Influence of the Molecular Weight on the Inverse Temperature Transition of a Model Genetically Engineered Elastin-like pH-Responsive Polymer

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Abstract

The pH-responsive elastin-like polymers, $[(PGVGV)_2-(PGEGV)-(PGVGV)_2]_n$, ($n=$ 5, 9, 15, 30, 45), were obtained using genetic engineering and microbial protein expression to study the effects of the molecular weight (MW) on the properties of their inverse temperature transition (ITT) and its dependence on pH. As a result, the transition temperature decreased and the transition enthalpy increased as the molecular weight increased, especially for the lowest MWs. More strikingly, the apparent pKa for the γ-carboxyl residue of the glutamic acid also depends on MW. The apparent pKa is lower for lower MWs. In summary, the modification in the ITT caused by changes in MW is similar than the one caused by changes in the mean polarity of the polymer as described in the literature. A reduction in the molecular weight is equivalent to a decrease in the mean hydrophobicity of the polymer.

Introduction

In the last years, the interest in protein-based polymers ("PBPs") is rapidly growing. This is especially true nowadays, where the incorporation of recombinant DNA technologies has revolutionized the design and production of novel PBPs[1]. From the point of view of macromolecular science, the use of tailored DNA templates as the base of their bioproduction provides a tight, practically absolute, control of the sequence and polymer architecture, with the inexistence of randomness in amino-acid stereochemistry, comonomer arrangement and molecular weight (MW). In this manner, the obtaining of complex and monodisperse polymers with a desired sequence and molecular architecture is possible in a quite natural way[2-4]. Additionally, the advantages of bioproduction include its environmental-friendly bioproduction and disposal[1,5].

Among PBPs, elastin-like polymers (ELPs) are emerging as a new class of polymers with exceptional properties[6]. These include mechanical properties ranging from excellent elastomers to plastics, outstanding biocompatibility[7] and acute smart behavior. This last attribute is caused by the so called "inverse temperature transition" (ITT). ITT has become the key issue in the development of new peptide-based polymers as molecular machines and materials. The understanding of the macroscopic properties of these materials in terms of the molecular processes taking place around the ITT has established a basis for their functional and rational design. Recently, the increased knowledge in the many aspects about the ITT has allowed the systematization and compilation of these topics in a set of five axioms[8]. Moreover, the usefulness of this set of axioms is not restricted to the design of new advanced materials since they can be used, for example, to understand the relationship between folding and function in native proteins or the principles behind amphiphilic macromolecular assemblies[8].

All of the functional elastin-like polymers exhibit phase transitional behaviour associated to the ITT[6]. In aqueous solution and below a certain critical temperature (T_t), the free polymer chains remain disordered, random coils in solution[9] that are fully hydrated, mainly by hydrophobic hydrationr[8,10]. On the contrary, above T_t, the chain hydrophobically folds and assembles to form a phase separated state[11] in which the polymer chains adopt a dynamic, regular, non-random structure, called a β-spiral, involving one type II β-turn per pentamer, and stabilized by intra-spiral inter-turn and inter-spiral hydrophobic contacts[6]. This is the product of the ITT. During the initial stages of polymer dehydration, hydrophobic association of β-spirals takes on fibrillar form that grows to a several hundred nm particle before settling into the visible phase separated state[6,12].

The design of smart polymers, responding to stimuli different than temperature changes, makes use of the "ΔT_t mechanism" and "amplified ΔT_t mechanism" [6,13-15]. This is also the case for the polymers studied in this work in which the γ-carboxylic function of the glutamic acid suffers strong polarity changes between its protonated and deprotonated state as a consequence of changes in pH around its effective pKa.

Taking advantage of the essentially monodisperse nature of these genetically engineered polymers, the influence of MW on the characteristics of the ITT displayed by this model polymer will be studied in this work. The effect of MW on T_t has been previously addressed by Meyer and Chilkoti[*1*] taking advantage of the development of a new gene construction based on recursive directional ligation in the production of unfunctionalized ELPs based on the sequence VPGXG, whith X= V, A or G.

