Chemical Modification of Microbial Poly(γ-glutamic acid): Progress and Perspectives

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ABSTRACT: Poly(γ-glutamic acid) (PGGA) is an edible polypeptide excreted by certain bacteria that is presently accessible on a semi-industrial scale. Chemically, it is a nylon 4 derivative bearing a carboxylic side group attached to the fourth carbon of the repeating unit. Although this biopolymer is being exploited in the food, agriculture and cosmetic sectors, its use in massive applications as packaging or coating is still unknown mainly due to its instability in wet environments and unsuitability to be processed by common techniques. Chemical modification of PGGA involving esterification or amidation of the carboxylic group is the approach explored the most to obtain materials with potential interest as fibers, films or hydrogels. Electrostatic coupling of PGGA with hydrophobic cationic species may also be profited to generate non-water-soluble stable derivatives. On the other hand, the interest of modified PGGA as a functionalized biomaterial for scaffold and drug delivery has increased incredibly in the last years. This paper summarizes the progress in the methodology available for synthesizing PGGA derivatives, the relevant features of the new materials that are synthesized, and the most outstanding advances achieved in the biomedical field when modified PGGA is used.

KEYWORDS: Microbial poly(glutamic acid), poly(gamma glutamic acid), polyglutamates

1 INTRODUCTION

Poly(γ-glutamic acid), abbreviated as PGGA or γ-PGA, is a bacterial capsular biopolymer produced by certain species of the genus *Bacillus*. Its isomer poly(α-glutamic acid), referred to in the literature as α-PGA, is obtained by chemical synthesis and is one of the most extensively studied polypeptides due to its outstanding biochemical relevance and suitability as model compound for protein structural studies.[1] The chemical formulae of these two polypeptides are depicted in Figure 1; α-PGA is an α-polypeptide and therefore a nylon-2 derivative, whereas PGGA is a γ-polypeptide related to nylon 4. Although much less familiar in the settled literature than α-PGA, PGGA is at present receiving great attention due to its enormous possibilities as biomaterial. What distinguishes PGGA is that it is a polyamide not only naturally-occurring but also biodegradable, biocompatible and even edible. Since it contains an asymmetric carbon and bears a free carboxylic function, PGGA affords a countless number of possibilities without parallel in the field of conventional polyamides. Several reviews covering different aspects of this biopolymer have been published in the last years.[2–6]

The natural occurrence of PGGA was accounted for Germany (Berlin/Heidelberg) in 1935 for the first time as a component of the capsule of *B. anthracis* [7], and five years later it was reported to be the main extracellular product excreted in the aerobic fermentation of organic substrates with *B. subtilis* [8]. In 1963 Fuji identified PGGA in *natto*, a mucilage naturally produced by partial fermentation of soybeans with the bacterium *nattō-ki* [9]. *Natto* contains between 0.1 and 1% of PGGA and has been known in Japan since antiquity. Its equivalent in China is known as *dan-douchi*. These fermentations are popularly recognized for their exceptional health benefits and have formed part of

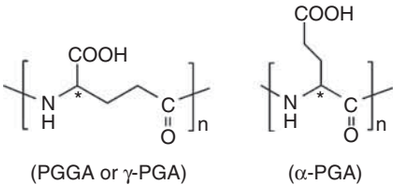

Figure 1 Chemical structure of polyglutamic acids.

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the daily diet of Asiatic people for hundreds of years (Figure 2). The PGGA produced by fermentation may reach several millions of daltons and its D:L enantiomeric composition largely varies from one species to another and is also dependent on both fermentation conditions and composition of the substrate.

PGGA is a polycarboxylic acid with pK_a = 2.27 that ionizes at increasing pH, so it becomes 50% ionized at pH 2.2 and fully ionized at pH 5.1. The solubility of PGGA is strongly dependent on its ionization degree. Only a few organic solvents are known to solubilize PGGA (DMSO, DMF), and its solubility in water is moderate and tends to diminish with age. Conversely, the sodium salt of PGGA is practically insoluble in organic solvents and very hygroscopic, being able to form gels containing more than 3000 times its weight in water. The conformation adopted by PGGA in solution becomes determined by concentration, pH, and ionic strength. In dilute solution (below 0.1% w/v) and pH above 7.0, the chain is arranged in an almost extended conformation. At an acidic pH with the polymer in the un-ionized state, the chain seems to adopt regular helical conformations [10–13].

Figure 2 Soybean fermented with *Bacillus natto.*

The presence of the carboxylic side group in PGGA makes it much more easily degradable than conventional unsubstituted polyamides (nylons). Thus, PGGA decomposes upon heating around 200°C through an unzipping depolymerization mechanism with release of pyroglutamic acid [14]. It is also much more sensitive to water than nylons due to the enhancing hydrolytic effect exerted by the carboxylic group on the main chain amide bond. As a result PGGA suffers from a rather environmental instability, particularly in high humid ambiences. Upon incubation in water at 37°C, a PGGA with a molecular weight of 1,500,000 drops down to half its original size in four days. The hydrolysis rate increases notably with temperature and is greatly accelerated under both acid and basic conditions; the hydrolysis in hot basic medium is quite fast and has been used to prepare PGGA fragments of low molecular weight [15]. At difference with its α-isomer, PGGA is not digested by the common proteases as pepsins, tripsins or quimotripsins. Conversely, it is rapidly degraded by the γ-glutamyl depolymerases present in their fermentations but that are known to be inactive to poli(α-aminoacid)s and proteins. Oppermann *et al.* carried out a systematic research on PGGA biodegradation and found twelve different species of bacteria able to degrade it [16]. PGGA is also degradable by ultrasounds. Pérez-Camero *et al* [17]. reported that by irradiation with ultrasounds in dilute solution, it is feasible to drastically reduce the molecular weight of the polymer in parallel with its dispersity and without essential changes in its chemical constitution. This method appears to be particularly convenient for the controlled preparation of low molecular weight fractions of PGGA from samples produced by biosynthesis.

2 CHEMICAL MODIFICATION OF PGGA

The high affinity of PGGA for water together with its well-proven nontoxicity towards humans and the environment have prompted the development of a diversity of industrial applications such as food additives, eco-friendly metal sequestrants, and cryoprotectant or flocculant agents, among others. Also, its use in biomedicine as a high water-retaining biogel is receiving increased attention. However, the poor stability of PGGA in humid environments as well as its incapacity to be processed by melting have hampered the spread of its utilization to massive consumer fields such as coatings and packaging. Although the possibility of preparing fibers and films of PGGA by wet spinning or molding has been reported [18, 19], chemical modification appears to be the most promising way to render PGGA materials with suitable properties for handling and processing.

All intended chemical modifications of PGGA avoiding alteration of the main chain will involve reaction on the carboxyl side group, or in the very special case in which block copolymers are pursued, on the amino or carboxylic end groups. The fact that the carboxylic side group is directly anchored to the

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polymer backbone and closely located to the amide main chain group makes its reaction difficult to perform because the attack by nucleophilic reagents is severely hindered. Furthermore, the reaction conditions cannot be strengthened without jeopardizing the integrity of the polymer chain, and the choice of the reaction medium is severely restricted by the scarce solubility of PGGA. Although the modification of the carboxyl group of PGGA is much trickier in principle, it can be presumed that a rich assortment of PGGA derivatives is available today. With the exception of certain ionic coupling reactions that have been recently reported, most chemical modifications carried out on the carboxylic side group involve the covalent attachment of organic compounds. In order to rationalize this topic and to allow a rather systematic study of the available methodology, the modification procedures have been grouped on the basis of the type of reaction implied.

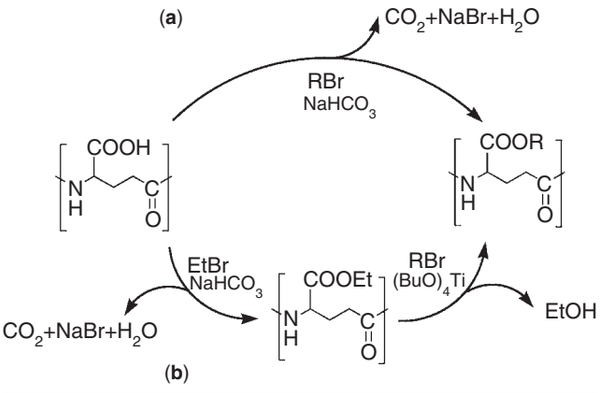

Figure 3 Esterification routes to poly(α-alkyl γ-glutamate)s a) Direct alkylation by reaction of PGGA with alkyl bromides. b) Two-step procedure including ethylation an transesterification with alkanols.

