ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA
INDUSTRIAL DE BARCELONA

UNIVERSIDAD POLITÉCNICA DE CATALUÑA

"Synthesis, characterization and biomedical applications of microbial polymalic and polyglutamic acids derivatives."

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Trabajo realizado bajo la dirección de los Drs.
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Barcelona, Febrero 2008

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Thermal decomposition of microbial poly(γ-glutamic acid) and poly(γ-glutamate)s

Summary

The thermal decomposition of poly(γ-glutamic acid), poly(α-methyl γ-glutamate) and ionic complexes of the polyacid with alkyltrimethyl ammonium salts was studied by TGA, GPC, and FTIR and NMR spectroscopy. It was found that both poly(γ-glutamic acid) and poly(α-methyl γ-glutamate) depolymerized above 200 ºC by an unzipping mechanism with generation of pyroglutamic acid and methyl pyroglutamate, respectively. On the other hand, the ionic complexes degraded through a two-stage process, the first one being cyclodepolymerization of the poly(γ-glutamate) main chain along with decomposition of the ionic complex promoted by the adsorbed water. Decomposition of the previously generated alkyltrimethyl ammonium compound followed by unspecific cracking of the resulting nitrogenated compounds accounted for the second degradation step happening at higher temperatures. Mechanisms explaining the decomposition processes of the three studied systems were proposed according to collected data.

7.1. Introduction

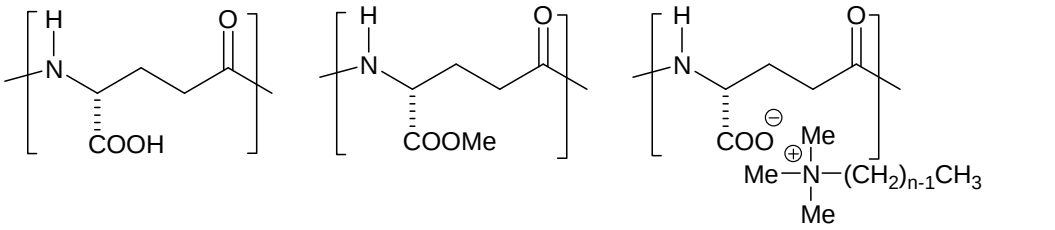
Poly(γ-glutamic acid) (PGGA) is a biosynthetic polymer consisting of a poly(γ-butyramide) main chain bearing a carboxylic group attached to the γ-carbon of every repeating unit. The naturally produced PGGA's contain approximately equal amounts of D and L units, and may attain very high molecular weights[1]. The high research interest that currently is developing for PGGA is due to its potential as a biomedical material with a high biocompatibility and a fair biodegradability. In fact, PGGA is extensively used as a food additive and it is known to be hydrolytically degradable by water with or without intervening of proteases[2,3]. An incessant number of publications dealing with the use of PGGA as drug delivery system are appearing in these last years[4,5] and several processes have been developed at large scale which are able to afford great amounts of this compound for industrial uses. [6]

Modification of PGGA by esterification[7,11] of the carboxylic side groups is the approach most frequently applied with the purpose of modifying the genuine properties of the biopolymer. A good number of poly(α-alkyl γ-glutamate)s (PAAG-n) covering a wide range of alkoxy sizes and constitutions have been reported. Additionally, polyglutamates of ionic nature have been also produced by coupling of PGGA with alkyltrimethylammonium surfactants with long alkyl chains[12,13]. These stoichiometric ionic complexes (*n*ATMA·PGGA) adopt a biphasic amphiphilic nanostructure that melts reversibly at 30-80 °C and are of interest as delivery systems because they are able to lodge fair loads of hydrophobic drugs within the paraffinic subphase[14].

Such a continuous development of new derivatives of PGGA and their expectable use in a near future as biomaterials should be backed by good knowledge on their physical properties, more specifically on their thermal behaviour. However, the information presently available on the thermal stability, the thermal degradation mechanism and the nature of the degradation products of polyglutamates is largely incomplete and it is almost inexistent as far as the polyacid and its ionic complexes are concerned. Some years ago we reported on the thermal degradation of some covalent poly(α-alkyl β,L-glutamate)s and a decomposition mechanism explaining the releasing of alkyl pyroglutamate was put forward[10]. To our knowledge this is the only account that has been published so far on this matter. This situation is in strong contrast with that concerning biodegradable polyesters as polylactic acid or polyhydroxybutyrate, for which a high amount of information covering multiple aspects of the pyrolysis of these compounds has been accumulated along the last decades[15-18].

In this work, a detailed study on the thermal degradation of PGGA, PAAG-1 and *n*ATMA·PGGA complexes is carried out using a combination of analytical techniques including thermogravimetry, size exclusion chromatography and infrared and NMR spectroscopy. The chemical formulae of these compounds are depicted in Scheme 1.

The purpose is to characterize their thermal behaviour and to understand as far as possible the reaction mechanisms underlying the degradation processes. The approach followed in this study is the same that we previously used in the analysis of poly(β, L-malic) acid and polymalates, whose results have been recently reported[19].



PGGA **PAAG-1** ***n*ATMA·PGGA** (*n* = 14 to 22)

Scheme I. Chemical structure of poly(glutamic acid) and poly(glutamate)s studied in this work.

7.2. Experimental

7.2.1. Materials. Sodium poly(γ,DL-glutamate) with a D:L enantiomeric ratio of 59:41 and a weight-average molecular weight of about 300,000, which was kindly supplied by Dr. Kubota of Meiji Co. (Japan), was used in this work. The polymer was changed to the protonated form by acidification with HCl followed by precipitation in 2-propanol. The final sodium content of the acidified polymer was 5.1% w/w as determined by inductively coupled plasma (ICP) in a multichannel equipment Thermo Jarrell-Ash model 61E Polyscan working under standard conditions.

