Ethylene Oxide Based Copolymers Functionalized with Terminal Alkynes: Structure Influence on their Micelle Formation

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Abstract

Copolymers from ethylene oxide (EO) and propylene oxide (PO) functionalized with glycidyl propargyl ether (GPE) are synthesized. The GPE allows further attachment of drugs but its influence on the polymeric micelle formation is unknown. In this work, the influence of the structure of these copolymers on their critical micellar concentration (CMC) in water and in the size and stability of obtained micelles is studied. For this purpose, the presence of GPE, the copolymer type (gradient or block), the EO:PO ratio and the initiator (water or ethanol) are modified. Gradient copolymers can be synthesized in a single step thanks to the different monomers reactivity, simplifying the process and obtaining similar CMC values to the block copolymers. The use of ethanol as initiator decreases the block copolymer CMC and increases the polydispersity. Besides, the presence of the GPE does not impede the micelle formation and has low effect on the copolymer CMCs. Finally, the higher the EO:PO ratio, the higher the CMC and the smaller the size of micelles. Moreover, Z-potential, DLS and HRSEM analyses show that the micelles are stable, spherical, capable to incorporate coumarin (a hydrophobic drug) and with apparent hydrodynamic sizes suitable to be absorbed by target cells.

Keywords: ethylene oxide; glycidyl propargyl ether; gradient copolymers; block copolymers; CMC; drugs delivery.

1. Introduction

One of the most important achievements in the field of polymer chemistry is the development of tailor-made copolymers with specific structure, chemical composition, functionality and low polydispersity index. The advantages of these copolymers come from the possibility to present properties from both of the combined homopolymers. The wide range of applications of block copolymers in advanced technologies such as nanotechnology [1] and biomedicine [2] has promoted their huge development in the last decades.

In order to control the characteristics of the copolymers, the ‘‘living” polymerization procedures were developed from the middle 50s, after the pioneering work of Szwarc in anionic polymerization [3]. Anionic ring opening polymerization (AROP) is used in this work since it allows the controlled synthesis of macromolecules, enabling their further functionalization [4].

The commercially available linear poly(ethylene oxide)-poly(propylene oxide) (PEO-PPO) block copolymers, also known as Pluronics®, have acquired significance due to their capability to load drugs, execute the delivery of the drugs on the site of action, improve the pharmacokinetics of the loaded drugs and reduce off-target cytotoxicity [5]. The presence of PEO (hydrophilic) and PPO (hydrophobic) blocks in the same molecule, is expected to allow the self-assembly of the copolymers in water, forming thermodynamically stable micelles, and improving the solubility of the hydrophobic drugs [6]. Besides, the ease modification of the amphiphilic character of this copolymers type, just varying the proportion between the PEO and PPO blocks [7], together with their biocompatibility and biodegradability [8], make them, and their derivatives, suitable for products formulation in industries ranging from agriculture to pharmaceuticals and controlled release of drugs [9, 10].

Due to all the mentioned before the main aim of this research is the design of a tailor-made polymeric drug carrier derivate from PEO-PPO copolymers, capable of incorporating poor soluble drugs in aqueous solution to improve the treatment of cancer and other human diseases in the future. Polymeric micelles are one of the most promising nanocarriers [11-13] since drug encapsulation in micelles may greatly increase aqueous concentrations of bioactive compounds in blood, prevent them from degradation during circulation and minimize the secondary effects of drugs. In addition, micellar

encapsulation is expected to favor the access to the target thanks to the enhancement of permeability and retention effect [14-16]. But, moreover, the synthesis of polymer-drug conjugates using click chemistry to the covalent attachment of the drugs in the copolymer chain, is another of the most currently used methods [17, 18] due to the several advantages that it provides, such as extended half-life in the blood, long-acting, simple administration and the decrease of the side effects of drugs, among others.

The introduction of a known amount of GPE (that contains an alkyne group) in the copolymer chain allows to obtain alkynyl-polyethers prepared for future click attachment of the hydrophobic drugs through the reaction between an azide group of the drug and the alkyne groups of the designed copolymers catalyzed by copper as in previous research [19, 20]. This way, the drugs can be covalently attached to the PEO-GPE-PPO copolymers with the objective to enlarge their lifetime in blood.

In the present work, tailor-made copolymers adding the monomers separately in three steps were synthesized, starting with the EO and finishing with the PO in order to leave the GPE in the middle of the copolymer chain. This way, copolymers with perfectly separated blocks or block copolymers were synthesized by sequential addition of the monomers. Additionally, considering the higher reactivity of the EO compared with that of the PO and GPE [21, 22], and in order to simplify the synthesis procedure, all the monomers were added in a single step. The objective of the single step process was to see if the difference between the monomers reactivity was high enough to allow the formation of gradient copolymers, with a gradual variation in the monomer distribution along their chains that allows them to present two domains with different hydrophilicity (a mainly hydrophilic domain and other mainly hydrophobic) [23, 24]. The capacity of the synthesized gradient copolymers to form stable micelles was also studied and compared to those of the block copolymers.

The study of the influence of the GPE presence on the critical micelle concentration (CMC) value and the comparison between the CMC of block and gradient copolymers of this copolymer type have not been reported before, being two of the key results that justify the novelty and interest of the manuscript.

The structure of the copolymers was also modified by changing the polymerization initiator (water or ethanol) and the EO:PO ratio. Then, this research was completed with the study of the influence of the copolymers structure (polymer type, the EO:PO ratio and

the polymerization initiator) on essential characteristics for the application of this kind of polymers as drug carriers such as the CMC, micelle size, stability and drug incorporation capacity were analysed. The drug capacity incorporation was also analysed using coumarin as hydrophobic drug.

