Expert Opinion

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Polypeptides and polyaminoacids in drug delivery

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Introduction: Advances achieved over the last few years in drug delivery have provided novel and versatile possibilities for the treatment of various diseases. Among the biomaterials applied in this field, it is worth highlighting the increasing importance of polyaminoacids and polypeptides. The appealing properties of these polymers are very promising for the design of novel compositions in a variety of drug delivery applications.
Areas covered: This review provides an overview on the general characteristics of polyaminoacids and polypeptides and briefly discusses different synthetic pathways for their production. This is followed by a detailed description of different drug delivery applications of these polymers, emphasizing those examples that already reached advanced preclinical development or have entered clinical trials.
Expert opinion: Polyaminoacids and polypeptides are gaining much attention in drug delivery due to their exceptional properties. Their application as polymers for drug delivery purposes has been sped up by the significant achievements related to their synthesis. Certainly, cancer therapy has benefited the most from these advances, although other fields such as vaccine delivery and alternative administration routes are also being successfully explored. The design of new entities based on polyaminoacids and polypeptides and the improved insight gained in drug delivery guarantee exciting findings in the near future.

Keywords: cancer therapy, drug delivery systems, gene delivery, nanomedicine, polyaminoacid, polypeptide

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1. An introduction to polypeptides and polyaminoacids: definition, properties and synthesis

Polyaminoacids (PAA) and polypeptides are polydisperse structures formed by condensation of amino acid monomers through amide bonds that, contrary to proteins, cannot fold into globular structures. They are regarded as important building blocks for the design of functional materials thanks to their ability to form well-defined secondary structures (i.e., α-helix and β-sheets). These secondary structures contribute significantly to the self-assembling character of polypeptide chains, leading to novel supramolecular structures with potential biomedical applications [1]. PAA and polypeptides are generally biocompatible and nontoxic. In addition, they can carry versatile reactive functional groups at their side chains (carboxylic acids, hydroxyl, amino and thiol groups) that allow for a variety of chemical modifications. All these features render PAA and polypeptides excellent polymers for drug delivery (DD) applications.

In this Expert Review, the term 'PAA' will be used when referring to synthetic materials. PAA usually incorporate a single amino acid monomer in their backbone. The cationic polylysine (PLL), polyarginine (PArg) and the anionic polyasparagine (PAsp) and polyglutamic acid (PGA) constitute typical examples of PAA. Conversely,

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Article highlights.

- Polyaminoacids and polypeptides are attractive biomaterials for drug delivery purposes due to their versatility, nontoxicity and biocompatibility.
- Synthesis improvements have allowed the achievement of more precise molecular weight polymers with lower polydispersity, which undoubtedly have a positive effect on their performance.
- The application of polyaminoacids and polypeptides to drug delivery has yielded the construction of new entities with interesting properties such as multidrug encapsulation, enhanced uptake or stimuli-responsiveness.
- Polyaminoacids and polypeptides have contributed to significant advances in biomedicine in the fields of cancer therapy and gene delivery. They also hold great promise for future developments in other areas such as vaccine delivery and tissue engineering.

This box summarizes key points contained in the article.

the term 'polypeptide' will be devoted to naturally occurring biopolymers of composite amino acid sequence such as protamine.

Interestingly, copolymers composed of PAA/polypeptides and synthetic polymers have recently gained much interest in different scientific areas, such as polymer and materials science, nanotechnology and biomedicine. These biomimetic hybrid polymers or 'molecular chimeras' combine the favorable properties of both polymer blocks and can form supramolecular structures with very interesting properties and applications otherwise unlikely with conventional materials. For instance, amphiphilic diblock copolymers of this type can form PAA-based micelles and vesicles in water, with potential applications in drug and gene delivery. In order to predict and direct the self-assembly behavior of these amphiphilic macromolecules, it is necessary to control a variety of chemical and physical parameters such as molecular weight, polydispersity, macromolecular architecture and chemical nature. The synthesis of such well-defined structures has been a major challenge for polymer chemists until recently.

1.1 Chemical synthesis of PAA

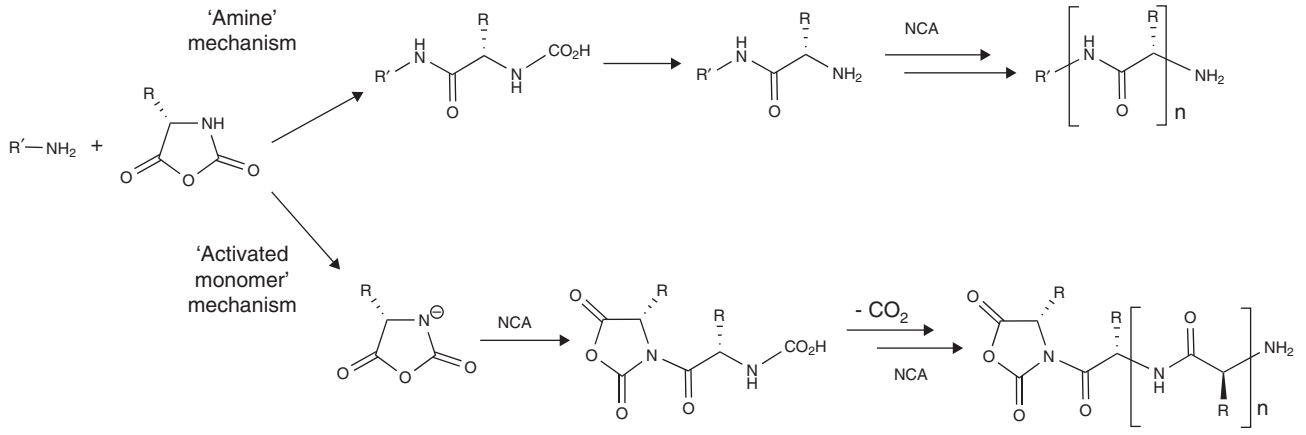
Traditionally, amino acids have been polymerized by conventional solid-phase peptide synthesis. A large number of oligomers and short peptides can be easily synthesized by this well-established method. However, the preparation of peptides larger than 100 residues usually results in undesired byproducts due to incomplete deprotection and coupling steps. To date, various alternative approaches to produce PAA and their block copolymers have been developed. Among them, the ring-opening polymerization (ROP) of α-amino acid-*N*-carboxyanhydrides (NCAs) is the most commonly applied technique (Scheme 1) [2,3]. This method is economic and usually leads to polymers in good yields and large quantities. In addition, NCAs are straightforwardly prepared [4-6].

Commonly, NCA polymerizations are initiated by different nucleophiles and bases, such as primary amines or alkoxide anions [2,3]. Unfortunately, no universal initiators and polymerization conditions have been identified and optimal results often require fine-tuning. Aspartic acid, glutamic acid and lysine appear to be the most suitable amino acids in terms of NCA preparation and subsequent polymerization [7]. Generally speaking, the main limitation of NCA polymerization has been the presence of side reactions that restrict control over molecular weight and usually lead to broad molecular weight distributions [8].

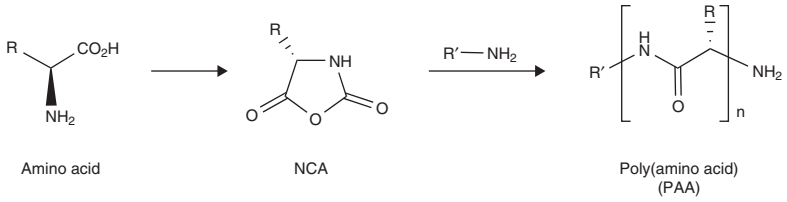
Two likely pathways for NCA polymerizations have been proposed, the 'amine' and the 'activated monomer' (AM) mechanisms (Scheme 2) [2,3]. The amine mechanism consists on a nucleophilic ROP initiated by species with stronger nucleophilic than basic character, typically primary amines. In the AM mechanism, the NCA is first deprotonated to become the nucleophile that initiates chain growth. It is generally attributed to strong bases such as metal alkoxides or tertiary amines.