Experimental section

Materials. *E. coli* strain BLR(DE3) and pET-25(+) were obtained from Novagen (Madison, WI). *Taq* DNA polymerase was purchased from Stratagene (La Jolla, CA),

T4 DNA ligase and all restriction enzymes were obtained from New England Biolabs (Beverly, MA). Synthetic oligonucleotides were purchased from IBA GmbH (Goettingen, Germany).

Synthetic gene construction. Cloning and molecular biology techniques were performed using standard procedures[16,17] and the sequence of all putative inserts was verified by automated DNA sequencing. A synthetic DNA duplex encoding the oligopeptide $(VPGVG)_2VPGEG(VPGVG)_2$ was generated by Polymerase Chain Reaction (PCR) amplification using synthetic oligonucleotides. The gene cloning, concatemerization and colony screening were performed as described in Ref. X*.

Expresion and Purification. Selected genes were subcloned into a modified pET-25(+) expression vector and transformed into the *E.coli* strain BLR(DE3). Expression conditions and the purification protocol are described in Refs. 18 and 5, respectively.Production yields for the five polymers of general formula $[(VPGVG)_2VPGEG(VPGVG)_2]_n$ selected for this work (n= 5, 9, 15, 30 and 45) ranged between 32 and 92 mg/L of culture, depending on the molecular weight. The final products were characterised by SDS-PAGE, MALDI-TOF mass spectrometry, NMR and amino-acid analysis. All the analysis confirms the correctness of the biosynthesis process in terms of sequence and MW.

Differential Scanning Calorimetry (DSC). DSC experiments were performed on a Mettler Toledo 822^e with liquid-nitrogen cooler. Calibration of both temperature and enthalpy was made with a standard sample of indium. For DSC analysis, 50 mg·mL^{-1} phosphate buffered (0.1 M) water solutions of the polymers were prepared at different pHs. In a typical DSC run, 25 μL of the solution were placed inside a standard 40 μL aluminium pan hermetically sealed. The same volume of water was placed in the

reference pan. All DSC samples were pretreated 15 minutes at 5 ºC inside the sample

chamber just before the beginning of the experiment.