Therefore, the topic of the work is of the highest importance since the understanding of the structure influence of the these copolymers type on the evaluated properties is basic for the further applications in drug delivery systems, which is a research field of major interest nowadays.

2. Experimental

2.1. Materials

Propylene oxide (PO; 99.9%, Praxair), ethylene oxide (EO; 99.8%, Fluka) and glycidyl propargyl ether (GPE; ≥ 97%; Chemos) were used as monomers. Potassium hydroxide (KOH; 90%, Scharlau) was used as catalyst. Ethanol (EtOH; 99.9%; Merck), anhydrous ethanol (absolute; VWR) and Milli-Q water were used as initiators and nitrogen (N_2; 99.999%; Praxair) was used as inert agent for the synthesis of the different copolymers. The hydrophobic drug used in the preparation of loaded micelles was 4-bromomethyl-7-methoxycoumarin ($C_{11}H_9BrO_3$; 97%; Acros Organics). These reagents were used without further purification. Milli-Q water was used as medium to prepare the copolymer solutions for the CMC determination and for the drug incorporation in polymeric micelles experiments.

2.2. Copolymer synthesis procedure

The PEO-GPE-PPO copolymers were synthesized through a three steps nucleophilic ring-opening polymerization or just in a single step, feeding all the monomers at the same time in the gradient copolymers case. All the polymerization reactions were carried out in a 0.6 L high-pressure reactor (Büchi BEP 280 type III, Switzerland), with digital control of stirring rate, temperature and pressure.

Regarding the polymerization in three different steps, in the first step, the initiator solution was separately prepared in a beaker; for that, H_2O or EtOH as initiators were added with KOH as catalyst (molar ratio of the catalyst-to-initiator = 0.1 and initiator-to-monomer = 0.02) to achieve a homogeneous mixture. KOH was used as catalyst since it is compatible with both monomers, EO and PO, and its activity and purification are well established

[25]. The initiator solution was introduced in the pressure reactor and, subsequently, EO was also fed.

This mixture was then pressurized with nitrogen ($3 \cdot 10^5$ Pa) in order to work under inert atmosphere; it was stirred at 1000 rpm and heated up to 80 ^{0}C. At the beginning of the EO polymerization reaction (Figure 1a), the pressure reactor increased as the temperature rose until the moment in which the set point temperature was reached; then, the pressure began to decrease as the monomer was being consumed. Finally, the initial value of pressure was achieved, indicating the end of the reaction. Lastly, in order to remove residual monomer, high vacuum (4000 Pa) was applied for 3600 s. The vacuum was controlled using a Divatronic DT (Köln, Germany) digital vacuum indicator-controller, acting on a solenoid valve.

In the second step, terminal alkyne groups were introduced in the polymer chain of PEO previously formed through the reaction shown in Figure 1b. In this step, GPE was fed into the reactor together with catalyst (but without initiator to avoid the formation of new chains). Molar ratio PEO:GPE = 1:1 was used in all the studied reactions. This mixture was pressurized with nitrogen, stirred and heated up to 120 ^{0}C to activate this monomer. In this case, the pressure reactor did not increase, so the reaction time was determined by taking samples at different times, finding the total consumption of GPE after 12600 s.

In the third step, the catalyst and the corresponding amount of PO to end the copolymer were fed into the pressure reactor and were led to the same reaction conditions that in the previous step (120 ^{0}C), but in this case, the pressure evolved as in the first step and high vacuum was also applied at the end of the reaction. Mass ratio EO:PO was modified in the different synthesized copolymers with the values 0.35:0.65, 0.5:0.5 and 0.65:0.35. The scheme of this reaction step is shown in Figure 1c.

In addition, PEO-GPE-PPO gradient copolymers were synthesized by adding all the monomers (EO, PO and GPE) at the same time in the reactor, simplifying the synthesis procedure to a single step reaction. These reactions were carried out in the same high-pressure reactor and with the same amounts of KOH as catalyst and H_2O or EtOH or anhydrous ethanol as initiator. The employed conditions were 120 ^{0}C with stirring rate of 1000 rpm and inert atmosphere. In order to avoid the presence of water impurities in the reactions initiated with anhydrous EtOH, the monomers were dried under high vacuum for 3 hours. As indicated before, it is expected a difference between the monomers

reactivity [21, 22, 26] enough to allow the formation of two polymeric domains with different hydrophilicity (gradient copolymers). According to literature, the reactivity ratios are r_{EO} = 3.1 and r_{PO} = 0.3 for the copolymerization reaction of EO and PO [26], which are the main monomers implicated; and r_{EO} = 14.8 and r_{GPE} = 0.076 for the copolymerization of EO and GPE [21]. These reactivity ratio values support our hypothesis of having gradient copolymers since it is known that when the reactivity ratios differ by a factor of around 10, the obtained copolymers behaves similar to block copolymers thanks to a tapered structure [27].

Considering the reactivity ratios and the monomer proportion in the mixture, it can be calculated the probability of adding a PO or GPE molecule to the chain when the end is an EO molecule and vice versa. This probability is expressed by equations 1 and 2 [27, 28]:

$$P\left({}^{PO}/_{EO}\right) = \frac{1}{1+r_{EO}.{}^{f_{EO}}/_{f_{PO}}} \quad (1)$$

$$P\left({}^{GPE}/_{EO}\right) = \frac{1}{1+r_{EO}.{}^{f_{EO}}/_{f_{GPE}}} \quad (2)$$

where P(PO/EO) and P(GPE/EO) are the probability of adding a PO or GPE molecule to the chain when the end is an EO molecule, respectively; and f_{EO}, f_{PO} and f_{GPE} are the monomer ratios on the reaction media.

