/spacer, polymer-drug conjugates can yield long-circulating macromolecular systems that display passive targeting based on the enhance permeability and retention (EPR) effect [17,18], and therefore lower systemic toxicity; limiting also its uptake to the endocytic route. Linker/spacer choice must ensure stableness in circulation and release under selected physiological conditions. When the bioactive molecule is a protein or a peptide, stability is remarkably increased and immunogenicity is reduced.

These nano-sized systems retain such features as (i) a tailored drug loading; (ii) an incorporation of drug combinations or (iii) rationally design spacers/linkers adapted to enzymatic, pH or self-immolative triggers [4]. Furthermore, the versatility of synthetic chemistry and the inclusion of bioresponsive

elements and biomimetic features under a rational design provide scope for polymer therapeutics in comparison with other nanopharmaceutics [3]. Central objectives are (i) to release therapeutics at the desired body site (targeting) and (ii) to maintain the drug concentration within a therapeutic window for a desired duration (controlled drug release) [19]. Not only therapy but also diagnostics are demonstrated possibilities for polymer therapeutics [20]. The multi-functionality of these systems allows the combination of imaging probes and drugs simultaneously to the same carrier, enabling early diagnosis through image-guided therapy.

Up to now, a first generation of polymer therapeutics has resulted in several products being introduced to the market (PEGylated proteins and polymeric drugs), and a growing number of polymeric micelles and polymer-drug conjugates are in clinical development mainly as anticancer agents via parenteral administration [4,21]. PEGylation is a well-established technology approved by the regulatory agencies [22] and consequently, has become one of the best-defined polymer platforms for biomedical applications [23,24]. Knowledge acquired on biodistribution, clearance, mechanism of action and stability *in vivo* of these polymer conjugates has contributed to design of an advanced second generation construct. Together with the aim of meeting regulatory demands, this second bundle is directed towards four main strategies: (1) the synthesis of novel biodegradable polymeric carriers with defined architectures; (2) the implementation of innovative physico-chemical characterisation methods; (3) the use of polymer-based combination therapy to enhance treatment specificity and efficacy always looking for drug synergism and (4) their application in diseases other than cancer with an important focus on pathologies related to the aging population and infectious diseases [25].

It is within these novel strategies that polypeptides have gained importance. For instance, poly(glutamic acid) (PGA), poly(lysine) (PLL) or poly(aspartate) have emerged as perfect candidates as they present the required carrier features, namely, biodegradability, high drug loading capacity and well-defined structures. These polymers have been catalogued as a novel family of materials due to their physical, chemical and biological properties derived from their controlled sequences and composition [26–30]. Polypeptides are able to adopt stable conformations and undergo hierarchical assembles to form highly organised supramolecular structures at the nanoscale. Their ordered sequences end up in a secondary (e.g., helices, sheets or turns) or even tertiary or quaternary structure [29], responsible for a defined functionality of the final material.

The trend to move towards biodegradable polymers in drug delivery is mainly justified by their use in chronic or infectious diseases, neurological disorders or tissue regeneration, where a prolong treatment is always required and, therefore, material safety is a limiting step to develop efficient therapeutics. Polypeptides can be degraded in the presence of specific proteases (*i.e.*, cathepsins) to yield small nontoxic metabolites that can be easily excreted through natural body mechanisms. Polypeptides have also merits of biocompatibility and low immunogenicity [31,32], and therefore might be suitable for repeated parenteral administration and allow the use of high molecular weight ($M_w$) carriers to optimise pharmacokinetics as well as high polymer doses [4]. Other clinically relevant non-polypeptide biodegradable carriers are dextrins [33] degradable by amylase or polyacetals, which display pH-dependent degradation [34]. These polymers are outside the scope of this review and will not be further discussed.

Polypeptides can be obtained by means of polymerisation techniques using amino acids or their derivatives, or through recombinant DNA techniques. Modern synthetic approaches have already

yielded novel and well-defined polymer structures which are beyond nature's possibilities, providing multivalent surfaces for the immobilisation of drugs and/or tracing agents, higher loading capacity and the possibility to exploit multiple pathways in cellular trafficking due to differences in conformation and sizes. Additionally, peptide-based synthetic polymer hybrid constructions have opened up novel opportunities [29]. On the other hand and as the main drawback, some specific amino acid sequences or homopeptides can trigger disease cascades or their accumulation as aggregates may result in disease promotion, *i.e.*, neurodegeneration disorders. Therefore, this possible impaired toxicity must be studied, and precise control of the selected amino acid composition and the final macromolecular architecture adopted have been defined as crucial design features [35–38]. It has to be emphasised that there is a growing effort to better understand the structure-activity relationships in order to better define safety and efficacy of these novel therapeutics [15]. At this point, it is worthy to remark an emerging class of pseudo-peptidic polymers: the polypeptoids [39,40]. Basically, these non-natural biomimetic oligomers present an N-substituted glycine backbone which, opposite to polypeptides, is achiral and the side-chain position differs. On the other hand, due to their structural similarities to polypeptides, possibilities for their use cover areas such as drug-discovery, diagnostics, material science and an understanding of protein folding. Comparable polymeric systems to polypeptides have been also synthesised [41,42].

Finally, it is important to note that first examples with polymer conjugates show that the panacea of enabling specific and individualised therapy through nanomedicine is becoming a feasible approach and polypeptidic carriers are major players in this role [43,44].

In this review, we aim to highlight the potential of polypeptides within the polymer therapeutics field, summarising the current techniques for their synthesis, the wide range of structure possibilities and their multiple clinical applications as drug/gene delivery carriers or imaging agents.

## 2. Polypeptide Design and Synthesis

Polypeptides belong to a family of macromolecules diverse both in applications and structural features. As a result, there are several strategies for their synthesis and preparation. In general, the methods for the design of polypeptides can be divided in two main different methodologies: the synthetic techniques, and the recombinant DNA techniques.

In the synthetic approaches, stepwise solid-phase polypeptide synthesis (SPPS) [45,46], native chemical ligation (NCL) [47] or ring opening polymerisation of α-amino-*N*-carboxyanhydrides (NCAs) [48–50] can be included. Those techniques are mainly based on the use of amino acids or their derivatives as monomers and are particularly useful in the design of hybrid architectures that combine sequences of peptidic and non-peptidic nature. Control on polymer chain length and stereochemistry have been one of the major challenges in synthetic approaches over the past years. However, recent reports have demonstrated how to overcome those limitations with precise controlled reactions followed by an adequate characterisation yielding to well-defined polypeptidic architectures [51].

Recombinant DNA techniques can be seen as an alternative—genetically encoded approaches for the synthesis of polypeptides. The main advantage for this technique is the inherent accuracy of the methodology, which includes high specificity in the sequence and stereochemistry of the newly synthesised polypeptide. On the other hand, not all polypeptides can be properly expressed in a

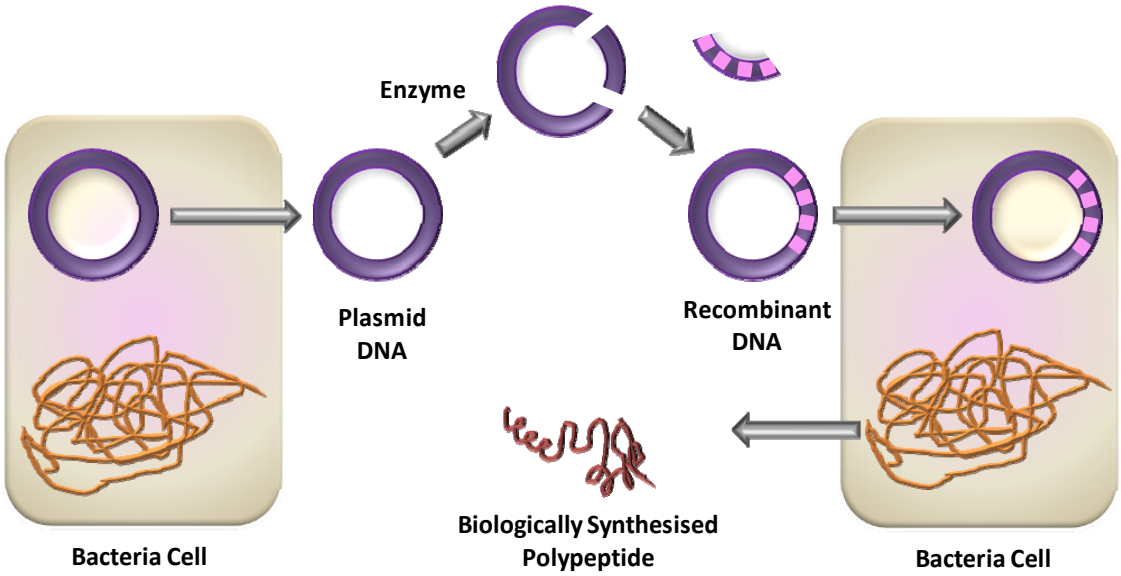
heterologous host, and non-peptidic moieties cannot be included unless post-expression modification techniques are applied [52].