2.1 Esterification

The PGGA alkyl esters poly(α-alkyl-γ-glutamate)s were considered derivatives of interest as soon as the chemical modification of PGGA started to be used as a means to alter their properties [20]. First PGGA esterifications were based on the reaction with alkyl bromides in the presence of sodium hydrogen carbonate using an organic solvent as DMSO or NMP [21–25]. This methodology has been extensively applied by different authors who introduced more or less important modifications in the reaction conditions in order to improve yields and conversions. However, kinetics studies revealed that alkylation did not increase steadily with reaction time due to the unavoidable reverse hydrolysis reaction that occurs induced by the water released in the alkylation reaction [26]. Since the hydrolysis of the alkyloxycarbonyl group is favored at higher conversions and also by the application of severe conditions, attaining 100% of PGGA esterification by this procedure is certainly difficult, especially where higher alkyl groups are concerned. Nevertheless, a good number of PGGA esterifications including a variety of alkyl groups carried out by this method have been reported and some of them covered by patent. On the other hand, methylation with diazomethane is also a valid akylation method that allows reaching full conversion under soft reaction conditions, although its applicability is restricted to the preparation of the methyl ester [23].

In recent years, a new procedure for the esterification of PGGA consisting of two steps, ethylation with ethyl bromide followed by transesterification with alcohols, has been developed [27,28]. In this procedure, the relatively easily accessible poly(α-ethyl γ-glutamate) is made to react with alcohols in the presence of titanium tetrabutoxide to afford fully converted polyglutamates with a wide variety of sizes and constitutions. Pérez-Camero *et al*. applied a slight variant of this method to prepare water-soluble polyglutamates by transesterification of poly(α-methyl γ-glutamate) with mono-, di- and triethyleneglycol methyl ethers [29]. A scheme of the procedures that have been used to date for esterification of PGGA is depicted in Figure 3.

Structure and properties of poly(α-alkyl-γ-glutamate)s. All the alkyl esters of PGGA are without exception non-water-soluble compounds that are soluble in organic solvents, which display thermal stability higher than the polyacid, and are able to melt before decomposing. Since the structure in the solid state and the thermal and mechanical properties of the alkyl polyglutamates are highly dependent on the length of the alkyl side chain, they are conveniently classified into groups according to such criterion. The "short" alkyl polyglutamates group embraces esters from methyl to butyl that are distinguished by being semicrystalline and which display significant hydrophylicity. They tend to crystallize with the polypeptide chain arranged in α-helix-type conformation with the short alkyl side chain integrated in the crystal lattice. A detailed structural study made on poly(α-benzyl γ-glutamate) revealed the occurrence of three polymorphs for this derivative, all three with the polypeptide chain in helical

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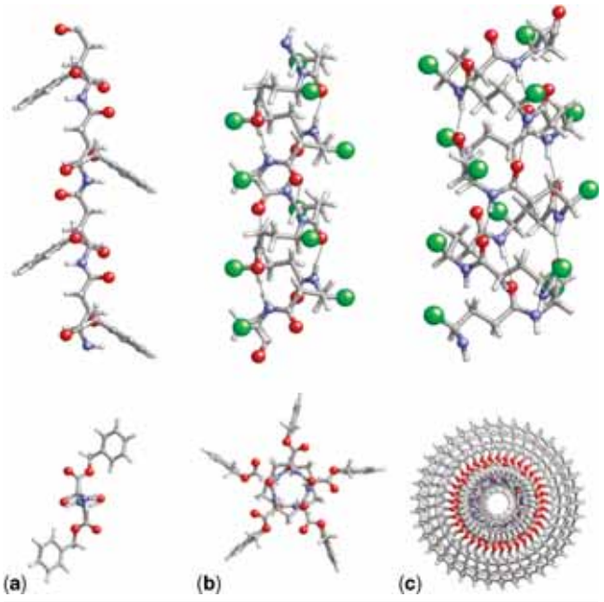

Figure 4 Schematic representation of PAB(D)G helices: (a) 2/1 helix present in form I; (b) right-handed 5/2 helix present in form II; (c) left-handed 37/10 helix present in form III. In the axial projections of parts b and c, the benzyloxy side groups have been replaced by green spheres for clarity. (From ref [30] with permission).

arrangement (Figure 4) [30]. Preliminary studies carried out on short alkyl esters of PGGA suggest that similar arrangements may be adopted in these cases [31].

The "short" alkyl PGGA esters have T_g between 80 and 140°C and T_m within the 250–280°C range, and they display a mechanical behavior typical of semicrystalline polymeric material. High crystalline fibers may be obtained from these polyglutamates by either stretching from the melt or by pulling out from a high concentrated solution in a volatile solvent (Figure 5).

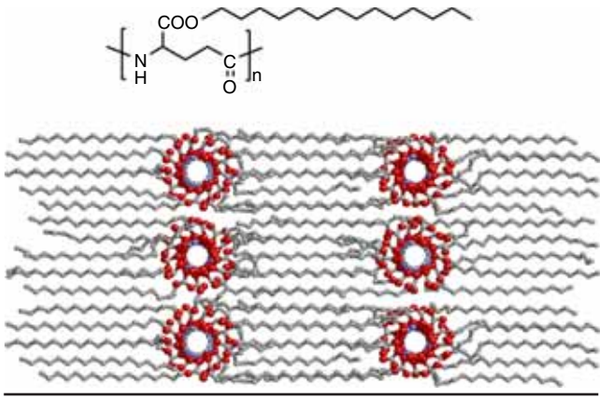

Figure 6 Chemical structure of "long" poly(alkylglutamate)s (top) and projection along the polypeptide axis of the type of layered structure that they adopt in the solid state (bottom).

Also, consistent colorless translucent films may be prepared by casting.

On the other hand, "long" alkyl polyglutamates are those bearing linear alkyl side chains equal or higher than dodecyl. These polyglutamates display a pattern of behavior characteristic of comb-like polymers made of a stiff main chain and flexible side chains able to crystallize under cooling. Comb-like polyglutamates are amphyphilic systems that tend to self-assemble with the polypeptidic main chains and the alkyl side chains alternating in a biphasic layered structure [32]. As schematized in Figure 6, in this structure, the polypeptide is in helical conformation and arranged side-by-side in sheets. The polymethylene chains protrude more or less perpendicularly to the sheet in an almost extended conformation to crystallize in a separate paraffinic phase. No T_g has been observed for these "long" alkyl polyglutamates,

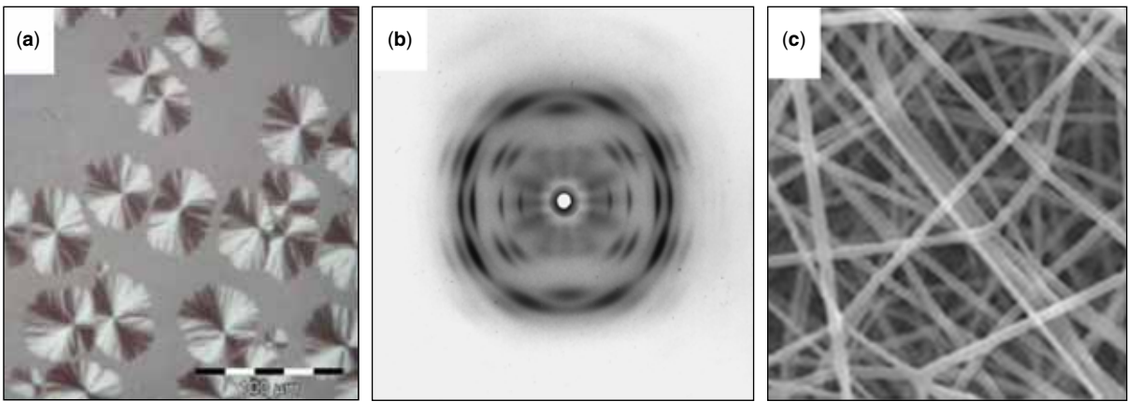

Figure 5 a) Spherulites of poly(α-ethyl γ,L-glutamate) crystallized from the melt as observed under POM. b) WAXS pattern of poly(α-ethyl γ,L-glutamate) recorded from a fiber prepared by stretching from the melt. c) Fiber of PGGA butyl ester produced by electrospinning (Adapted from reference [51] with permission).

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but they display well-defined melting peaks in the 30–80°C temperature range associated with the fusion of the paraffinic phase. Heating above melting results in a change in the mechanical behavior of the material from hard and brittle to rubbery, but without flowing; this change is quite apparent and reversible upon cooling.

Alkyl polyglutamates with alkyl side chain lengths from hexyl to decyl have an ambiguous behavior with structure and properties intermediate between "long" and "short" types; for example, poly(α-hexyl γ-glutamate) has a melting temperature of 255°C but its glass transition temperature appears at nearly 20°C.