The probabilities are calculated for the initial moment of the polymerization, obtaining initial values of P(PO/EO) and P(GPE/EO) of 0.24 and 0.005, respectively. They will increase with the monomer consumption since the kinetic of EO is faster and, thus, the proportion in the reaction media will vary. This will cause the evolution of the copolymer composition throughout the copolymer chain, passing from high concentration of EO to high concentration of PO, expecting different domains.

2.3. Copolymer characterization

Gel Permeation Chromatography (GPC) was used to determine the number/weight average molecular weights (M_n and M_w) and polydispersity index (PDI) of the synthesized copolymers. Measurements were performed with a Viscotek chromatograph with two columns (Styragel HR2 and Styragel HR0.5) at 35 ^{0}C with a flow of 1 mL·min$^-$

[1] and THF as eluent. The calibration curves for GPC analysis were obtained with poly(ethylene glycol) standards (from Waters).

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) was used to verify the incorporation of GPE in block copolymers and to know the distribution chains and their accurate M_w. Measurements were carried out using a Bruker Autoflex II TOF/TOF spectrometer (Bremen, Germany) using dithranol (1, 8, 9-trihydroxyanthracene) as matrix material. Samples co-crystallized with matrix in a ratio of 100:1 on the probe were ionized in positive reflector mode. External calibration was performed using Peptide Calibration Standard II (covered mass range: 700-3200 Da) and Protein Calibration Standard I (covered mass range: 5000–17500 Da). ^{1}H NMR spectra were measured with a Varian Gemini FT-400 spectrometer using CDCl3 as solvent in order to confirm the incorporation of GPE in gradient copolymers. Tetramethylsilane was used as an internal standard for calibrating the chemical shifts.

2.4. Determination of the copolymers CMC

Several solutions with different copolymer concentrations were prepared with Milli-Q water as solvent and the concentration values are expressed in terms of %(w/v). Absorbance measurements for the different concentrations of the copolymers were taken at 36 ^{0}C, since this is approximately the corporal temperature and it is expected that the formed micelles will serve as vehicle of drugs for the treatment of human diseases in the future [29, 30].

Absorbance measurements were performed with the iodine-iodide (KI/I_2) method previously used by other authors [5], using Agilent 300cary series UV-visible with matched pair of stoppered quartz cells of 1 cm optical path length. This method is based on the color change of iodine that takes place when non-ionic association micelles are added to an iodine solution [31]. The 25 μL of KI/I_2 standard solution was added into each copolymer solution and equilibrated at 36 °C before each measurement. To prepare standard KI/I_2 solution, 0.5 g of iodine and 1 g of potassium iodide were dissolved in 50 mL of Milli-Q water. Absorbance at varying concentrations of each copolymer was measured at 366 nm in all cases. The absorbance of the copolymer solutions in water decreases with the increase of the copolymer concentration until a value from which the absorbance remains constant. This concentration value is considered the CMC of the copolymer.

2.5. Preparation of drug-loaded micelles

Coumarin was chosen as hydrophobic drug due to its many pharmacological properties such as anticancer agent among others [32]. The coumarin-loaded micelles were prepared according to bibliography [5]. An appropriate amount of PEO-PPO-GPE copolymer was dissolved in milli-Q water to obtain 1 w/v % aqueous solutions of copolymers at 36 ºC (concentration over their CMC values to ensure the micelles formation at this temperature). Then, coumarin was added to obtain desired drug to polymer ratio (1:10). The mixture was stirred vigorously with a magnetic stirrer and placed in a water bath to maintain the temperature in the desired value. Besides, to facilitate the drug incorporation in the micelles, the mixture of the drug and the copolymer in aqueous solution was agitated in an ultrasonicator for 3600 s. The resulting micellar solution was then centrifuged at 3800 rpm for 1 hour to separate the undissolved drug.

2.6. Micelles characterization

The entrapment efficiency (EE%) of PEO-GPE-PPO copolymeric micelles loaded with coumarin was determined by measuring the amount of free coumarin (unentrapped drug). The micellar solution and unentrapped drug were separated through centrifugation at 3800 rpm for 1 hour, and later, the amount of free coumarin was dried overnight at 100 ºC and weighed.

The EE% was calculated based on the amount of the drug encapsulated into the polymeric micelles versus the amount of drug initially added or total drug, using the equation 3:

$$EE\% = \frac{(Amount\ of\ total\ drug - Amou\quad of\ free\ drug\ )}{Amount\ of\ total\ drug} \times 100 \qquad (3)$$

The apparent hydrodynamic size of micelles and their zeta potential or stability were obtained using dynamic light scattering (DLS) measurements with a Zetasizer Nano ZSP of Malvern. The light source was 10mW He-Ne laser operating as a fixed wavelength (633 nm), with a scattering angle of 173° and a constant temperature of 36 ^{0}C (because this is the corporal temperature approximately). Each measurement was repeated at least three times.

The morphology of the micelles and their size were also checked using a Zeiss high resolution scanning electron microscope (HRSEM) operated at 2.00 kV and under high

vacuum conditions. Samples were prepared by depositing several drops of micellar solutions onto silicon wafers and were dried overnight.

3. Results and discussion

3.1. Structure and properties of PEO-GPE-PPO copolymers

The experiments carried out for studying the influence of the initiator, copolymer type (block or gradient) and EO:PO ratio on the micelle formation of the copolymer are summarized in Scheme 1.

As can be seen in Scheme 1, water and ethanol were employed as initiators since they confer different functionality and polarity to the linear polyether obtained. The relative polarity of the EtOH respect to water is 0.654 and the functionality values are 2 and 1 for the H_2O and EtOH, respectively. In the case of the water as initiator, a linear polyether hydroxyl terminated in both extremes is obtained; this polyether is able to react by both ends. On the contrary, in those polymers synthesized from ethanol as initiator, only one of their ends is hydroxyl terminated.