### *2.1. Recombinant DNA Expression of Proteins*

Recombinant DNA expression of proteins is often used for the synthesis of proteins with different purposes. Examples include, the identification of the polypeptide coded by a DNA sequence, the analysis of the biological activity of a protein, the study of structure-activity relationships or the 3D-structure, the production of specific antibodies, the development of a target-specific drug or a therapeutic protein, vaccine or biotechnological enzyme, and protein and peptide engineering and design, which is the one that will be reviewed herein.

In recombinant techniques (genetically encoded synthesis) three steps can be described. First of all, the creation of a recombinant gene segment that encodes for the protein of interest: a protein target is identified and translated into the corresponding genetic code. Then, the target oligonucleotide is synthesised; secondly, the insertion of this segment into a DNA vector, which is classically a plasmid from bacteria to produce a recombinant DNA molecule; and finally, transformation of this recombinant DNA molecule into a host cell. Cells that are successfully transformed with the recombinant DNA molecule are grown in culture. This gene produces large amounts of the desired protein, which is later isolated from the cells (Figure 2). Due to low cost and convenience, bacteria [such as *Escherichia coli* (*E. coli*) or *Bacillus subtilis*] are the most frequently used. It is important to mention that the achievement of the recombinant gene is the rate-limiting step, in particular if the polypeptidic product of interest is based on a large number of repeats.

**Figure 2.** Recombinant DNA technique for protein synthesis. Redrawn from [53].




Short sequences up to 100 nucleotides can be produced by chemical synthesis using an automated solid phase DNA synthesiser. To produce larger sequences that will encode for repetitive polypeptides of high Mw, these chemically synthesised short DNA sequences must be assembled. There are different methods to gather the small sequences such as concatenation of oligonucleotides [54], recursive

directional ligation (RDL) [55], and mutagenesis or amplification of existing gene segments using polymerase chain reaction (PCR) [56]. However, it is not within the scope of this review to discuss in detail all the methods, please check [54–56] for further details.

The recombinant synthetic techniques display several advantages over the chemical synthesis:

- Polypeptides produced by genetically encoded synthesis have a defined sequence, stereochemistry, and Mw based on a genetic template.
- Once optimised, transformed cells can provide a continuous supply of the polypeptide.
- If the polypeptide has a particular secondary or tertiary structure, the *in vivo* folding machinery of the cell can assist to ensure the correct conformation.

However, they also present some drawbacks that must be mentioned:

- The technique is, in general, time- and effort- consuming, mainly due to the synthesis optimisation of the gene (especially if large Mw are desired); and to the optimisation of the expression levels in the host cell.
- Multidomain proteins are much more challenging to express than proteins smaller than 30 kDa.
- Incompatibility between protein and bacteria leads to toxicity, reducing protein production.

Only the 20 natural L-amino acids can be incorporated using standard cellular components. Incorporation of artificial amino acids, stereochemically unique amino acids and β-amino acids, all of which can prevent protease degradation is in principle not possible. Although, this problem is slowly being surpassed [57–59].

### *2.2. Synthetic Approaches*

There is a growing interest in developing synthetic strategies that could overcome the limitations of the genetically encoded synthesis to produce polypeptides with similar control of size and uniformity [48–50]. Among the advantages of the synthetic approaches, the following can be pointed out:

- Unnatural amino acids can be used to produce a vast number of novel architectures with different properties and structure-function relationships.
- It enables the combination of polypeptides with other synthetic polymers such as poly(ethylene glycol) (PEG).
- Easier and faster methodology compared with the genetically encoded techniques.
- Higher yields and large scale synthesis.

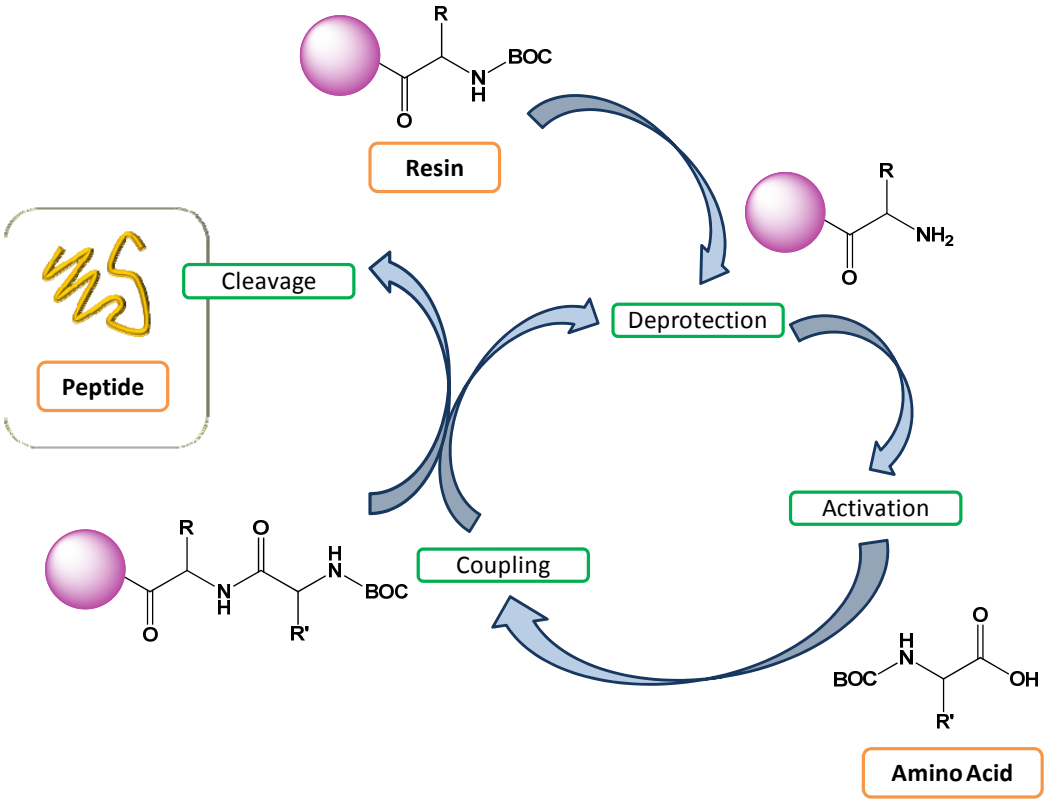
However, as mentioned above, controlled chain length and stereochemistry might be an issue.

#### 2.2.1. Solid-Phase Peptide Synthesis (SPPS)

The stepwise Solid-Phased Peptide Synthesis (SPPS) was first reported by Merrifield in 1963. Initially, it was described as a method for the preparation of short peptides with a potential application in the preparation of high Mw polypeptides [45,60]. In his protocol, Merrifield attached the first N-protected amino acid group through an ester bond to a polystyrene resin partially chlorinated. After that, the protecting group was removed yielding a free amino group ready to react with the next N-protected amino acid. Thus, the general principle of SPPS consists on repeating cycles of

coupling-wash-deprotection-wash. The peptide is 'immobilised' onto the solid-phase and can be retained during a filtration process, whereas liquid-phase reagents and synthesis by-products are flushed away. Finally, the resin can be removed and the peptide isolated (Figure 3). The main amino protecting groups usually used are 9-fluorenylmethyloxycarbonyl (FMOC) and t-butyloxycarbonyl (BOC), and each of them requires different resins and amino acid side-chain protection and, consequently, different cleavage/deprotection steps. The FMOC group is the most routinely used as it can be easily removed using piperidine (without the need of trifluoroacetic acid as for BOC), it produces higher quality and in greater yield peptides as compared with the BOC method. Nevertheless, the BOC chemistry is sometimes indispensable when complex peptides or non-natural amino acids are required.

**Figure 3.** Schematic representation of Merrifield solid-phase peptide synthesis (SPPS).




In general, the advantages of this approach are listed below:

- It allows the synthesis of natural peptides that are difficult to express in bacteria, the incorporation of unnatural amino acids, peptide/protein backbone modification, and the synthesis of D-polypeptides, which consist of D-amino acids.
- Total control over peptide composition is achieved.
- The possibility to perform wash cycles after each reaction, removing reagents in excess with all of the growing peptide of interest remaining covalently attached to the insoluble resin.
- The process can be automated using a peptide synthesiser.