Interestingly, a given system can switch back and forth between the amine and AM mechanisms during polymerization, which results in side reactions. Thus, the propagation step for one mechanism is a side reaction for the other one. As a result, block copolymers prepared from NCAs using, for instance, amine initiators reveal structures different from predicted and a considerable homopolymer contamination. These side reactions also prevent control of chain-end functionality, which is of crucial importance in the preparation of hybrid copolymers.

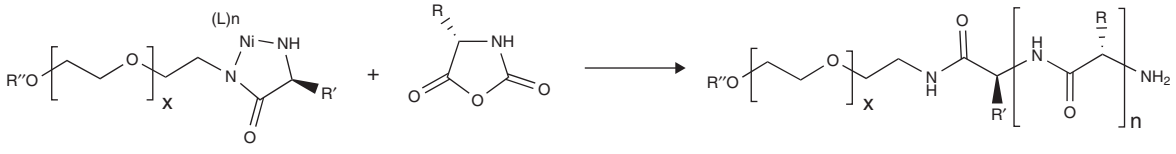
These potential side reactions in the polymerization of NCAs have made the synthesis of PAA with controlled molecular weight and low polydispersity unsuccessful until 1997, when Deming discovered novel NCA initiators based on transition metal complexes [9]. Since then, the efficiency and fidelity in the synthesis of PAA materials have greatly improved. In another seminal contribution, Hadjichristidis and coworkers reported in 2004 the preparation of PAA with control over chain length and length distribution under high-vacuum conditions [10]. Also in 2004, Olivia Giani and coworkers lowered the temperature of the polymerization process to 0°C as a strategy to obtain well-defined PAA [11]. Another innovative strategy to control amine-initiated NCA polymerizations has been described by Schlaad and coworkers, who employed primary amine hydrochloride salts as initiators to obtain well-defined PAA segments of narrow chain length distribution ($M_w/M_n < 1.03$) [12]. More recently, Lu and coworkers have reported on the controlled living polymerization of NCAs mediated by hexamethyldisilazane (HMDS) [13]. These authors have identified trimethylsilyl carbamate (TMS-CBM) as an efficient chain-propagating group and demonstrated that alternative TMS-protected amines were also efficient initiators, allowing the introduction of different functionalities for further chemical transformations [14], including 'click reactions' [15].



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



Scheme 2. 'Amine' and 'activated monomer' mechanisms.



Scheme 3. Nickelacycle macroinitiators in a controlled α-amino acid-*N*-carboxyanhydride (NCA) polymerization.

1.2 Synthesis of PAA copolymers

Since the first reports on the synthesis of PAA hybrid block copolymers in the mid-1970s, the preparation of numerous PAA–synthetic polymer (AB type) and PAA–synthetic polymer–PAA (ABA type) block copolymers has been described [16]. Usually, these copolymers are prepared in two steps because of the chemical incompatibility of the two polymerization processes. This approach benefits from the fact that most synthetic polymers can be prepared with controlled chain length, low polydispersity and a high degree of amine functionalization at the chain ends. In most of these examples, the PAA block is composed of lysine or glutamate derivatives, since they form α-helixes with good solubility properties.

As in the case of PAA homopolymers, several improvements have been described for the controlled polymerization of hybrid PAA copolymers. For instance, the amine hydrochloride initiators developed by Schlaad and coworkers have been efficiently employed in the preparation of well-defined protected polystyrene-PLL (PS-PLL) block copolymers [12], and for the copolymerization with γ-benzyl-L-glutamate and β-benzyl-L-aspartate [17]. Deming and coworkers have also demonstrated that amido-amine nickelacycle end groups can be incorporated into synthetic polymers and subsequently used as macroinitiators in a controlled NCA polymerization (Scheme 3) [18]. The application of this technology renders copolymers where the length of the PAA segment can be tuned with good control and no unreacted homopolymers are detected. This methodology for the preparation of block copolymers seems to be of general scope and has been used with a wide range of amino-terminated polymers.

An alternative approach to obtain PAA block copolymers is the convergent coupling of pre-synthesized polymer segments. Among the various reactions used for this goal, the Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC) [19] has revealed

as an extremely powerful coupling technology [20]. This orthogonal high-yielding reaction is compatible with a broad range of functional groups and hence is particularly attractive for the synthesis of biopolymer conjugates.

2. DD applications of PAA and polypeptides

Drug delivery systems (DDS) have provided great benefits to current therapies by i) increasing stability and bioavailability of therapeutic molecules, ii) decreasing their side effects and/or iii) enabling alternative and better administration routes. This field offers a whole range of structures with different properties that can be tuned according to the therapeutic needs. For example, multi-encapsulation of drugs, bioadhesion, enhanced uptake properties, biocompatibility, drug sustained release or surface modification with target moieties to achieve a localized delivery of the drug transported are some of the appealing properties that DDS can offer [21,22]. In this sense, composing polymers play a crucial role and, therefore, need to be thoughtfully selected in the design process of new entities. Polypeptides and PAA have significantly contributed to the formulation of novel DDS, and their applications are going to be disclosed in the following lines, not only focusing mainly on major achievements of recent years, but also including some of the pioneering early developments where appropriate. The authors are aware of the advances related to the use of proteins as biomaterials in this field; however, these are out of the scope of the present work.

2.1 Polymer therapeutics

Synthetic polymers have been explored as therapeutic agents over more than 50 years. The term 'polymer therapeutic' is used to describe polymer-based constructs that are considered as new chemical entities by regulatory agencies [23]. In the mid-1970s, Ringsdorf [24] proposed that polymer–drug conjugates could enhance the delivery of anticancer drugs to tumors. He envisioned that when an anticancer drug is conjugated to a polymeric carrier, its pharmacological properties could be manipulated by changing the physicochemical properties of the polymer. In addition, it was later recognized that polymer–drug conjugates tend to accumulate in solid tumors thanks to the so-called enhanced permeation and retention (EPR) effect [25]. To date, many polymers have been investigated as candidates for the delivery of different drugs, and a growing number of polymer therapeutics have been transferred to the market. In general, the ideal polymer for DD should be water-soluble, biodegradable (or have adequate molecular weight to allow elimination from the body), have a low polydispersity and be non-immunogenic. In this context, biodegradable PAA such as PLL and PGA have revealed as promising candidates with several formulations in clinical trials. In addition, PGA and PLL have a high drug-loading capacity derived from the presence of reactive side groups (carboxylic acid and amine), which have been employed for the attachment of different chemotherapeutic agents such as doxorubicin (DOX), paclitaxel (PTX) and camptothecin (CPT). To date, one of the most advanced PAA formulations is Opaxio® (formerly known as Xyotax®), currently in Phase III for the treatment of ovarian and non-small cell lung cancer (NSCLC) (**Figure 1**). This polymer therapeutic results from the attachment of PTX to PGA through ester linkages. This system has an impressive drug loading (~ 37 wt%) and is able to circulate for prolonged times in the bloodstream and subsequently accumulate in tumors, where the drug is released [26]. This long circulation half-life and low toxicity seem to be key factors for the clinical success not only of Opaxio™ but also of CT-2106, a poly(glutamate)-glycine CPT polymer therapeutic currently in Phase I/II [27].

In addition, PGA is well-known to be biodegraded by cysteine proteases (cathepsin B) at the lysosomes, an essential property to avoid accumulation in the organism after repeated administration. Interestingly, clinical results suggest that this degradation pathway is behind the increased activity of Opaxio in women with NSCLC [28]. These studies sustain that the presence of estrogen may influence the metabolism of Opaxio, due to the enhanced activity of cathepsin B and subsequent drug release.

Another type of polymer therapeutics is constituted by well-defined multivalent dendrimers and dendritic polymers, which may act as nanocarriers or as a drug themselves. VivaGel® is an L-lysine-based dendritic microbicide decorated with anionic groups, in which the dendrimer is not a carrier but an active ingredient. It has been evaluated in Phase II as a vaginal gel for preventing/reducing transmission of HIV and genital herpes (**Table 1**) [29,30]. As another example of dendritic polymer therapeutics, it is worth mentioning those based on PGA, recently evaluated for the delivery of DOX. This dendritic delivery system can be further modified with biotin for targeting purposes and deliver the anticancer drug by the cleavage of the pH-sensitive bonds that attach the drug to the structure [31].