The given account shows that PGGA esterification affords an assortment of materials with thermal and mechanical properties of interest for their potential as bioplastics suitable for applications as coating and packaging. These properties are tunable by selecting the size of the alkyl group and could be improved by optimum processing or proper additivation. Comb-like poly(alkylglutamates) are particularly appealing due to their peculiar biphasic structure dimensionally sensitive to heating in a thermal range not far from room temperature. The structural changes taking place in such a structure upon heating-cooling imply a disordering-ordering transition in the paraffinic phase, probably with beneficial consequences on the permeability of the material to gases or other non-polar small molecules. Further research to address optimization of the esterification procedures, utilization of alcohols from renewable resources, or even from food or agricultural wastes, and evaluation of specific properties of the modified materials according to their intended uses, such as barrier or rheological properties, should be carried out for the industrial development of these PGGA derivatives.

Copolyglutamates PGGA alkyl esters bearing two different alkyl side chains will display properties in between their parent homopolyglutamates which may be exigible for some specific applications. The preparation of "long-long" copolyglutamates with predetermined compositions is feasible by transesterification of poly(α-ethyl γ-glutamate) with the corresponding mixture of alkanols [33]. On the other hand, "short-long" copolyglutamates with ethyl glutamate being one of the comonomeric units are readily obtained by partial transesterification of poly(α-ethyl γ-glutamate) with the alkanol of choice. Transesterification is actually a very flexible method for preparing copolyglutamates of any composition provided that the replaced alcohol is easily removable. Successive alkylations with selected alkyl bromides may also be a valid option of course, but in this case the probability of breaking the main chain is greater. However, this has been the method of choice for preparing copolyglutamates bearing allyl or propargyl groups diluted with inert alkyl chains, which are needed to carry out grafting by click chemistry [34]. In general the microstructure of copolyglutamates obtained by these methods is not as fully random in as expected in principle, particularly where short and long alkyl groups are concerned. This is because the compositional microheterogeneities generated in the forming copolymer give rise to microphases in the reaction mixture that favor the growth of homogeneous sequences. Nevertheless, the esterification strategy as well as operational conditions can be handled to achieve a more or less randomized chain.

Among copolyglutamates, those containing glutamic units, i.e., partially esterified PGGA, are of singular interest because they combine hydrophobic alkyl chains with hydrophilic carboxylic groups. Moreover, since they retain part of the reactive carboxylic group in the free state, they continue being functional and therefore susceptible to further chemical modification. Such amphiphilic functional polyglutamates are particularly suitable for the synthesis of conjugates useful for the manufacture of structured micro- and nanoparticles of interest as active DDS. These partially alkylated PGGAs may be prepared either by alkylation with alkyl bromides or by direct esterification with alkanols since full conversion is not required in this case. A scheme illustrating the diverse routes practicable to prepare copolyglutamates is depicted in Figure 7.

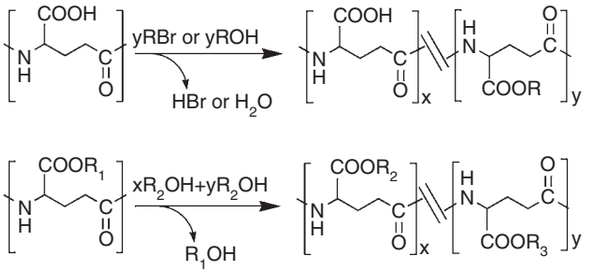

Figure 7 Strategies leading to copolyglutamates. a) Partially alkylated polyglutamates. b) Fully alkylated copolyglutamates (partial replacement of R1 will lead to terpolymers containing R_1, R_2 and R_3).

Developing simple polymer formulations able to combine optimized drug loading and delivery properties with long shelf life and low toxicity is one of the challenges facing the field of controlled release. In this regard, amphiphilic copolyglutamates appear to be appropriate materials. Partially esterified PGGA containing 75% of ethyl and 50% of hexyl, dodecyl or octadecyl glutamate units have been prepared and have been successful in the preparation of spherical nanoparticles with a diameter of 200–300 nm and a

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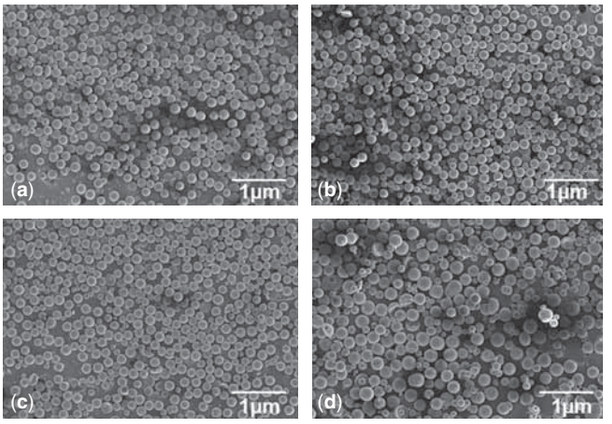

Figure 8 SEM images of nanoparticles made of a) *co*PAAG-($Et_{75}H_{25}$), b) *co*PAAG-($Hex_{50}H_{50}$), c) *co*PAAG-($Dod_{50}H_{50}$), and d) *co*PAAG-($Octd_{50}H_{50}$). (From reference [35] with permission).

narrow distribution (Figure 8). The surface of these nanoparticles is slightly negatively charged, and they are degraded hydrolytically upon incubation in simulated physiological medium at a rate dependent on both the alkylation degree and the length of the alkyl group. They were all proven to be able to efficiently encapsulate erythromycin and α-chymotrypsin with satisfactory efficiency [35].

Upon incubation, encapsulated proteins in these nanoparticles were essentially released following the weight-loss *vs* time profile of the copolyglutamate, which is indicative of the dependence of the delivering process on the degradation of the matrix (Figure 9a). Since the degradation rate can be precisely tuned by adjusting both the esterification degree and the constitution of the alkyl group, copolyglutamate nanoparticles offer outstanding possibilities in the development of delivery systems for drug controlled release. Furthermore, it is worthy to note that the use of polymer particles for protein delivery carrier systems promotes frequently severe perturbations in the secondary structure with a consequent loss of protein functionality. On the contrary, loading of α-chymotrypsin in copolyglutamate nanoparticles was found to help the preservation of the enzymatic activity over time (Figure 9b), revealing the suitability of these systems for encapsulating organic active compounds sensitive to conformational changes.

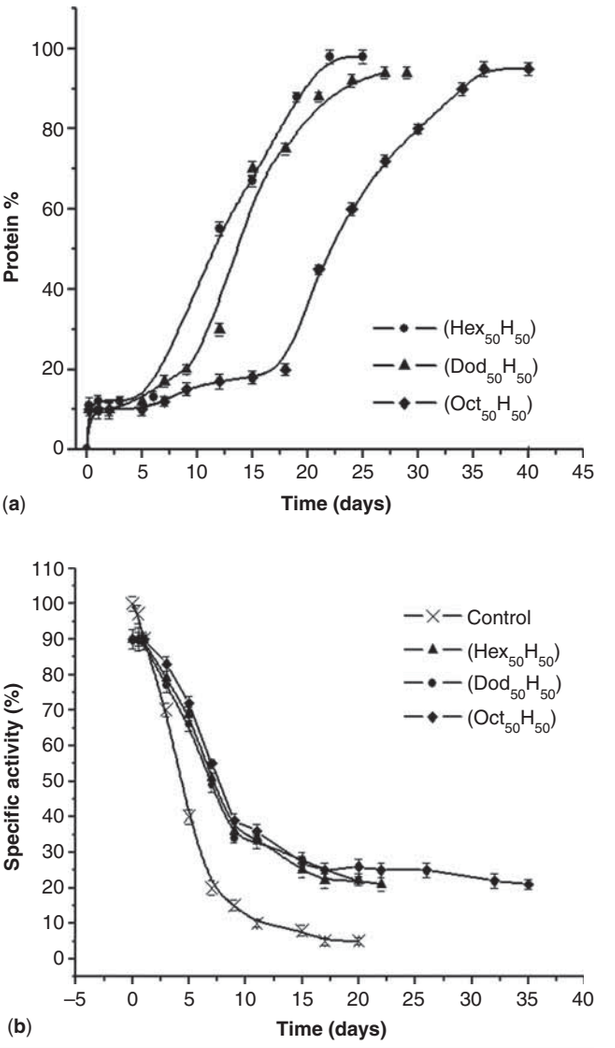

Figure 9 Delivery of quimiotripsin from copolyglutamates. a) Protein release profiles at pH 7.4 and 37°C b) Decrease of the specific activity of α-chymotrypsin as a function of releasing time.(From reference [35] with permission).