Methylation of PGGA was accomplished by esterification with diazomethane according to our previously reported method[20]. Stoichiometric complexes *n*ATMA·PGGA were prepared by mixing PGGA and the corresponding alkyltrimethyl ammonium bromides (*n*ATMA·Br) with *n* =14,16,18, 20 and 22 by applying the experimental procedure previously described by us[12,13]. Main features of all these compounds which are relevant to the study carried out in this work are given in Table 1.

Table 1. Main characteristics of polymers studied in this work.

Compound	Mw[a]	PD[a]	Tm[b] (ºC)	$^{0}T_d$[c] (°C)	$^{m}T_d$[d] (°C)	W [e] (%)
PGGA	190,000	2.1	220	237	290	2
PAAG-1	45,000	2.7	228	270	297	6
*n*ATMA·PGGA						
14ATMA·PGGA	735,000		n.o.	198	267/315	18/6
16ATMA·PGGA	776,200		n.o.	200	270/330	21/5
18ATMA·PGGA	817,400		53	198	274/352	24/6
20ATMA·PGGA	858,700		63	199	279/364	28/6
22ATMA·PGGA	899,900		69	198	281/382	31/5
18ATMA·Br	404	-	107	200	278/370	20/5

[a] Weight-average molecular weight (Mw) and polydispersity (PD) determined by GPC. Values for complexes calculated from PGGA data taking into account the increment of mass due to stoichiometric complexation. [b] Melting temperature for complexes refers to melting of the paraffinic phase. [c]Onset decomposition temperature measured for 5% loss of the initial weight. [d]Maximum rate decomposition temperature for the two decomposition steps. [e] Remaining weights at the end of the respective decomposition stages.

FTIR spectra were registered with a FTIR Bomem Michelson MB100 spectrophotometer with a resolution of 4 cm^{-1}. An attenuated total reflection accessory with a diamond crystal (Golden Gate Heated Single Reflection Diamond ATR, Specac-Teknokroma) was used.

NMR spectra were recorded on a Bruker AMX-300 NMR instrument with samples dissolved in $CDCl_3$ either pure or added with trifluoroacetic acid, and using TMS as internal reference. Sample concentrations of about 1% (w/v) were used for these analyses. Gel permeation chromatography (GPC) was performed using a Waters equipment. Both PGGA and PAAG-1 were chromatographied using 0.005M sodium trifluoroacetate-hexafluoroisopropanol (NaTFA-HFPI) and all the chromatograms were calibrated against PMMA.

Thermogravimetric (TGA) experiments were carried out in a thermobalance Perkin-Elmer TGA6 under a circulating nitrogen flow. Sample weights of about 15 mg were used in dynamical experiments whereas weights up to 40 mg were used in the isothermal treatments in order to have enough amount of residual product for spectroscopy and chromatography analyses. For the analysis of volatile emanating from decomposition, pyrolysis was carried out using much larger amounts of samples in a flask provided with a cold-finger to collect condensed gases in the needed amounts.

7.3. Results and discussion

7.3.1. Thermal degradation of poly(γ-glutamic acid) and poly(α-methyl γ -glutamate). The TGA traces of PGGA and PAAG-1 recorded at a heating rate of 10 ºC·min^{-1} under circulating nitrogen are compared in Figure 1. The two profiles reveal the occurrence of a first decomposition step taking place in the 250-300 ºC range and involving a weight loss of around 50 and 70% of the initial mass, respectively. The small weight loss (< 3%) observed on the PGGA trace below 200 ºC is the release of the water absorbed during sample handling because of the highly hydrophilic nature of the polyacid.

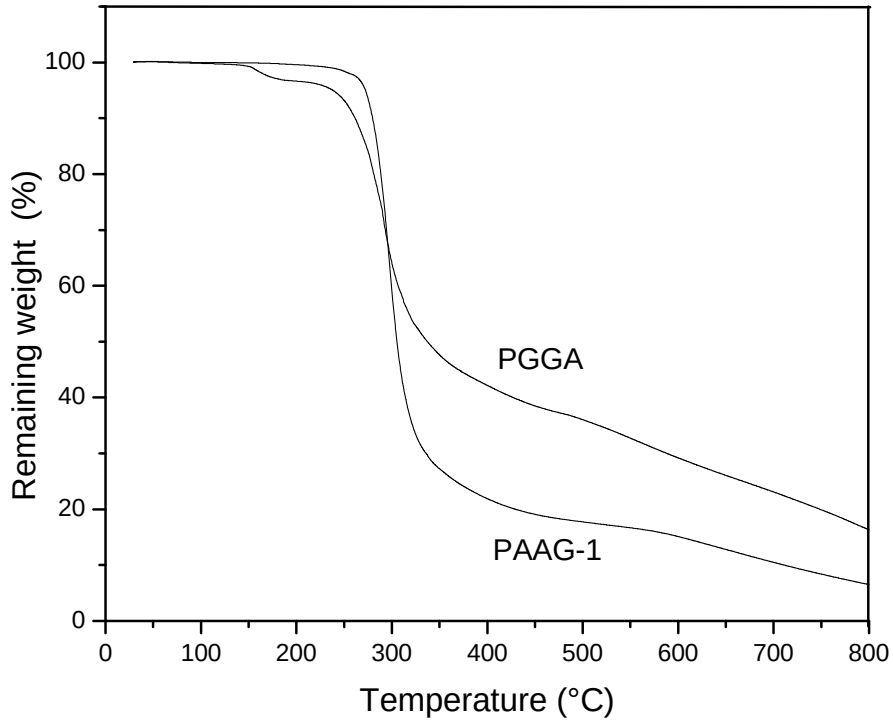


Figure 1. TGA traces of PGGA and PGGA-1 recorded at the heating rate of 10 ºC·min^{-1} under nitrogen atmosphere.

The ^{1}H NMR spectra of the original sample, the residual polymer and the volatiles collected upon heating PAAG-1 at 250 ºC are depicted in Figure 2.

