DIFFERENT DRUG INCORPORATION ROUTES IN ETHYLENE OXIDE BASED COPOLYMERS

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ABSTRACT

Coumarin is successfully incorporated in poly(ethylene oxide)-poly(propylene oxide) block copolymers functionalized with terminal alkynes, PEO-b-PPO-b-GPE, by click reactions at atmospheric pressure and in CO_2 supercritical conditions ($scCO_2$). The presence of glycidyl propargyl ether (GPE), an alkynyl-terminated monomer, in the copolymer chain allows the covalent attachment of the cumarin by click chemistry, obtaining polymer-drug conjugates. First, the most suitable synthesis procedure for the copolymers abovementioned is stablished. Then, the click reactions are carried out confirming the coumarin attachment by FTIR and ^{1}H-NMR analyses, achieving good yields in both cases with a coumarin content of about 9.3 wt% and avoiding the use of toxic solvents in case of $scCO_2$. Besides, thanks to the amphiphilic character of the copolymer due to the presence of a hydrophilic (PEO) and hydrophobic (PPO) segments, micelle formation is also possible and was confirm by DLS and HRSEM. Finally, the coumarin incorporation was also achieved by micelle formation using the direct dissolution method in order to compare the polymer-drug systems properties. This second route allows to reach a drug entrapment efficiency of a 14 wt %. In both cases, the size of the polymeric micelles obtained is in the suitable range to enable its permeability. However, an interesting point is the reduction of the size of micelles with the increase of the GPE percentage and with the covalent attachment of the coumarin to the copolymer, what is supposed to improve their permeability.

Keywords: Block copolymers; GPE; micelles; click chemistry; supercritical CO_2; coumarin

1. INTRODUCTION

Aqueous solubility is a critical property that influences the absorption of orally administered drugs and restricts the exposure level to the active ingredient. This property is mainly determined by the equilibrium solubility of compound but many other molecular (like crystallinity, particle size, etc.) and physiological factors (like transit time, gastric motility, etc.) have large effect on it. The membrane penetration depends on the lipophilicity and permeability properties [1]. For these reasons poorly soluble drugs have mostly to be administered parenterally (intravenously, subcutaneously or intramuscularly). Their tissue specificity is first confined to the cells of the reticuloendothelial system (RES) which recognizes them as foreign microparticles and transports them to liver and spleen [2]. Among the alternatives to overcome these inconveniences, the inclusion of drugs into polymeric micelles and the synthesis of polymer-drug conjugates by click chemistry are the most attractive options nowadays [3-5].

On the one hand, polymeric micelles as carriers for hydrophobic drugs are being investigated in many research groups [4, 6] in order to improve the solubility of these drugs due to, among others, the simplicity in general of micelle formation methods. In addition, block copolymer micelles have diameters between 10-200 nm [7] typically, due to this size neither renal filtration nor uptake by the RES occurs so that the micelles circulate in the blood stream for a long time and therapeutic efficacy is enhanced [8]. Owing to the low critical micelle concentration (CMC), block copolymers aggregate into micelles even when highly diluted in blood. The low-concentrated unimers are excreted via the kidneys [9].

On the other hand, among different techniques for polymer functionalization, click chemistry has emerged as one of the most promising reactions because it is classified as a very specific, efficient and versatile reaction which allows to obtain high products yields [10]. Within the reactions included in the field of click chemistry, Huisgen 1,3-dipolar cycloaddition is the most employed in polymer chemistry. It consists on the reaction of an azide group to an alkyne group (AAC) catalyzed by copper (CuAAC) in organic media, where DMF or THF are the most common solvents used to achieve the functionalization [11, 12]. This way, the drugs are attached by chemical bonding (covalent) to the copolymer chain, enlarging their lifetime.

Nevertheless, considering that the usage of organic solvents brings the disadvantage of their possible incorporation or presence of toxic residues into the active pharmaceutical ingredient (API) [13, 14], if they cannot be completely removed, the amount must be controlled or limited to levels safe to the patient. Thus, significant efforts in substitution of traditional organic solvents are focused on using green solvents to carry out environmental friendly processes which can eliminate this problem [15]. In this context, the use of supercritical carbon dioxide ($scCO_2$) as solvent in the click reaction between azide and alkyne groups appears as a solution due to its lack of reactivity, high diffusivity, zero surface tension, good transport properties and sterilization capacity [16, 17].

The possibility of synthesizing tailor-made PEO-b-PPO copolymers functionalized with terminal alkynes through the incorporation of GPE in the chain has been demonstrated in previous research [18, 19]. Carrero et al. [18] reported the synthesis of PEO-b-GPE-b-PPO block copolymers with different initiators and studied the influence of hydrophobic/hydrophilic ratio in the

micelle formation. This previous work compared the addition of all the monomers together with the sequential addition of EO, then GPE and finally PO. In this work, a new initiator is used and the influence of the order of hydrophobic monomer addition is studied. Moreover, once selected the best synthetic route, the maximum GPE incorporation is studied. Besides, different possibilities for hydrophobic drug incorporation into them are explored and compared, studying the efficiency of the entrapment and micelles size. Coumarin is chosen as hydrophobic drug due to many and well-known pharmacological properties of these compounds (anti-inflammatory, antibacterial, antifungal, etc.) and mainly, due to their demonstrated anticancer effects [20]. The analyzed incorporation routes are: micelle formation and click chemistry in atmospheric and supercritical conditions. Ethylene oxide based copolymers are one of the most common ones used as drug carriers; however, to the best of our knowledge, they have never been used as support for drug incorporation by click chemistry in supercritical CO_2, what justifies the novelty and interest of this work.

2. MATERIALS AND METHODS

2.1. Materials

Propylene oxide (C_3H_6O; 99.9 %, Praxair), ethylene oxide (C_2H_4O; 99.8 %, Fluka), glycidyl propargyl ether ($C_6H_8O_2$; ≥97 %; Chemos), potassium hydroxide (KOH; 90 %, Scharlab), ethylene glycol anhydrous ($C_2H_6O_2$; 99.8 %; Sigma-Aldrich) and nitrogen (N_2; 99.999 %; Praxair) were used in the synthesis of the different copolymers.

Potassium iodide (KI; 99 %; Sigma-Aldrich) and iodine (I_2; 99 %; PANREAC) were the compounds used to prepare standard KI/I_2 solution for the critical micellar concentration (CMC) determination of the copolymers. Milli-Q water was used as medium to prepare these solutions, for the drug incorporation in polymeric micelles experiments and as solvent or in purification processes.

The hydrophobic drug used in the preparation of loaded micelles was a coumarin-type, 4-bromomethyl-7-methoxycoumarin ($C_{11}H_9BrO_3$; 97 %; Across Organics) or bromo-coumarin.

The required reagents in the synthesis of 4-azidomethyl-7-methoxycoumarin or azide-coumarin for the click reaction were the previously described bromo-coumarin, sodium azide (NaN_3; ≥99.5 %; Sigma-Aldrich), acetone (C_3H_6O; ≥99.5 %; PANREAC), acetonitrile (C_2H_3N; ≥99.9 %; Merck) and heptane (C_7H_{16}; ≥99 %; Honeywell). These were used without previous purification.

For click reaction at atmospheric pressure, sodium ascorbate ($C_6H_7NaO_6$; ≥99 %; PANREAC), copper (II) sulfate pentahydrate ($CuSO_4$; 99.995 %; Sigma-Aldrich), Tetrahydrofuran (C_4H_8O; ≥ 99.0 %; Honeywell) and ethanol (C_2H_6O; ≥99.9 %; Merck) were used as received. For click reaction in supercritical CO_2 conditions, copper (II) acetate monohydrate ($C_4H_6CuO_4 \cdot H_2O$; ≥ 99 %; Sigma-Aldrich) used as catalyst and carbon dioxide (CO_2; 99.5 %; Carburos Metálicos S.A., Spain) used as solvent were of analytical grade and used as delivered.