2.2 Amidation

The carboxylic group of PGGA is susceptible to being amidated with a variety of amino compounds provided that the reaction is assisted by a convenient activating agent such as a carbodiimide. Although different amino compounds have been covalently attached to PGGA, conjugation with naturally-occurring amino acids is undoubtedly the most preferred grafting approach. Nontoxic hydrophobic esters of amino acids, in particular the ethyl ester of L-phenylalanine (L-PAE), have been extensively explored for producing amphiphilic PGGA derivatives able to self-organize in internally structured nanoparticles. The degree of amidation of PGGA-L-PAE determines the type of molecular association that operates in the building of the particle and is therefore critical in establishing its size [36]. Small unimer nanoparticles stabilized by intrapolymer

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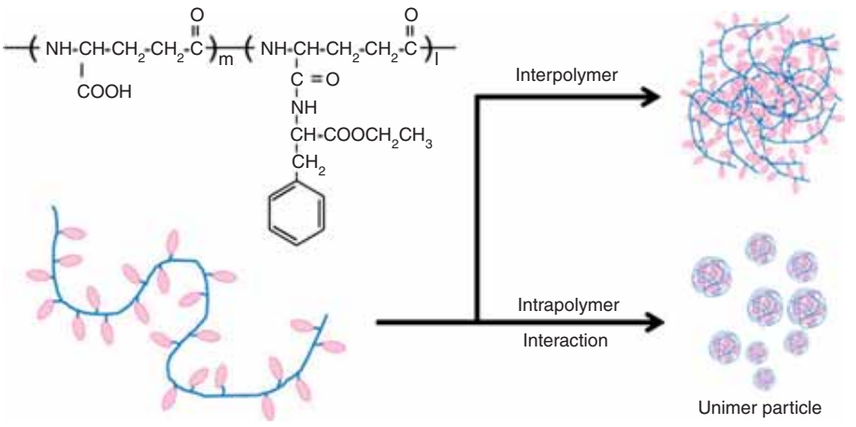

Figure 10 Chemical structure of PGGA-LPAE and the formation of microaggregated polymer particles and unimer nanoparticles by intermolecular and intramolecular interactions, respectively. (From reference [37] with permission).

hydrophobic interactions are formed for a wide range of intermediate amidation degrees; for low values the PGGA-L-PAE is water soluble whereas for high values it tends to form microaggregates due to the predominance of interpolymer interactions (Figure 10) [37]. Moreover, the sensitivity of the PGGA chain to the ionic strength makes it possible for the nanoparticle size to be additionally controlled by changing the salt concentration. PGGA-L-PAE is fairly resistant to hydrolysis under physiological conditions but very sensitive to biodegradation, not only by glutamyl-γ-peptidase but also by lipases [38]. The fully natural origin of the two components along with the flexibility of conditions that may be used for building the particles, make this modified PGGA an excellent platform for the fabrication of biodegradable functionalized nanosystems with adjustable particle size.

Functional proteins may be encapsulated in PGGA-L-PAE nanoparticles with an entrapping efficiency oscillating between 25 and 50%. These nanoparticles are able to enter into macrophages at enhanced rates and deliver the encapsulated proteins via cytostolic translocation from the endosomes, which is a key process for the actuation of nanoparticle-based vaccines. The membrane disrupting activity of PGGA-L-PAE is attributed to the occurrence of conformational transitions taking place in the modified PGGA chain when pH falls below 7.0 [39]. As illustrated in Figure 11, the size of the nanoparticles critically affect not only the cellular uptake but also the intracellular degradation undergone by the encapsulated protein. Such a pattern of behavior is really outstanding because it provides a valuable tool to design vaccine delivery systems on the basis of manipulating the particle size [40, 41].

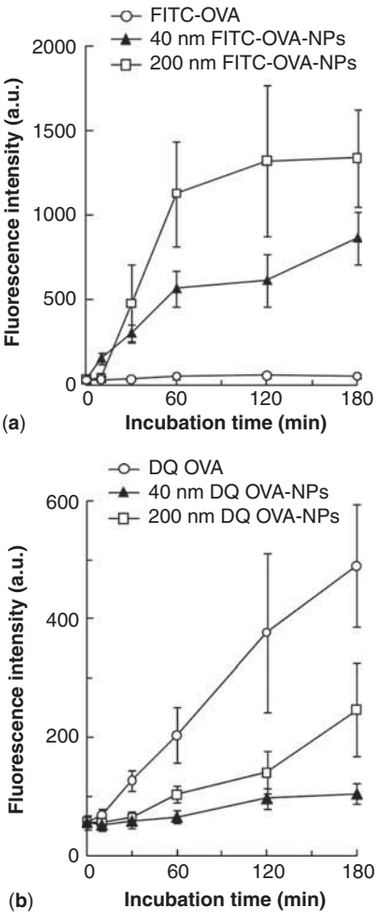

Figure 11 a) Fluorescence intensity measured for intracellular labeled ovoalbumin (OVA) as a function of incubation time for the isolated protein and for the protein encapsulated in PAAG-*graft*-L-PAE nanoparticles of two different sizes. a) Fluorescence indicative of the uptake of OVA; b) Fluorescence indicative of OVA degradation.(From reference [39] with permission).

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The sulfonated amino acid taurine (2-aminoethane-1-sulfonic acid) has also been inserted in PGGA via an amide linkage to the carboxyl side group. The objective of this modification is to replace the weak carboxyl group with the strong sulfonic group in a degree that can be adjusted by carefully selecting the reaction conditions [42]. The presence of the sulfonic group provides PGGA with anticoagulant activity with a delay in clotting time that increases for higher sulfonation degrees. The combination of such a property with the biocompatible nature of PGGA makes these derivatives extremely interesting as components of biomaterials for those medical applications in which anticoagulation is a prime requisite.

Partial amidation of PGGA with linear α,ω-aminoalkanols (C4 to C6) renders polymers that are stimulus-responsive to both pH and temperature [43]. Thermo-responsive polymers are today's materials of interest for their potential in the manufacture of intelligent devices. Among them, those showing a critical solution temperature in the proximities of the physiological values (LCST) are investigated with particular attention due to their suitability for controlled drug delivery systems, biomimetic actuators, and active biocatalysts supports. However, most of the intelligent polymeric systems studied so far are nonbiodegradable, and to our knowledge, none displaying well-proven nontoxicity have been technically developed. The reaction of PGGA with appropriate combinations of α,ω-amino alcohols containing from 4–6 carbon atoms provides water-soluble derivatives exhibiting a sharp LCST in the range from 21–50 °C in water and are also highly sensitive to pH changes (Figure 12).

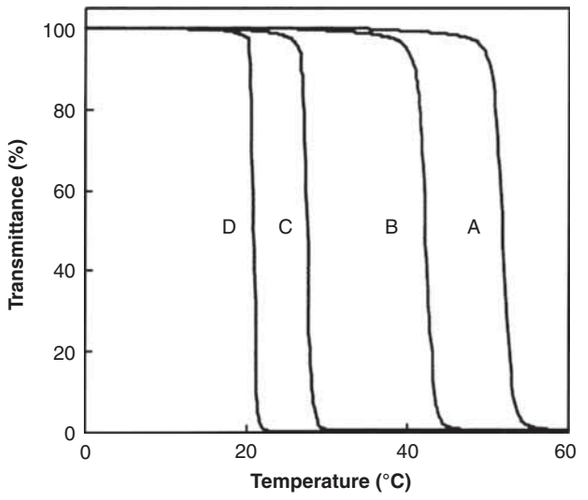

Figure 12 Effect of temperature on turbidity of 1 wt % aqueous solution of PGGA amidated with mixtures of aminoalkanols at different ratios and compositions (A to D) (see reference for meaning of labels). (From reference [43] with permission).

Furthermore, since these PGGA derivatives possess hydroxyl and carboxylic acid side groups, a wide variety of functional molecules such as drugs and probes can be easily attached to them. The combination of thermo-responsiveness with functionalization makes these systems unique candidates for the design of intelligent drug delivery systems and bioconjugates.

Crosslinking of PGGA. Although hydrogels have been widely studied for years, the interest in biodegradable biocompatible hydrogels has recently increased because of their potential as scaffolds designed to contain repairing human cells. Additionally, environmentally sensitive hydrogels, also known as "smart gels" or "intelligent gels", are being looked at with much attention; these gels are able to notice small changes in pH, temperature, or concentration of metabolites, and release their load in reply to such changes. The amount of water that PGGA takes up is critically dependent on pH and this property may be accurately modulated by slight crosslinking with polyfunctional compounds. PGGA hydrogels are readily prepared by esterification or amidation with dihaloalkanes, alkanediols or alkanediamines. The degree of swelling of these hydrogels is extremely sensitive to variations in the molecular weight of the polyacid, the nature and concentration of the crosslinker, and the solution used for the swelling (ionic strength, pH) [44–48].

Crosslinking of PGGA is also a valuable chemical tool for the stabilization of particles, fibers and films made of this biopolymer. High hydrophilic nanoparticles with 20–90 nm sizes have been made of PGGA crosslinked with 2,2′-(ethylenedioxy) diethylamine [49]. On the other hand, water-soluble nanofibers of PGGA with diameters oscillating between 50 and 500 nm made by electrospinning have been crosslinked with cystamine, a diamine containing disulphide linkages, in the presence of carbodiimide (Figure 13) [50]. These crosslinked PGGA fibrous nonwovens are non-water soluble and show good adhesion by fibroblasts and excellent cell proliferation. Since the network is based on -S-S- bonds, it can be easily decomposed under physiological conditions using L-cysteine, a biocompatible reducing compound. Therefore much is expected of these nonwovens as bio-assimilable materials for use in tissue engineering without causing any inflammatory reactions after implantation. Although a variety of vinyl polymers have been explored and are even being used for preparing nonwoven scaffolds, they still imply a toxicity risk to the body; nonwoven matrices based on biodegradable and biocompatible polymers, as it in the case of those made of PGGA, are much more preferable for tissue engineering [51].