Polymer therapeutics with stimuli responsive functions have also been described in the literature. These should undergo physical or chemical modifications in response to small changes in the environmental conditions. The benefits of stimuli-responsive systems are especially important when these stimuli are unique for disease pathology, allowing a specific response to pathological 'triggers.' Typical examples of biological stimuli are pH, temperature, ionic strength and redox microenvironments.

In a recent approach, Shen and coworkers combined both pH-sensitive groups and disulfide bonds that were reduced in the intracellular reductive environment for the nuclear delivery of CPT by a PLL-based conjugate [32]. In order to avoid the toxicity and problems associated to *in vivo* applications of cationic PLL, they converted PLL's primary amines into acid-labile carboxylic amides (PLL/amide). CPT was introduced into the polymer by means of an intracellular cleavable disulfide bond, and folic acid was attached to the PLL skeleton in order to obtain a cancer-cell-targeted

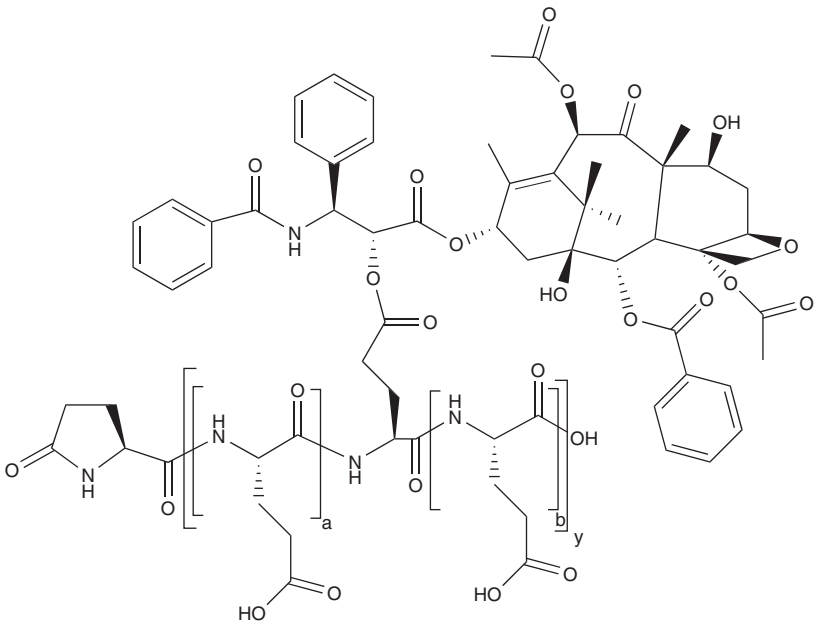


Figure 1. Chemical structure of Opaxio.
a, b, y denominate different numbers of monomer units.

nuclear-localization polymer–drug conjugate. The conjugate efficiently entered folate-receptor overexpressing cancer cells and moved to their nuclei with improved cytotoxicity.

2.2 Self-assembled nanostructures

A fascinating area in materials science and nanochemistry is concerned with the creation of supramolecular architectures with well-defined shape and function. Self-assembled nanostructures are held together through non-covalent forces, such as van der Waals forces, electrostatic interactions, hydrogen bonding and metal complexation [33]. Various self-assembled morphologies with spherical, rod-like and lamellar structures have been described and offer numerous possibilities to tailor their physical, chemical and biological properties by variation of their chemical structure or by conjugation to biomolecules. In this section, we will focus on self-assembled nanostructures most commonly employed in drug and gene delivery, which will be classified according to their supramolecular structure.

2.2.1 Polymeric micelles

Amphiphilic block copolymers with a large solubility difference between the hydrophilic and the hydrophobic segments can spontaneously form polymeric micelles in aqueous media. These micelles are characterized by a sub-100 nm core–shell structure, which provides a reservoir for hydrophobic drugs enveloped by a hydrophilic shell that improves drug solubility and suppresses protein adsorption (stealth effect) [34]. Furthermore, polymeric micelles can be functionalized with targeting ligands and biomarkers to provide control over biodistribution and site-specific cellular uptake (active targeting).

Among the various amphiphilic copolymers leading to micellar structures, block copolymers of PAA and poly(ethylene glycol) (PEG-PAA) obtained *via* ROP of NCA are especially appealing as they allow chemical modification at the amino acid side chains [35]. Within this context, Kataoka and coworkers have pioneered the use of PEG–PAA block copolymers for DD [36-38]. For instance, they prepared polymeric micelles from PEG–poly(aspartate) (PEG–PAsp) carrying DOX covalently conjugated at the side chains through amide bonds. This enhances the hydrophobicity of the PAsp block and facilitates the spontaneous formation of micelles in aqueous media [37].

Additionally, unbound DOX molecules could be physically entrapped in the micellar core by hydrophobic interactions. Physically entrapped DOX displays the major cytotoxic function, while conjugated DOX molecules work mainly by increasing the micelle stability [39]. This optimized PEG–PAsp (DOX) micelle has been studied in Phase I clinical trials as NK911 in Japan (**Table 1**) [40] and its structure is shown in **Figure 2**. Similarly, PTX has been physically entrapped in the core of hydrophobically modified PEG–PAsp polymeric micelles [41]. The resulting micellar system, known as NK105, is under Phase II clinical studies in Japan (**Table 1**) [42]. PEG–PGA micelles have also demonstrated to be useful for the delivery of the water-insoluble anticancer drug 7-ethyl-10-hydroxycamptothecin (SN-38), an analog of CPT. With this aim, Matsumura and coworkers covalently modified PEG–PGA with SN-38 at the PGA block, which self-assembled into micelles [43]. These SN-38-loaded micelles showed enhanced antitumor activity compared with the free drug and are currently under Phase I clinical trials as NK012 (**Table 1**) [44].

Table 1. Polyaminoacids (PAA)- and polypeptide-based drug delivery systems currently in clinical trials.

Polymer composition	Carrier type	Loaded drug	Clinical development	Identifier*/ref.
Polyglutamate	Polymer conjugate	Paclitaxel	Phase I (completed January 2009)	NCT00060359
			Phase II (completed September 2006)	NCT00069901
			Phase III (recruiting participants)	NCT00108745
		Methotrexate	Phase IV (completed December 2010)	NCT00695188
		Camptothecin	Phase I (completed July 2008)	NCT00059917
Polyglutamate–PEG	Micelles	SN-38	Phase II (recruiting participants)	NCT00951054
		Cisplatin	Phase I/II (recruiting participants)	NCT00910741
		Oxaliplatin	Phase I (Japan)	[144]
Polyaspartate–PEG	Micelles	Doxorubicin	Phase I (Japan)	
		Paclitaxel	Phase II (Japan)	
Polyglutamate–vitamin E	Nanoparticles	Insulin	Phase I	[145]
		Interferon β-1a	Phase I	[145]
		Interferon α	Phase II (recruiting participants)	NCT01010646
Polylysine	Dendrimers	SPL7013 Gel (VivaGel)	Phase II (completed June 2011)	NCT00740584
	Physical mixture/PEC	MUC-1 peptide vaccine	Phase 0 (recruiting participants)	NCT00986609
	Physical mixture /PEC	Poly-ICLC	Phase I (completed April 2011)	NCT00880867
			Phase II (recruiting participants)	NCT01245673
Polyarginine	Physical mixture/PEC	Vaccine for chronic hepatitis C virus	Phase II (completed July 2008)	NCT00601770

*According to clinicaltrials.gov [146].