2.2. Different pathways of copolymers synthesis

The PEO-b-PPO-b-GPE triblock copolymers were synthesized through nucleophilic ring-opening polymerization in different steps as in previous works [18]. Nevertheless, in this work, the

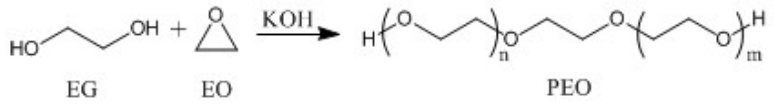
synthesis procedure was modified comparing three different pathways, changing the order of addition of hydrophobic monomers with the objective to choose the most suitable procedure to increase the molecular weight of the copolymer (higher yield) and its GPE content and availability for further click reaction. In any case, all these routes started with a first step of PEO synthesis (hydrophilic segment of the copolymer). Subsequently, the hydrophobic segment (PPO and GPE) of the copolymer was added, all at the same time (way A), or in separate steps varying the order of addition of the hydrophobic monomers (way B1 when PO was introduced firstly, or way B2 being GPE the first). Scheme 1 shows the reaction steps for each route.

The different polymerization steps 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. A mass ratio hydrophilic:hydrophobic segments of 1 was maintained for all the copolymers. However, in the hydrophobic portion, the amount of GPE was varied with respect to the final weight of the copolymer.

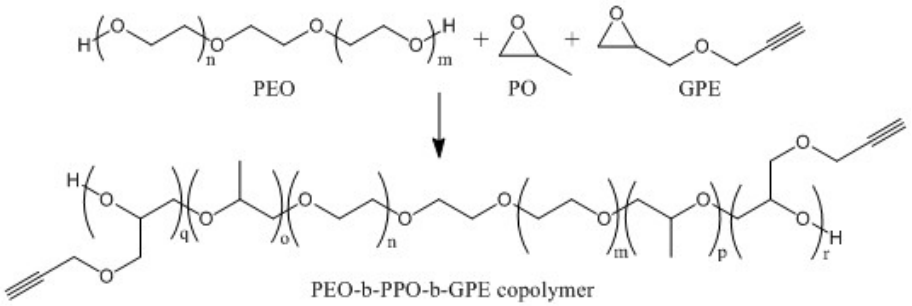
In the first step of the reaction, the initiator solution was separately prepared in a beaker. The initiator used in this work, was ethylene glycol. Ethylene glycol was added with KOH as catalyst and stirred to achieve a homogeneous mixture. KOH was used as catalyst since it is compatible with polymerization of both monomers, EO and PO, and its activity and purification are well established [21]. The initiator solution was introduced in the pressure reactor and subsequently, monomer EO was also fed. This mixture was pressurized with nitrogen (3 bar) to achieve an inert atmosphere, stirred at 1000 rpm and heated up to 80 °C.

During the course of this step, it was observed that the pressure of reactor increased as the temperature rose. When the set point temperature was reached, the pressure began to decrease as the monomer was being consumed. Finally, the initial value of pressure was achieved, indicating that the reaction was finished. Lastly, in order to remove residual monomer, high vacuum (40 mbar) was applied for 1 h. The vacuum was controlled using a Divatronic DT (Köln, Germany) digital vacuum indicator-controller, acting on a solenoid valve.

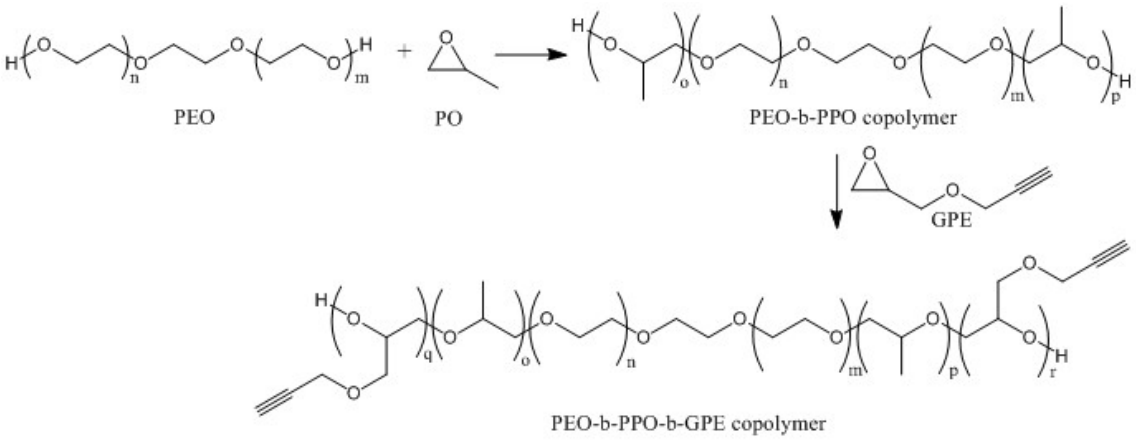
In the second and third steps as the case, the corresponding amounts of hydrophobic monomers were fed into reactor with catalyst (but without initiator to avoid the formation of additional chains). This mixture was heated to 120 °C and was led to the same reaction conditions that in the previous step. When GPE was in the reactor (alone or mixed with PO), the pressure did not increase, so the reaction time was determined by taking samples at different times, until the total consumption of the monomers. In the case of adding just PO (without GPE), the pressure in the reactor evolved as in the first step. Finally, to avoid the presence of remaining monomers embedded in the copolymer, high vacuum was also applied at the end of the reaction.


KOH
EG
EO
PEO

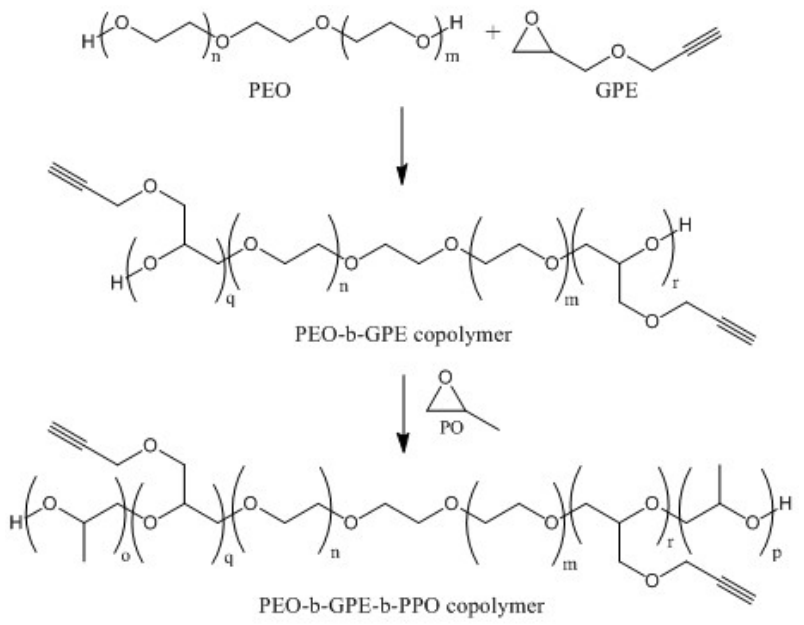
(a)


PEO
PO
GPE
PEO-b-PPO-b-GPE copolymer

(b)


PEO
PO
PEO-b-PPO copolymer
GPE
PEO-b-PPO-b-GPE copolymer

(c)



(d)

Scheme 1: Steps of the different copolymer synthesis routes: a) common first step, b) route A, c) route B1 and d) route B2

2.3. Determination of the copolymers CMC values

The critical micellar concentration (CMC) of the different copolymers can be determined measuring the variation in the behavior of the absorbance. This parameter decreases with the increase of the copolymer concentration for concentrations below the CMC, while for concentrations higher to CMC value, the absorbance remains constant. Hence, the CMC can be easily determined by measuring the absorbance using an UV-visible spectrophotometer. Absorbance at varying concentrations of each copolymer was measured at 366 nm in all cases.