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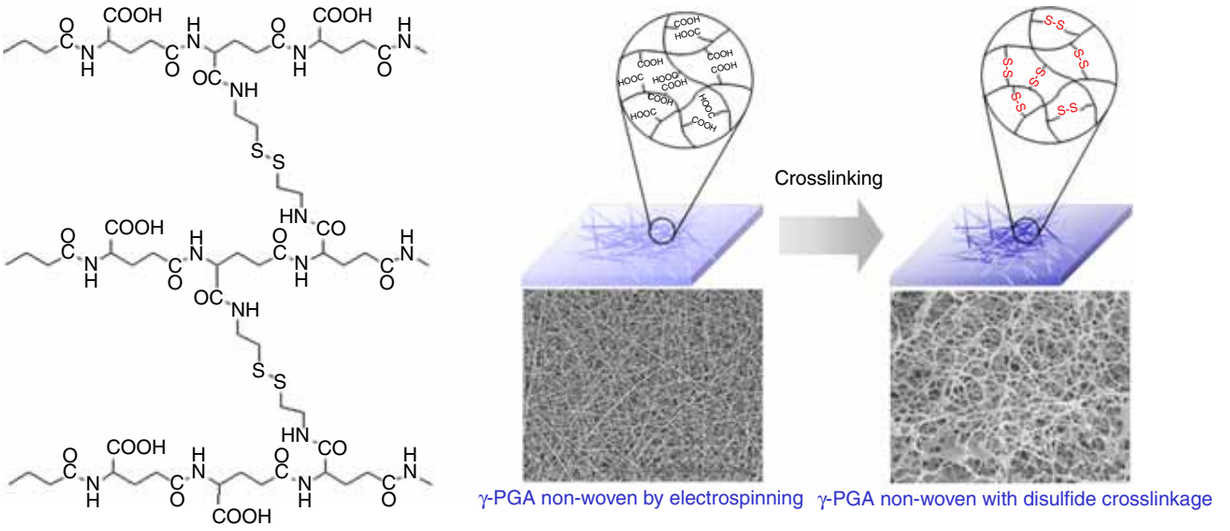

Figure 13 a) Network of PGGA crosslinked with cystamine. b) Schematic illustration of the fabrication of PGGA nonwovens and cell proliferation on the substrate. (From reference [50] with permission).

In order to maintain the sustainability of the crosslinked systems and avoid the introduction of unfriendly reagents, hydrogels of PGGA have also been produced by crosslinking with microbial poly(ε-lysine) (PL) by means of irradiation with γ-rays. The properties of these gels can be controlled by adjusting the ratio of the two biopolymers. Thus, the mixed hydrogel is biodegradable by proteases at a rate that decreased with increasing values of the PGGA/PL ratio. The PGGA-PL gels are outstanding because they are fully sustainable, biodegradable and biocompatible. Unfortunately their mechanical properties are poor and only comparable to those displayed by 2% agar [52, 53]. The development of new crosslinking procedures and formulations able to provide PGGA networks with improved mechanical behavior while retaining their swelling flexibility and good biodegradability and biocompatibility, is a research challenge to which notable efforts will be devoted in the coming years.

2.3 Block and Grafted PGGA Copolymers

In addition to the random copolyglutamates described above, copolymers containing long homogeneous sequences of PGGA linked to other homopolymeric segments of dissimilar constitution are being intensively explored for their potential application in biomedicine. The carboxylic side groups of PGGA may serve as excellent points to build graft copolymers by either attaching polymeric side chains (grafting-on) or initiating the growth of polymeric branches (grafting-from). On the other hand, PGGA may be used as a building block for copolymers by coupling with other active end polymers through its amine or carboxylic end groups.

The grafting of vinyl monomers onto PGGA by cationic or anionic polymerization proceeds with difficulty [54]. Conversely, methyl methacrylate can be grafted on PGGA bearing side azo groups through a free-radical mechanism. The PGGAs grafted with PMMA (30 and 65%) do not display crystallinity and are markedly hydrophobic [55]. On the other hand, the reaction of PGGA with other water-soluble polymers such as poly(propylene glycol) and poly(ethylenimine) in the presence of the condensing agent dicyclohexylcarbodiimide affords grafted copolymers the ability to retain their high water affinity and displays an excellent solubility in volatile alcohols at the same time [56].

Although copolymerization with conventional monomers leads to modified PGGA with new properties of certain interest at the industrial scale, it is the combination with other biodegradable polymers that opens up exciting possibilities for the application of PGGA copolymers as biomaterial. Thus, grafting of polycaprolactone on PGGA [57] leads to amphiphilic copolymers able to produce core-shell nanoparticles with negative *Z*-potential and sizes comprised between 130 and 220 nm. Polycaprolactone is previously attached to the antitumor agent doxifluoridine providing the nanoparticles with the potential capacity for cancer therapy. Another example of the unlimited capacity of PGGA as a source for the preparation of nanomaterials is provided by the systems consisting of PGGA partially grafted with poly(ethylene glycol) and functionalized

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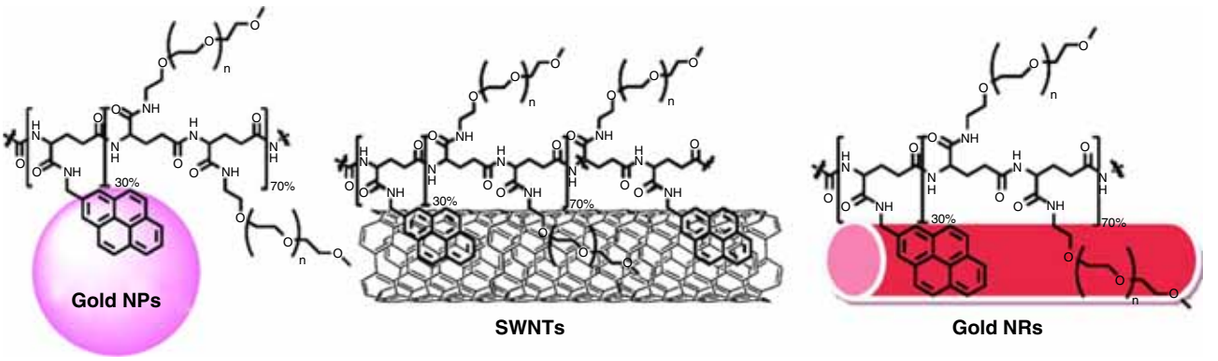

Figure 14 Gold nanoparticles, single-wall nanotubes and gold nanorods coated with PEG-*graft*-PGGA functionalized with pyrene. (From reference [58] with permission).

with either hydrophobic moieties such as pyrene or phospholipids [58]. These three-component systems efficiently coat SWCN, Au-NP and Au-NR by absorption of the hydrophobic moieties in the particle surface (Figure 14) thereby stabilizing their suspension in serum. The coated nanomaterials also exhibit a remarkable circulation time in blood suggesting a greatly delayed clearance by the reticuloendothelial system, a highly desired property for *in vivo* applications of nanomaterials, including imaging and drug delivery.

Polylactides are well-known biodegradable and biocompatible polyesters whose easy accessibility and display of a good overall pattern of properties make them widely used in multiple applications as biomaterials. They exhibit high efficiency in the encapsulation of hydrophobic active compounds, but for an optimum exploitation as DDS they need to be combined with hydrophilic functional moieties in order to generate active shell-core nanoparticles. Coupling of polylactic acid (M_n ~10,000) with low molecular weight PGGA (M_n~10,000) by means of carbodiimide produces an amphiphilic block copolymer that is self-assembled in nanoparticles constituted of a compact PLA core and a hairy shell made of PGGA (Figure 15) [56]. The presence of the outer PGGA counterpart confers affinity for water to the nanoparticle and makes it possible to attach galactosamine to the surface via amidation of the carboxylic side groups. Paclitaxel, a polyvalent hydrophobic anticancer drug, can be efficiently encapsulated in these nanoparticles [57]. The galactosylated nanoparticles display specific interaction with HepG2 hepatic cells via ligand-receptor making them a potential DDS for the targeted delivery of Paclitaxel to liver cancers [58–61].

2.4 Ionic Complexes of PGGA with Cationic Species

The capacity of polyelectrolytes to form more or less stable complexes upon coupling with opposite charged ionic compounds is well known. In the case of the counterion having a noticeable hydrophobicity, the water solubility of the original polyelectrolyte is lost and the complexes become soluble in organic solvents.