Kataoka and coworkers have also developed PAA-based micelles containing cisplatin (cis-dichlorodiammineplatinum [II], CDDP). In this case, CDDP was introduced into PEG–PAsp block copolymer by metal complexation between platinum and carboxyl groups of the PAsp block. This complex spontaneously formed polymeric micelles that exhibited a higher accumulation in tumor sites than free CDDP [45]. The micelle composition was further modified to regulate the CDDP release and to extend the blood circulation time by using the more hydrophobic PEG–PGA copolymer instead of PEG–PAsp. The PEG–PGA(CDDP) micelle is currently undergoing a Phase I clinical trial as NC-6004 in the UK (**Table 1**). Alternative platinum-drug-loaded polymer micelles based on dichloro(1,2-diaminocyclohexane)platinum(II) (DACHPt) and PEG–PGA have been obtained and shown longer circulation times and more than 20-fold higher accumulation in tumors than the free drug [46].

Further studies aimed to improve the biodistribution profile of PEG–PAA polymeric micelles have shown a crucial effect of the PAA length on biological performance. Micelles composed of a copolymer PEG–PGA with 20 glutamic acid units have resulted in reduced accumulations in the liver compared with longer PGA blocks. It has been proposed that shorter PGA segments may decrease the micellar core size and achieve a more efficient PEG shielding of the core, which helps to decrease hepatic toxicity and improve antitumor activity by increasing accumulation in tumor tissue [47].

In addition, stimuli-responsive polymeric micelles sensitive to different pH of specific cell compartments or tissues, as well as the differences in the concentrations of reductive agents (e.g., glutathione) outside and inside the cell, have been reported in the literature. For instance, pH-sensitive micelles that exploit acidic environment at tumor tissue to unload its contents have been prepared by different approaches, such as the employment protonatable groups (amines or carboxylic acids) or of pH-sensitive linkers (hydrazone, *cis*-aconityl or acetal groups, etc.) In this context, Kataoka's group has also prepared stimuli-responsive micelles containing DOX by conjugating the drug to the PAsp segment of a PEG–PAsp block copolymer through a hydrazone bond, which is stable under physiological conditions but cleavable under acidic intracellular environments [48] In addition, the micelles were modified with a folate moiety at the distal end of PEG, allowing for an active targeting to cancer cells and

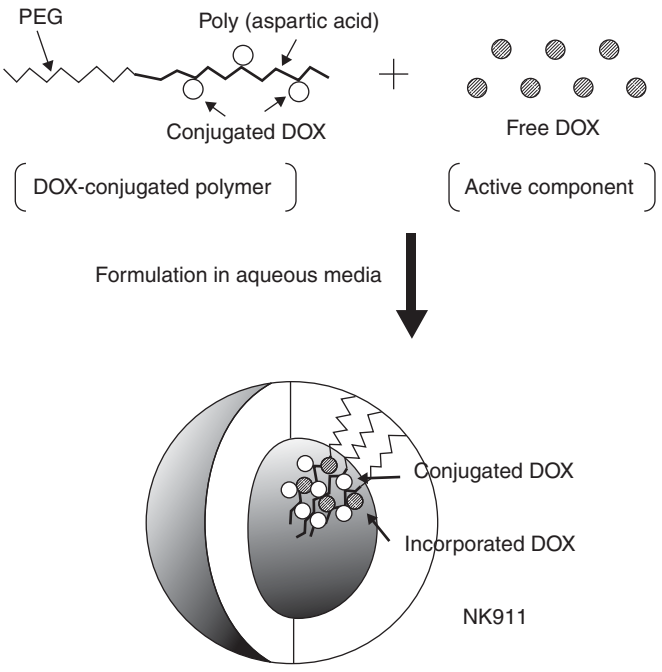


Figure 2. Schematic structure of NK911, a polymeric micelle consisting of a block copolymer of PEG and polyaspartic acid.
Reproduced with permission from Nature Publishing Group [40].
*= statistical differences of CP-Dox vs Dox at p=0.03 and vs PBS at p=0.00002
‡= statistical differences of CP-Dox vs Dox at p=0.0001 and vs PBS at p=0.00004

showing a significantly improved therapeutic effect compared with free DOX [49].

Another example to pH-triggered drug release was reported by Bae and coworkers [50]. They employed a pH-sensitive polymeric micelle composed of a mixture of PEG–poly(L-histidine), a pH-sensitive PAA with pKa values around physiological pH and biodegradable PEG–poly(L-lactic acid) block copolymers intended to encapsulate DOX. The resulting drug-loaded mixed micelles were stable under physiological pH but destabilized at the acidic pH of the target tumor site. When these mixed micelles were conjugated to folic acid, they resulted more effective in killing tumor cells due to accelerated drug release in the tumor region and folate receptor-mediated tumor uptake. Furthermore, the fusogenic activity of poly(L-histidine) in the endosomes facilitated the cytosolic delivery of DOX to achieve improved cytotoxicity.

Recently, as a result of the in-depth knowledge gained on protein structure and function, a new family of polymers called 'engineered peptide-based biopolymers' has been established [51]. Among them, those based on elastin-like polymers (ELP) have found application in DD. ELP self-assemble into polymeric micelles in aqueous solution in a reversible temperature-dependent way. Above a critical solution temperature (T_t) that is sequence specific, ELP assemble from a soluble expanded state to a controlled micellar collapsed state [52]. This behavior can be tuned depending on the polymer sequence to obtain polymeric carriers with customized properties [53]. Moreover, ELP have a typical phase-transition behavior, which can be also used for self-assembly purposes into nanostructures by selective desolvation of specific blocks of the recombinant ELP block copolymers [54].

The application of ELP to DD has yielded promising results in cancer therapy. Chilkoti and coworkers have recently published the formation of monodisperse nanostructures from a chimeric polypeptide–DOX conjugate that self-assembles in aqueous media. The chemical attachment of DOX to ELP through hydrazine linkages enables its encapsulation and its selective delivery at pH 5 by the cleavage of the acid-labile hydrazone bond. This pH-sensitive behavior assures that the drug remains within the structure while in the blood circulation, but it is released after internalization and exposure to the acidic endolysosomal environment. This phenomenon is shown by the improved pharmacokinetics of the system, showing long-circulating times for the micelles and a clear 3.5-fold enhanced drug concentration in tumor compared with free DOX at the same dose. Moreover, the accumulation at non-tumor sites is also reduced, being especially remarkable the decrease in drug concentration in the heart, which allows to achieve higher maximum-tolerated dose for the micelles compared with the free drug. In addition, an impressive remission of tumor burden was observed after a single injection of these DOX-loaded micelles (**Figure 3**) [55].

2.2.2 *Polyion complex micelles*

Another interesting type of micelles that can be prepared from PAA block copolymers is constituted by polyion complex