The released gases were found to contain exclusively methyl pyroglutamate and the residue appeared spectroscopically undistinguishable from the original polymer. Similar results were obtained in the ^{1}H NMR analysis of PGGA, the volatiles released in this case consisting exclusively of pyroglutamic acid.

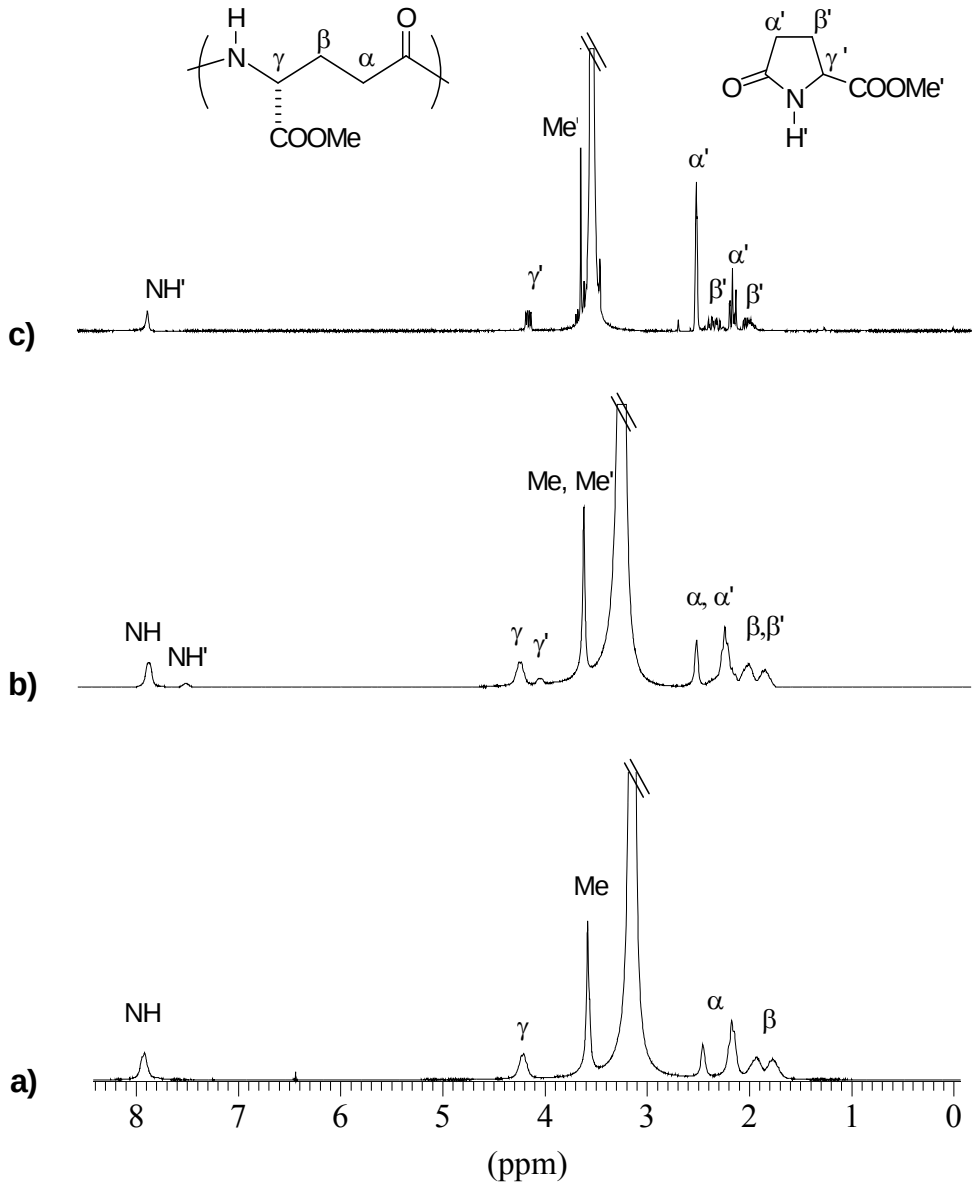


Figure 2. ^{1}H RMN spectra of products resulting from the thermal degradation of PAAG-1. a) Original polymer. b) Residual polymer. c) Released gases. The peak at 3.3 ppm corresponds to the water present in the solvent.

Additional evidences on the decomposition mechanism were attained by IR analysis (Figure 3) which showed that spectra of the residues left by both PGGA and PAAG-1 for 50% of weight loss were fully coincident with the spectra of their respective initial polymers.