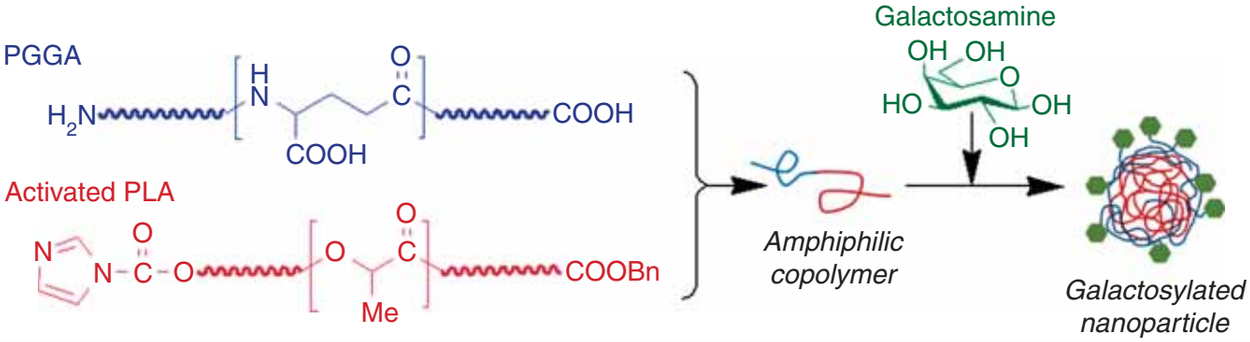

Figure 15 Synthesis of PLA-*block*-PGGA copolymer and structure of their shell-core nanoparticles functionalized with L-galactosamine. (Adapted from reference [61] with permission).

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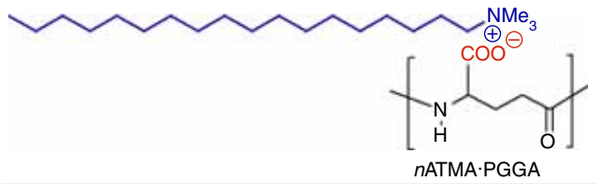

Figure 16 Stoichiometric complexes of PGGA with alkyltrimethylammonium surfactants.

Furthermore, the complexes usually display a pattern of physical properties largely dissimilar to those of the parent polyelectrolyte, which is in many cases the objective of their preparation. PGGA is able to form stoichiometric complexes with alkyltrimethylammonium surfactants with a precise composition and satisfactory stability. Several recent papers have described the synthesis, characterization and properties of *n*ATMA·PGGA complexes (Figure 16), which are the stoichiometric compounds constituted by anionized PGGA and alkyltrimethylammonium cations bearing long alkyl chains (*n* =12–22, even values) [62–64].

Coupling in *n*ATMA·PGGA complexes is established between the carboxylate and ammonium groups involving not only ionic interaction but also multiple hydrogen bonding (Figure 17a) [65]. These complexes are non-water soluble, soluble in chloroform, and start to decompose when heated at temperatures near 200°C. They display thermal properties very similar to the comb-like poly(α-alkyl γ-glutamate)s described above, with melting temperatures increasing from 30 to 70°C as the number of carbons in the alkyl chain *n* increases from 14 to 22. The structure adopted by these complexes in the solid state is also the layered biphasic structure described for polyglutamates, with a periodicity linearly increasing from ~30 to ~45 nm with the length of the alkyl side chain (Figure 17b) [66]. These complexes

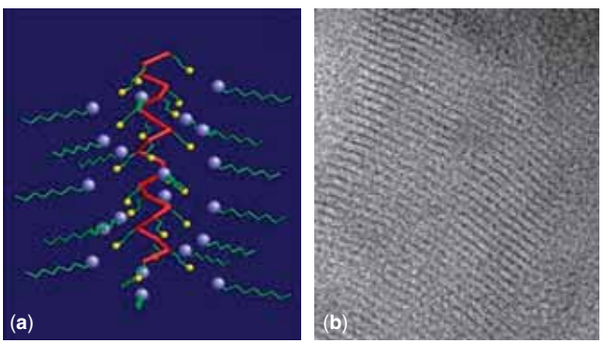

Figure 17 a) The molecular structure of *n*ATMA·PGGA complexes. b) TEM micrograph of the 22ATMA PGGA complex. (From reference [66] with permission).

are remarkable for their extremely good accesbility; they are readily prepared in high yields by simple mixing of the components, and separated by spontaneous precipitation from the aqueous mixing media.

The *n*ATMA·PGGA complexes are hydrolyzed under physiological conditions at a rate that decreases for longer alkyl groups, but in all cases with the total degradation of the complex in a period of time from 1–2 months (Figure 18a) [67]. The degradation mechanism is in bulk due to the resistance of the amide main chain group to be hydrolyzed compared to the relatively fast decomposition taking place in the complex by the action of water. Incorporation of erythromycin in the complexes was achieved with the rather lipophilic antibiotic microcrystallized and lodged into the amorphous paraffinic subphase. Erythromycin is released after a lag time that extends beyond the fifth day of incubation and that corresponds

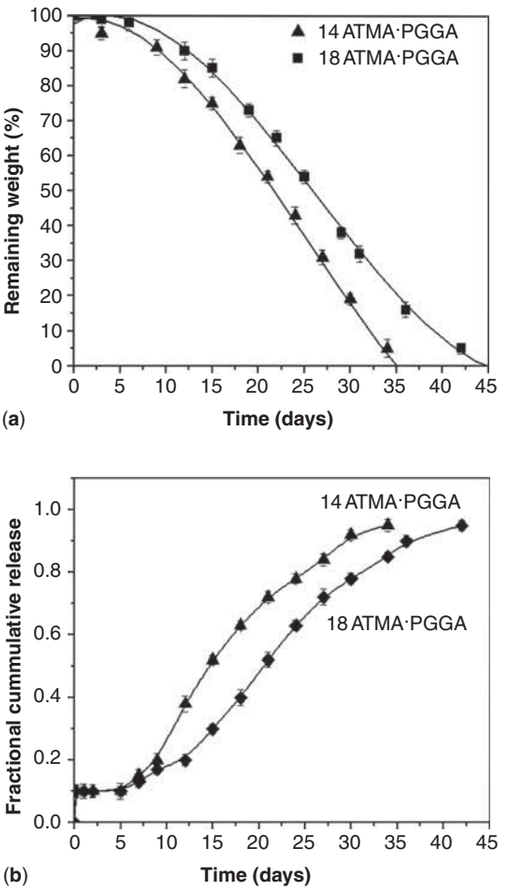

Figure 18 a) Hydrolytic degradation profiles of *n*ATMA·PGGA complexes incubated at pH 7.4 and 37°C, and b) cumulative releasing profile of erythromycin from complexes loaded with 10% of drug. (From reference [67] with permission).

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approximately to the time needed for observing appreciable weight loss in the complexes due to hydrolysis. Temperature and pH effects on the erythromycin releasing rate were found to be similar to those observed for degradation indicating that drug delivery in *n*ATMA·PGGA complexes is a process concomitant to their decomposition [67].

The interest of these complexes in their application as drug delivery systems relies mainly on two facts: a) their extreme ease of preparation, and b) the great assortment of hosting and delivery possibilities they offer due to the wide variety of counterion characteristics that are available; the chemical nature and size of the groups attached to the alkyltrimethylammonium cation will be those mainly determining both the hosting efficiency and release rate. The studies carried out so far are preliminarily in the exploration of these complexes as DDS and a considerable amount of research needs to be done in order to appraise their actual potential. The utilization of naturally-occurring counterions with a well-proven nontoxicity, as well as the preparation of nanoparticles and micelles able to encapsulate drugs efficiently with controlled stability in the aqueous environment, are the main challenges facing the development of these systems as DDS.