On the other hand, some drawbacks must also be taken into account:

- Large, complex polypeptides/proteins cannot be prepared, due to a low coupling efficiency as the length of the peptide increases.

- High purity is not usually obtained when large polypeptides are synthesised. Significant quantities of resin-bound by-products can be often detected.
- It is necessary to use an excess of amino acids and coupling reagents to ensure the maximum coupling efficiency.
- Solubility of protected peptide segments is sometimes challenging.
- Complete deprotection of long peptides can be difficult to accomplish.

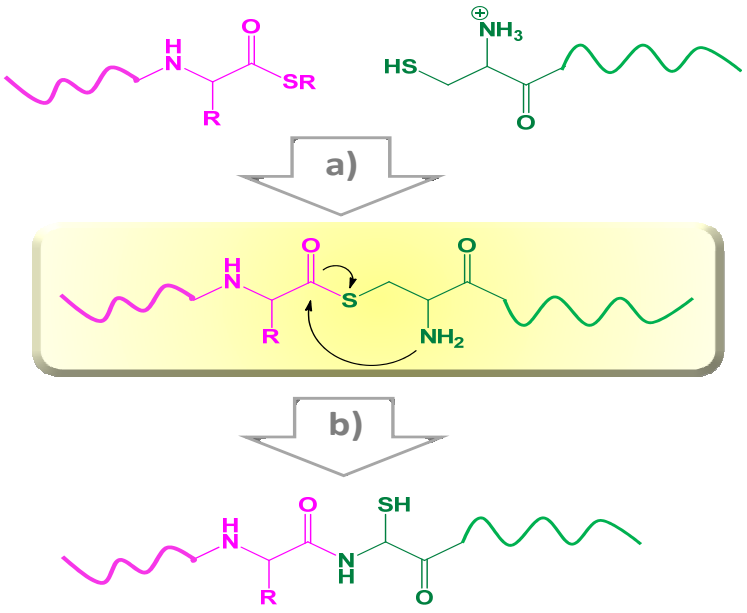
Due to all these drawbacks, SPPS is typically restricted to the synthesis of polypeptides of less than 50 amino acid residues in order to not compromise purity.

### 2.2.2. Native Chemical Ligation (NCL)

The chemical ligation can be considered as a simple technique that allows the direct synthesis of native backbone proteins of moderate size. It is based on the chemoselective reaction of two unprotected peptide segments that will react exclusively among them diminishing the potential side reactions and generation of undesirable by-products [61,62]. It is a useful tool to integrate unique functional groups that are not typically present in amino acids.

This methodology was first introduced in the field of polypeptide synthesis in 1991 by Schnölzer and co-workers [63]. Chemical ligation is a procedure based on the use of chemoselective reactions which form a non-amide bond at the ligation site, such as the reaction of a peptide-R COSH with a bromoacetyl peptide yielding a thioester-linked product (Figure 4). The introduction of a non-native linker within the protein was well-tolerated in folded proteins. Nevertheless, in order to mimic natural proteins and enzymes, it is always better if possible, the use of peptidic bonds as linkers.

**Figure 4.** Native chemical ligation. (**a**) water, pH = 7; (**b**) rearrangement of thioester intermediate amide linked product.




In 1994, the chemical ligation approach was adapted to allow the formation of a native peptide bond at the ligation site by Dawson and co-workers [47,64]. Known as native chemical ligation (NCL),

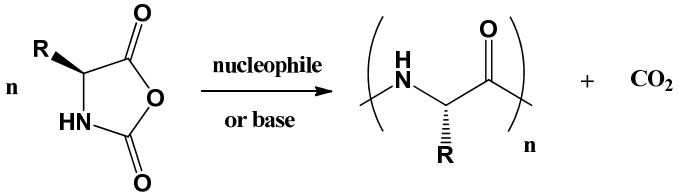
this robust approach has been widely used to produce a series of model peptides [65], protein inhibitors [66], and a vast variety of other proteins [67].

It consists on the reaction of an unprotected peptide-thioester with a second unprotected peptide containing an N-terminal cysteine residue giving rise to an unstable covalent bound thioester-linked intermediate. The new thioester bond that links the two segments spontaneously rearranges forming the most thermodynamically favoured amide bond at the ligation site, without any change in the reaction conditions. The main advantage of this strategy is the absence of complex combinations of protecting groups as unprotected amino acids can be directly linked using this technique, thus, avoiding the problems caused by the limited solubility of many synthetic intermediates. However, its use is restricted to link peptide segments. Thus, it is necessary to synthesise those segments using other alternative technique such as SPPS in general bases.

### 2.2.3. Ring-Opening Polymerisation (ROP) of α-Amino Acid *N*-Carboxyanhydrides (NCAs)

The ring-opening polymerisation of amino acid-*N*-carboxyanhydrides (NCA) is the most commonly applied polymerisation technique to produce polypeptides and polypeptide-based block copolymers on a multigram scale (Scheme 1). Although the obtained polymers are less defined than natural peptides, the polymerisation method enables access to polypeptidic architectures, which are beyond nature's possibilities. The ROP of NCAs has already been used for the synthesis of polypeptides with various applications that range from drug delivery systems [68], tissue engineering [69], sensing [70] to catalysis [71]. The first NCA [72–74] were synthesised by Leuchs in 1906. Since then, a plethora of polypeptides has been created due to the variety of natural and non-natural amino acids and the versatility of the polymerisation method, as it has been reviewed in the following excellent literature [48–50].

**Scheme 1.** Ring-opening polymerisation of α-amino acid *N*-carboxyanhydrides (NCAs).



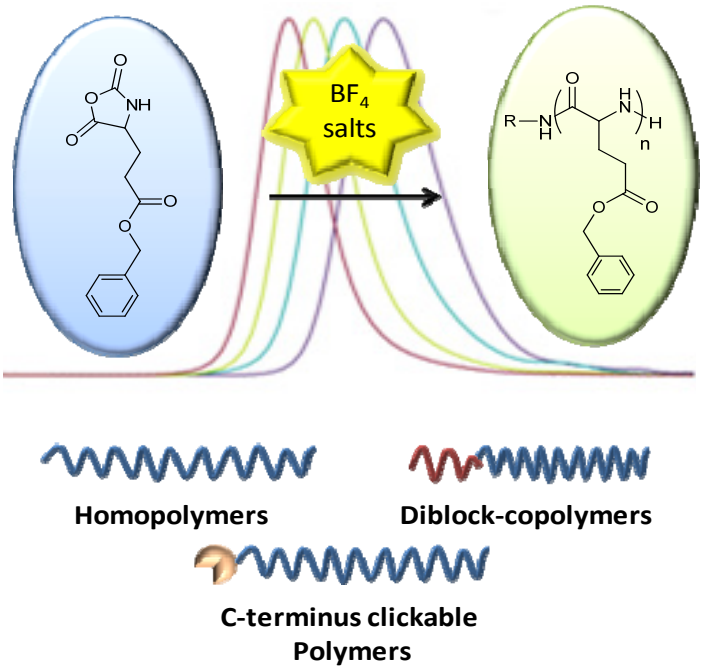

The opportunity to use functional non-natural polymers in combination with the scalable synthesis makes the ROP of NCA a great choice to reach well-defined polypeptides. Besides their multiple functionalities, adjustable $M_w$s (1–1000 KDa) and structural homogeneity favor self-assembly into defined nanostructures with potential biomedical and pharmaceutical applications. One of the trends in the development of polymeric based nanomedicines is the building of well-defined, reproducible and homogenous architectures. Thus, the synthesis of narrowly distributed polypeptides for their potential use in biomedicine is a desirable goal, which turned out to be rather demanding. ROP of NCA can proceed via two mechanisms: the normal amine mechanism (NAM, induced by nucleophilic substitution) and the activated monomer mechanism (AMM, induced by deprotonation). AMM should be diminished to yield well-defined homo and block copolymers as the control over polymer end

groups is essential for the synthesis of multiblock architectures or end group functionalisation. In addition, this mechanism leads to polymer propagation by step as well as chain growth, promoting a broad molecular weight distribution.

This occurs even when initiated by primary amines, leading in most cases to reduced control of the polymerisation process itself. Especially, whenever a higher degree of polymerisation or complex architectures are desired, the occurring side reactions interfere. For that reason, efforts have been devoted to develop new approaches in order to overcome these drawbacks, such as, the use of heavy metal catalysts [75], high vacuum techniques (HVT) [76], primary amine hydrochloride salts [77], the combination of low temperature with primary amines [78], the use of silazane derivatives as initiators [79] or the optimisation of reaction conditions (pressure, temperature, *etc*.) [80,81]. However, all methods present limitations: HVTs require a complex and expensive experimental setup; hexamethyldisilazane (HMDS) amines are sensitive to hydrolytic reactions, or heavy metal catalysts must be carefully removed to avoid non-specific toxicity in biomedical applications.