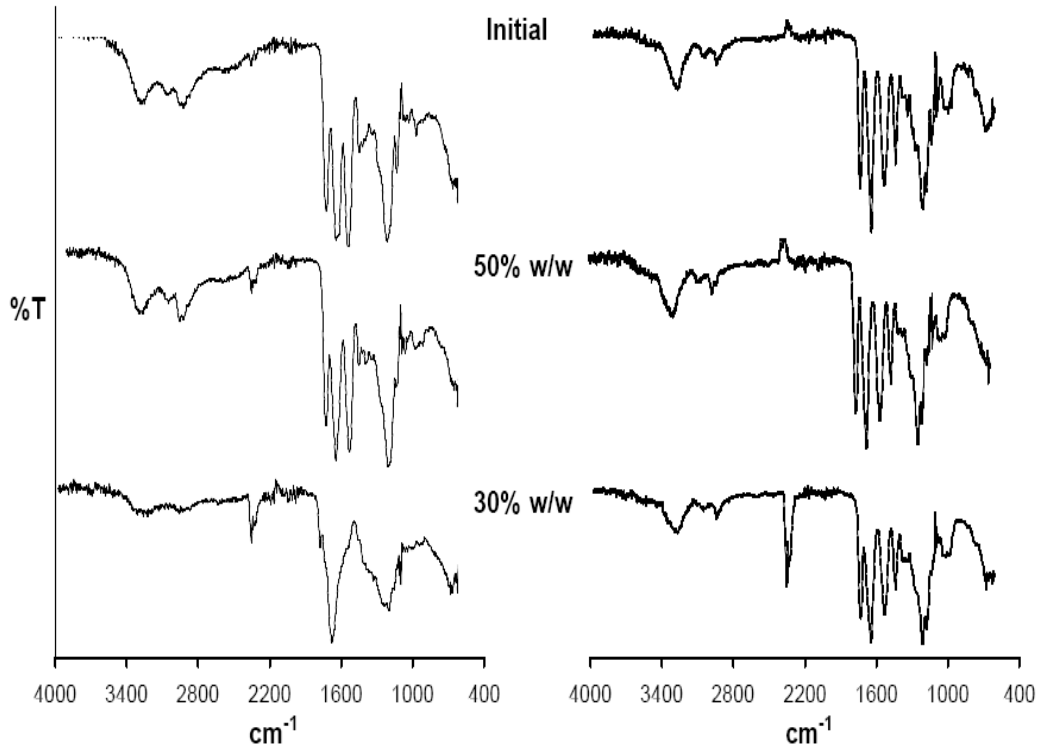


Figure 3. Infrared spectra of the residual material left by PGGA (left) and PAAG-1 (right) at the indicated remaining weights.

All these results are fully consistent with the occurrence of a cyclodepolymerization reaction entailing the release of γ-lactam. It should be noticed however that the weight remaining at the end of this step is significantly lower for PAAG-1 than for PAAG, a difference that can not be just accounted for by the higher molecular weight that the released compound has in the former case. Furthermore, the IR analysis of the residues left after 70% of weight revealed notable differences between the acid and the ester. Whereas the spectrum of the residue of PAAG-1 continued being practically undistinguishable from that of the original polymer, the spectrum produced by the residue left by PGGA lacks the characteristic NH stretching absorption band, and the 1700-1550 cm^{-1} carbonyl region appears significantly distorted.

Such differences in both weight loss and infrared spectra can be accounted for by taking into account the ~5% (w/w) of sodium present in the PGGA sample. Since sodium glutamate units are unable to render volatile compounds, the weight lost by PGGA in the depolymerization step decreased in an amount approximately corresponding to their molar content in such units. The second decomposition step taking place above 300 ºC evolves following a similar weight loss profile for the two polymers. According to antecedents on the thermal decomposition of poly8 (γ-glutamate)s at temperatures above 300 ºC [21], a decarboxylation process leading to unsaturated chain fragments should happen in this stage. The final residue left is approximately 20% and 5% for PGGA and PAAG-1 respectively, which is agreement with the presence in PGGA of non-volatile metallic compounds as discussed above.

The GPC analysis of the residue remaining after the first decomposition step afforded extremely valuable information on the molecular mechanism operating in the thermal degradation of PGGA and PAAG-1 in this stage. In Figure 4, the number-average molecular weight M_n of the residual material left after heating at 220 ºC is plotted against the remaining weight of the initial sample.

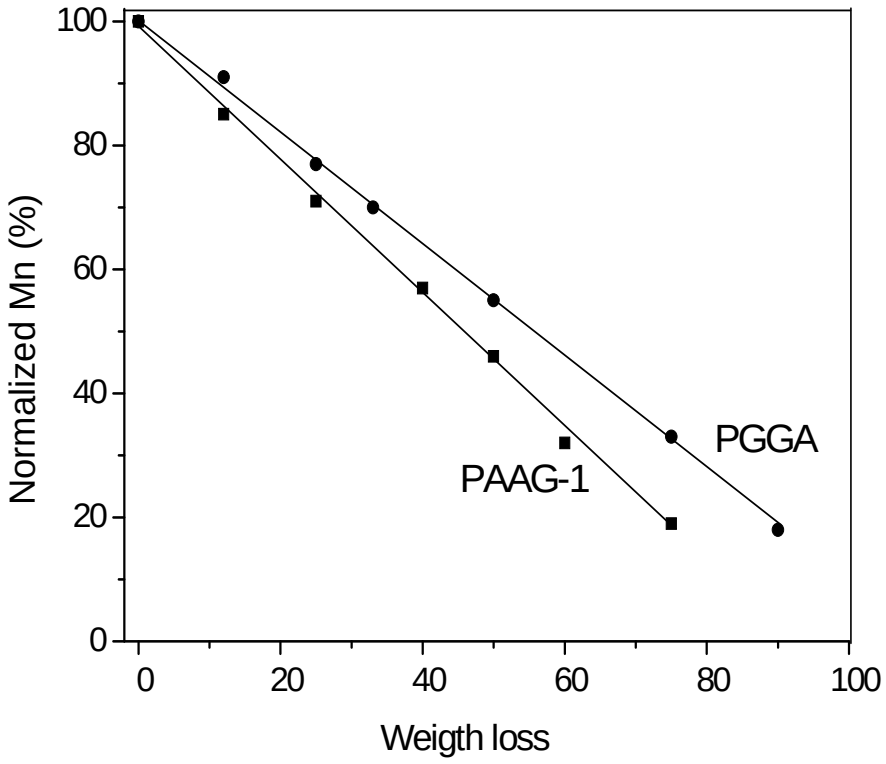
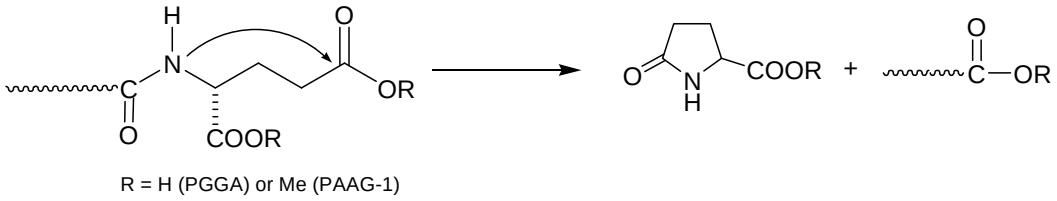


Figure 4. Evolution of the normalized number-average molecular weight of the residual product left after heating PGGA and PAAG-1 from 30 ºC to 800 ºC at 10 ºC·min^{-1}.