Ionic coupling of PGGA with cationic polyelectrolytes is a step ahead in the formation of complexes with functional interest, particularly if naturally-occurring polycations are used and fully sustainable systems are therefore generated. Chitosan [β-(1-4)-2-amino-2-deoxy-D-glucose] (CS) is a partially deacetylated derivative of chitin, the prime structural biopolymer of the arthropod exoskeletons. Chitosan becomes protonated in dilute acids allowing the formation of insoluble complexes with water-soluble polyanionic species. Direct mixing of PGGA with chitosan produces biocompatible CS-PGGA hydrogels with properties that may be partially controlled by adjusting the pH at which the complex is formed [68]. The morphology, swelling degree and mechanical properties of these hydrogels depend on the ratio of the two components. In all cases, they form highly permeable networks with a porous size decreasing with the content in chitosan (Figure 19). The open texture displayed by

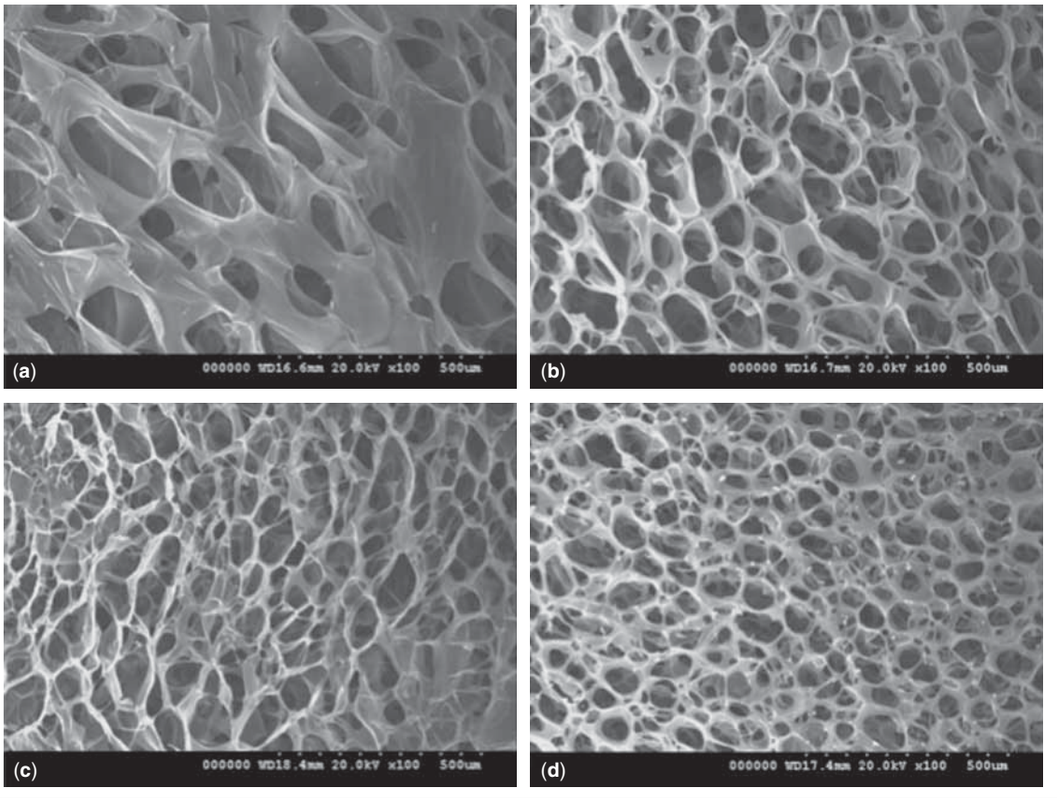

Figure 19 SEM micrographs of the surface of the hydrogel made of CS-PGGA complex for different PGGA/CS ratios (w/w): a) 0.1, b) 0.2, (c) 0.6, and (d) 1.0.(From reference [68] with permission).

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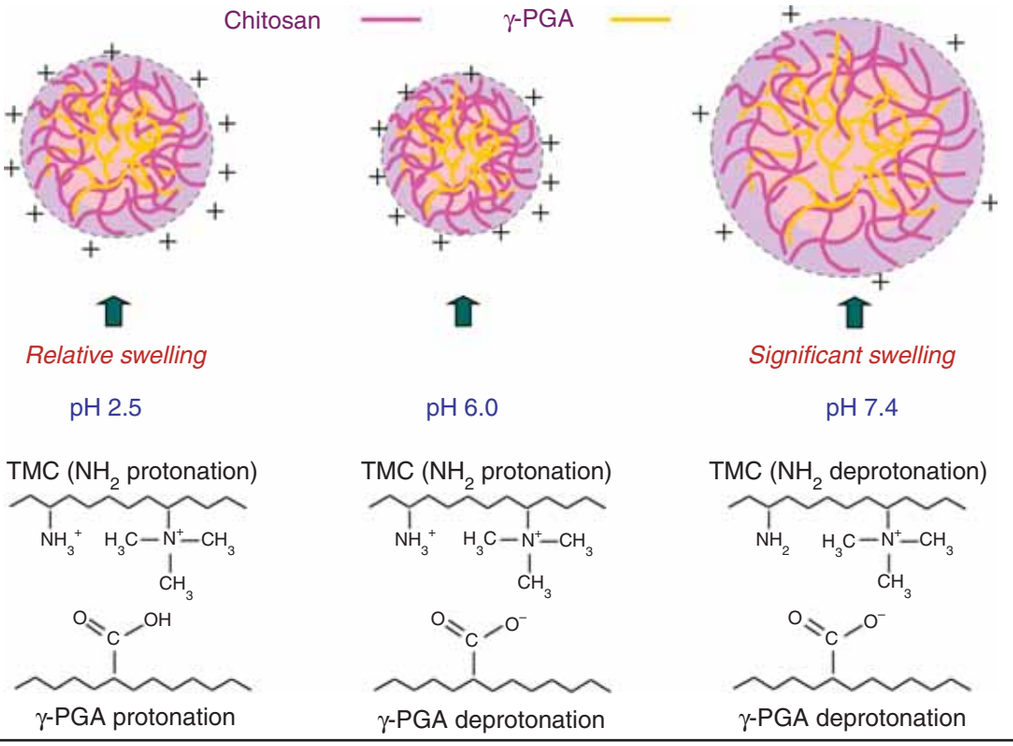

Figure 20 Nanoparticles made of CS-PGGA with chitosan quaternized at 40%.(From reference [69] with permission).

this material will be beneficial in diverting the fluid when applied as substrate for cell growth. The possibility of adjusting the porosity by formulation will allow flexible use of these cytocompatible hydrogels as tissue engineering scaffolds for a wide variety of applications.

At present CS-PGGA complexes are being investigated as a potential platform for the design of DDS with exceptional responsive delivery properties. Particularly, the dependence of the stability of these complexes on pH may be exploited for carrying proteins through aggressive environments to then be delivered under specific conditions. The pKa values of CS (amine groups) and PGGA (carboxylic groups) are 6.5 and 2.9, respectively. At pH between 2 and 6, both polyelectrolytes are ionized and nanoparticles made of stable ionic complexes CS-PGGA are formed. At pH below 2.0, most carboxylic groups on PGGA are protonated so the electrostatic interaction with the amine groups of CS is weak and nanoparticles disintegrate. The same effect is observed at pH values above 6.6 since under such conditions the CS amine groups are deprotonated. Quaternization of chitosan as *N*-trimethyl chitosan increases the stability of the complexes in the pH range of 6.6–7.4 and extends their solubility over a much broader pH range. Nanoparticles made of PGGA with chitosan quaternized at 40% (CS-TM40-PGGA) swell significantly with increasing pH value (Figure 20) and still retain a positive surface charge. These nanoparticles are pointed out as suitable carriers for transmucosal delivery of protein/peptide drugs within the entire intestinal lumen [69]; when they are loaded with insulin and orally administrated, they are able to reach the intestinal tract and open the tight junctions of cell monolayers to allow the paracellular transport of the protein. A more recent work reports on the preparation of an enteric-coated capsule loaded with freeze-dried nanoparticles made of CS-PGGA complex for the delivery of insulin mediated by environmental pH changes. Upon oral administration, the capsule remains intact in the acidic environment of the stomach, but dissolves rapidly in the proximal segment of the small intestine [70].

A second example of the application of PGGA to the design of DDS sensitive to pH is provided by

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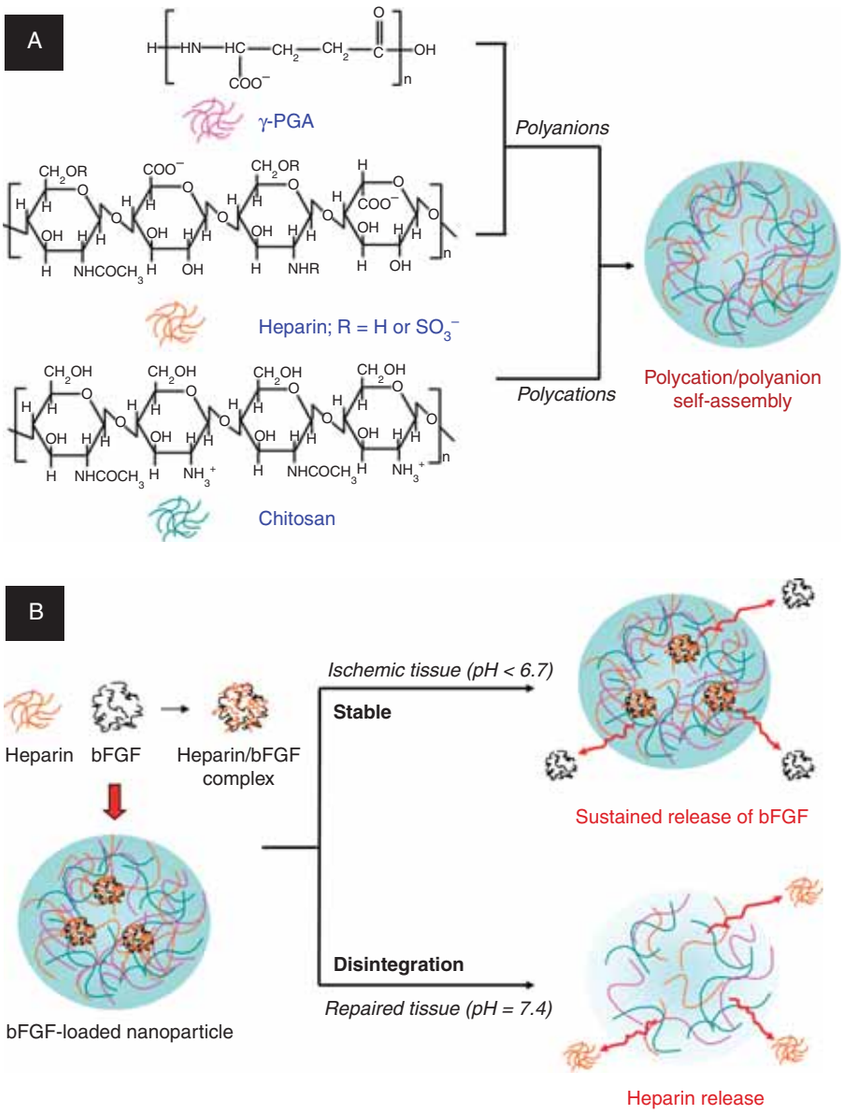