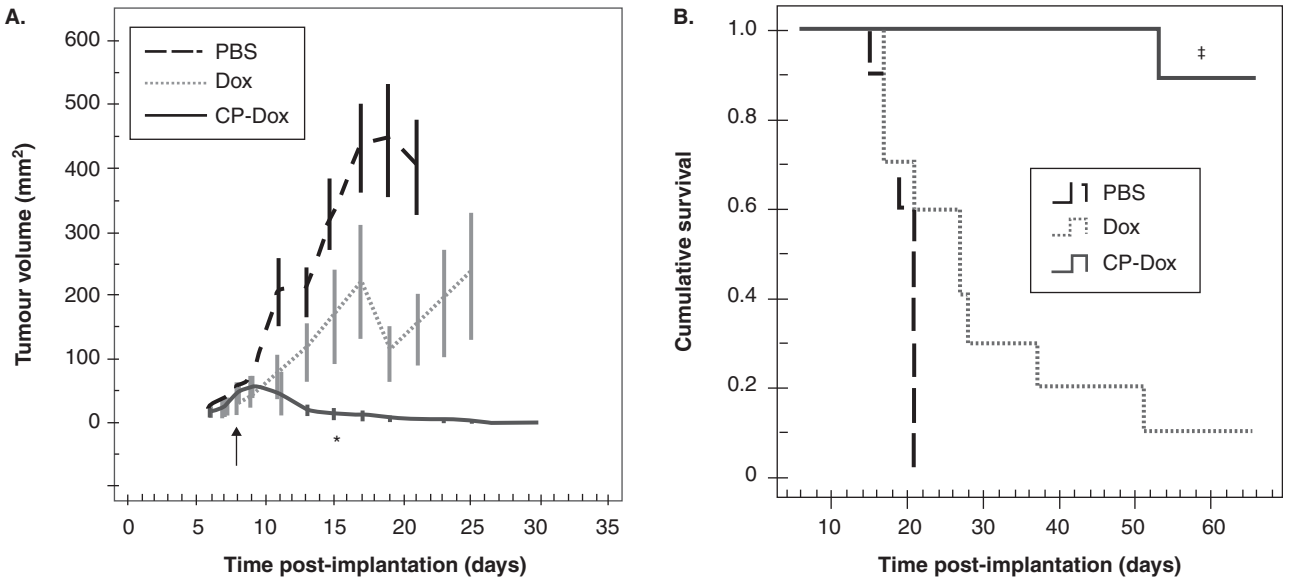


Figure 3. Antitumor activity of self-assembled polypeptide–doxorubicin nanoparticles. A. The decrease in tumor volume with camptothecin–doxorubicin (CP–Dox) at day 15. **B.** The high cumulative survival of mice with CP–Dox vs Dox and PBS.
Reproduced with permission from Nature Publishing Group [55].
*= statistical differences of CP-Dox vs Dox at p=0.03 and vs PBS at p=0.00002.
‡= statistical differences of CP-Dox vs Dox at p=0.0001 and vs PBS at p=0.00004.

(PIC) micelles [56,57]. Originally described by the groups of Kataoka and Kabanov, PIC micelles are formed by electrostatic interaction between oppositely charged polyion copolymers, usually in a stoichiometric charge ratio [58]. Similar to classical polymeric micelles, PIC micelles have a core-shell structure with a core of ionic blocks surrounded by a neutral hydrophilic corona, typically of PEG (**Figure 4**). It is well known that, at charge neutrality ratios, electrostatic interactions between oppositely charged polyelectrolytes result in phase separation and precipitation. By contrast, if a neutral segment such as PEG is linked to one of the interacting polyelectrolytes, soluble colloidal particles (PIC micelles) are formed instead. Indeed, the vast majority of examples of PIC micelles reported in the literature comprise PEG–PAA block copolymers.

Although research on PIC micelles is still in its infancy compared with more classical polymeric micelles, their therapeutic applications are rapidly increasing. Advantage has been taken from the charged nature of various types of biopharmaceuticals. Cationic PAA block copolymers have been used in the preparation of PIC micelles with DNA, siRNA, proteins and even viruses [58]. In the case of nucleic acids for gene delivery applications [59-62], the PEG segments surrounding the core have proven to prevent the complex from precipitation and to render the system with high resistance against DNase I [63,64] (also see Section 2.2.3.). Similarly, block copolymers of anionic PAsp have been used in the preparation of PIC micelles with proteins [65,66]. PIC micelles containing dendritic photosensitizers have been also reported for photodynamic therapy and as light-harvesting sensitizers [67-70]. *In vivo* results indicate that these dendritic micelles may constitute innovative formulations for the treatment of ophthalmologic diseases. More recently, remarkably stable PIC micelles prepared from PEG-dendritic block copolymers and PAA have been described by the group of Fernandez-Megia and Riguera [71]. These micelles are envisioned as attractive delivery systems for low-molecular weight drugs, proteins, nucleic acids and imaging agents.

In addition, similar to conventional micelles, the surface of PIC micelles has been modified with ligands for targeting. Lactose [72] and a cyclic arginine–glycine–aspartic acid peptide [73] have been recently introduced into PIC micelles for active gene delivery.

2.2.3 *Polyelectrolyte complexes*

Polyelectrolyte complex (PEC) dispersions result from strong electrostatic interactions between charged microdomains of at least two oppositely charged polyelectrolytes. These systems can generally be obtained by simple mixing of polyanions and polycations, leading to the spontaneous formation of stable PEC under certain conditions [74,75]. Although electrostatic interaction is the main driving force in the formation of PEC and PIC micelles, they differ in their supramolecular structure (**Figure 4**). As detailed above, PIC micelles are organized in a core-shell structure, which resembles polymeric micelles, and their hydrophilic corona renders them stable even under charge stoichiometric conditions. By contrast, stoichiometric mixtures of oppositely charged polyelectrolytes lead to precipitation in aqueous media (**Figure 4**). Therefore, PEC needs to be formed with an excess of one of the charged species in order to confer water solubility to the complex. Thus, for instance, in the case

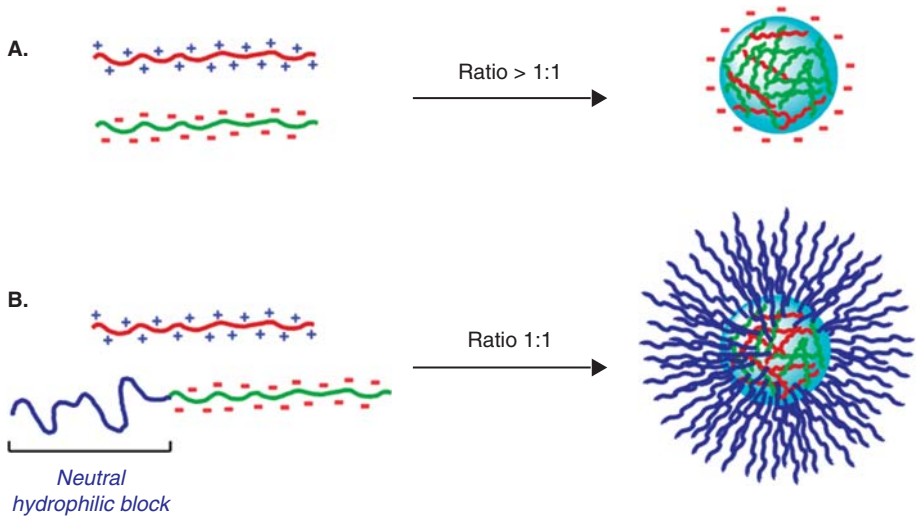


Figure 4. Schematic representation of (a) polyelectrolyte complex (PEC) and (b) polyion complex (PIC) micelle formation.

of PEC composed of polycations and nucleic acids, a large excess of the polycation is generally employed.

Although there have been a few reports on PEC for the delivery of therapeutic molecules or vaccines, this type of delivery systems has been mainly explored for gene delivery due to their capacity of binding and condense nucleic acids reversibly. Some of these materials also combine this capacity with i) cellular and/or tissue specificity (essential for *in vivo* gene delivery), ii) membrane fusogenic or disruptive activities and/or with iii) the promotion of nuclear translocation [76]. Due to these characteristics, a great number of polypeptides and PAA have been investigated for the formation of PEC complexes for gene delivery. In particular, polypeptides and PAA rich in basic residues such as lysine, arginine or histidine have received most attention as these are able to efficiently condense genetic materials into small, stable and compact PEC systems [77].

One of the first and most investigated cationic PAA for complexing nucleic acids has been PLL. PLL may be synthesized with different molecular weights (typically from 1 up to 300 kDa). Its cationic structure is able to protect the associated genetic materials against nuclease digestion and enhance their cellular uptake via nonspecific endocytosis. Nevertheless, its significant molecular weight-dependent cytotoxicity has led to intensive studies in order to achieve effective gene transfer combined with low cytotoxicity and to balance the polymer cationic density with endosomal escape moieties [78].

A relevant example of current polylysine-based gene carriers is those developed by Dr. Hilton Levy for delivery of dsRNA (e.g., polyI:C) for the induction of interferon production in antiviral and anticancer treatments [79]. As shown in **Table 1**, some of these formulations have already entered clinical evaluation for the treatment of malignant brain tumors [80-82].

Other efforts have been directed to enhance the specific cellular uptake of PLL-based complexes through the attachment of diverse ligands to the polymer backbone. Relevant examples of such targeting moieties include asialoorosomucoid proteins, carbohydrates such as fucose, mannose and galactose, epidermal growth factor, folic acid, transferrin, steroids and viral or bacterial proteins.