The concentration values are expressed in terms of w/v %. Absorbance results for the different concentrations of each copolymer were taken at 36 °C, since this is approximately the corporal temperature and it is expected that in the near future, the formed micelles will serve as vehicle of drugs for the treatment of human diseases.

The CMC value for different synthesized copolymers in aqueous solution were determined by iodine-iodide (KI/I_2) method as in previous studies [22] using Agilent 300cary series UV-visible spectrophotometer 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 [23]. 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 Milli-Q water.

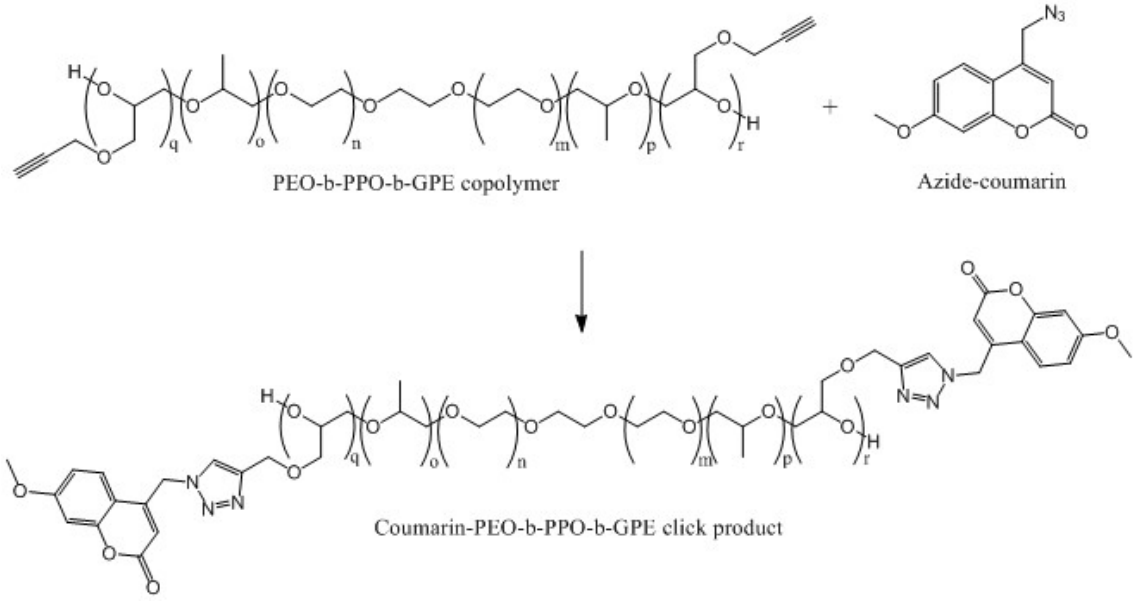
2.4. Synthesis of 4-azidomethyl-7-methoxycoumarin (azide-coumarin).

The synthesis of this compound was carried out according to previous research [24, 25]. A mixture of NaN_3 (1.2 g, 0.018 mol) and 4-bromomethyl-7-methoxycoumarin (1 g, 0.0037 mol) in acetone/acetonitrile (1:1, 120 mL) solution was added to a 250 mL flask. The mixture was stirred at 500 rpm and heated at 50 °C for 48 h. Then, solvents were removed under vacuum in a rotary evaporator. The organic extracts were washed with water to dissolve the sodium azide in excess and filtered under vacuum to retain the mixture bromo-coumarin (reagent without reacting)/azide-coumarin. Subsequently, this mixture was separated by washing with warm heptane, since the original coumarin is soluble in it while the azide-coumarin is insoluble. Finally, the synthesized azide-coumarin was dried at 80 °C for 1 h [26, 27].

2.5. Click reaction at atmospheric pressure

The previously obtained azide-coumarin was incorporated by click chemistry in the synthesized PEO-b-PPO-b-GPE copolymers, obtaining a polymer-drug conjugate with the drug covalently attached as shown in Scheme 2. Within the reactions included in the field of click chemistry, it has been chosen in this work the Huisgen 1,3-dipolar cycloaddition of an azido group to an alkyne group (AAC) catalyzed by copper (CuAAC). This reaction is usually carried out using copper sulfate pentahydrate as source of Cu(II) and sodium ascorbate as a reducing agent [28-31]. A solution of azide-coumarin and copolymer in the suitable amounts to have mol azide-coumarin/mol alkynyl groups ratio of 1:1 in tetrahydrofuran (THF)/ethanol/water (1:2:2) [19] was added to a flask. This mixture of solvents was used to maintain the solubility of all components. Copper sulfate pentahydrate (0.1 $mol \cdot L^{-1}$) in water and sodium ascorbate (0.1 $mol \cdot L^{-1}$) in water were added. The reaction mixture was stirred at 600 rpm for 48 h at room temperature.

After the reaction, solvents were removed in a rotary evaporator. Ethyl ether was added to dissolve the original copolymer and precipitate the click-copolymer; then, the mixture was decanted and several washes with ether were carried out. Finally, click product was filtered and dried at 40 °C for 2 h.



Scheme 2: Click reaction between synthesized copolymer and azide-coumarin

2.6. Synthesis of click product at supercritical conditions ($scCO_2$)

Click product at supercritical conditions was synthetized again by the Huisgen 1,3-dipolar cycloaddition but using Copper (II) acetate monohydrate as catalyst in this case. The procedure of synthesis was the following: an equimolar quantity of alkynyl groups of the PEO-b-PPO-b-GPE copolymer and azide-coumarin were added into a stirring tank reactor using a molar loading of catalyst of 11.5 %. Once the sample was introduced into the reactor, it was heated and loaded with CO_2 up to 47 °C and 110 bar, optimal values of temperature and pressure respectively according to previous research for functionalization of PLA [25]. When reaction time was completed, 24 h later, the reactor was depressurized to remove CO_2 in order to obtain click product free of solvent.

2.7. Preparation of coumarin-loaded micelles

Among the many drug-loaded micelle preparation methods, direct dissolution method was used in this work because is a simple technique in which the polymer and drug are dissolved in an aqueous solvent (or water) that leads to micellization. Moreover, this technique is used mainly for moderately hydrophobic polymers, such as PEO-PPO and drugs [32].

The drug-loaded micelles were prepared dissolving an appropriate amount of PEO-b-PPO-b-GPE copolymers in milli-Q water to obtain 1 w/v % aqueous solutions of copolymers (concentration over their CMC values to ensure the micelles formation at body temperature). Then, coumarin was added to obtain desired drug to polymer ratio (1:10 w/w). The mixture was stirred vigorously with a magnetic stirrer and placed in a water bath to maintain its value at body temperature (36°C). 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 1 h. The resulting micellar solution was then centrifuged at 3800 rpm for another hour to separate the undissolved drug [22].

The entrapment efficiency (EE %) of polymeric 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 h, and later, the amount of free coumarin was dried overnight at 100 °C and weighed.

The EE % was calculated based on the amount of the drug incorporated into the polymeric micelles versus the amount of drug initially added or total drug, using the Equation 1:

$$EE\ \% = \frac{(Amount\ of\ total\ drug\ - Amount\ of\ free\ drug\ )}{Amount\ of\ total\ drug} \times 100 \qquad (1)$$

2.8. Characterization techniques

Gel Permeation Chromatography (GPC)

Gel Permeation Chromatography was used to determine the molecular/number weights (M_W, M_n) and polydispersity index (PDI) of the PEO-b-PPO-b-GPE copolymers. Measurements were performed with a Viscotek chromatograph with two columns (Styragel HR2 and Styragel

HR0.5) at 35 °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).