Results and Discussion

Figure 1 shows the DSC analysis of the five polymers in phosphate buffer at pH

2.5. This pH is considered to be low enough to yield a complete protonation of all γ-

carboxyl residues in the polymers. Those thermograms show the typical endotherm

associated with the ITT.
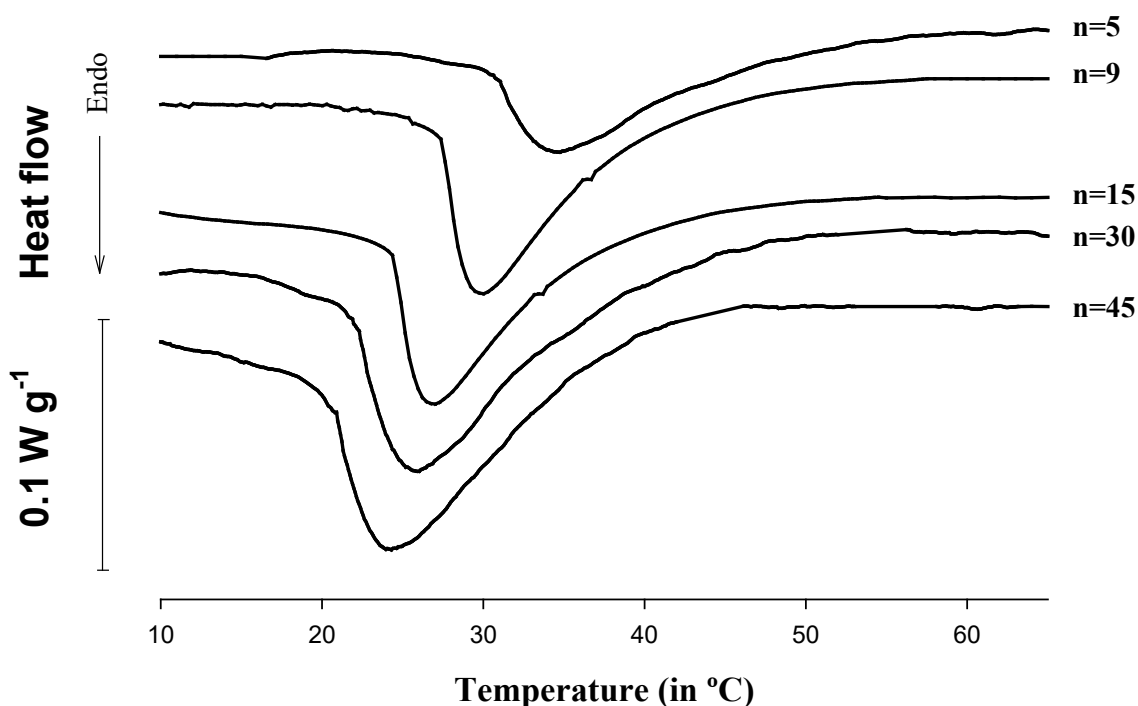
Figure 1. DSC thermograms of 50 mg·mL^{-1} phosphate buffered (0.1 M, pH 2.5) water
solutions of the studied polymers. Their polymerization degree (n) is shown on the right
side of the plot.

According to previous work, these endotherms are the net result of two concurrent

events with distinct thermal contribution; the endothermic component due to loss of

hydrophobic hydration and an oppositely signed exothermic component due to the

physical association of chains; being the endothermic component predominant[18].

Qualitatively, we can appreciate from this figure some differences between the five MWs. In general, the peak temperature of each endotherm, which can be identified with the transition temperature at this pH (T_{t0}), tends to be higher for lower MW. On the contrary, the transition enthalpy at this same pH (ΔH_0) tends to increase with the increasing MW. Due to the dynamical nature of the DSC experiments, while ΔH_0 values are not affected by the dynamical conditions of the DSC experiment, those T_{t0} values are affected by thermal lags[19]. In addition, although not likely, some differences could happen on the C_p values among the different polymer samples. That would give rise to different thermal lags among the samples making the comparison of the observed T_{t0} values not satisfactory. To evaluate the effect of this possible artefact on our data, we have estimated the so-called true T_{t0} (T_{t0}^{true}) for the five MWs studied. According to literature, this temperature must be understood as the true physical point of the transition[19] and adequate values of T_{t0}^{true} can be obtained by extrapolation of the linear sections of the resulting plot of T_{t0} vs. the square root of the heating rate ($v^{1/2}$) to $v=0$ (see Ref. 19). The results of this analysis are given in figure 2 and a plot of T_{t0}^{true} vs