The use of PEGylated PLLs has also been widely studied with the aim of providing better physical and biological stability to the complexes. It is possible to bind to the PLL–PEG other molecules and form thereby new copolymers with further improved characteristics (e.g., lower cytotoxicity, better endosomolytic capacity or better ability to condense pDNA) and mediate effective gene transfection in various cell lines [83]. Relevant examples of these combinations include materials as polyethylenimine, polylactic acid, lactose or galactose, among others.

Another interesting example of such combined approaches is the modification of PLL/DNA complexes with PEG and the tripeptide (Glu-Lys-Glu), and by incorporating folate moieties [84]. These new complexes showed extended systemic circulation times following intravenous administration to mice with up to 2000-fold more DNA measured in the bloodstream compared with simple PLL/DNA complexes. This study also showed that DNA uptake via the folate receptor is dependent on PEG spacer length, with the transgene expression relatively independent of the level of internalized DNA [84].

More recently, other PAA similar to PLL have also been explored as vehicles for gene delivery. One relevant example is polyornithine (PLO), a polycation that differs from PLL in a methylene (-CH2-) unit in its side chain. This difference affects the interaction with pDNA and the stabilization of the PAA/DNA complex with profound effects on the processing of the associated pDNA molecules. These include cellular uptake, intracellular trafficking and nuclear localization, and as such may well contribute to the disparity in cell transfection efficiency observed between these complexes [85], indicating

that a relatively slight change in the structure of such linear polymers may have a significant effect on their efficacy [86,87].

Arginine-rich compositions represent another important group of biomaterials for nucleic acid complexation [88]. Polyarginine (PArg) itself is a cationic polymer that has shown an ability to translocate through mammalian cell membranes. These cell-penetrating properties of PArg have been attributed to the presence of the guanidine moiety in its side chain, which interacts directly with cell surface domains and subsequently facilitates cellular internalization [89]. This interesting feature has been the rationale of its use in gene therapy. Recently, PArg has also been explored for siRNA delivery in the form of polyionic complexes with hyaluronic acid [90] and as a covalent conjugate with cholesterol. In this latter case, the complexation of siRNA with this new entity resulted in an effective inhibition of VEGF production in colon adenocarcinoma cells (CT-26) *in vitro* and it was able to suppress tumor growth *in vivo* following local administration [91]. PArg also forms stable complexes by electrostatic interaction with CpG ODNs, resulting in complexes with potential interest in immunization strategies as vaccine adjuvants [92].

Other arginine-rich compositions have also been extensively studied for gene delivery due to their potential fusogenic or penetration-enhancing properties. Protamine is a naturally occurring substance synthesized in late-stage spermatids of many animals, with the physiological function of condensing the spermatid genome into an inactive state [93]. Due to this natural affinity for nucleic acids, protamine has been investigated in a great number of different gene therapy-related applications [94-96]. For example, this polypeptide in combination with antisense ODN spontaneously forms compact nanoparticulate complexes called 'proticles,' and this specific association increases the cellular uptake of the ODN (up to eightfold) compared with the free ODN, showing very low cytotoxicity at the same time [97]. Other studies have shown that RNA condensed on protamine is protected from RNase-mediated degradation. In particular, the complexation of messenger RNA with protamine seems to be a promising strategy for genetic vaccination approaches as this complex efficiently activates immune cells and stimulates the secretion of cytokines such as TNF-α and IFN-α [98].

The detailed analysis of binary protamine-nucleic acid nanosystems revealed two major disadvantages: i) aggregation of particles within a few minutes in the presence of salt and ii) low intracellular dissociation between protamine and ODN. To resolve these problems, a ternary system of albumin–protamine–ODN has been developed. This system showed better stability under isotonic conditions and facilitated the intracellular dissociation between ODN and protamine [99].

It is also possible to conjugate protamine with polysaccharides (e.g., dextran) for enhanced transfection properties [100] or to conjugate the ODN first with a polysaccharide and then form the complexes with protamine [101].

Other arginine- and histidine-rich molecules such as TAT, KALA or LAH peptides have also been widely used for their cell penetration enhancer and fusogenic properties. The potential interest of these short sequences for DD and targeting is revised elsewhere [102-104].

As it can be concluded from this section, most PAA and polypeptides used in gene delivery are characterized by a high number of positively charged amino acid monomers (lysine, arginine, histidine, etc.). In fact, a serious drawback associated to their use is that the strong positive charge of the complexes may lead to hemolysis and toxic effects after their intravenous administration. Interestingly, the well-known biocompatibility shown by another, negatively charged polyaminoacid PGA has been the rationale behind its use as an additional coating agent for previously described cationic complexes with nucleic acid [105]. The PGA coating was meant to shield the positive charge of the system and efficiently decreased its hemotoxicity compared with uncoated systems while maintaining high transfection efficacy (**Figure 5**).

2.2.4 *Other self-assembled nanostructures*

This section describes further self-assembled delivery systems incorporating polypeptides, PAA and peptides. PGA and PAsp have been the subject of many strategies for the design of new nanoparticulate entities. Among these, it is worth to highlight the Medusa® technology developed by Flamel Technologies. This company has developed a whole pool of products that are currently in the pipeline for various applications such as diabetes, hepatitis or cancer. The basis of the Medusa technology is PGA, optionally further modified with L-leucine in order to obtain amphiphilic block polymers, or with hydrophobic molecules such as α-tocopherol (vitamin E) to produce randomly grafted polymers [106]. Both generations of polymers are known to self-assemble in aqueous media into nanoparticles, exposing the hydrophilic glutamate chains to the outer media, while the hydrophobic domains constitute the core of the structure. Therapeutic molecules such as proteins and peptides can be associated within the polymer matrix and subsequently delivered in a controlled manner after subcutaneous injection [107].

Another interesting system has been developed by Deming and coworkers, who obtained polymeric vesicles from a self-assembling polymer formed by PArg and polyleucine segments. In this study, PArg performs the synergistic role of being a structural component and promoting the cellular uptake of the system by its cell-penetrating properties [108]. A recent improvement of these PAA-based vesicles is the achievement of covalent cross-linking by the incorporation of oxidatively cross-linkable residues, in an attempt to enhance the stability of the system [109]. Similar vesicles have also been obtained by combining PAA with binding agents for biological targets, which also work as hydrophilic blocks constituting the nanostructure. This is illustrated in the work published by Lecommandoux and coworkers by the formation of DOX-loaded poly(γ-benzyl glutamate)-*block*-hyaluronan polymersomes [110].

Self-assembly is also a very attractive strategy to construct nanoscale materials for application in regenerative medicine