All the assayed copolymers were successfully synthesized with polymerization yields higher than 90% after all the steps (see Table 1). The copolymers were analyzed by GPC, observing that the use of EtOH as initiator resulted in a copolymer with two molecular weight distributions as shown in Figure 2 and with higher polydispersity index than the one initiated with H_2O (see Table 1).

In order to discard that the bimodal distribution of the copolymers initiated with EtOH was due to water impurities, the monomers were dried under high vacuum for 3 hours and anhydrous absolute ethanol was used. Figure 3 shows the GPC analysis of the copolymer obtained with the referred conditions and the molecular weights and PDIs for the copolymers with different structure obtained by GPC are gathered in Table 1.

As shown in Figure 3, in the case of using anhydrous EtOH, the final copolymer also revealed a second molecular weight distribution and the PDI value presented almost the same value than when working with ethanol with purity of 99.9%, which is considerably higher (1.33) than when using H_2O as initiator (1.09) (see Table 1). Thus, the hypothesis about the two molecular weight distributions due to the presence of water when using EtOH as initiator was discarded. The reason for the broad molecular weight distribution, with the appearance of two different distributions although overlapped can be a side

reaction of transfer with the initiator (EtOH) during the polymerization, as has already occurred in other studies [4].On the other hand, as can be seen in Table 1, similar molecular weights were found for the case of block copolymers independently of using H_2O or EtOH as initiators. However, all the molecular weights increased when gradient copolymers were synthesized, especially for the case of using EtOH as initiator. This is so because the addition of the all monomers in a single step hampers the control of the final structure and the molecular parameters, such as the molecular weight, the length of each block and the monomer distribution as in the case of block copolymers [33].

Regarding PDI values, the gradient copolymers presented higher PDIs than the block ones, and in both copolymer types, block and gradient, the use of EtOH as initiator promoted higher PDIs than that initiated with H_2O. As commented before, this can be an evidence of a side reaction of transfer with the initiator (EtOH) during the polymerization, as has already occurred in other studies, resulting in a broad molecular weight distribution [4]. Regarding the higher value of the PDI of gradient copolymers, it can be a consequence of the considerably lower GPE reactivity compared to that of EO and PO. When all the monomers are added together, once the GPE is incorporated into a chain, it grows more slowly than the rest. Thus, there is higher difference between length chains than when the monomers are added sequentially. This has been observed to affect significantly to the PDI and molecular weight.

As indicated in the experimental section, MALDI-TOF was used to characterize the distribution of chains of the copolymers and to verify the incorporation of GPE in the copolymer chain in block copolymers. This incorporation was checked by comparing the first and second step of the block copolymer synthesis. As an example, Figures 4a and b show the MALDI-TOF MS spectra of the first and second steps of a block copolymer initiated with H_2O and with EO:PO ratio 0.5:0.5, respectively.

In Figure 4a the chains distribution of the PEO polymer obtained from the first step is analyzed in order to identify the starting chains before reaction with GPE and, consequently, be able to confirm its further incorporation comparing it with that shown in Figure 4b. A Gaussian distribution of molecular weights with a series of peaks ranging from a mass of 674.87 Da to a mass of 1381.60 Da can be seen for the first step.

From these MALDI-TOF results, the number/weight average molecular weights of the obtained polyethylene oxide polymer were calculated according to equations 4 and 5,

obtaining values of 1029 and 1058, respectively. Additionally, the PDI was calculated as a function of the molecular weights [34], obtaining a value of 1.03 with equation 6. In these equations, N_i is the measured peak intensity of a molecular ion with degree of polymerization i and M_i is the mass of the i oligomer.

$$M_n = \sum(N_i \cdot M_i) / \sum N_i \quad (4)$$

$$M_w = \sum(N_i \cdot M_i^2) / \sum(N_i \cdot M_i) \quad (5)$$

$$PI = M_w / M_n \quad (6)$$

Besides, as expected, the spacing between the main peaks was 44.05 Da, corresponding to the EO repeat units.

On the other hand, comparing both spectra (Figures 4a and 4b), it was recognized that, for the main signals, the difference between the peaks obtained for the first and second steps was 112.13 Da, which corresponds to the incorporation of one molecule of GPE. This way, the incorporation of GPE in the polyethylene oxide polymer chains has been demonstrated. Besides, the comparison between the chains masses indicated that the number of units of GPE joined to each chain of the polyethylene oxide polymer of the first step was just one.

Finally, the PDI of the second step was also determined, obtaining a value of 1.03 again. This may be because the GPE molecules were added exclusively to the previously formed PEO chains, and in the same proportion to each chain. Besides, the PDI value of this second step reaction is very similar to the final PDI value of copolymer (1.09). The lower PDI value can be due not only to differences in the further PO incorporation in the chains; but also to the underestimation of the PDI by the MALDI results, since in this technique, the small chains usually fly worse than the large ones.

Regarding the gradient copolymers, the GPE incorporation was checked by means of ^{1}H-NMR analyses. Figure 5 shows the ^{1}H-NMR spectrum of the gradient copolymer initiated with water and with a monomer EO to PO proportion of 0.5:0.5. It can be clearly observed the peak corresponding to the terminal alkynes signal at 2.1 ppm. Thus, the functionalization of the gradient copolymers with terminal alkynes was also achieved.

3.2. CMC determination

Experiments to determine the CMC of the different synthesized copolymers were carried out at 36 ^{0}C with a UV-vis spectrophotometer, varying the copolymers concentration (w/v %) in aqueous solution.

Figure 6 shows the absorbance as a function of the concentration in an aqueous solution for all the synthesized copolymers with the ratio EO:PO of 0.5:0.5.