The decay in molecular size with conversion is approximately linear with a slope close to one in both cases. This fact together with the spectroscopical evidence that the constitution of the residual polymer remained essentially unaltered throughout the degradation process, lead us to put forward a mechanism for the first thermal degradation step of PGGA and PAAG-1 consisting of an end-of-chain unzipping cyclodepolymerization reaction. Degradation would take place with releasing of pyroglutamic acid or methyl pyroglutamate according to the case and gradually decrease of the polymer chain length, as depicted in Scheme II.



R = H (PGGA) or Me (PAAG-1)

Scheme II. Mechanism of thermal decomposition of PGGA and PAAG-1.

This mechanism is the same we proposed some years ago for the thermal degradation of poly(γ-glutamate)s bearing longer alkyl side chains[10]. The present results confirm the validity of the unzipping mechanism for the whole series of poly(α-alkyl γ,L-glutamate)s and extend it to the thermal degradation of the polyacid itself. It is worthy mentioning that certain polyesters such as poly(caprolactone)[22] and poly(lactic acid)[23] are known to degrade also by such a type of mechanism with generation of caprolactone and lactide, respectively. Decomposition of poly(β,L-malic) and poly(methyl β,L-malate) is known to occur through unzipping depolymerization too, although in this case the released products are mixtures of fumaric and maleic acids[19].

In order to make a comparative kinetics analysis of the degradation process occurring in the first stage of PGGA and PAAG-1, the activation energy was evaluated by using TGA traces recorded at different heating rates from 2 to 30 ºC·min^{-1}. As expected, the TGA traces are moved to higher temperatures for increasing heating rates whereas their overall profiles remain practically unmodified. Firstly, the isoconversional method of Ozawa, Flynn and Wall (OFW) was used.[24] This method assumes that the conversion function f(α) does not change with the heating rate and temperatures corresponding to fixed values of α in experiments carried out at different heating rates β are measured.

Plotting log(β) against 1/T according to Equation 1 (A is a pre-exponential factor independent of T and R is the gas constant) should give a straight line, the slope of which are directly proportional to the activation energy *E*.

$$\log\beta = \log\left[\frac{AE}{g(\alpha)R}\right] - 2.315 - 0.4567E/RT \qquad (1)$$

The Ozawa plots obtained for different degrees of conversion ranging from 0.1 to 0.8 for PAAG-1 are shown in Figure 5 (left). Isoconversional lines are nearly parallel assessing the applicability of the method to the system under study. The Kissinger method[25], which calculates the activation energy from plots of the logarithm of the heating rate versus the inverse of the absolute temperature, at which the decomposition rate is maximum for each stage, was also applied and the corresponding plot obtained there from is depicted in Figure 5 (right).

Similar results were obtained in the analysis of PGGA within the 0.1-0.5 conversion range. Kinetic parameters resulting from this analysis are summarized in Table 2 showing that similar *E* values were obtained by both methods for the two polymers, which is in full agreement with the occurrence of a common degradation mechanism for the two polymers. It should be noted that the *E* values resulting in this study are of the same order than those reported for polymalic acid and poly(methyl β,L-malate) which were determined using the same thermogravimetric method[19].

Table 2. Kinetic parameters of thermal degradation of PGGA and PAAG-1.

Polymer	$\Delta\alpha$ [a]	T_i [b] (ºC)	T_f [b] (ºC)	E [c] (kJ mol^{-1})	E [d] (kJ mol^{-1})
PGGA	0-0.5	248	295	110.6	102.3
PAAG-1	0-0.8	278	323	108.2	100.2

[a]Interval of conversion. Conversion calculated in term of mass loss as $\alpha = (W_0-W)/(W_0-W_\infty)$, where W_0, W and W_∞ are, respectively, the initial weight, the actual weight and the final weight at the end of the degradation process. [b]Initial and final temperature of the step determined at 10 ºC·min^{-1}. [c]Average activation energy determined with the Ozawa method. [d]Activation energy determined with the Kissinger method.