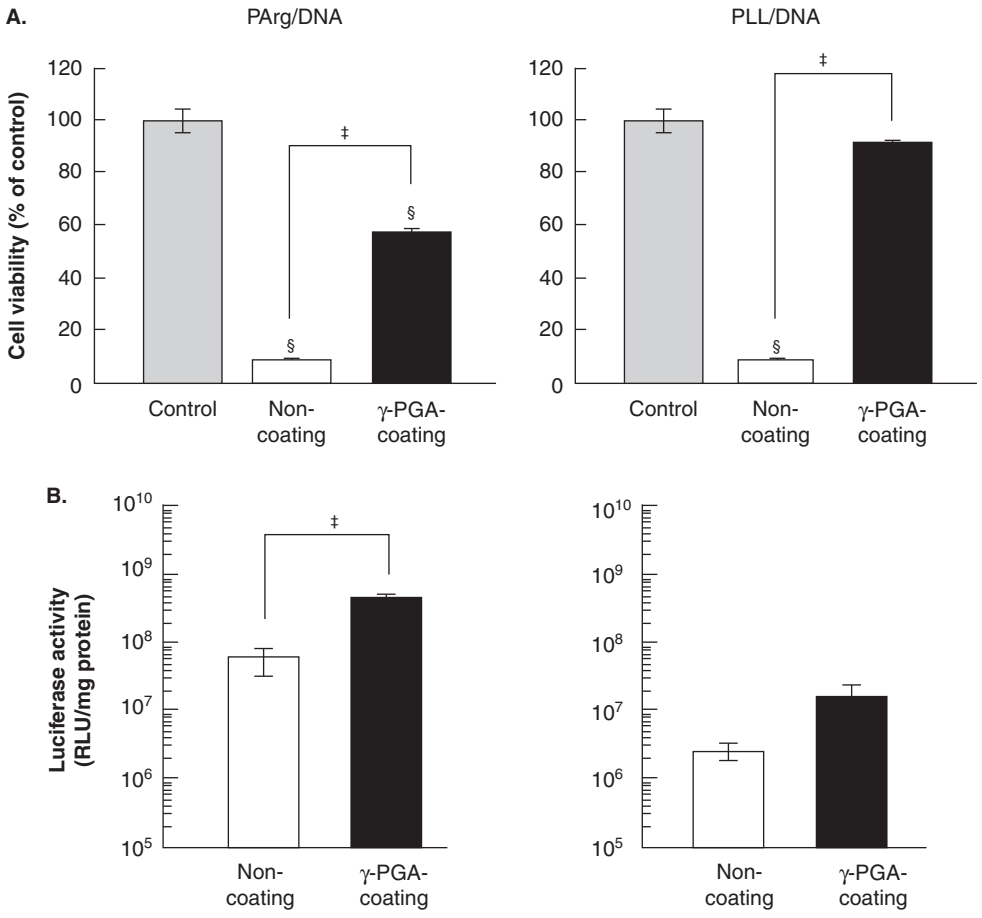


Figure 5. Effect of polyglutamic acid coating on the cytotoxicity (a) and transfection efficiency (b) of polyarginine and polylysine-based polyplexes.

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§=statistical differences with the control at $P < 0.01$.

‡=statistical differences between the formulations at $P < 0.01$.

due to its simplicity in application and its unique capacity to produce a variety of diverse nanostructures [111]. In this specific area, it is necessary to produce biologically compatible scaffolds that might be readily adopted by the body without harm and be suitable platforms for cell growth, including stem cells or primary cells that can effectively replace damaged tissues [112]. In this context, self-assembling peptides have been studied for diverse purposes such as cartilage tissue engineering [113], substrate for neurite outgrowth and synapse formation in nerve regeneration [114], functionalized with bone marrow homing peptides for stimulating neural stem cell adhesion and differentiation [115], for the formation of confluent cell monolayers of human aortic endothelial cells [116], as hydrogel scaffolds for controlled release of functional proteins [117].

Polypeptide-based nanofibers are also of great interest in tissue engineering and wound repair. Among the different materials that have been used for the formation of nanofibers, polypeptides and peptides are particularly promising as they resemble extracellular matrix proteins. More information on these applications can be found in the work of Barnes *et al,* [118] Silva *et al.* [119] or Ellis-Behnke *et al.* [120].

2.3 Particulate supramolecular DDS

DDS are extremely versatile carriers with respect to their composition and structure. This facilitates their formulation process by adjustment to the specific requirements. The following subsections will review further PAA and polypeptide-based structures applied in DD. In contrast to the others, self-assembled nanostructures disclosed in the previous section, the systems below are characterized by a more complex arrangement, in terms of their structure and/or shape, generally as a result of diverse methods applied for material processing (melt or solvent casting, mechanical forces, etc.).

2.3.1 Liposomes

Liposomes are spherical structures formed by one or several concentric lipid bilayers comprising inner aqueous phases.

From the early first attempts to the present times, liposome research has considerably evolved toward more optimized and sophisticated structures. One of the main drawbacks that DDS faced at their beginnings was their recognition by the cells of the reticuloendothelial system and their subsequent elimination as foreign to the organism. This limitation was successfully overcome by the design of PEG shell that covered the liposomes, enhancing their circulation times [121]. PEG has since then widely been used as a golden standard for many years for giving stealth properties to nanostructures. Nevertheless, there are some concerns about the use of PEG, arising from possible side effects such as hypersensitivity reactions or altered pharmacokinetics [122]. Consequently, different polymers such as PAA have been investigated as alternatives for PEG. Surface modification of liposomes with poly(hydroxyethyl L-glutamine) and poly(hydroxyethyl L-asparagine) has effectively prolonged liposome circulation times achieving similar levels to the ones obtained with PEG [123]. This prolonged circulation behavior can be adjusted by modifying the physicochemical characteristics of the liposomes, an attractive feature to achieve the optimization of the system [124]. Despite this promising research, there are some aspects like the activation of the complement system after intravenous administration or the application of other PAA for liposome coating that will need further study [125]. By coating liposomes with PAA, it is also possible to decrease the toxicity of the formulation. Despite their efficient transfection, cationic liposomes may induce cytotoxicity and hemagglutination due to their strong positive charge. A recent work of Sasaki and coworkers describes an approach to decrease the toxicity of cationic liposomes by coating with PGA. These authors have shown a significant reduction in the toxicity profile and a high transfection efficacy in coated liposomes compared with non-coated ones [105]. Another interesting approach has been reported by Li and Huang, entrapping protamine/DNA and protamine/siRNA complexes in the interior of liposomes. These liposomes were further modified with anisamide, a molecule with affinity for sigma receptors, which are overexpressed in a variety of human tumors [126]. The biological evaluation of the system showed significant accumulation of siRNA in tumors (up to 70 – 80%), obtaining large differences with the amount that could be found in liver and lung (10 and 20% respectively). The efficiency of the system was evaluated by monitoring the tumor growth; the treatment with the liposomes achieved a 40% tumor growth inhibition, which was even enlarged by the coadministration of the anticancer drug cisplatin. The results exposed provide the first evidence of such high tumor delivery efficiency reported so far in the literature [127].

Protamine has also been used as adjuvant in liposome-based vaccine formulations. The hybrid composition of liposome–protamine–DNA particles developed by Sloat and Cui carrying a protective anthrax antigen achieves strong mucosal and systemic immune responses in mice following nasal immunization. These responses are comparable with subcutaneously injected protective antigen adjuvanted with aluminum hydroxide (a classical adjuvant used in commercial formulations) [128].

2.3.2 *Particulate polymeric micro- and nanostructures*

These delivery systems include micro/nanospheres and micro/nanocapsules with a matrix or vesicular structure, respectively, and a size typically in the range from 0.1 to 250 μm. This difference in size between nanoparticles and microparticles entails variations in parameters such as drug encapsulation efficiency, release rates, stability issues, administration route and cellular recognition and processing [129]. These parameters can also been controlled by the nature and structure of the matrix as it will be illustrated in the following examples. Due to their size, in general, microspheres can be taken up by only phagocytic cells, which entail them as excellent vehicles for passive targeting in vaccine delivery to antigen-presenting cells. Surface modification of biodegradable poly(D,L-lactic-co-glycolic acid) and non-biodegradable polystyrene microspheres with PAA and polypeptides has been extensively studied for this purpose [130]. A recent work of Merkle and coworkers highlights the potential of PLL–PEG-modified microspheres for the delivery poly(I:C) as a potent immunoadjuvant. These results show that poly(I:C) exhibits a strongly enhanced immune cell maturation and activation when assembled on the surface of these microparticles [131].

Surface modification of microparticles with protamine has also been reported, showing that the presence of this polypeptide (also see Section 2.2.3.) promotes the stimulation of stronger and more specific immune responses and enhances cytokine secretion in a dose-dependent manner. The success of this system may rely on combining the prolonged antigen release from the microparticles with the internalization properties of protamine [132].