Results shown in Figure 6 confirmed that the difference between the monomers reactivity was high enough to obtain, by a single step, gradient copolymers with two domains with different hydrophilicity capable of forming micelles. Moreover, based on these data, it can be concluded that in principle, the polymerization initiator did not affect the CMC value (0.11 w/v% for the case of water and 0.12 w/v % for EtOH). Nevertheless, this variable is reviewed in detail with block copolymers since they have a well-known structure and the interference of other structural differences is avoided. In the case of gradient copolymers, the addition of the all monomers in the same step and their different reactivity can make it difficult to obtain copolymers with specific final characteristics.

On the other hand, the CMC of the PEO-GPE-PPO block copolymers initiated with H_2O, EtOH of 99.9% purity and absolute EtOH were 0.151% (w/v%) and 0.08% (w/v%) and 0.073 (w/v%), respectively. Based on these results, the use of EtOH as initiator of the polymerization decreases the CMC value, what can be interesting depending on the application. It can be explained by the fact that the EtOH is less polar than water and that the final copolymer presents just a functionality of 1 while those initiated with water presents a functionality value of 2. Furthermore, these CMC values are very similar to the gradient copolymers with the EO:PO ratio 0.5:0.5 on contrary to what found for other copolymer types derived from EO by some authors [35].

Finally, the influence of the EO:PO ratio on the CMC value was studied for copolymers initiated with H_2O due to their lower polydispersity compared with those initiated with EtOH. The results obtained from this study and a summary of all CMC values of the copolymers synthesized in this research are shown in Table 2.

According to these results, as expected, the higher the EO:PO ratio, the higher the CMC value. This increase can be explained by the fact that the EO is hydrophilic and thus, a larger amount of this monomer increases the copolymer solubility in water, hampering

the micelle formation and being necessary a higher amount of polymer to reach the CMC [36].

A very interesting point of this study is the fact that, independently of the presence of GPE in the studied percentage (around 7.2 wt%), the CMC values are similar to those reported for commercial PEO-PPO-PEO copolymers. For instance, the commercial copolymer L43 with a molecular weight of 1850 and a EO:PO ratio of 0.36:0.64 has a CMC value of 0.407 w/v % [37]. Thus, this monomer can be added to functionalize this type of amphiphilic copolymers with percentages around 7.2 wt% without altering their micelle formation capacity and making possible, at the same time, their use for the covalent incorporation of some drugs by a further click reaction [20, 38].

3.3. Size, stability, morphology and EE% of micelles

The apparent hydrodynamic sizes of polymeric micelles obtained from the synthesized copolymers with different structure measured by DLS are summarized in Table 3. It is known that the micelles with small sizes (10-200 nm) have less probability to be cleared by reticuloendothelial system (RES) and are more likely to accumulate at tumor site through enhanced permeability and retention (EPR) effect [14, 39]. Considering that fact, in the case of H_2O as initiator, the copolymers with EO:PO ratio 0.5:0.5 or 0.65:0.35 seem to be more appropriate for this purpose due to their small size. Moreover, it also can be said that the larger the hydrophobic chain domain or core-forming block (PPO) in the copolymers initiated with H_2O, the bigger the apparent hydrodynamic size of micelles, consistent with previous reports [40, 41]. In the case of EtOH, the apparent hydrodynamic size of micelles was smaller than those initiated with H_2O. This fact could be explained by the lesser polarity of ethanol (compared with water) and its monofunctionality, what allows it to self-assemble only from one side, forming smaller micelles than the obtained with water (bifunctional) as initiator. Besides, there is no significant differences between the micelle apparent hydrodynamic size of block and gradient copolymers.

Table 3 also shows the zeta potential values of micelles obtained with the different copolymers, which are a measure of their stability. The zeta potential values were large, indicating a good stability in all cases [42]. Gradient copolymers presented also zeta potential far from zero, confirming once again that these copolymers were able to form stable micelles in the same way that the block ones due to the presence of two domains with different hydrophilicity.

Taking into account that empty micelles obtained from copolymers with EO:PO ratio 0.5:0.5 and water as initiator had a proper size for their use as therapeutic micelles loaded with drugs in the human body, this copolymer was selected to incorporate coumarin by micelle formation. Figure 7 sustains the spherical morphology of loaded-micelles by HRSEM. Besides, HRSEM results showed that there are some big micelles which average value is comparable to the empty micelles apparent hydrodynamic size obtained by DLS (about 237 nm) and others of smaller size (between 20-50 nm).

The entrapment efficiency of these micelles was found to be about 14%, which is similar to that obtained with Pluronics® with a close molecular weight although different hydrophilic/hydrophobic ratio [5].

4. Conclusions

PEO-GPE-PPO block copolymers were successfully synthesized by means of three steps ring-opening polymerization process, using KOH as catalyst and H_2O or EtOH as initiators with polymerization yields higher than 90% after all the steps. Moreover, it was confirm that the difference between the monomers reactivity was high enough to allow the formation of two polymer domains with different hydrophilicity, a mainly hydrophilic domain and other mainly hydrophobic, resulting in PEO-GPE-PPO gradient copolymers in a single step procedure with similar yields. This fact allowed to simplify the synthesis of the amphiphilic copolymers for the hydrophobic drugs incorporation by micelles formation. However, the characteristics of these copolymers were more difficult to predict.

The presence of the GPE did not impede the micelle formation; while incorporated the advantage of functionalizing the copolymer chain with alkyne groups for further covalent incorporation of drugs by click chemistry, forming the named polymer-drug conjugates. The use of EtOH as polymerization initiator, instead of water, reduced the block copolymer CMC value, but also had an important effect on the polydispersity index, increasing it. Finally, as expected, the higher EO:PO ratio, the higher the CMC since the EO is more hydrophilic than PO.