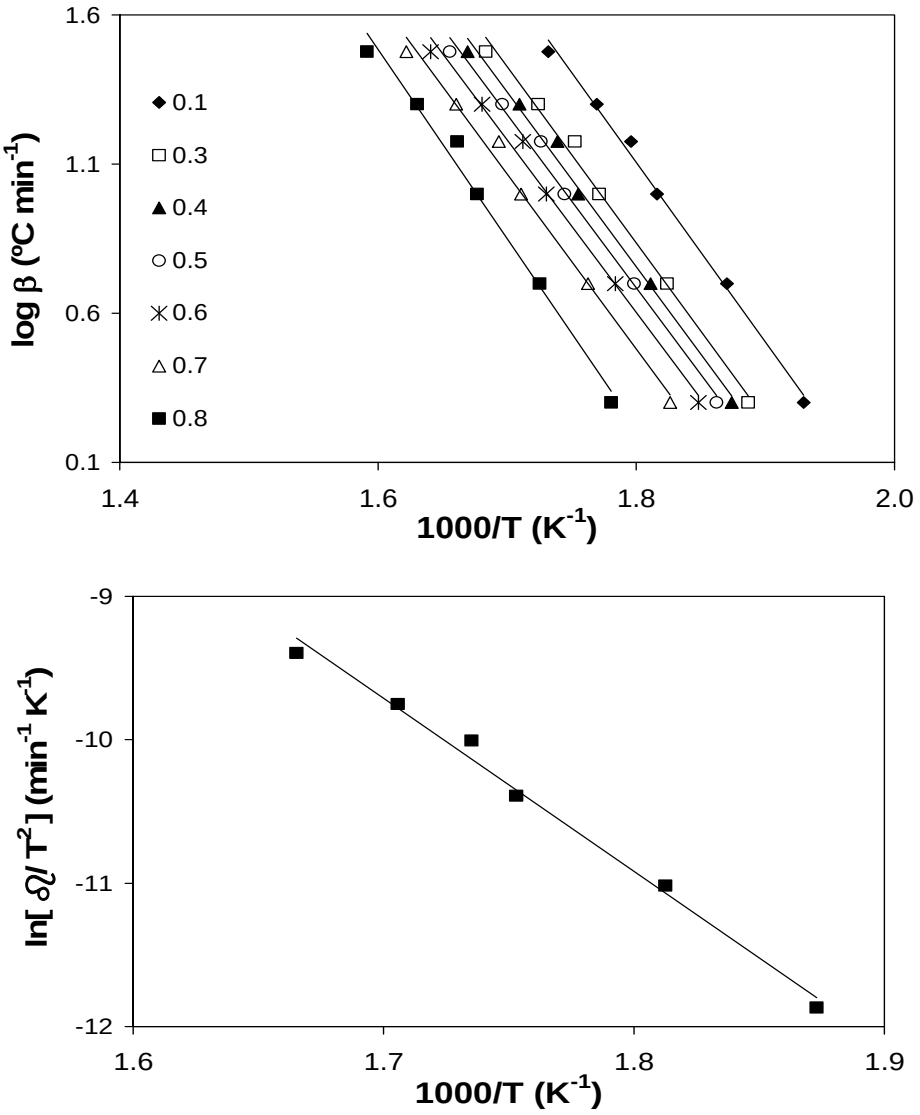


Figure 5. Ozawa (top) and Kissinger (bottom) plots for the thermal decomposition (first stage) of PAAG-1.

7.3.2. Thermal degradation of n-alkyltrimethylammonium poly(γ-glutamate)s. The TGA traces for the series of *n*ATMA·PGGA showing the mass loss as a function of temperature recorded at a rate of 10 ºC·min^{-1} are compared in Figure 6a.

All they display a main decomposition step taking place within the temperature range of 250–285 ºC, the maximum decomposition rate temperatures, $^{m}T_{d}$, slighttly increasing with *n* values. Conversely, mass losses taking place in this stage were observed to decrease slightly with the size of the alkyl side chain.

For all complexes a second decomposition step involving a minor amount of weight loss appeared at steadily increasing temperatures from 315 up to 385 ºC for increasing values of *n*, as depicted in the inset of Figure 6a.

The residual material remaining at the end of the whole degradation process amounts 5-6 % of the original mass for all the complexes. The two decomposition stages become clearly discriminated in the respective derivative curves, as it is illustrated in Figure 6b for the case of 18ATMA·PGGA.

By analogy with the TGA trace recorded for PGGA, the main decomposition step of *n*ATMA·PGGA should be made to correspond to the decomposition of the polypeptide main chain. In fact, the ^{1}H NMR analysis of the gases released upon heating 18ATMA·PGGA at 230 ºC were found to be pyroglutamic acid (spectrum not shown). The ^{1}H NMR spectrum of the residual material revealed that it consisted mainly of octadecyltrimethylammonium hydroxide (Figure 7) indicating that decomposition of the complex takes place simultaneously to depolymerization of the main chain.

Since *n*ATMA·PGGA complexes are known to retain a significant amount of strongly bounded water, the formation of *n*ATMA·OH in the thermal decomposition of the *n*ATMA·PGGA complexes may be explained by assuming the concourse of the absorbed water in the decomposition process.

A minor amount of octadecyldimethylamine and octadecylmethylamine is observed together with the ammonium salt in the spectra shown in Figure 7b. Decomposition of *n*ATMA·PMLA showed concomitances with the first decomposition stage observed for surfactant salt 18ATMA·Br. The pyrolysis of this compound is known to occur in two steps taking place at 280 ºC and 370 ºC, respectively.[19] The first stage is a complex process entailing approximately (70-80%) of mass loss and leaving a residue composed mainly of a mixture of methyl and dimethyloctadecylamines. These amines evaporate slowly upon further heating so that the residual material left at 350 ºC is composed almost exclusively the less volatile octadecylmethylamine.

Thermal decomposition of the trimethyloctadecylammonium hydroxide is expected to proceed by a reaction mechanism based on the Hofmann elimination reaction with generation of trimethylamine and 1-octadecene.