MW can be seen in figure 3. As suspected from the qualitative analysis of the DSC
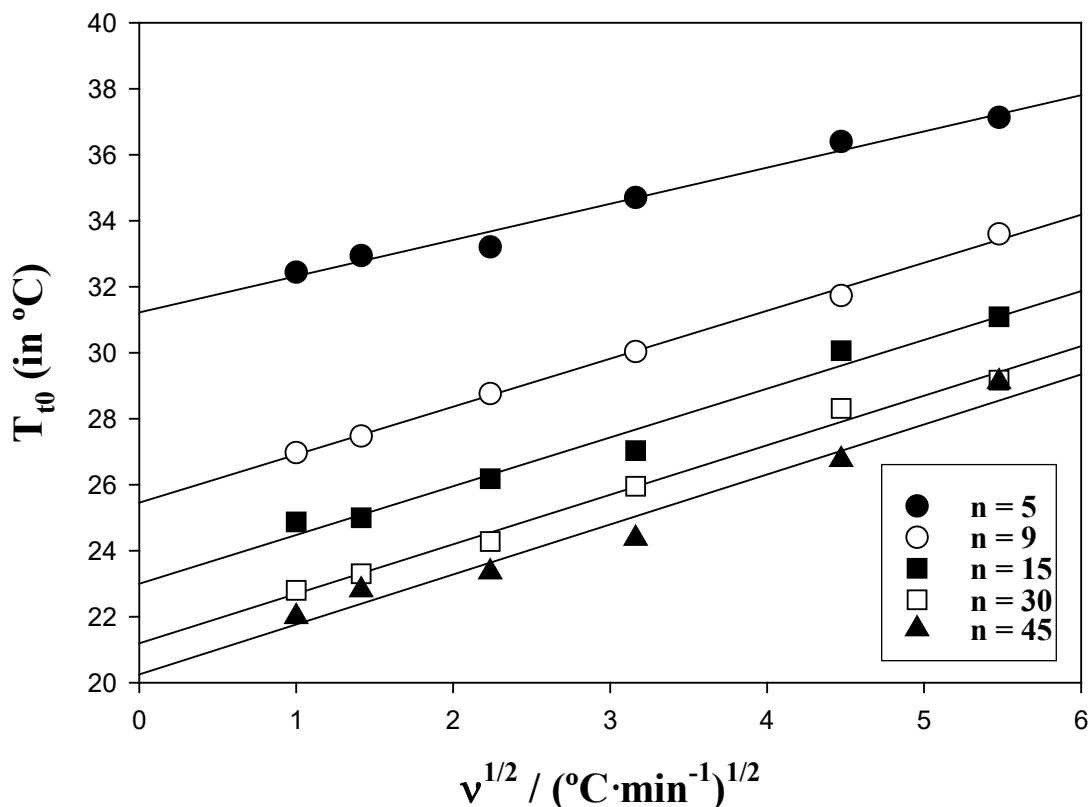
Figure 2. Dependence of T_{t0} on the square root of the heating rate for the studied polymers. The corresponding polymerization degree (n) is indicated in the plot. The lines represent the least square linear regressions of the data for each n.

results, the dependence of T_{t0} on the polymer MW was not an artefact. There is a strong

dependence that scores a difference of about ten degrees between the lowest (n=5) and

highest molecular weight (n=45) tested in this work, being T_{t0} lower for higher MWs.

This dependence is not linear. The strongest variations in T_{t0} are found for the lowest

MWs. Figure 3 also shows the dependence of ΔH_0 on MW for the samples in phosphate

buffer (pH=2.5). As it happened with T_{t0}, there is also a clear dependence of this

parameter on MW; ΔH_0 decreases in a non-linear manner as MW is smaller. The highest