Fourier Transform Infrared Spectroscopy (FTIR)

Infrared spectroscopy was used to check the incorporation of GPE in the copolymer chain and also to verify the presence of azide group in azide-coumarin obtained, and the 1,2,3-triazole ring signal in the product after click reaction. Measurements were recorded on a Varian 640-IR type FTIR spectrophotometer. The spectra data were acquired by using ResolutionsPro™ software, version 5.2.0, with 16 scans per experiment at a resolution of 8 cm^{-1} in the range of 4000 to 400 cm^{-1}.

Proton Nuclear Magnetic Resonance (^{1}H-NMR) and Carbon NMR (^{13}C-NMR)

Also, in order to substantiate the success of the GPE incorporation in the copolymer chain and of the click reaction, ^{1}H-NMR analyses were used. The success of the azide-coumarin production method was measured by ^{13}C-NMR too. The ^{1}H-NMR and ^{13}C-NMR spectra were measured with a Bruker 500 MHz spectrometer, using $CDCl_3$ as solvent. Tetramethylsilane (TMS) was used as an internal standard for calibrating the chemical shifts.

Dynamic light scattering (DLS)

The apparent hydrodynamic size of empty and coumarin-loaded micelles was measured using dynamic light scattering with a Zetasizer Nano ZSP of Malvern. The light source was 10mW He-Ne laser operating as a fixed wavelength (633 nm) at scattering angle of 173 ° and at 36 °C of temperature, because this is the body temperature approximately. Each measurement was repeated at least three times and the average value is given.

High Resolution Scanning Electron Microscope (HRSEM)

The spherical morphology of the micelles and their size were also checked using a Zeiss 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. Determination of copolymers synthesis procedure

The first step of this research was to stablish the most suitable synthesis procedure to obtain copolymers with the desired molecular weight, low polydispersity index and the specific architecture. The aimed molecular weight was about 6500 Da because is in the range of many of the current commercial polymers derived from EO that are used as drug carriers [33] and also in the range to be filtered by the kidney and cleared in urine [34].

In order to confirm the best route for the synthesis, the three pathways (A, B1 and B2) were assayed for a copolymer with a 3 wt % of GPE. The molecular weight, polydispersity index and process yield are gathered in Table 1.

After GPC analyses, as shown in Table 1, the addition of the hydrophobic monomers in separate steps and the GPE at the end (synthesis route named as B1) was the one that allowed

to obtain a copolymer with the lowest PDI, a molecular weight more similar to the objective and with the highest yield (92.5 %). Thus, this was the synthesis route stablished to obtain the copolymers with increasing percentage of GPE. The fact of obtaining worst results in the other cases can be explained by the low reactivity of the GPE compared to the reactivity of the other two monomers; r_{EO}=14.8 and r_{GPE}=0.076 for the copolymerization of EO and GPE, while the values are r_{EO}=3.1 and r_{PO}=0.3 for the copolymerization reaction of EO and PO [35, 36]. The low reactivity of the GPE causes a limitation of the growth of the copolymer chain when it is added at the same time that the PO, which is in agreement with previous research [35-37].

Moreover, as can be seen, the copolymer with lower yield and more different to the objective was the one obtained adding both hydrophobic monomers at the same time (way A), as noted in previous studies where all the monomers were added simultaneously [18].

3.2. Incorporation of different GPE contents into the polymer chain

Once stablished the optimal sequence of synthesis steps, the next step was to study the influence of the incorporation of different GPE amounts in order to increase the functionality of the polymer for the further incorporation of the drug.

In view of the GPC results of the copolymers obtained with different percentages of GPE (Table 2), the molecular weight of the copolymer without GPE was closer to the objective value (6500 Da). When adding GPE between 3-10 wt % under the same conditions, similar molecular weights were obtained, but for the 15 wt % a significant decrease was obtained and also a reduction of the yield. These results may be due to the reactivity differences between monomers as commented before, that can have caused such a reduction of the reaction rate, that part of the monomer has not been incorporated in the reaction time. A further study about reaction conditions (temperature or catalyst) would be necessary to achieve an increase of the GPE content. Besides, the polydispersity of the copolymers obtained increased with the increase of the percentage of this monomer. For all these reasons, high deviation from the desired molecular weight, reduction of the yield and increase of the PDI, the copolymer with 15 wt % was discarded for future coumarin incorporation studies.

In order to demonstrate the incorporation of the alkynyl groups provided by GPE in the copolymer chain, the copolymers synthesized were analyzed using FTIR and ^{1}H-NMR.

Figure 1 shows the infrared spectrum of GPE used as reagent in the copolymer synthesis. This monomer has its own characteristic signal as a weak peak around 3000 cm^{-1}. Comparing this spectrum with the spectrums of the copolymers, the incorporation of GPE in the copolymer chain was demonstrated due to the existence of a peak around 3000 cm^{-1} in the copolymer with GPE, that not appears in the copolymer without GPE.