The recently reported use of primary amine tetrafluoroborate ($BF_4$) salts by Conejos-Sanchez *et al*. [51,82] as an improvement of the Schlaad method represents a powerful alternative to the above-described methodologies, since the non-nucleophilicity of the $BF_4$ salts in contrast to the Cl counteranions cannot lead to initiation steps (Figure 5). This newly reported strategy allows a multigram scale [83] polyglutamate synthesis with defined molecular weight (up to 800 units), low polydispersity (<1.2), controlled chain end functionality and adequate stereoselectivity and absence of any trace of toxic impurity to allow biomedical applications. Conformation and polydispersity are relevant parameters in the named methodology. Recently, Huesmann *et al.* have addressed both topics in polylysine synthesis [84].

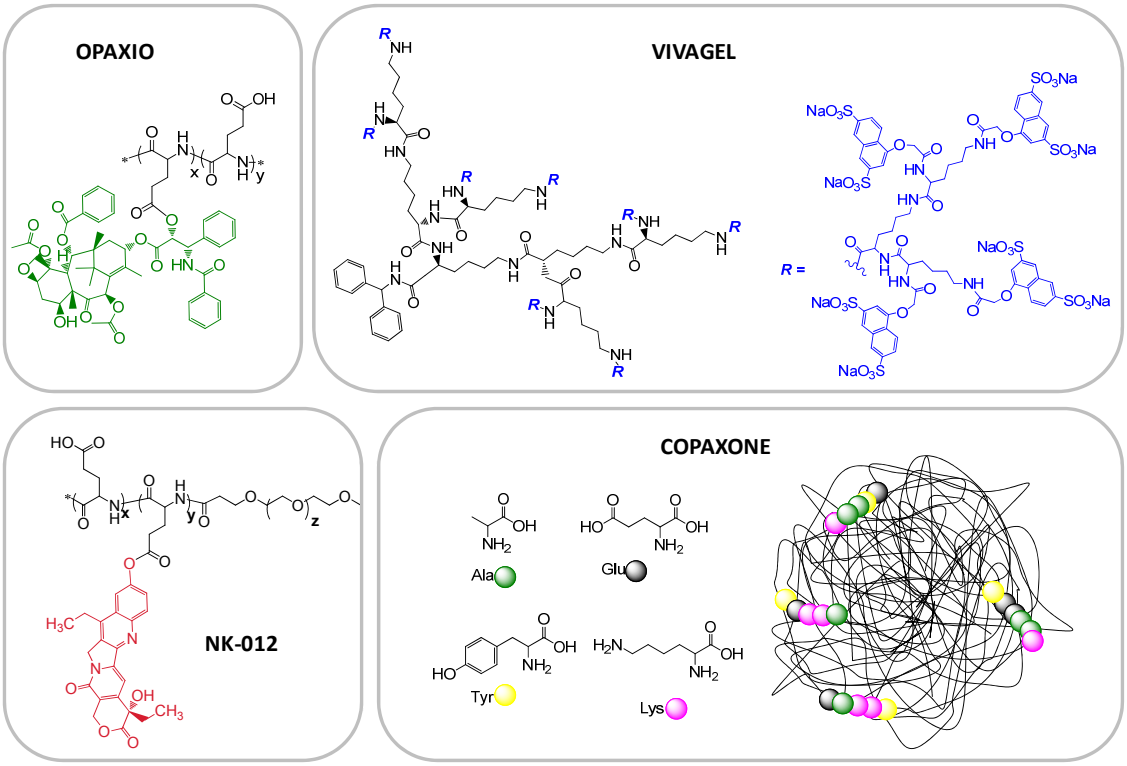
**Figure 5.** Schematic representation of the NCA polymerisation methodology developed by Conejos-Sanchez *et al*. (Adapted from Conejos-Sanchez *et al*. [51]). Reproduced by permission of The Royal Society of Chemistry.



## 3. Polypeptides as Polymer Therapeutics

The opportunity of using amino acids as building blocks to create single polymeric systems or hybrid block copolymers together with diverse macromolecular architectures offers numerous possibilities to adapt the final nanovector to a specific therapeutic application. Some of the inherent characteristics of polypeptides make them excellent candidates for drug delivery, such as the presence of functional groups in their sequence that provide specific sites for direct attachments or charged-induced interactions with different biomolecules. The most successful first generation polymer therapeutics is mainly represented by linear polymers and polymeric micelles (Figure 6). This is composed by homopolymers, diblock or triblock copolymers formed overall by PGA, PLL or poly(aspartate). Polypeptides can degrade enzymatically within a safe profile and non-toxic metabolites, which are remarkable properties to turn them into a market product. Herein, we review their different applications as drug/gene delivery carriers and imaging agents. Polypeptide applications cover many other areas such as their use in implants, nanogels or coatings, which are out of the scope of this review [27,30,85].

**Figure 6.** Examples of polypeptide-based polymer therapeutics in the clinics.




### *3.1. Drug and Gene Delivery*

#### 3.1.1. Drug Delivery

Initial steps of polymer therapeutics were focused on anticancer agent development. Conjugation of orthodox chemotherapeutic agents, e.g., Doxorubicin (Dox), to water-soluble carriers was performed and lead candidates to the clinical arena [8]. These nanosize constructs are of interest for cancer treatment due to their favoured accumulation within the tumour, facilitated by the EPR effect and a

demonstrated tissue penetration [17,18]. In respect to this, it is worth highlighting Opaxio™ due to its advanced position in clinical trials. This is a PGA-Paclitaxel (PTX) conjugate in Phase III for the treatment of various cancers including ovarian, prostate or head and neck carcinomas. Recently, it has been designated as an orphan drug in combination with radiotherapy for the treatment of glioblastoma multiforme (GBM) [86]. Polyglutamates are highly biocompatible, biodegradable and multifunctional polymers, which have been successfully used as building blocks in polymer-drug conjugates and polymeric micelles for various medical applications [4,14,25,87]. In the body, such degradability is triggered by cysteine proteases (particularly cathepsin B), which play a key role in the lysosomal degradation of this polymer [88]. A similar system is PGA conjugated with camptothecin (CPT), a drug that belongs to the new generation of topoisomerase I inhibitors [89]. PGA-CPT (formerly CT-2106) is in Phase II for colorectal and ovarian cancers, although the company Cell Therapeutics Inc. (CTI, Seattle, WA, USA) has no immediate plans to conduct any further clinical studies [90]. Continuing with PGA, Eldar-Blok *et al*. have designed a PGA-PTX conjugate which incorporates an integrin-targeted moiety: a cyclic RGD peptidomimetic which significantly improved tumour accumulation and increased anti-tumour efficacy [91]. It is also worth mentioning PGA-Dopamine conjugate that has significantly increased the short half-life of this molecule which displays a vital role in the regulation of angiogenesis, setting up the basis for possible treatments of angiogenesis-dependent diseases [92]. Finally, synthetic polypeptides have also demonstrated great potential in vaccination, e.g., PGA can act as an adjuvant itself. Thus, only the antigen needs to be presented within the polymer to generate a directed immune response [93,94].

One of the trends in polymer therapeutics, and in particular with polymer-drug conjugates, is their application towards new molecular targets [25]. As an example, a hydrophobic pro-apoptotic sphingolipid: *N*,*N*-dimethylsphingosine (DMSP) has been linked to PGA through an ester linkage. This drug induces apoptosis competing with sphingosine kinase (SpK), a key regulator of tumour angiogenesis [95]. Although pro-apoptotic approaches are related with cancer therapy, anti-apoptotic strategies have also applications in regenerative medicine [96]. Vicent and coworkers designed the first anti-apoptotic polymeric nanomedicine: a PGA-based Apaf-1 inhibitor conjugate [97,98], which has shown to promote regeneration in the course of inflammation-induced tissue injury [99].