variations are found again for the lowest MWs.
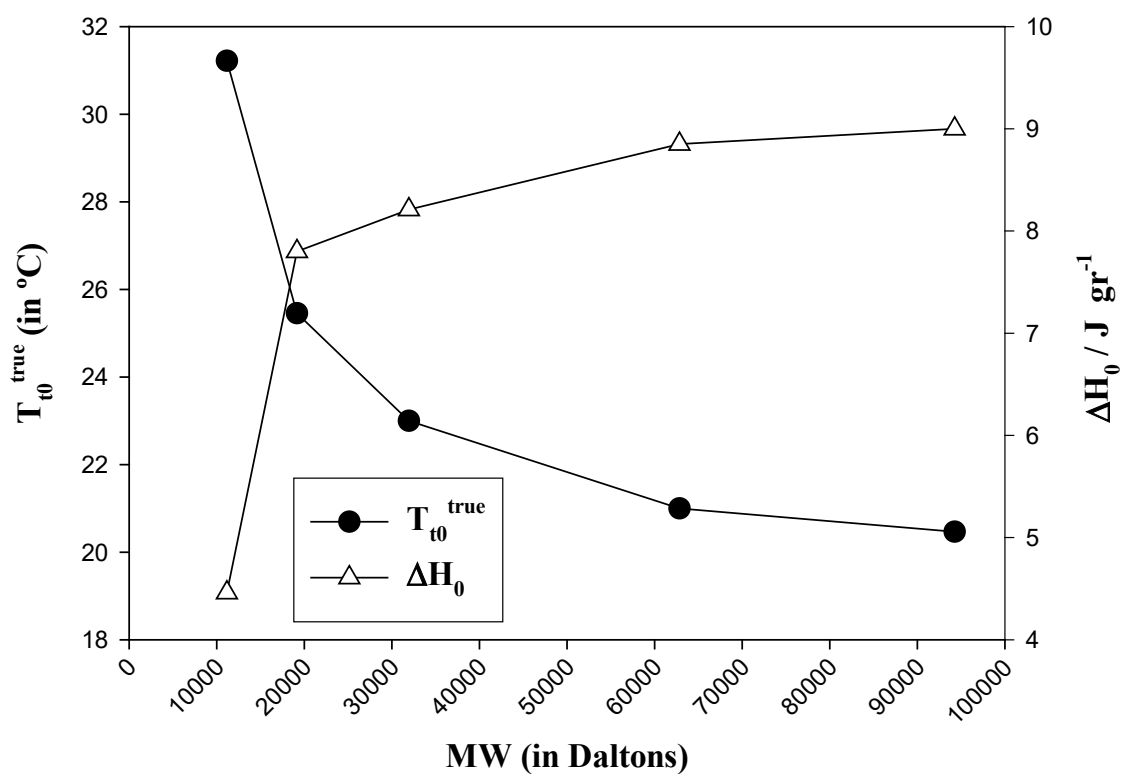
Figure 3. Dependence of T_{t0}^{true} and ΔH_0 on the molecular weight.

It is of interest to study the influence of the pH on the ITT (T_t and ΔH) for this set of polymers. To do this, DSC analysis of phosphate buffered solutions has been carried out for pH ranging from 2.0 to 5.5-6.0. The results for T_t as a function of pH are plotted in figure 4.
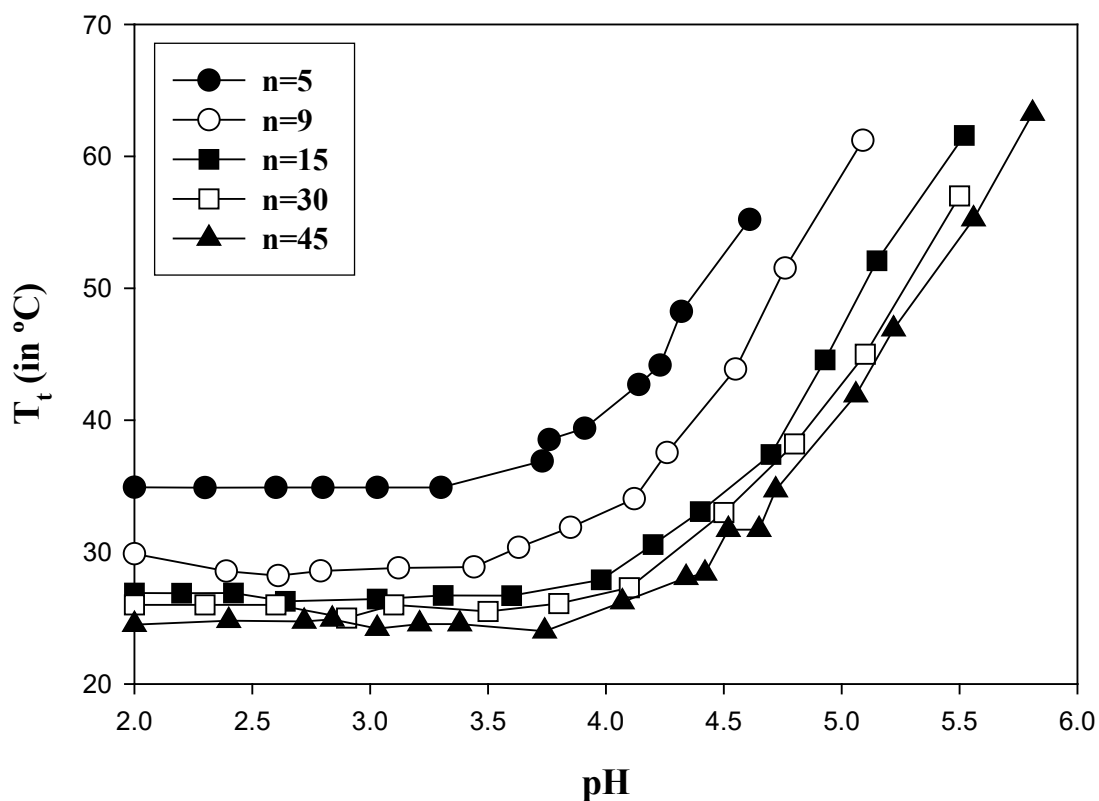
Figure 4. Dependence of T_t on the pH for the studied polymers (as indicated in the plot).