Protamine microspheres have also been successfully tested for tissue engineering applications by Nakamura and coworkers; the results show that fragmin/protamine microspheres promote vascularization and fibrous tissue formation after their *in vivo* administration. The role of protamine in the microspheres is to diminish the anticoagulant effect of fragmin, a heparinoid used to stabilize and promote the activity of encapsulated growth factors [133]. Protamine and PArg have also been used in combination with polysaccharides (e.g., dextran sulfate or sodium alginate) for the formulation of multilayered microcapsules. These microcapsules are constituted by successive adsorption of the two oppositely charged polymers onto the surface of a preformed particle core that can subsequently be dissolved under mild conditions [134,135]. The benefit of such multilayered microcapsules is the controlled release of the entrapped molecules as a function of the coating nature and thickness. This aspect has attracted much interest from the pharmaceutical industry, as it is illustrated by the ongoing research of Flamel Technologies on microparticle formulations for the oral delivery of

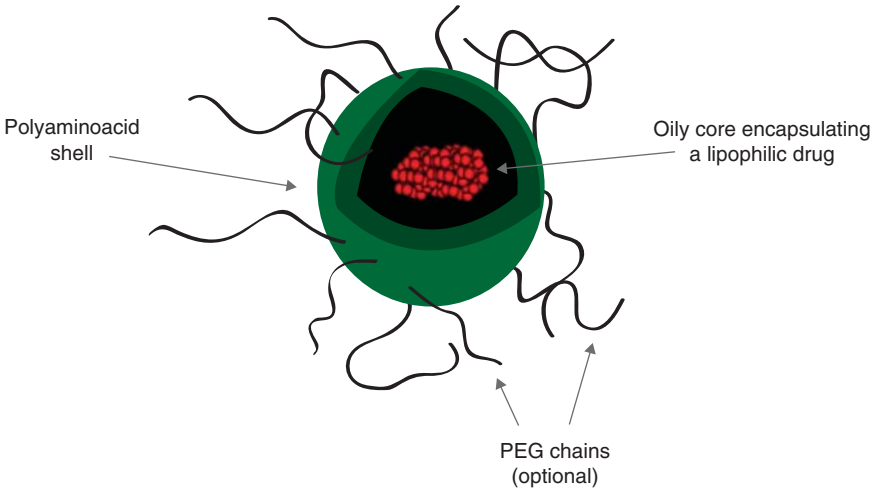

Figure 6. Schematic representation of polyaminoacid nanocapsules.

peptide drugs based on the Medusa technology, which has been commented in the previous section.

On the other hand, nanometric size is a powerful feature with important consequences in the properties of DDS and their activity. Indeed one of their main characteristics is a high surface/volume ratio, which makes them highly reactive, clearly affecting the performance of the system. In addition to the great number of nanostructures described in the previous sections, recent work performed in our research group indicates that PAA are promising biomaterials for the formulation of nanocapsules. The additional values that nanocapsules hold are i) great versatility due to the wide range of polymers that can be used as shell, from polysaccharides to polyesters [136,137], allowing the adaptation of prototypes as a function of the biological barriers to overcome [138]; ii) enhanced physicochemical stability due to the presence of the polymer corona [139]; and iii) co-encapsulation of different types of drugs in the inner core and the surrounding polymeric shell (**Figure 6**) [140]. During the last years, our group has intensively worked in the development of new formulations of nanocapsules, relying on the potential of PAA and polypeptides as polymeric coating materials. PGA and polyasparagine (PAsn) were selected as examples of negatively charged or neutral coating materials due to their biocompatibility and long-circulating properties. Moreover, PAsn nanocapsules may also benefit from the high PAsn demand of solid tumors that could be potentially used as an active targeting strategy. Based on this rationale, we have extensively investigated PAA-based nanocapsules as novel DDS for cancer treatment [141]. The versatility of this nanocapsule technology has been also proved by the formulation of positively charged, PArg nanocapsules. This nanosystem gathers the cell-penetrating properties of PArg [142] with the ability for co-encapsulation of drugs with different properties, such as docetaxel (in the inner core) and plasmid DNA (assembled onto the cationic surface), which seems to be a promising combination therapy in cancer [140,143]. *In vivo* experiments are currently ongoing with the aim of further exploring the potential of these PAA nanocapsules for cancer therapy.

3. Conclusions

The results exposed throughout this work provide a brief overview of the most important achievements of PAA and polypeptide research in DD. Synthesis methods have been improved from the early solid-phase technique toward new synthesis pathways that yield optimized products with improved purity. Several novel formulations based on these biomaterials are currently in clinical trials, followed by many others with interesting results. Altogether, the information collected herein illustrates the potential of these polymers and predicts exciting advances for clinical applications in the future.

4. Expert opinion

During the last two decades, PAA and polypeptides have emerged as interesting building blocks for the design of functional materials thanks to their well-defined secondary structure, biocompatibility and lack of toxicity. They have demonstrated great capacity for self-assembling into well-defined supramolecular structures and show promising applications in DD. These interesting applications have been fueled by recent advances in the synthesis of PAA by ROP of NCAs. The use of initiators based on transition metal complexes and amine hydrochloride salts, or high-vacuum/low-temperature polymerizations, represents breakthroughs in the synthesis of PAA and their block copolymers with precise molecular weight and low polydispersity.

PAA and polypeptides have demonstrated a great versatility as polymeric materials for DD, with applications ranging from polymer therapeutics (covalently bound drugs) to

various types of self-assembled nanostructures (micelles, vesicles, capsules, fibers) with the ability to encapsulate a plethora of different drugs. The possibility of locating positive and negative charges on the amino acid side chains results in PAA and polypeptides with polyelectrolyte character that have found application in the delivery of oppositely charged biopharmaceuticals such as nucleic acids and proteins. Also advantage has been taken from the low pKa of some PAA (PGA, PAsp, polyhistidine) for the preparation of block copolymers with PEG that have found application in the preparation of pH-sensitive DDS.

In particular, the application of polypeptides and PAA to cancer therapy has been attracting great interest and is yielding highly original and promising results. The appealing properties of these polymers, such as biodegradability or their versatility for chemical modifications, turn them into suitable candidates for the design of novel delivery systems for cancer treatment and diagnosis. Interestingly, the nanometric size of these constructs benefits from a tendency to accumulate in solid tumors thanks to the EPR effect. The potential of these polymers in cancer research is reflected by the increasing number of formulations in preclinical and clinical studies.

Among the different systems mentioned above, polymer therapeutics and micelles have shown to be so far the most relevant concerning their clinical development for cancer therapy. Accordingly, as shown in **Table 1**, more than 50% of the formulations in clinical trials belong to these types of structures carrying antitumor agents. In general, the data collected from the clinical evidence show improved pharmacokinetic profiles compared with well-established reference formulations in use. Most of the formulations are well tolerated after their administration to the patients, although some adverse reactions such as hypersensitivity could be observed in some cases. This and other issues related to toxicity and others regulatory considerations have been carefully revised elsewhere.

From the perspective of new nanotherapeutics, novel DDS are proposed by merging the appealing properties of polypeptides and PAA with already known possibilities offered by classical DDS. An interesting example of these new strategies is the recent development of PAA-based nanocapsules. These nanosystems provide a flexible platform with adjustable composition, size, surface charge, according to specific needs. The promising results obtained during the past few years indicate that possible applications of these novel nanosystems include i) encapsulation of hydrophobic drugs in the inner oily core of the system (e.g., anticancer drugs, lipophilic immunoadjuvants), ii) delivery of hydrophilic macromolecules embedded in the surrounding PAA shell (e.g., nucleic acids, antigens) and (iii) the co-delivery of different therapeutic molecules within the same system.

Nevertheless, and despite all the exciting findings obtained so far, it has to be taken into account that these strategies are in their early development stage and, therefore, their potential will have to be fully assessed in subsequent studies. In addition, the vast majority of the delivery systems described in this review have been conceived for parenteral administration. Taking into account the accumulated knowledge, we believe that PAA and polypeptides could also be interesting biomaterials for alternative routes such as the oral or nasal administration of drugs and vaccines. Within this regard, it is clearly necessary to gather more information on the *in vivo* behavior of these biomaterials. In particular, their interaction with the biological environment as well as their biodistribution and immunotoxicology profile should be properly evaluated for different administration routes in order to fully explore the potential of polypeptide and PAA-based systems for DD applications.

Declaration of interest

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