As introduced before, combination therapy is a well-established strategy used towards the design of a second generation polymer conjugates. This approach is focused on multi-agent therapy, capable to modulate different signalling pathways in diseased cells in order to maximise therapeutic output and overcome drug resistance. Pioneered by Vicent *et al*, synergistic effects were observed with a synthetic polymer carrying a chemotherapy agent (Dox) and an endocrine therapy (aminogluthetimine, AGM) designed for the treatment of postmenopausal breast cancer patients [100]. Aiming to improve this strategy by using a biodegradable and multivalent polymer, Deladriere *et al.* have designed and evaluated a family of PGA-AGM-Dox conjugates with demonstrated activity in an orthotopic 4T1 breast cancer mouse model [101]. In recent years, several studies have corroborated the therapeutic potential of this application [102] and, as expected, polypeptides have followed this trend. For instance, Wadhwa and Mumper have conjugated D-penicillamine and idarubicin to PGA, proving *in vivo* longer residence time in blood and a significant enhancement in the survival of athymic mice bearing NCI-H460 tumour xenografts [103].

Not only are homo-polyamino acids able to display drug carrier functions. Engineering of elastin-like peptides (ELPs) has also been explored for drug delivery. ELPs are 5–1500 amino acids in length based on the pentamer sequence Val-Pro-Gly-X-Gly, where X represents any amino acid [104]. Synthesis is achieved through recombinant DNA techniques (Section 2.1). Their most significant property relies on a temperature-conformation relationship: ELPs remain disordered below their transition temperature and become ordered β-turns above it. Through local hyperthermia, this phase transition has been used to target ELPs to solid tumours. As an example, Geldanamycin has been conjugated to the block polymer lysine 8-elastin-like polypeptides (K8-ELP) using their thermal responsive properties for drug targeting. Cell experiments showed higher cytotoxicity combining heat and K8-ELP-GA [105]. Chilkoti *et al*. have reviewed multiple alternatives for these systems [106,107]. ELPs have been successfully fused to a peptide (H1) inhibitor of an oncogene [108] together with penetratin (Pen) in order to allow membrane translocation demonstrating that Pen-ELP-H1 inhibits cell growth and more importantly, that hyperthermia significantly enhances treatment effectiveness. The well known chemotherapeutic agent Dox has been also conjugated to ELPs [109–111] alone or in combination with cell penetrating peptides [112], positioning the ELP carriers as promising candidates for thermally responsive polymer-drug conjugates in cancer treatment.

Synthetic polypeptides have resulted in a number of successful products as polymeric drugs (Table 1). For instance, Copaxone® (glatiramer acetate) was the first polymeric drug reaching the market and it was considered one of the top 10 selling drugs worldwide in the first quarter of 2013. Copaxone® is a random copolymer based on four amino acids (L-alanine, L-lysine, L-glutamic acid and L-tyrosine) with Mw within the range of 5000–10000 Da for the treatment of multiple sclerosis [113,114]. After subcutaneous administration, it reduces frequency of relapse and disease progression [115]. In the early 90s, Poly(Arg-Gly-Asp) was demonstrated to inhibit lung metastasis and migration of B16-BL6 melanoma in mice [116,117]. VivaGel® is another example of a polymeric drug under clinical development and it is based on a multivalent lysine-dendrimer. Polyionic drugs have yet to realise a market product. However, this topical polypeptide-based dendrimer developed by Starpharma could be the first one, as a vaginal virucide to prevent HIV-1 infection [118].

As explained before, there has been a rising interest in self-assembled block copolymers forming micelle structures as advanced carriers for bioactive molecules. Nowadays, several examples are under clinical evaluation waiting to promptly become a market reality [14]. These polymeric structures have been described as promising candidates for tumour-targeted therapy as well as non-viral gene vectors [119,120]. Driving forces of one block (hydrophobic or electrostatic interactions, metal complexations or hydrogen bonds) promotes the molecular assembly into core-shell architectures. Commonly, the hydrophobicity of the drug itself is used to achieve the amphiphilicity needed for micellisation. The majority of these micellar systems include a biodegradable polypeptide block, such as poly(aspartate), PGA or PLL and a variety of hydrophilic polymers, mostly PEG [24]. The first example was reported by Ringsdorf *et al*. by conjugating cyclophosphamide (CP) sulfide in PEG-PLL copolymer [121]. The hydrophilic palisade helps the system to stay unrecognised along blood circulation. PEG is usually selected as the shell-forming segment because of its physicochemical characteristics, including high water solubility through steric stabilisation, significant chain mobility, lower toxicity and a stealth effect on the polymeric micelles preventing the adsorption of proteins [122]. Polymeric micelles have demonstrated good stability at low concentrations, adequate size for systemic drug

delivery, useful out-shell emplacement for active targeting and feasibility for multiple drug incorporation towards combination therapy applications [123]. Kataoka and co-workers are pioneers in this area with several block co-polypeptide micelles in clinical trials as anticancer agents [124] (Table 1), and many similar analogues in preclinical evaluation, e.g., NC-6301 (analogue of NK911 with docetaxel) and NC-4016 [analogue of NC-6004 with oxaliplatin (DACH-platinate)] [31,125]. Inoue and co-workers conjugated Dox to the aspartic acid residue of PEG-block-poly(aspartic acid) copolymer. The polymeric micelles were delivered intravenously to the brain through conventional-enhanced delivery (CED). According to their report, micellar Dox infused by CED resulted in prolonged median survival compared with free Dox [126].

Once again, under the concept of combination therapy and including pH-response stimuli, PEG-poly(aspartate-hydrazide) (PEG-PAH) block copolymers have been conjugated to Dox and wortmannin, alone or in combination to achieve pH-sensitive polymeric micelles [127]. However, wortmannin is not a FDA approved drug and its synergistic activity with DOX was not fully demonstrated in this work. On the contrary, with the same structure and under the same concept, NC-6300/K-912 releases epirubicin at low pHs [128] and is currently in phase I clinical trials.

Despite the big efforts done on block co-polymer micelles, the market is yet to come. Present challenges are low drug loading capacity, reduced tumour targetability, short *in vivo* stability and premature drug release [129–133]. The future of these systems implies novelties in their design, such as cross-linking by reversible bonds with stimuli-responsive futures at the site of action [134] and the inclusion of targeting moieties which could enhance cellular uptake, in particular diseased cells through receptor-mediated endocytosis. All these systems are defined as a second generation of polymer therapeutics where their intrinsic characteristics may further improve the therapeutic index and reduce side effects [122].

#### 3.1.2. Gene Delivery

PT present the conditions to build up non-viral vectors for emerging macromolecular drugs (e.g., siRNA, oligonucleotides) enhancing the cytosolic delivery required to achieve a selected therapeutic output. In the case of gene therapy, nuclear localisation is an extra requisite. Polypeptides with inherent ionic character on their pendant chain groups can complex opposite charged molecules as, e.g., DNA, siRNA carriers. Several examples are found in the literature [135–137] although it is still early to certify its viability as nanomedicines. The only example so far within PT transferred to the clinics is not a polypeptide but a cyclic oligosaccharide, polymer-cyclodextrin nanoparticles-siRNA (CALAA-01) polyplexes which were evaluated in Phase I for solid tumours treatment [138,139] although the company decided not to advance into Phase II.

Most of the systems under investigation are block copolymers-forming micelles that have been named polyion complex micelles (PIC micelles). First evidence was reported by Kataoka *et al*. with the systems PEG-PLL and PEG-poly(Asp) [140]. Several examples are compiled in Table 1. The complexation of positively charged proteins is possible by taking advantage of the electrostatic interactions with negatively charged amphiphilic polymers. For instance, the positively charged lysozyme form a PIC within PEG-poly(Asp) at physiological pH [141]. Not only proteins but also low Mw drugs such as cis-dichlorodiamineplatinum II (CDDP) have also been used to form PICs [142–144].