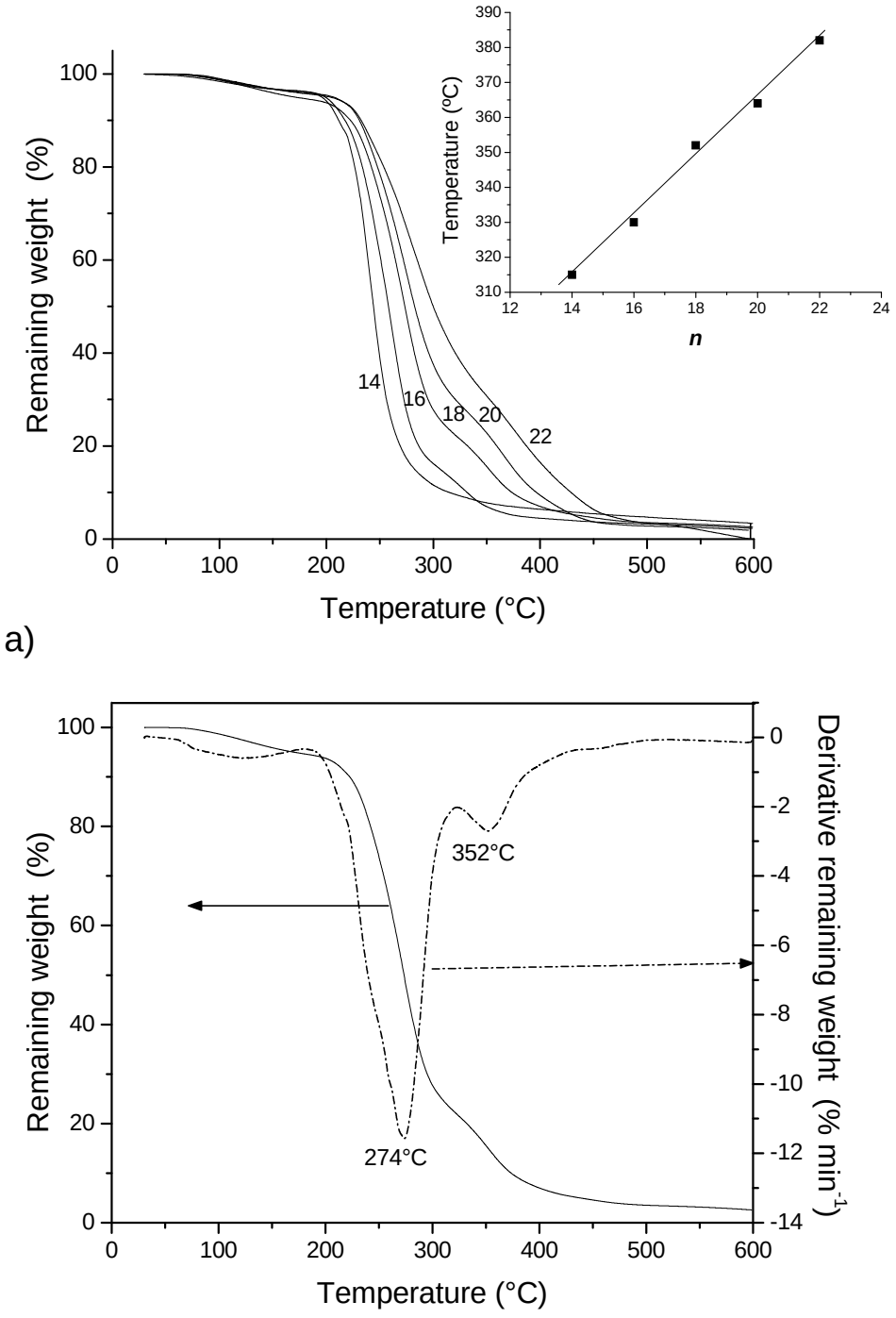


Figure 6. a) TGA traces of *n*ATMA·PGGA complexes recorded at the heating rate of 10 ºC·min^{-1} (*n* values indicated at labels) and (inset) maximum rate decomposition temperature in the second decomposition step of *n*ATMA·PGGA complexes plotted against *n*. b) TGA trace of 18ATMA·PGGA and its corresponding derivative curve.

The presence of this alkene was detected in the volatiles released upon heating the complex at 260 ºC, which evidenced the occurrence of the Hofmann reaction during the second decomposition stage (Figure 7c). The residual material left after this treatment consisted of a complex mixture of nitrogenated compounds of difficult spectroscopic assignment.

This process occurs above 300 ºC and is though to be caused by cracking of the nitrogenated products left after the second stage. On the basis of the results obtained in this study, a comprehensive mechanism can be reasonably proposed for the decomposition of *n*ATMA·PGGA complexes (Scheme III), this mechanism is essentially similar to that proposed for the thermal decomposition of *n*ATMA·PMLA complexes which was investigated by us recently.[19]