Moreover, the polymeric micelles obtained from all the synthesized copolymers were in the suitable apparent hydrodynamic size range to enable its absorbability by the target cells. A reduction of the micelles apparent hydrodynamic size was observed with the

increase of the hydrophilic domain and with the use of ethanol as initiator. Moreover, all the copolymers formed stable micelles solutions since Z-potencial values far from 0 were obtained.

Finally, the micelles presented spherical shape and were capable to incorporate coumarin in the hydrophobic core according to HRSEM results, with an entrapment efficiency of 14%.

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Figure captions

Scheme 1. Variables modified for the synthesis of different PEO-GPE-PPO copolymers in order to study the influence of their structure in the CMC value.

Figure 1. Chemical reaction schemes of (a) 1st, (b) 2nd and (c) 3rd steps of synthesis of the PEO-GPE-PPO block copolymers.

Figure 2. GPC chromatograms of PEO-GPE-PPO (a) block and (b) gradient copolymers with EO:PO ratio 0.5:0.5 and water and ethanol as initiators.

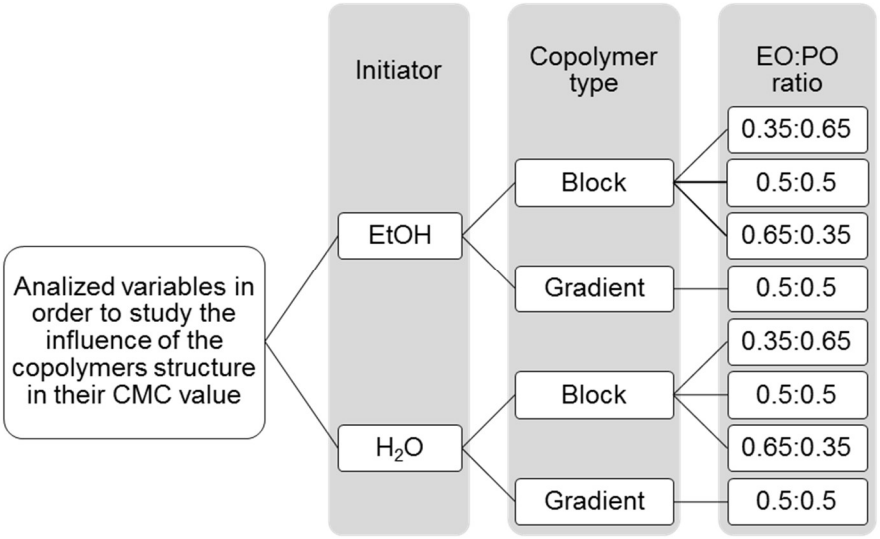
Figure 3. GPC chromatogram of the PEO-GPE-PPO block copolymer with EO:PO ratio 0.5:0.5 and anhydrous EtOH as initiator.

Figure 4. MALDI-TOF MS spectra of (a) first and (b) second steps products of the synthesized block copolymer initiated with H_2O and for the final EO:PO ratio of 0.5:0.5.

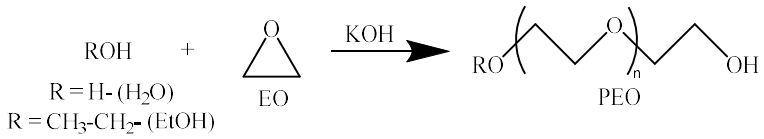
Figure 5. ^{1}H-NMR spectrum of the gradient copolymer initiated with water and EO:PO ratio of 0.5:0.5.

Figure 6. CMC determination for all the PEO-GPE-PPO copolymers with 0.5:0.5 of EO:PO ratio.

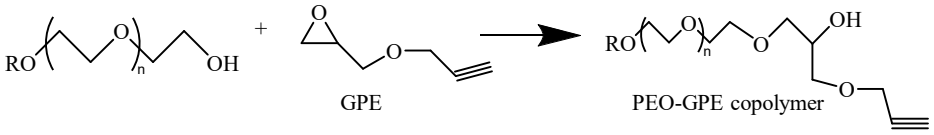
Figure 7. HRSEM picture of coumarin-loaded micelles for the PEO-GPE-PPO block copolymer with EO:PO ratio 0.5:0.5 and initiated with H_2O.


Analized variables in order to study the influence of the copolymers structure in their CMC value
Initiator
EtOH
H2O
Copolymer type
Block
Gradient
Block
Gradient
EO:PO ratio
0.35:0.65
0.5:0.5
0.65:0.35
0.5:0.5
0.35:0.65
0.5:0.5
0.65:0.35
0.5:0.5

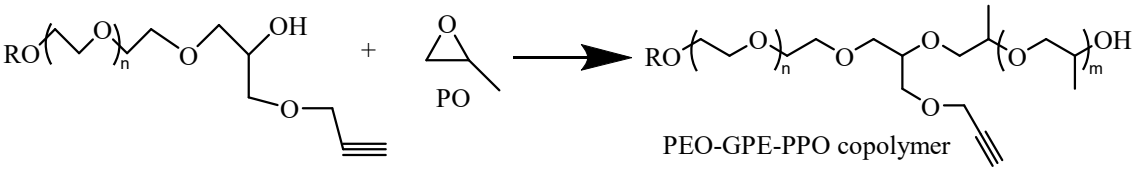
Scheme 1


ROH
+
R = H- (H2O)
R = CH3-CH2- (EtOH)
EO
KOH
RO
n
OH
PEO

(a)