The five polymers showed a similar trend. Up to a given pH, T_t values remain practically constant. Above this particular pH the transition temperature rapidly raises. A parallel inverted behavior is found for ΔH; the plot ΔH vs pH can be seen for the polymers with extreme MWs; n=5 and 45 (the other polymers are not plotted for clarity purposes)(Figure 5). Again, ΔH remains rather constant below the characteristic pH. However at this given pH the enthalpy rapidly drops to zero. This behavior is the expected according to the literature[6] and can be easily explained. As a result of the increase in the pH, the γ-carboxyl group of the glutamic acid starts to become deprotonated. This deprotonation yields an increase in the mean polarity of the polymer which causes a T_t shift to higher temperatures and a drop in ΔH, since this parameter

depends primarily on the amount of hydrophobic hydration around the polymer and this hydration mode is not possible in the vicinities of the charged carboxyl[6]. Thus, the pH pointing the onset of the increase in T_t and the decrease in ΔH is related with the onset of the γ-carboxyl deprotonation. As it can be seen in figure 5, this pH is different for the different   polymers.
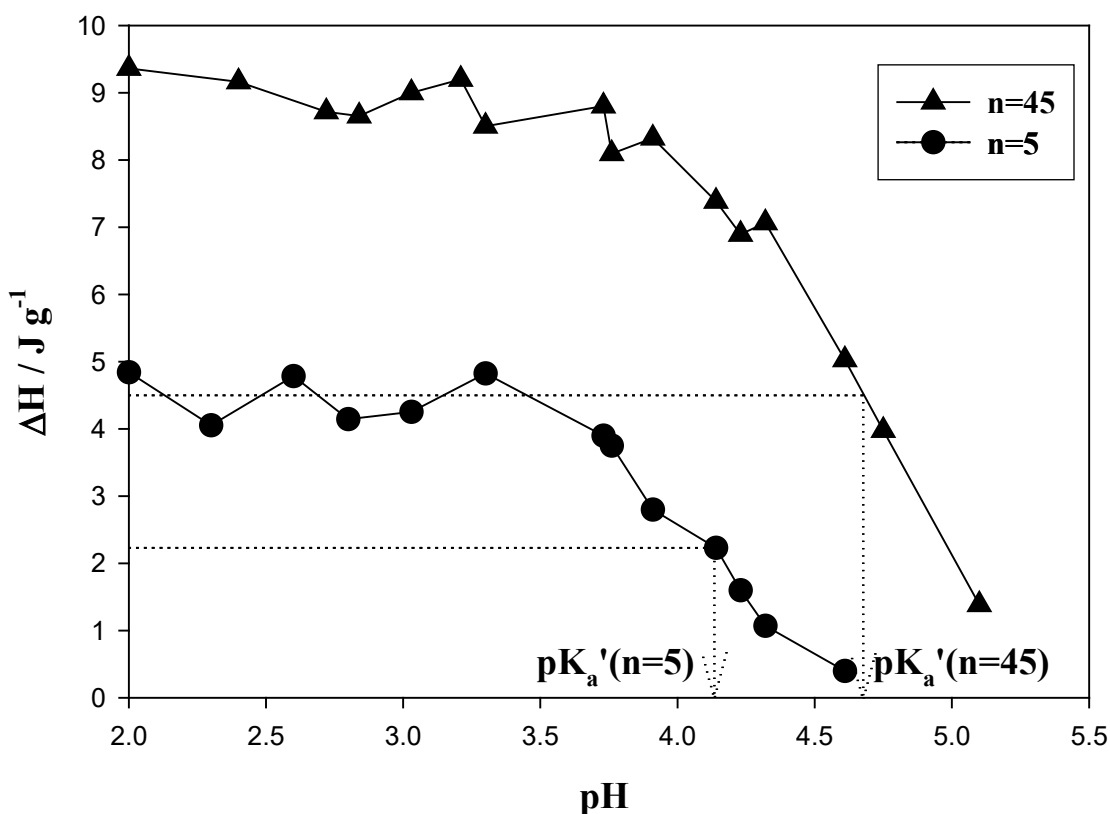
Figure 5. Dependence of ΔH_0 on the pH for the polymers with N= 5 and 45 (as indicated in the plot). Dotted lines illustrate the procedure to estimate the apparent pK_a values (pK_a') for the γ-carboxyl group of the glutamic acid (see text).

Strikingly, this fact would imply that the equilibrium constant for the protonation-deprotonation of the γ-carboxyl group would depend on the MW. We have estimated the apparent pK_a (pK_a') of the γ-carboxyl group as the pH yielding a 50% drop in ΔH, which, according to the molecular interpretation of ΔH, can be roughly considered as

the point with 50% of deprotonated carboxyls. The dependence of pK$_a$' on MW can be seen in figure 6. Again, the lowest MWs showed the strongest pKa shifts to lower values, ranging from 4.13 for n=5 to 4.68 for n=45.
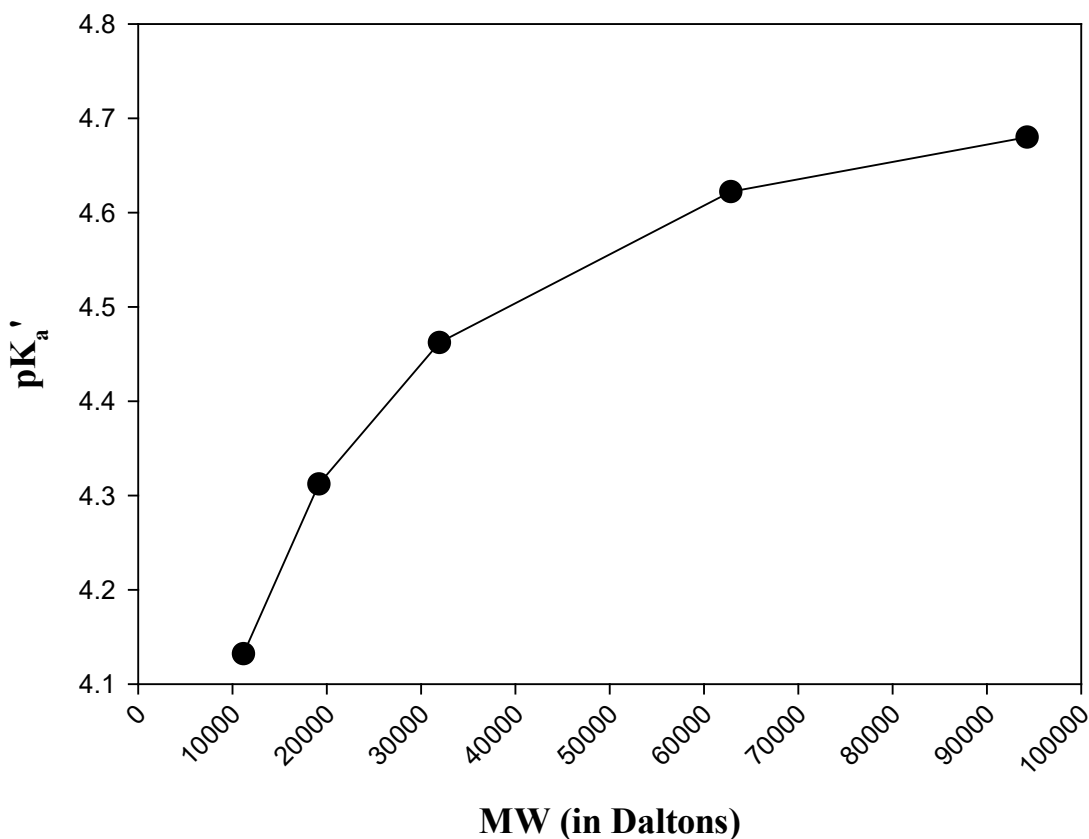
Figure 6. Dependence of pK$_a$′ for the γ-carboxyl group of the glutamic acid on the molecular weight.