**Table 1.** Examples of polypeptide-based Polymer Therapeutics in the clinics.

| Product Name | Technology | Indication | Stage | Info Source |
|---|---|---|---|---|
| | | **Drug Delivery** | | |
| ***Polymer-Drug Conjugates*** | | | | |
| CT-2103; Xyotax; Opaxio | Poly glutamic acid (PGA)-Paclitaxel | Cancer—NSCLC, ovarian, various, other cancers and combinations | Phase III, | Cell Therapeutics Inc. |
| CT-2106 | Poly glutamic acid (PGA)-Camptothecin | Cancer -colorectal and ovarian | Phase II | Cell Therapeutics Inc. |
| – | Poly glutamic acid (PGA)-Paclitaxel-RGD | Cancer-glioblastoma, breast cancer, pancreatic adenocarcinoma | Preclinical | [91] |
| – | Poly glutamic acid (PGA)-N,N-dimethylsphinosine (DMSP) | Cancer-Breast adenocarcinoma | Early stage | [95] |
| – | Poly glutamic acid (PGA)-Apaf 1 Inhibitor | Regenerative medicine. Regeneration in the course of inflammation-induced tissue injury | Early stage | [99] |
| – | Poly glutamic acid (PGA)-d-Penicillamine and idarubicin | Cancer-non-small cell lung cancer | Preclinical | [103] |
| – | Block copolymer lysine 8-elastin-like polypeptide (K8-ELP)-Geldanamycin | Cancer therapy in general | Early stage | [127] |
| – | Elastin-like polypeptide-Penetrating peptide-oncogen inhibitor peptide H1: Pen-ELP-H1 | Cancer therapy in general | Early stage | [108] |
| – | ELP-Doxorubicin, and Pen-ELP-Doxorubicin | Cancer therapy in general | Early stage | [109–111] |
| ***Polymeric Drugs*** | | | | |
| Copaxone | Glu, Ala, Tyr copolymer | multiple sclerosis | Marketed | Teva |
| – | Poly(Arg-Gly-Asp) | Cancer: lung metastasis | Preclinical | [115] |
| Vivagel | Lysine-based dendrimer | Microbiocide | Phase II/III | StarPharma |
| ***Polymeric Micelles*** | | | | |
| NK911 | mPEG-poly(aspartic acid)-doxorubicin | Solid tumors | Phase I | Nippon Kayaku Co |
| NK012 | PEG-poly(glutamic acid)-SN38 | SCLC and triple negative breast cancer | Phase II | Nippon Kayaku Co |
| NK105 | mPEG-poly(aspartic acid) PTX | Recurrent or meta-static breast cancer | Phase III | NanoCarrier Co.-Nippon Kayaku Co |
| NC-6004 | mPEG-poly(glutamic acid) with cysplatin | Locally advanced or metastatic pancreatic cancer | Phase I/II | NanoCarrier Co. TOUDAI |
| NC-6301 | mPEG-poly(aspartic acid)-docetaxel | Cancer therapy | Preclinical | NanoCarrier Co. TOUDAI |
| NC-4016 | mPEG-poly(glutamic acid) with oxaliplatin | Cancer therapy | Phase I | NanoCarrier Co. TOUDAI |
| NC-6300/K-912 | mPEG-poly(aspartate-hydrazide)-epirubicin | Cancer therapy | Phase I | NanoCarrier Co. TOUDAI |
| – | mPEG-poly(aspartate hidrazide)-Dox and Wortmannin | Cancer therapy | Early stage | [127] |

**Table 1.** *Cont.*

| Product Name | Technology | Indication | Stage | Info Source |
|---|---|---|---|---|
| ***Polymer-Protein conjugates*** | | | | |
| Zinostatin stimalmer® | Styrene maleic anhydride-neocarzinostatin, (SMANCS) | Cancer- hepatocellular carcinoma | Marketed | Yamanouchia Japan |
| Oncaspar® | PEG-asparaginase | Cancer -acute lymphocytic leukaemia (ALL) | Marketed | Enzon |
| Peg-intron® | PEG-Interferon alpha 2b | Hepatitis C | Marketed | Schering-Plough |
| Pegasys® | PEG-Interferon alpha 2a | Hepatitis C | Marketed | Roche |
| Neulasta™ | PEG-hrGCSF | Chemotherapy-induced neutropenia | Marketed | Amgen |
| Adagen® | PEG-adenosine deaminase | Severe combined immune deficiency syndrome | Marketed | Enzon |
| Somavert® | PEG-HGH antagonist | Acromegalia | Marketed | Pfizer |
| Mircera® | PEG-EPO (polyethylene glycol-epoetin beta) | Treatment of anemia associated with chronic kidney disease | Marketed | Roche |
| KrystexxaTM (pegloticase) | PEG-uricase | Chronic gout | Marketed | Savient farmaceuticals |
| Cimzia (certolizumab pegol) | PEG-anti-TNF Fab | Rheumatoid arthritis, Crohn's disease | Marketed | UCB |
| ADI-PEG 20 | PEG-arginine deaminase | Cancer-hepatocellular carcinoma, melanoma | Phase I/II | Phoenix Pharmalogics-Polaris Group |
| Hemospan® MP4OX | PEG–haemoglobin | Delivery of O2 in post surgery and trauma patients | Phase I/II | Sangart |
| CDP 791 | PEG-anti VEGFR-2 Fab | Cancer-NSCLC | Phase II | UCB Pharma |
| ADI-PEG 20 | PEG-arginine deaminase | Cancer-hepatocellular carcinoma, melanoma | Phase I/II | Phoenix Pharmalogics-Polaris Group |
| | | **Gen Delivery** | | |
| – | PEG-poly(lysine) block copolymer-siRNA | Nephrin protein silencing for renal diseases | Preclinical | [145] |
| – | Poly(phenylalanine)coPoly(ethylene imine) multiarm copolymer-DNA | DNA delivery | Early stages | [146] |
| – | Poly(lysine-co-phenylalanine) | DNA/siRNA delivery | Early stages | [147] |
| – | Poly(lysine) dendritic scaffold with HIV tat protein sequences | DNA/siRNA delivery | Early stages | [148] |
| – | Poly(lysine) dendrimers containing lipidated units | DNA/siRNA delivery | Early stages | [149] |
| – | Poly(lysine) dendrimers containing Cell Penetrating Peptides (CPPs) | DNA/siRNA delivery | Early stages | [150,151] |
| – | Poly(lysine-co-hystidine) dendrimers-DNA | DNA delivery | Preclinical | [152–154] |

**Table 1.** *Cont.*

| Product Name | Technology | Indication | Stage | Info Source |
|---|---|---|---|---|
| | | **Imaging and monitoring** | | |
| Gadomer 17 | Poly(lysine) dendrimer-DOTA-Gd | MRI contrast agent | Retired from clinics due to non adequate PK profile | [155,156] |
| – | Poly(lysine) dendrimer-RGD-DTPA-Gd | MRI contrast agent | Preclinical | [157] |
| – | Poly(glutamic acid)-DTPA-Gd | MRI contrast agent | Preclinical | [158] |
| – | Poly(glutamic acid)-DTPA-Gd-NIR813 | MRI contrast agent + fluorescence agent | Preclinical | [159] |
| | | **Other applications** | | |
| – | Dendrimeric Peptides with microbial surface recognition tetra and octapeptides | Antimicrobial agents | Early studies | [160] |
| – | Dendrimeric peptide $(RW)_{4D}$ | Antimicrobial agents | Early studies | [161] |
| – | Branched His-Lys dendrimers | Antimicrobial agents | Early studies | [162] |
| – | Peptide dendrimers based on His-Ser and diaminopropionic acid (Dap) | Enzyme mimic applications | Early studies | [163–166] |
| – | MAPs (Multiple Antigenic peptides), poly(lysine dendrimers) | Peptide antigens and inmunomodulating molecules to be used as vaccines | Early studies | [167] |

PLL have shown initial success in interacting and favouring the entrance of genetic material into the nucleus [168,169]. Targeting renal diseases, a block co-polymer containing PEG and PLL has been synthesised by Dhal *et al*. based on the use of a siRNA sequence to silence the protein nephrin which is localised in the podocytes (a type of renal cell). Significant differences among treated and non-treated animals were observed, validating them as a possible therapy in glomerular diseases [145]. Poly(phenylalanine) has been also used for this purpose, either as part of an amphiphilic multi-armed copolymer using poly(ethyleneimine) (PEI) as macroinitiator achieving gene transfection *in vitro* [146] or co-polymerised with other molecules such as PLL (poly(lysine-co-phenylananine) observing translocation across the lipid bilayer and subsequent drug release into the cytoplasm [147].

In order to avoid dissociation and to stabilise the structure of the block co-copolymer micelles, cross-linking of the core or the shell can be performed with reversible linkages. PEG-PLL cross-linked by disulfide bonds has been described and characterised [134] although the promised benefits in drug efficacy have not yet been reported.

### *3.2. Molecular Imaging and Theranostics*

Polymer conjugates also spans the field of diagnostics. Through a variety of imaging agents, several have been proposed as nanoprobes for disease monitoring. By means of tracer probe labelling, it is possible to build a single targeted conjugate to simultaneously report pharmacokinetics, targeting and clearance data *in vivo* and could also be used to report cellular and even subcellular location of a biopsied sample *ex vivo* [170]. Radiolabelled polymer based patient gamma camera imaging agents developed by Duncan *et al*. more than one decade ago have already been tested clinically [171]. Other techniques, such as magnetic resonance imaging (MRI), positron emission tomography (PET) or optical imaging using near infra-red (NIR) fluorescent and luminescent probes are approaches under study in the area [172–178].