Figure 21 (A) Scheme of polyelectrolyte self-assembly of the polyanions and polycations into heparinized CS-PGGA nanoparticles, and (B) release of bFGF or heparin from the nanoparticles, depending on the environmental pH variation. (From reference [71] with permission).

heparinized CS-PGGA nanoparticles (Figure 21) [71]. This system is designed as a multifunctional carrier for fibroblast growth factor and heparin delivery. The HP-CS-PGGA nanoparticles can sustain bFGF release at the pH of ischemia tissue (pH<7) but are rapidly disintegrated at repaired tissue pH 7.4. The released bFGF from the nanoparticles enhances the proliferation of HFF cells, whereas the released heparin maintains the anti-factor Xa activity in blood plasma. This mutilfunctional controlled delivery system is a potential therapeutic technique for regeneration of ischemic tissue and prevention of harmful clot formation.

Nanoparticles made of the binary complex CS-DNA are known to transfect cells more efficiently than naked DNA but less than commercially available liposome formulations. Nevertheless, the strong electrostatic interactions between chitosan and DNA prevent the dissociation of the complex within the cells, thus precluding transcription of DNA and resulting in low transfection. With incorporation of PGGA as a third component of the nanoparticles, the cellular uptake and transgene expression of CS-DNA complexes are significantly enhanced [72,73]. Although the detailed mechanisms operating in the endocytosis

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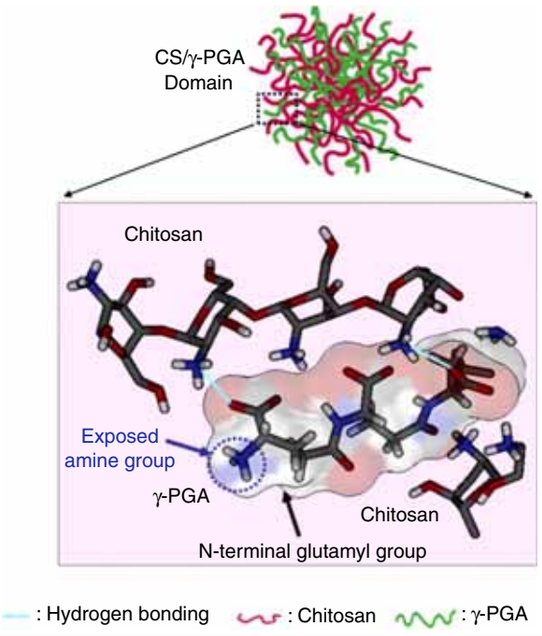

Figure 22 Structure of the CS-PGGA complex resulting from molecular dynamic simulations showing intermolecular hydrogen bonding and the exposed *N*-terminal amine group in PGGA. (From reference [72] with permission).

mediated by PGGA remain understood, recent studies suggest that it is the free *N*-terminal γ-glutamyl unit of PGGA exposed on the surface of the complex (Figure 22) which enhances its interaction with the enzyme γ-glutamyl transpeptidase (GGT) localized in the cell membrane. This specific interaction favors the endocytosis processes and hampers the formation of lisosomes with the consequent improvement in transfection efficiency.

Although the nanoparticles made of polyionic complexes are promising carriers for DDS, their sensitivity to the environment (ionic strength or pH) causing their disintegration can be a limitation for those applications where a highly stability of the particle is required. An interesting approach towards the formation of highly stabilized complexes is to create additional hydrophobic interactions. Nanoparticles composed of biodegradable PGGA-L-PAE and ε-PL are quite stable under physiological conditions, exhibiting no aggregation, precipitation, or dissociation for a prolonged period of time. The exceptional stability of these nanoparticles is attributed to the formation of the hydrophobic domain in the core of the nanoparticles (Figure 23). The possibility of modifying the hydrophilicity of PGGA is therefore a very useful tool for the stabilization of nanoparticles and provides a novel concept for DDS particle design [74].

The ability of PGGA to form ionic complexes may be exploited in its simplest version to synthesize complexes with drugs that may be directly used as particulate DDS. A recently reported example of this approach is the direct ionic coupling of Doxorubicin, an anthracycline antibiotic widely used as antineoplasic agent, with PGGA [75]. However, the encapsulation of the drug may be much improved by adding an amphiphilic trimethylammonium cation as a compatibilizing agent. The three-component nanoparticles are readily formed by precipitation from the aqueous mixture with a diameter of about 500 nm and 90% of trapped Doxorubicin in respect to the added amount [76]. Matching the compatibilizer with drug for an optimum design of these DDS affords a broad span of possibilities that are still to be explored.

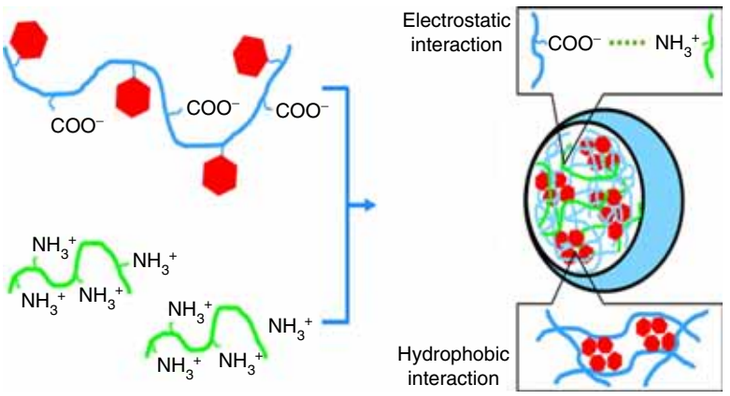

Figure 23 Nanoparticles made of ionic complex of ε-PL and PGGA modified with hydrophobic L-PAE. (From reference [74] with permission).

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3 CONCLUSIONS

Poly(γ-glutamic acid) (PGGA) is a water-soluble biopolymer that is not only biodegradable and biocompatible but even edible. At present it is commercially used in applications where water affinity and biodegradability are well appreciated advantages. However, its exploitation in packaging or coating materials is hampered by its marked sensitivity to water as well as by its incapacity to be processed by conventional procedures.

The chemical modification of the carboxyl side group of PGGA is the method of choice for yielding derivatives of practical interest. Esterification with linear alkanols or alkyl halides lead to poly(α-alkyl γ-glutamate)s, both homopolymers and copolymers that are non-water soluble and melt before decomposing. The physical properties of polyglutamates may be tuned by selecting the constitution of the attached alkyl side chain and by adjusting the copolymer composition. Copolymerization also provides processable materials, and the application of click chemistry appears to be a very promising tool towards the preparation of grafted PGGA copolymers.

PGGA is receiving increasing attention in biomedicine because it combines a well-proven nontoxicity with the possibility of conjugating diverse active agents and drugs via the carboxyl side group. Of particular interest is its potential as DDS due to the capacity of PGGA derivatives to form structured nanoparticles. Partial amidation with hydrophobic amino acids renders stable nanospheres able to efficiently encapsulate proteins that are conveyed by mechanisms appropriate for the design of vaccine delivery systems.

Modification by ionic coupling with organic cations constitutes another option to generate PGGA derivatives with new potential applications. Stoichiometric complexes made of PGGA and tetraalkylammonium surfactants are of interest as DDS, and temperature-responsive membranes may be readily prepared. Complexes with polycationic polymers such as poly (ε-lysine) or chitosan are suitable for building drug and protein carrier systems able to respond intelligently to pH changes.

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