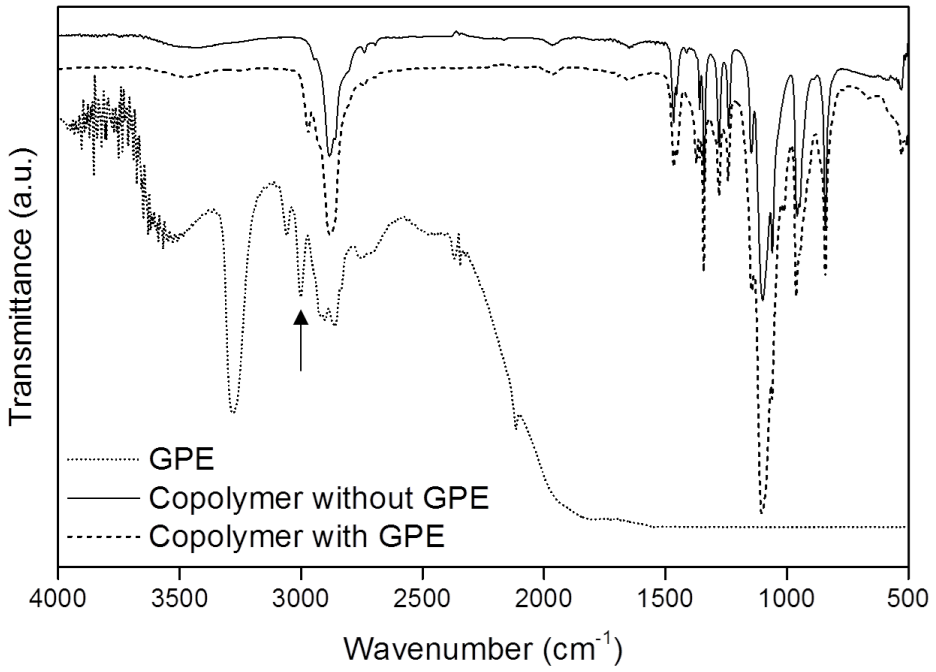
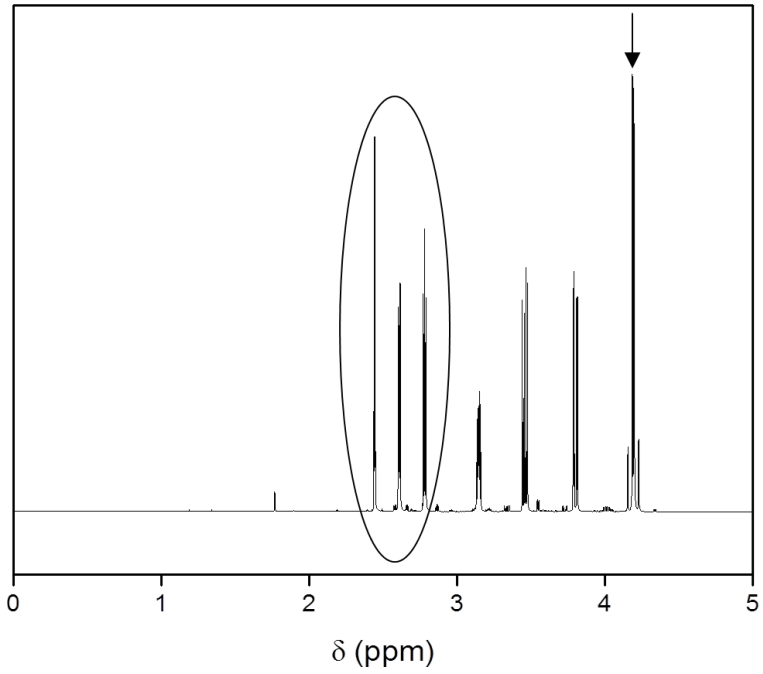
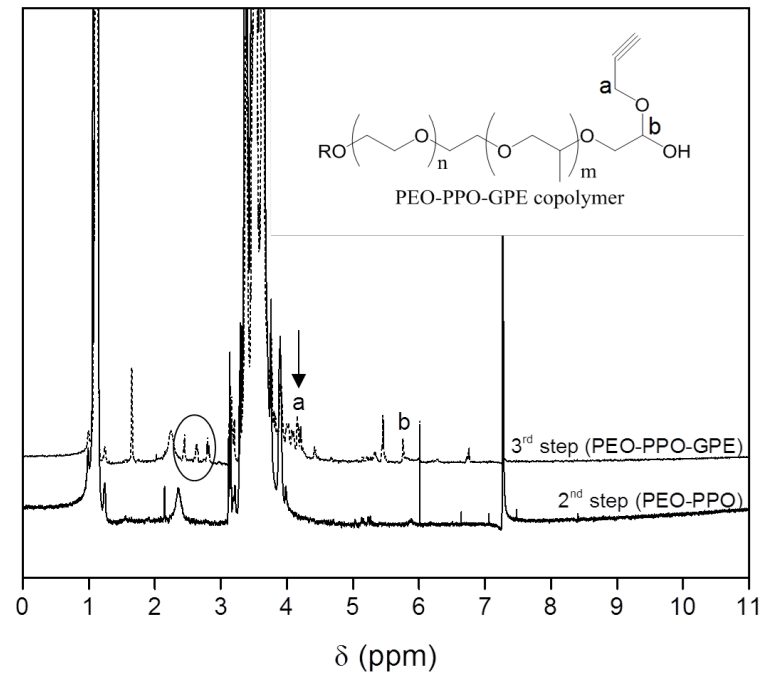


Figure 1: FTIR spectra of the GPE and the copolymers without and with GPE

Moreover, the ^{1}H-NMR spectra shown in Figure 2 also verified the functionalization of the copolymer chain, because the spectrum of the copolymer after the GPE incorporation has a strong signal around 4.2 ppm and weak signals around 2.5 ppm that do not appear in the spectra of the copolymer before of the GPE addition (Figure 2(b)), these signals can be also clearly seen in the pure GPE spectrum (Figure 2(a)). Thus, it can be said that the incorporation of this monomer was carried out successfully [35, 38].



(a)



(b)

Figure 2: ^{1}H-NMR spectra of (a) pure GPE and (b) 2nd and 3rd steps of the PEO-b-PPO-b-GPE copolymer synthesis reaction for a 10 wt % of GPE content

3.3. Study of the GPE content influence in micelle formation

Once synthesized and characterized the copolymers with different mass percentages of GPE, the influence of this monomer content on the CMC values was evaluated.

Figure 3 shows the absorbance as a function of the concentration in water for a PEO-b-PPO copolymer without GPE in order to determine its CMC value by KI/I2 method, obtaining a value of 0.0028 w/v %. According to bibliography [39], the CMC of the commercial Pluronic P105, which has the same characteristics of molecular weight and PEO:PPO ratio, is around 0.005 w/v % at this temperature. Thereby, it has been demonstrated the suitability of the used CMC determination method.

In Table 3, the CMC values determined for the copolymers with all percentages of GPE studied are summarized. In view of these results, the CMC values were almost equal for the different amounts of GPE incorporated. Consequently, it can be concluded that the addition of GPE in the hydrophobic segment up to a 10 wt % did not affect the micelle formation of the copolymers in a different way that the PPO (commercial hydrophobic segment), and its presence had low effect on the copolymers CMC values as has been demonstrated before [18].

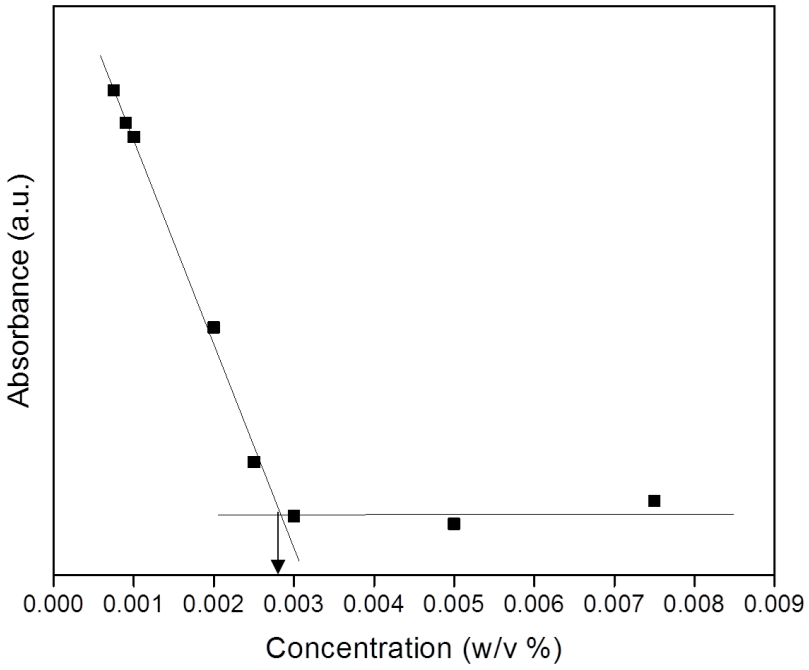


Figure 3: Determination by UV-vis/KI/I_2 method of CMC value for a PEO-b-PPO copolymer without GPE

3.4. Evaluation of drug incorporation

3.4.1. Synthesis of 4-azidomethyl-7-methoxycoumarin

Taking into account that the GPE forms a stable triazole linkage by 1,3-dipolar cycloaddition with functionalized azide groups [31, 40] for the future click reactions, in this point of the research, the bromine end groups of bromo-coumarin were replaced by azide groups according to the procedure of section 2.5.

The success of this reaction was confirmed by FTIR analysis, observing the appearance of the azide group (N_3) at 2110 cm^{-1} [41]. This signal does not appear in the starting bromide compound as noted in Figure 4.