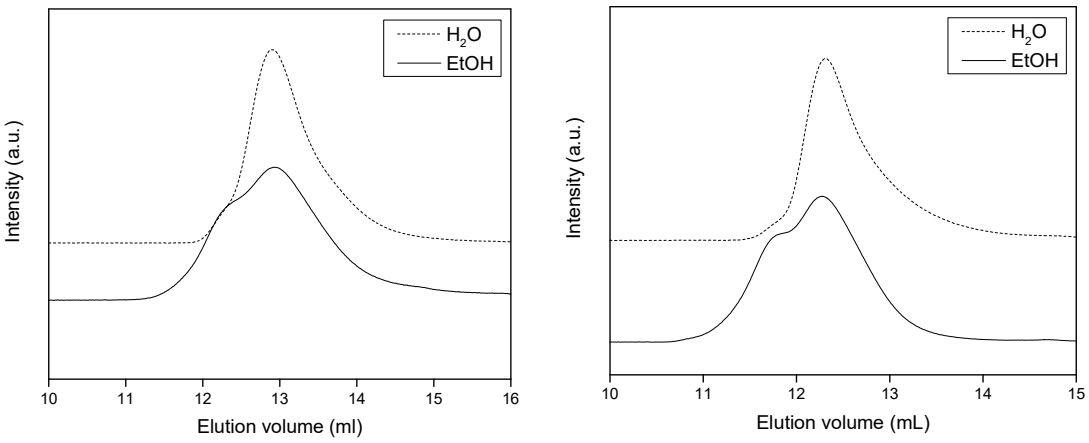

RO
n
OH
+
GPE
PEO-GPE copolymer

(b)


RO
n
OH
+
PO
PEO-GPE-PPO copolymer

(c)

Figure 1


H2O
EtOH
Intensity (a.u.)
10
11
12
13
14
15
16
Elution volume (ml)
H2O
EtOH
Intensity (a.u.)
10
11
12
13
14
15
Elution volume (mL)

(a) (b)

Figure 2

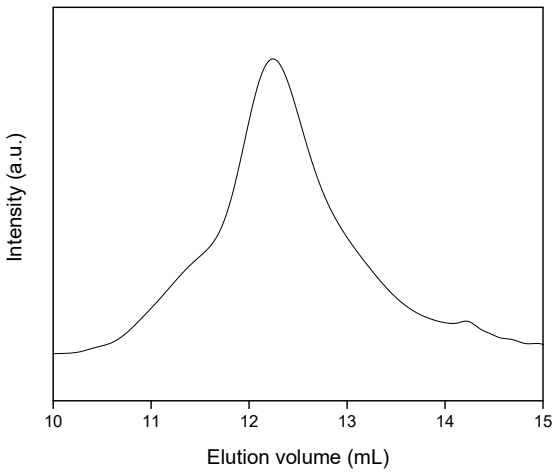
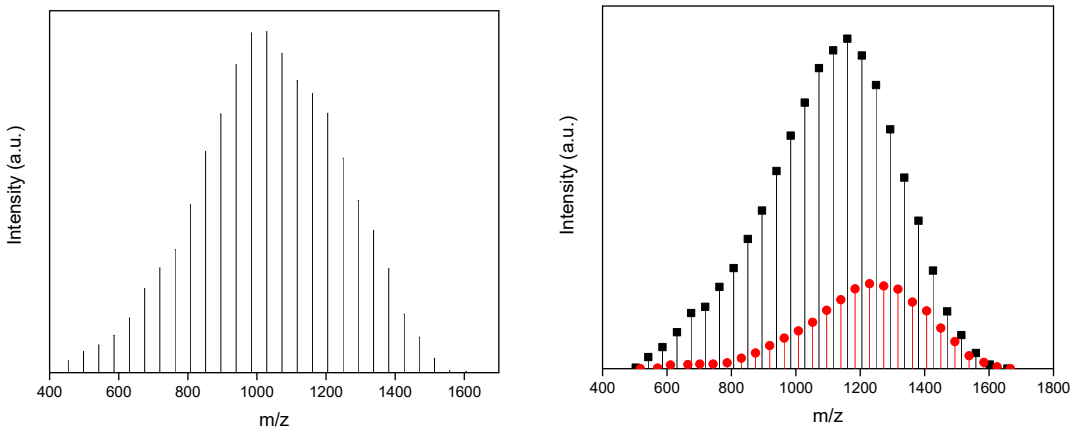


Figure 3


Intensity (a.u.)
400
600
800
1000
1200
1400
1600
m/z
Intensity (a.u.)
400
600
800
1000
1200
1400
1600
1800
m/z

(a) (b)

Figure 4

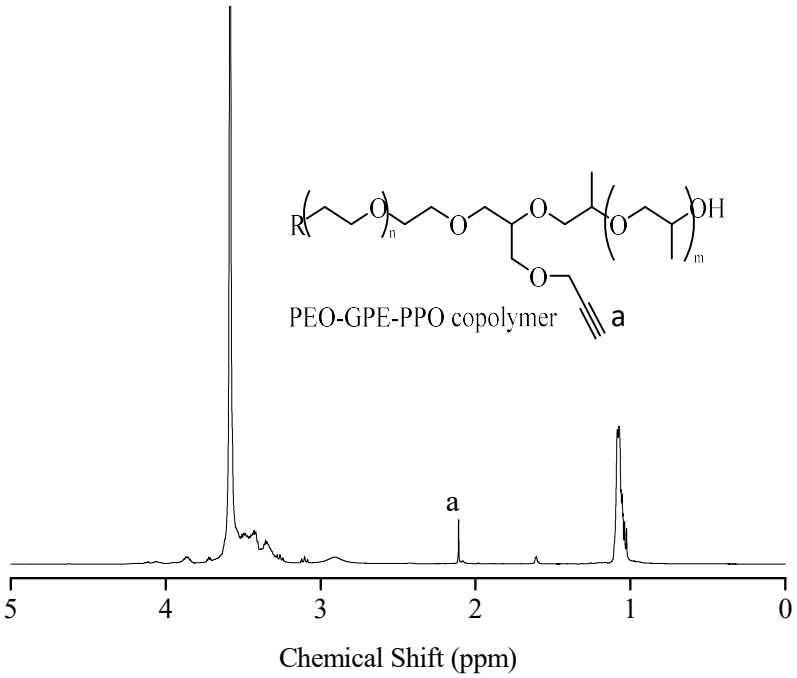

R
O
n
O
O
O
O
m
OH
PEO-GPE-PPO copolymer
a
a
5
4
3
2
1
0
Chemical Shift (ppm)