This is not the first reported case where a pK$_a$ value of a free carboxyl moiety is shifted as a consequence of changes in the intrinsic characteristic of the elastin-like polymer where this carboxyl is present. In ref. 20, the effect of increasing the mean hydrophobicity in a sequence of polymers with formulas poly[0.8(PGVGV), 0.2(PGEGV)], poly [0.8(PGVGI), 0.2(PGEGI)] (I stands for isoleucine) and poly[0.75(PGFGV), 0.25(PGEGV)] (F stands for phenylalanine) caused 1.7 units pKa positive shift. In a later work from the same authors, by appropriate nanometric design

of the polymer compositions, hydrophobic-induced pKa shifts for aspartic acid as high as 6.2 units have been reported[21]. As compared the pKa shifts found in this work as a consequence of changes in the MW and those found as consequence of changes in the mean polarity of the polymer, again a clear similarity between MW and the mean hydrophobicity is found. The pKa shift observed as MW increases is equivalent to that caused by an increase in the mean hydrophibicity, in a similar way to that previously found for T_{t0} and ΔH_0.

Concluding Remarks

In summary, from the study of this set of model elastin-like polymers one can conclude that MW has an evident influence in the most outstanding molecular characteristic of this kind of smart polymers, the ITT. The decrease in MW causes an increase in T_t, a decrease in ΔH and a pKa shift to lower values. All these effects have been already reported for other elastin-like polymers as being caused by a reduction of the mean hydrophobicity of the polymer by selected substitution of the amino acids in the polymer chain. Therefore, there is certain equivalence between polymer length and polymer hydrophobicity. This equivalence can be partially explained by the influence of the polar end groups, being this influence higher for lower molecular weights. Thus, the mean hydrophobicity would be lower for lower MWs. However, the sole effect of the polarity of the chain ends seems not to be enough to account for the strong influence reported in this work. We believe that a large part of the effect of MW in the ITT is caused by the inter and intrachain cooperativity of the hydrophobic self-assembly taking place in the ITT[9]. In this sense, it is reasonable to think that short chains do not show an efficient cooperativity so their self-assembly is hindered, just like the presence of polar

moieties in longer polymer chains hinders the characteristic hydrophobic folding and self-assembly during the ITT.

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