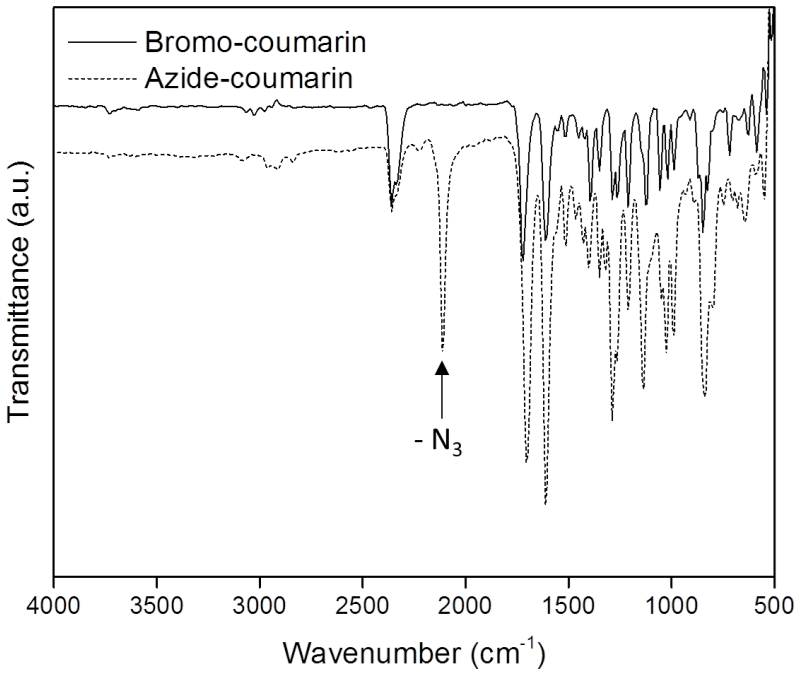


Figure 4: FTIR spectra of bromo-coumarin and azide-coumarin

Moreover, as can be seen in Figure 5, comparing the ^{13}C-NMR spectrum of azide-coumarin with the product before azidation, the success of the reaction was also confirmed through this technique due to the disappearance of the characteristic peak of the carbon in $–CH_2$-Br at 26.82 ppm and the appearance of a new peak at 50.84 ppm, characteristic of the carbon of $–CH_2$-N_3 [42]. Thus, it could be concluded that the azide group was incorporated into the coumarin molecule.

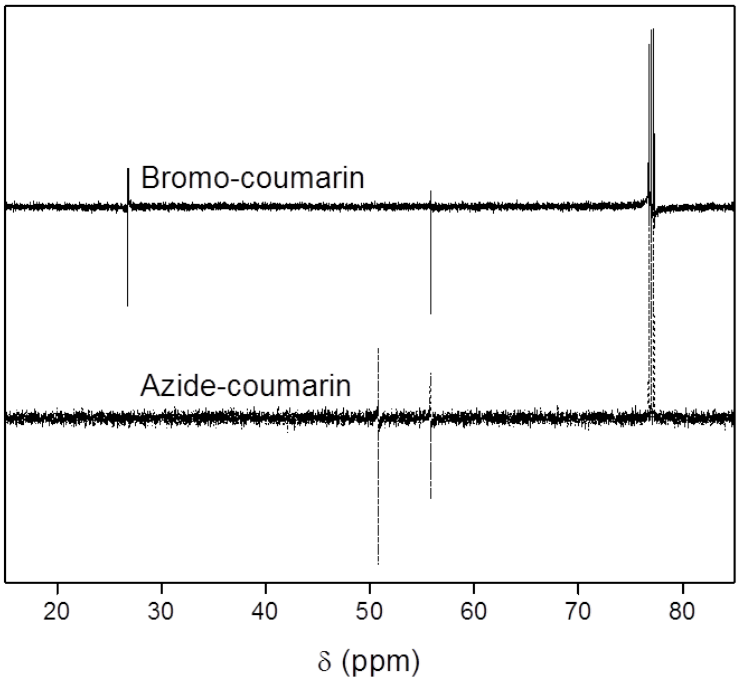
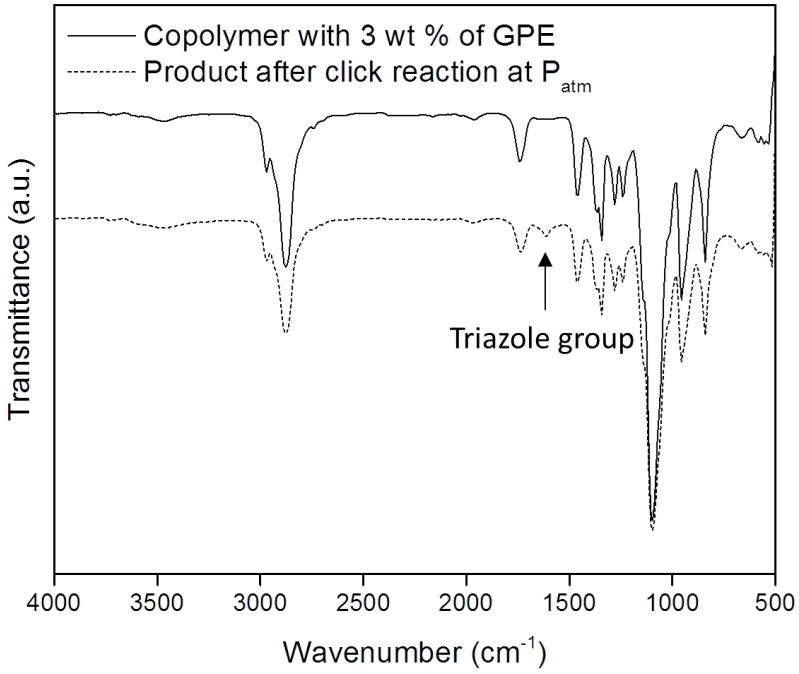


Figure 5: ^{13}C-NMR spectrums of bromo-coumarin and azide-coumarin

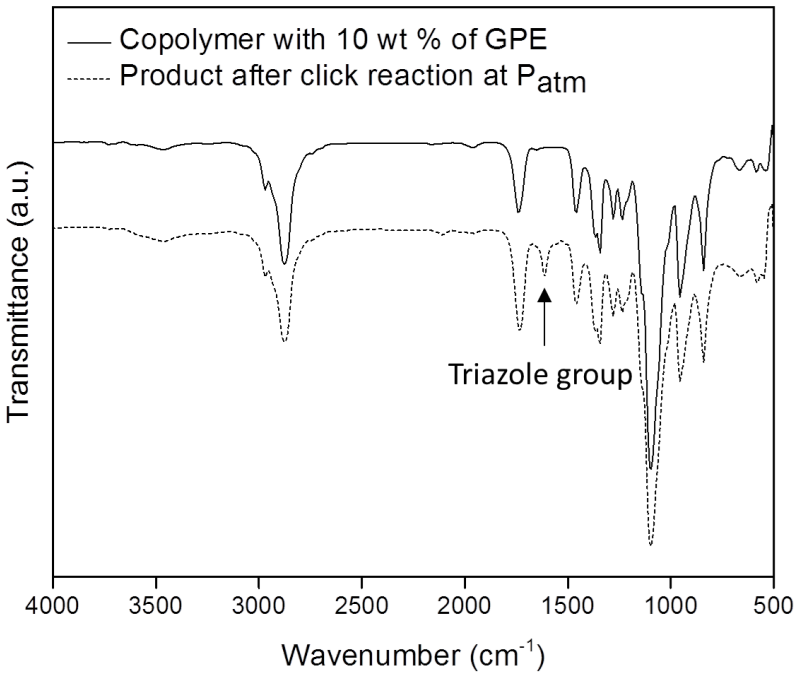
3.4.2. Coumarin incorporation into the copolymer by click reaction at atmospheric pressure

Azide-coumarin previously obtained was reacted with PEO-b-PPO-b-GPE copolymer by click chemistry to obtain a polymer-drug conjugate. This reaction was carried out using quantitative mol azide-coumarin/mol alkynyl groups as commented before [19]. The real concentration of terminal alkyne groups in the copolymers synthesized was determined using UNE 53985-4:2004, giving a value of 0.11028 mmol alkynyl groups/g of polyol and 0.44112 mmol alkynyl groups/g for the copolymers with a 3 and 10 wt % of GPE, respectively.