Figure 5

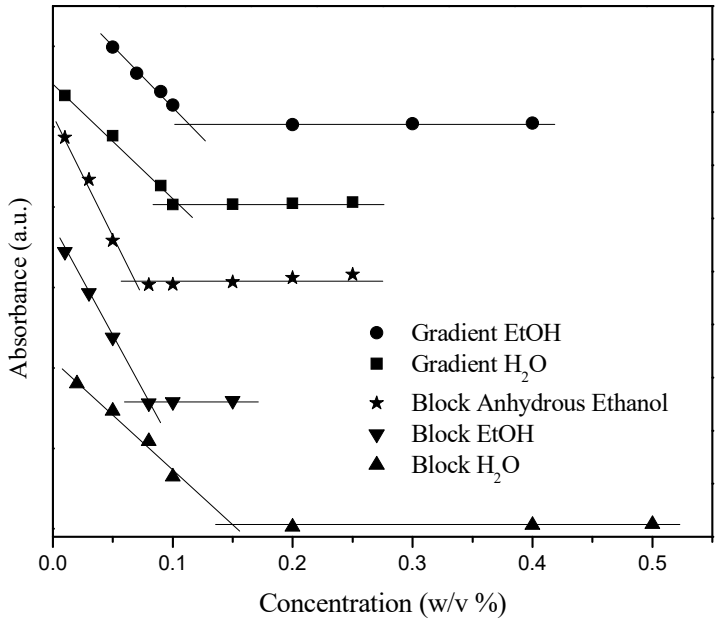

Absorbance (a.u.)
Concentration (w/v %)
0.0
0.1
0.2
0.3
0.4
0.5
Gradient EtOH
Gradient H2O
Block Anhydrous Ethanol
Block EtOH
Block H2O

Figure 6

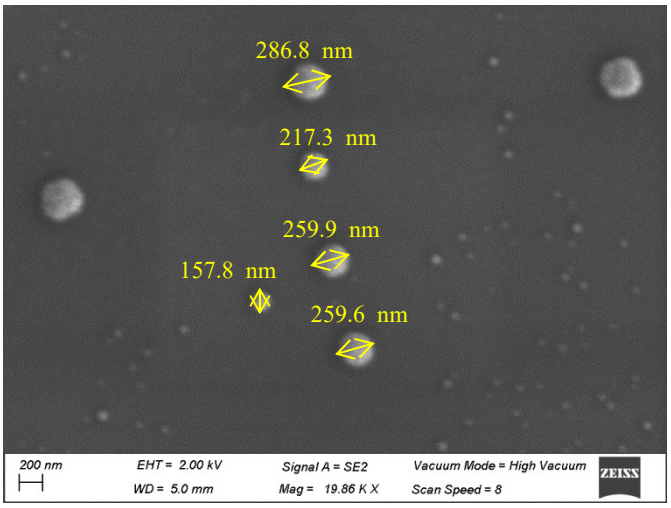

286.8 nm
217.3 nm
259.9 nm
157.8 nm
259.6 nm
200 nm
EHT = 2.00 kV
WD = 5.0 mm
Signal A = SE2
Mag = 19.86 K X
Vacuum Mode = High Vacuum
Scan Speed = 8
ZEISS

Figure 7

Table 1
Polydispersity indexes, number/weight average molecular weights and yield of the synthesized copolymers depending on their structure.

Copolymer type	EO:PO	Initiator	M_n (Da)	M_w (Da)	PDI	Yield (%)
Block	0.35:0.65	H_2O	2100	2500	1.16	93.8
		EtOH	1300	1600	1.20	92.9
	0.5:0.5	H_2O	1600	1700	1.09	93.0
		EtOH	1650	2130	1.29	93.6
		Anhydrous EtOH	1600	2100	1.33	92.8
	0.65:0.35	H_2O	1800	2000	1.12	92.5
		EtOH	1350	1660	1.24	91.5
Gradient	0.5:0.5	H_2O	2150	2600	1.21	92.7
		EtOH	3100	4700	1.52	93.6

Table 2
Summary of CMC values obtained by absorbance measurements for synthesized PEO-GPE-PPO copolymers with different structure.

Copolymer type	EO:PO	Initiator	CMC (w/v %)
Block	0.35:0.65	H_2O	0.11
	0.5:0.5	H_2O	0.15
		EtOH	0.08
		Anhydrous EtOH	0.07
	0.65:0.35	H_2O	0.18
Gradient	0.5:0.5	H_2O	0.11
		EtOH	0.12

Table 3. Apparent hydrodynamic size and stability of polymeric micelles formed from different copolymers synthesized at 36 ºC.

Initiator	H_2O				EtOH	
Copolymer type	Gradient	Block			Gradient	Block
EO:PO	0.5:0.5	0.35:0.65	0.5:0.5	0.65:0.35	0.5:0.5	0.5:0.5
Micelle size (nm)	249.8	443.9	237.0	215.7	140.5	140.5
Zeta potential (mV)	-31.5	-50.0	-40.7	-39.4	-32.5	-36.1

Graphical abstract