Even more interesting is the concept of "theranostics" based on the "find, fight and follow approach" that offers the possibility of an early detection, disease targeting and treatment [20]. Relevant scientific studies related to this topic have been already reported [20,179,180]. The presence of end functionalities in the polymer carrier allows site-specific conjugation of drugs in parallel with imaging probes and/or targeting moieties, enabling the design of advanced theranostics or polymer-based combination nanopharmaceutics, limelight topics in this area due to current clinical needs. By means of non-invasive methodologies, conjugated imaging probes allow *in vivo* visualisation helping to elucidate drug mechanism of action, cell trafficking, biodistribution and conjugate fate. Li *et al*. have carried out biodistribution studies in solid tumour models using PGA-DTPA-Gd by MRI [158,181]. They were able to observe the localisation of the PGA based probe in the necrotic zone of the tumours. Recently, they have added a second label for multi-modal imaging by incorporating the near-infrared dye NIR813 [159]. The authors were able to ratify an increased uptake of PGA-Gd-NIR813 into the tumour and a selective accumulation into the necrotic/apoptotic region of the tumour. This dual-imaging approach allowed an accurate mapping of the intratumoral distribution of the conjugate. In this area, polypeptide-based micellar MRI contrast agents are under current development ensuring the validity of these constructs for imaging purposes [182,183].

Polymer conjugates for theranostic purposes are expected to facilitate non-invasive monitoring of drug delivery efficiency, a great opportunity through patient individualisation therapy.

### *3.3. Other Roles of Polypeptides in Polymer Therapeutics*

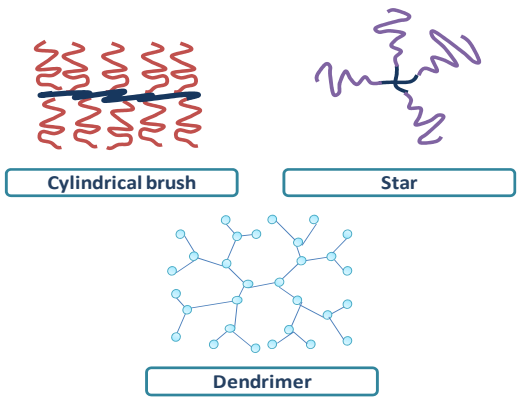
As it has been pointed out in the introduction, polypeptides such as proteins or specific peptide sequences can display biological functions, acting themselves as drugs, and their conjugation to a polymer carrier facilitates their development towards disease treatments [8,14]. Apart from displaying roles as a carrier or a bioactive counterpart, polypeptides may act as targeting moieties (e.g., antibodies [184,185], cell penetrating peptides [186,187]) or linkers. Within this last function, this type of biodegradable bond between the polymer and the bioagent can be adapted to specific conditions [188]. For instance, there are specific peptidyl sequences recognised by enzymes present at the disease site, *i.e.*, the tumour-associated protease legumain [189] or the well-known matrix-metalloproteinases (MMP) [190], which are associated enzymes with a number of types of cancer and other diseases that are essential in disease progression. Creativity in the field has built self-immolative linkers [191] or bioresponsive coiled-coil peptide linkers [192] with promising applications.

## 4. Polypeptide-Based Complex Architectures

The development of new and more defined architectures with higher $M_w$ (to enhance passive targeting by the EPR effect), predictable structure and conformation, lower heterogeneity, higher drug loading capacity and greater possibility for multivalency are main research lines in polymer therapeutics in particular, and in nanomedicine in general. As already stated polypeptide-based architectures are considered good starting points, however, there are still many unexplored areas in terms of design and synthesis of new polymeric constructs, their physicochemical characterisation, conformational studies, and specially their potential biological applications.

The first efforts within this context will be described herein, mainly focusing on the description of polypeptide-based branched polymers including, dendritic-like polypeptides, star and branched polymers, dendrimers and cylindrical brushes (Figure 7). They present different properties as for the linear polypeptides due to their intrinsic multivalency, and their significantly different architectures, shapes, sizes and solution conformations.

**Figure 7.** Schematic representation of branched polypeptide based complex architectures described within this review.



### *4.1. Star and Branched Polypeptides*

Star polypeptides are branched polymers, which consist of various linear chains linked to a central core. There are two main synthetic strategies for their synthesis: the core-first approach or multifunctional initiators and the arm-first approach or the use of multifunctional linking agents.

The first one, the core-first approach, is also known as divergent approach, and is based on the use of a multifunctional core that simultaneously initiates the polymerisation of several arms. In order to achieve control in the polymerisation for the preparation of homogenous products, it is necessary that all the initiating sites are equally reactive, and that the initiation step is faster than the propagation. The main drawback of this method is that the products obtained are more difficult to characterise as the arm Mw cannot be directly measured. Nevertheless, this strategy is the preferred one in the synthesis of star polypeptides. The second approach, the arm-first or convergent approach, is based on the previous synthesis of living macromolecular chains that will react with a multifunctional core. The main advantage is that characterisation should be easier since the living arms can be analysed independently before the linking step. However, steric hindrance is a major drawback and the use of an excess of living arms in the linking step is required. This fact yields to an unavoidable purification-fractionation step in order to obtain star polymers with good purity. Apart from these two well-known approaches, there is a recent classification including another strategy based on the reaction of living macroinitiators (MI) (or macromonomers) with a multifunctional cross-linker to provide stars. The resulting constructs are named core cross-linked star (CCS) polymers.

Using these methodologies and a combination of different polymerisation techniques, various polypeptide-based star polymers have been synthesised over the years. For example, Klok *et al*. [193] used perylene derivatives with four primary amine groups as initiators to lead 4-arm PBLG and PZLL and Inoue *et al*. [194,195] used hexafunctional initiators for the synthesis of 6-arm PBLG star polymers both taking profit of the NCA polymerisation techniques. Other examples are provided from the work of Aliferis *et al*. [196] who used 2-(aminomethyl)-2-methyl-1,3-propanediamine as a trifunctional initiator for the synthesis of P(BLL-*b*-BLG)$_3$ 3-arm star-block co-polypeptides; or the studies of Karatzas *et al*. [197] in the synthesis of 4-arm (PEO-*b*-PBLG)4 hybrid star block co-polymers using four-arm PEO stars end-functionalised with primary amines as initiators for the polymerisation of BLG-NCA among others.

Despite the fact that there is a growing interest in the development of hybrid and peptide-based star polymers as prospective advanced materials for biological applications in the field of nanomedicine, most of the studies undertaken to date have been focused on the synthesis of new and varied star polymers and their study in terms of self-assembly under a variety of conditions [198]. Only recently, peptide-based star polymers have been considered and explored as drug delivery systems. Sulistio *et al*. [199,200] synthesised peptide-based CCS polymers composed entirely of amino acid building blocks. These constructs were composed of PLL arms emerging from a poly(L-cysteine) (PLC) core and could be core-functionalised via reaction with primary amines bearing different functional groups (e.g., pyrene, alkyne) ending up always in water soluble, biocompatible, and biodegradable star polymers. These types of stars were able to entrap hydrophobic drugs, such as the anti-cancer drug pirarubicin, through physical interactions with the pyrene moieties of the core. Moreover, due to the presence of disulfide bonds at the core, the stars could also be cleaved by reducing agents such as

dithiothreitol, yielding redox-sensitive polymers. Apart from that, the same group developed CCS polymers with PLL arms, PLC cores and peripheral allyl functionalities by using an allylamine initiator. Those allyl groups allowed the conjugation of the stars with thiol-ended PEG via thiol-ene click chemistry. Furthermore, the other PEG terminus could be conjugated with folic acid moieties for cancer cell targeting. They were able to perform *in vitro* studies against breast cancer cells showing the absence of toxicity and an enhanced cell uptake for those CCS bearing folic acid residues. Xing *et al*. [201] prepared CCS polymers using $MeOPEG_{1900}–NH_2$ as a macroinitiator for ROP cystine and benzyl glutamate NCA derivatives which resulted in the formation of nanogels where the polymers had 9700 PEG arms and a $M_w$ of $4.2 \times 10^7$ Da. Those nanogels were tested *in vitro* in terms of cytotoxicity, being non toxic and biocompatible. In addition, they were capable of encapsulating the hydrophobic drug indomethacin, and release studies using gluthathione revealed that 100% of the drug was released after 200 h in physiological media. Although these examples are based on the encapsulation of hydrophobic drugs and not on a chemical conjugation, they are considered good examples to point out the possible use of star-shaped polypeptides in drug delivery applications.