After the click reaction at atmospheric pressure, the chemical structure of the polymer-drug conjugates was characterized using FTIR and ^{1}H-NMR analyses (Figure 6). FTIR spectrum shows the triazole group band at around 1612 cm^{-1}, while this signal does not appear in the spectrum of the original copolymers. As examples, FTIR spectra of copolymers with 3 and 10 wt % of GPE before and after the click reactions are shown in Figure 6, indicating the success of the reaction [43]. Besides, as expecting, the signal of triazole group in the products after click reaction is more noticeable in the case of copolymer with more GPE content.



(a)



(b)

Figure 6: FTIR spectra of products before and after click reactions at P_{atm} for PEO-b-PPO-b-GPE copolymers with (a) 3 wt % and (b) 10 wt % of GPE

^{1}H-NMR analysis was also used to confirm the success of the click reaction under these conditions. Figure 7 shows the spectrums of polymer-drug conjugate obtained for the copolymer with 3 and 10 wt % of GPE. The spectrums confirmed again the formation of this conjugate, due to the appearance of characteristic signals associated with the presence of the heterocyclic ring (triazole group), that demonstrates unequivocally the linkage of the coumarin with the copolymer. These were the signal at 7.42 ppm corresponding to the proton of the 1,2,3-triazole ring and the signals at around 6.35 ppm and 7.74 ppm assigned to the protons of the coumarin group [44, 45].

Furthermore, this comparative between the ^{1}H-NMR spectra of the products obtained from the two "clicked" copolymers (with 3 and 10 wt % of GPE) at atmospheric pressure, presents the same signals in both cases, with the unique difference that, as expected, they were more pronounced in the click product with 10 wt % of GPE indicating that is able to incorporate more amount of drug.

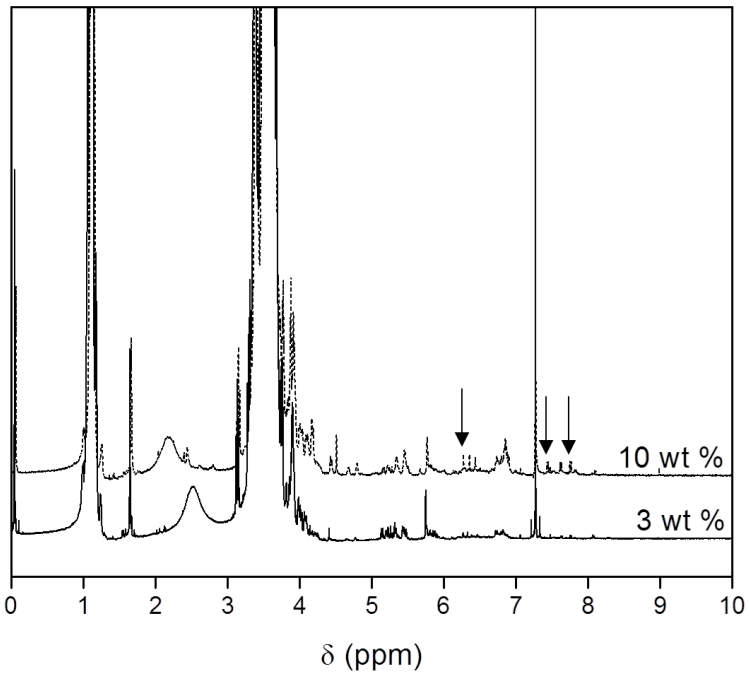


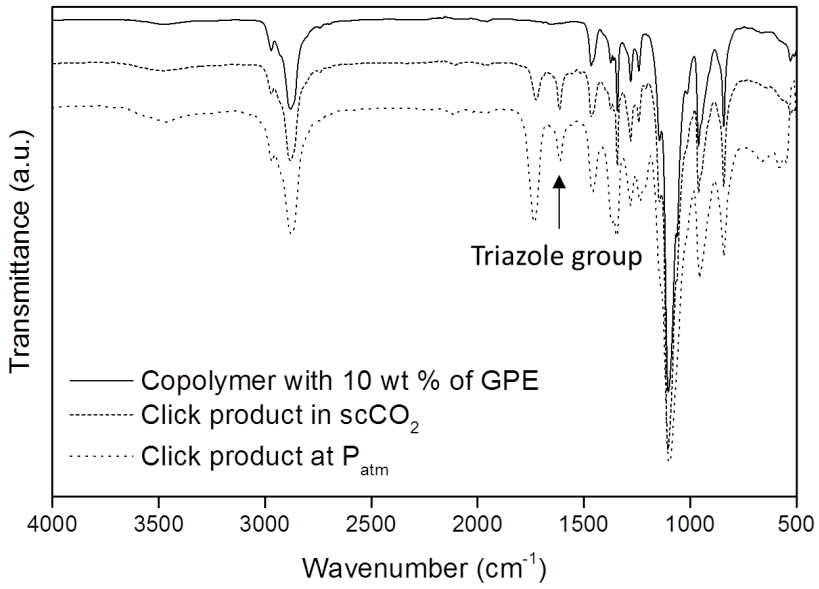
Figure 7: ^{1}H-NMR spectra of products after click reactions at P_{atm} for PEO-b-PPO-b-GPE copolymers with 3 wt % and 10 wt % of GPE content

3.4.3. Incorporation of coumarin by click reaction in $scCO_2$

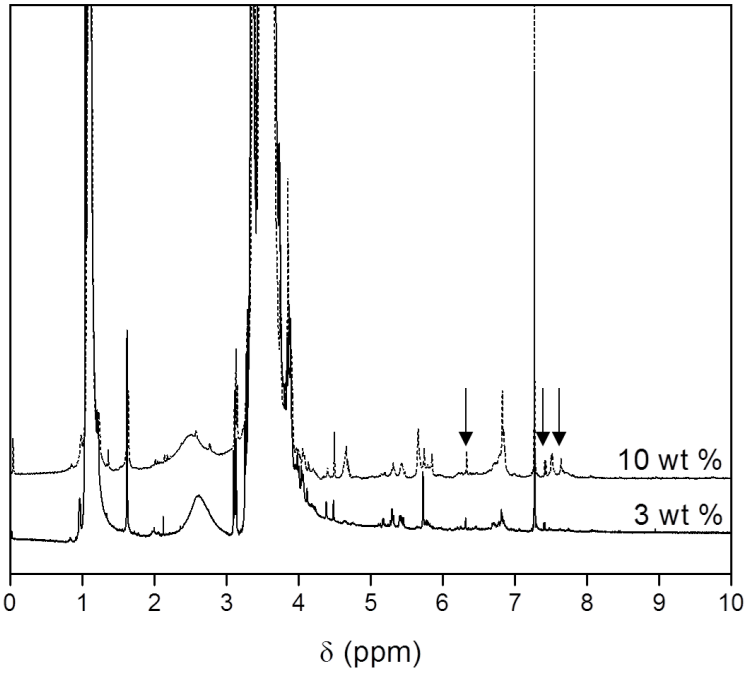
Coumarin incorporation by click chemistry was studied in supercritical conditions with $scCO_2$ as solvent, with the objective of achieving a simple purification process and a click product completely free of organic solvents, which is important for a further pharmacological application. Besides, the lack of organic solvents is also an environmental advantage, since these solvents can cause contamination problems.

In this case, the copolymers tested were the same and the products were also analyzed by FTIR and ^{1}H-NMR (Figure 9(a) and (b)).

In the FTIR analyses for both click products, at P_{atm} and in $scCO_2$ conditions, the spectra were identical and appeared the triazole group signal at around 1612 cm^{-1}, that is not present in the original copolymer, as can be seen for example in the case of the polymer with a 10 wt % of GPE in Figure 8(a). Moreover, in Figure 8(b) the ^{1}H-NMR results of click products for the copolymers with 3 and 10 wt % of GPE in $scCO_2$ conditions, can also be seen the same signals corresponding to the triazole ring and to the coumarin group that in the click products obtained at atmospheric pressure.