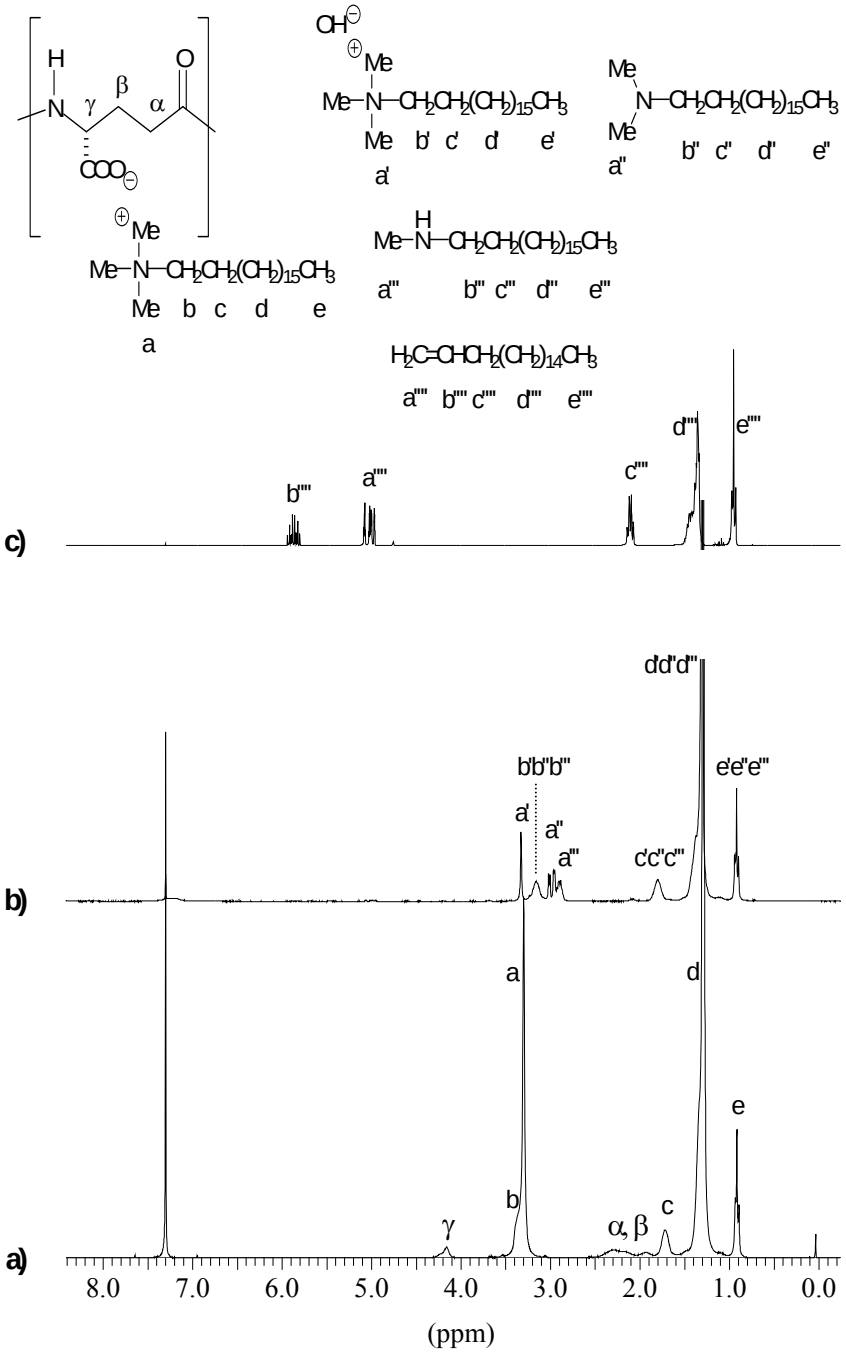
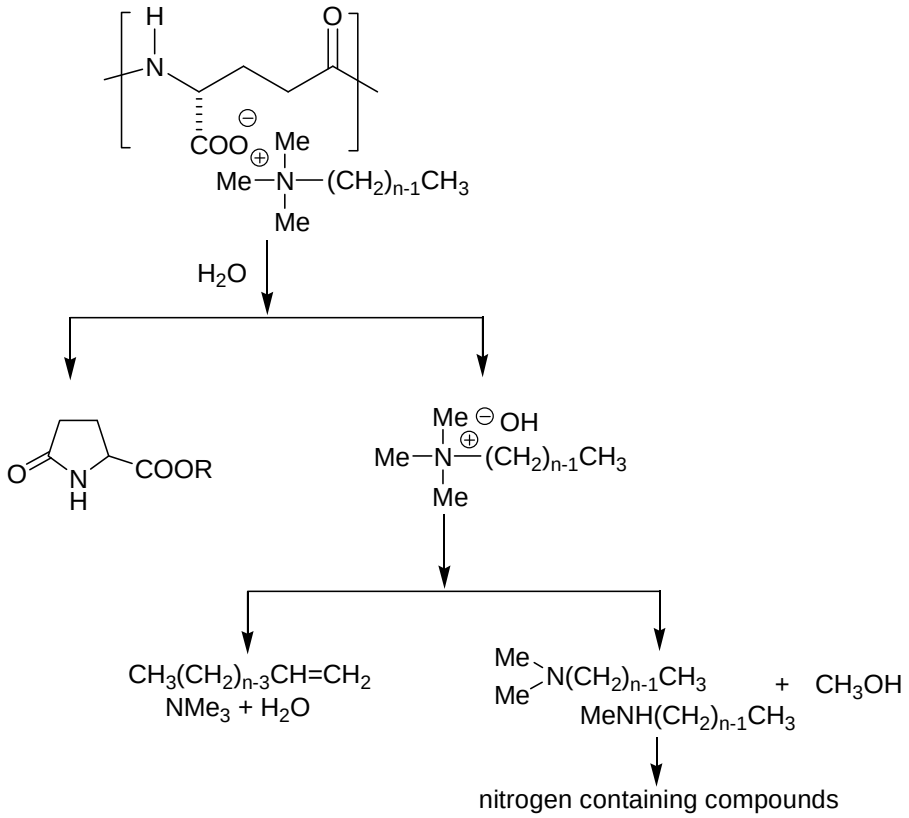


Figure 7. [1]H NMR spectra of 18ATMA·PGGA. a) Original product. b) Residual products left after heating at 250 ºC. c) Gases released upon heating at 280°C.



Scheme III. Mechanism of thermal decomposition of ionic complexes *n*ATMA·PGGA.

7.4. Conclusions

The thermal degradation processes that occur upon heating poly(glutamic acid) and polyglutamates have been characterized by combining experimental data afforded by TGA, GPC, FTIR and NMR spectroscopy. The underlying molecular mechanisms have been partially unveiled. The main conclusions derived from this study are the followings:

(a) Decomposition of poly(γ-glutamic acid) (PGGA) and poly(α-methyl γ-glutamate (PAAG-1) by heating under inert atmosphere starts at temperatures near 250 ºC and evolves an unzipping depolymerization mechanism with generation of pyroglutamic and methyl pyroglutamate, respectively. The activation energies measured for the thermal decomposition of PGGA and PAAG-1 are very similar with values within the 100-110 KJ·mol^{-1} in full agreement with the occurrence of back-biting depolymerization mechanism common for the two polymers.

(b) Thermal decomposition of *n*ATMA·PGGA complexes proceeds along two differentiated steps. Firstly, decomposition of the polyglutamate involving cyclodepolymerization of the main chain together with decomposition of the ionic complex promoted by absorbed water takes place. In the second stage, the quaternary alkyltrimethylammonium hydroxide formed in the first stage decomposes through a Hoffman elimination reaction and formation of alkyldimethyl and alkylmethylamines.

(c) It can be concluded with general validity for poly(γ-glutamic acid) and poly(γ-glutamate)s that the cyclodepolymerization reaction of the polypeptide main chain is the basic molecular mechanism operating in the thermal decomposition of these compounds when heated under a inert atmosphere. This behaviour reflects the strong tendency displayed by the polybutyramide chain towards cyclization, which is known to be a consequence of the high stability of the 5-membered lactam that is generated in the process. In the present case the tendency is enhanced by the attached carboxyl group, which favours the cyclization reaction by entropy reasons.

7.5. References

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