In conclusion, there is a wide range of opportunities in the field of polymer therapeutics for these relatively new and interesting architectures. Preliminary results suggest that they are non-toxic entities, validating them as possible carriers for drug delivery among other applications. However, there is still a long way to go in this respect and the true potential of these constructs has not yet been realised. In addition, *in vivo* studies must be accomplished in order to confirm the absence of an immunogenic response and an adequate fate. Many other possible architectures, with the use of natural and unnatural amino acids, are yet to be unexplored, which may increase the versatility and applicability of these nanostructures.

### *4.2. Cylindrical Brushes*

Cylindrical brushes are branched polymers where a single linear polymer (primary chain) has secondary polymer chains grafted to it.

The advances in polymer chemistry have allowed the preparation of brush-like polymers using controlled polymerisation techniques, such as anionic, radical, and ring-opening metathesis polymerisation (ROMP) [202,203]. Brush-like polymers can be designed to contain flexible side-chain polymers, such as poly(methyl methacrylate), poly(styrene), elastin-like polypeptides, and PEG [204–206].

There are three main methodologies for the synthesis of brush-like polymeric architectures in general basis: the grafting onto, the grafting from, and the grafting through approach. The grafting onto approach is based on the attachment of already synthesised secondary chains onto a polymeric backbone with reactive sites usually randomly distributed. In the grafting from approach, the polymer backbone containing initiating sites is used as macroinitiator for the polymerisation of a second monomer. Finally, in the grafting through approach, or macromonomer approach, a polymer chain bearing a monomer unit is polymerised with a second monomer to lead graft co-polymers.

The incorporation of polypeptides with intrinsic secondary structures to the brush-like architectures provides an open door to materials with unexpected properties and behaviour. Despite the fact that it is an attractive approach to explore, the reports about brush-like polymers containing polypeptide chains are not many, and the majority are devoted to the grafting of oligo or polypeptides to a polymer.

Polymer-brush-grafted cargos have attracted increasing interest in controlled drug release systems. However, to our knowledge, there are not yet examples in the literature of these promising architectures within the polypeptide field.

### *4.3. Dendrimers*

Amino acid based dendrimers can be described as molecules based on branched amino acids (*i.e*., lysine, glutamic acid, aspartic acid, cis-4-amino-L-proline [207–212]) or other building blocks, such as cyclic peptides, poly(amido amines) (PAMAM) [213], polypropylene imines (PPI) [214], poly(aryl ethers) [215,216] and a large variety of other branched moieties including carbohydrates [217,218]. In this review, we will focus on examples coming from amino acid based dendrimers exclusively composed of amide bonds. Dendrimers with other branched scaffolds as core will be only briefly mentioned.

As for the other branched system, there are two possible synthetic routes to achieve peptide-based dendrimers: the divergent and the convergent approaches. In the divergent approach, dendrimers are built from their core and grow with a subsequent number of reactions, being expanded from one generation to the next. The convergent approach, developed by Frechet *et al*. [215,216], is based on the synthesis of the dendrimeric units that will be finally attached to the central core.

Their applications in the field of nanomedicine cover a broad range e.g., antimicrobial agents, protein/enzyme mimics, ion sensors and MRI contrast agents, DNA/RNA delivery vectors, among others.

The already mentioned VivaGel developed by Starpharma under clinical evaluation [118] is the most advanced polymer therapeutic with dendrimeric architecture showing promising results as a vaginal virucide to prevent HIV-1 infection. In addition, the development of Gadomer 17 must be mentioned here [155]. Gadomer 17 is a dendritic MRI contrast agent based on a trimesoyltriamide core with 18 lysine residues attached to it which serve as conjugating points for 24 $Gd^{3+}$-DOTA (tetraazacyclododecanetetracetic acid) units. This intravascular contrast agent was transferred to the clinics although recently retired due to a non-adequate PK profile [156]. Another example which combines the use of targeting ligands RGDs and $Gd^{3+}$-DTPA (diethylenetriaminepentaacetic acid) units has been recently developed as MRI contrast agent. This system is based on a polylysine dendrimer prepared via chemical ligation, in which in one dendritic edge it is conjugated to RGDs and the other to MRI contrast agents [219].

In the late 80s, Tam and co-workers tried to develop potential multiple antigenic peptides (MAPs) by using PLL dendrimers [167,220,221]. The aim was to produce a rational system in order to link different types of peptide antigens and immunomodulators to be used as vaccines. However, apart from some articles reporting promising preliminary results, there is still no vaccine based on them. In respect to their application as antimicrobial agents, Tam *et al*. have synthesised various families of dendrimeric peptides with different amounts of copies of tetrapeptides and octapeptides which where demonstrated to recognize a putative microbial surface recognition motif [160]. These peptidic structures were able to be active against a broad spectrum of bacteria and fungi, without being toxic to human erythrocytes (the main problem of naturally occurring antimicrobial peptides). Later on, Kallenbach *et al*. [161,222,223] developed more simple dendrimeric peptides based on four copies of a dipeptide RW $(RW)_{4D}$ which also demonstrated efficient activity against *Escherichia coli* and *Staphylococcus aureus*, but diminishing the haemolytic activity when compared the linear chains RW

($n$ = 3–5). Zhu *et al*. [162] has recently tested the antimicrobial activity of a set of branched His-Lys dendrimers on *Candida* species and other fungi, finding that the four-branched dendrimer H2K4b was the most active among all. There are many other dendrimers that have been tested to be effective against a variety of bacterial strains, including multidrug-resistant strains [157,224–227].

As mentioned, dendrimers might be useful as enzyme mimetics. One example for this application is the work done by Reimond *et al*. [163–166]. They have identified a set of peptide-based dendrimers, firstly made from His-Ser and diaminopropionic acid (Dap) as branching units. These dendrimers showed a catalytic activity following Michaelis-Menten kinetics in the hydrolysis of pyrene trisulfonate esters. It was reported that the catalytic efficiency increased with dendrimer generation and a five-fold improvement was achieved by using mutational models substituting the amino acid Ser by Thr.

Cationic peptide dendrimers can in principle be considered as DNA/RNA delivery vectors since they accomplish many of the requirements. They bind the negatively charged molecules by strong electrostatic interactions protecting them from enzymatic degradation. There are many examples for this application. The main efforts have been directed towards the incorporation of different moieties (such as long lipid chains, cell penetrating peptides, *etc.*) in order to overcome the problems with cell internalization (membrane interaction and cell penetration). For instance, the modification of a polylysine dendritic scaffold with HIV tat protein sequences, which are described to be able to facilitate cellular and nuclear transport [148]; the modified polylysine dendrimers containing lipidated units developed by Bayele *et al*. [149], which were able to efficiently deliver oligonucleotides into the nucleus; and the cell penetrating peptide bearing polylysine dendrimers developed by Toth and Minchin's group [150,151]. On the other hand, Mixson *et al*. studied the effect of the histidine loading of different His/Lys-based dendrimers [152–154]. This was a development of his significantly enhanced DNA delivery in a variety of cell lines. These systems have been tested in tumour bearing mouse models with promising results.

## 5. Conclusions and Future Perspectives

Within this review, a general idea of the advantages of the use of polypeptides as carriers and the main strategies for their design have been described, pointing out the different architectures built and the current research in polymer therapeutics. Amino acid building blocks and polypeptide structural characteristics represent a good choice to overcome some of the limitations of the current nanomedicines such as their biodegradability, biocompatibility and multifunctionality, making these constructs suitable for clinical applications.

A second generation of polymer therapeutics is focused on the construction of novel and heterogeneous architectures with higher $M_w$ (to improve the EPR effect), predictable structure and conformation in order to achieve greater possibility of multivalency to tailor loading capacity. Progress into the polymer chemistry field is addressed in this issue, firstly applying the ring opening polymerization of NCAs as the method for the obtention of well defined polypeptide based architectures and secondly introducing a wide range of functionalities in the resulting structures, by the use of natural and/or synthetic amino acids bearing different side-chain functional groups for later selective bio-conjugations.

Current efforts and examples of the biological applications of polypeptide-based materials summarised in this review lay the foundations for their promising future as drug/gene carriers or imaging probes among other applications, and open the door for a second generation of improved architectures. However, there is still a whole field to explore in terms of design and synthesis of new polymers and polymeric structures with a wide range of tunable properties, their physicochemical characterisation, conformational studies, and in particular, their potential as nanomedicines in different clinical applications.

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## Conflicts of Interest

The authors declare competing financial interest (see Polymers website for details). Declaration: Aroa Duro-Castano, Inmaculada Conejos-Sánchez and María J. Vicent are inventors on a patent that has been filed by Centro de Investigación Príncipe Felipe [83]. María J. Vicent is co-founder of Polymer Therapeutic Solutions spin off company.

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