(a)



(b)

Figure 8: (a) FTIR spectra of products before and after click reaction at P_{atm} and in $scCO_2$ conditions for PEO-b-PPO-b-GPE copolymer with 10 wt % of GPE and (b) ^{1}H-NMR spectrums of products after click reaction in $scCO_2$ for PEO-b-PPO-b-GPE copolymers with 3 wt % and 10 wt % of GPE content

The yields obtained for the click reactions in both conditions were very similar, being around 96 % in the case of click reactions at P_{atm} after purification procedure, and close to 95 % in the case of $scCO_2$ conditions. Thus, up to a content of about 9.3 wt % of azide-coumarin has been incorporated in the PEO-b-PPO-b-GPE copolymers by click chemistry reaction with their alkynyl groups.

Therefore, the polymer-drug conjugates were also successfully obtained by the CuAAC 1,3-dipolar cycloaddition in $scCO_2$, proving the effectiveness of this method to incorporate drugs into ethylene oxide based copolymers in a media free of organic solvents.

3.4.4. Coumarin incorporation by micelles formation

Finally, the incorporation of the coumarin in the polymeric micelles obtained using direct dissolution method was carried out. This fact was feasible as a result of amphiphilic character of the different PEO-b-PPO-b-GPE copolymers synthesized.

The entrapment efficiency of these micelles was found to be about 14 % independently on the GPE content. This parameter was calculated according to Equation 1.

The apparent hydrodynamic sizes of the micelles obtained with the different copolymers before and after the entrapment of the coumarin by micelle formation method were measured by DLS, obtaining the results shown in Table 4. Based on these results, the physical entrapment of the coumarin in the core of polymeric micelles was also proved owing to the increase in the size of coumarin-loaded micelles. In addition, the results show that the presence of more amount of GPE in the copolymers reduces the size of the micelles, which benefits the permeability [77]. This reduction of the hydrodynamic radii can be due to the higher hydrophobicity of GPE compare to the PO.

Moreover, the capacity of micelle formation of the polymer-drug conjugates obtained after click reaction was tested. In the case of the click copolymers, it is just studied the micelle formation, not the drug incorporation by the micelle since the drug have been already joined to the polymers backbones. The micelles sizes obtained were 233.37 and 188.57 nm for the polymer-drug conjugates with a 3 and 10 wt % of GPE, respectively.

Thus, the drug incorporation by forming the polymer-drug conjugates by the click chemistry reaction allowed to form micelles in aqueous solution and with smaller size than that obtained by direct solution what benefits the permeability.

Figure 9 shows the spherical morphology of loaded-micelles by HRSEM. The HRSEM shows that there are big micelles, due to copolymer aggregation tendency, and another small ones of about 50 nanometers. Besides, this image shows that the obtained average size of big micelles by this technique is comparable to the micelles apparent hydrodynamic size obtained through DLS.

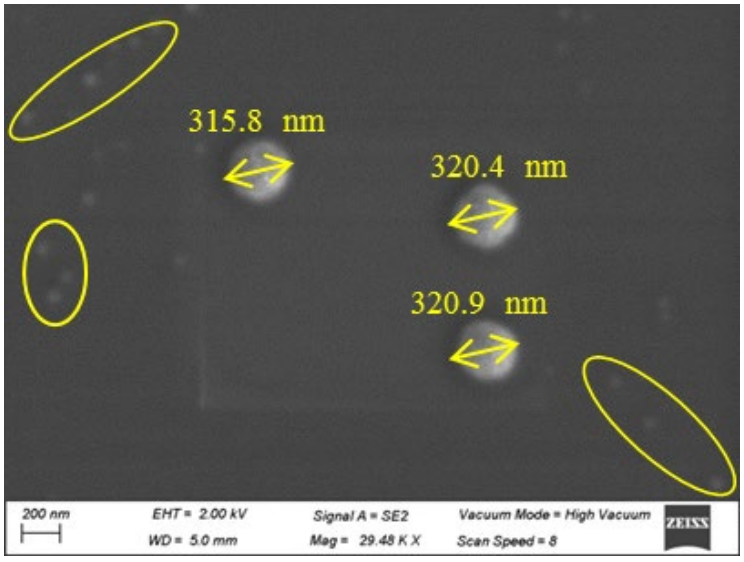


Figure 9: HRSEM image of coumarin-loaded micelles obtained by physical entrapment for the PEO-b-PPO-b-GPE copolymer with 3 wt % of GPE

4. CONCLUSIONS

Ring opening polymerization with ethylene glycol as initiator in three different steps, starting with EO and ending with the GPE, was the most suitable synthesis procedure to obtain PEO-b-PPO-b-GPE copolymers with the desired characteristics of hydrophilic:hydrophobic mass ratio, molecular weight and PDI, according to GPC analyses. The maximum GPE content for the stablished polymerization conditions was 10 wt % in order to avoid a high deviation from the desired molecular weight and an important yield process reduction. On the other hand, the CMC values obtained for the copolymers with the different mass percentages of GPE studied were very similar between them, and also to the value of a commercial PEO-b-PPO copolymer of equal architecture. Thus, the incorporation of GPE to functionalize the copolymer chain up to a 10 wt % does not affect significantly to their capacity of micelle formation.

On the other hand, the azide-coumarin synthesis process with NaN_3 in excess and the subsequent click reactions between this product and the functionalized copolymers at atmospheric pressure and in $scCO_2$ conditions, were carried out successfully according to FTIR and NMR analyses. Therefore, the formation of polymer-drug conjugates with coumarin covalently attached and by an environmentally friendly process were possible, obtaining similar yields in both ways. This way, up to a content of about 9.3 wt % of azide-coumarin has been incorporated in the PEO-b-PPO-b-GPE copolymers. Besides, the capacity of these polymer-drug conjugates to form micelles in aqueous solution was also confirmed according to DLS results.

Finally, the direct dissolution method was suitable for micelle formation because the size and spherical morphology of the polymeric micelles obtained according to DLS and HRSEM results respectively were in the proper range to enable its absorbability by the target cells. This size increased when coumarin was added, confirming its entrapment in their cores. Additionally, these results showed that the size of polymeric micelles was smaller for the copolymer with higher GPE content, even for the case of having the coumarin to the copolymer, what is expected to favor the polymer-drug conjugate permeability in the body.

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TABLES

Table 1: Mw, PDI and polymerization yield of copolymers with hydrophilic:hydrophobic mass ratio of 1 and 3 wt % of GPE depending on the synthesis route

Copolymer synthesis route	M_w (Da)	PDI (M_w/M_n)	Yield (%)
A	5032	1.188	87.6
B1	5643	1.101	92.5
B2	4942	1.159	88.9

Table 2: GPC results of copolymers with hydrophilic:hydrophobic mass ratio of 1 and different % (mass) of GPE

GPE Content (wt %)	M_w (Da)	PDI	Yield (%)
0	6250	1.086	96.6
3	5640	1.100	92.5
7	5330	1.160	90.8
10	5580	1.190	86.0
15	4470	1.200	82.9

Table 3: CMC values of copolymers with different wt % of GPE obtained by KI/I_2 method

GPE content (wt %)	CMC (w/v %)
0	0.0027
3	0.0014
7	0.0015
10	0.0014

Table 4: Size of empty and coumarin-loaded micelles

	Empty micelles size (nm)	Loaded-micelles size (nm)
Copolymer with 3 wt % of GPE	230.37	351.5
Copolymer with 10 wt % of GPE	160.47	225.34

FIGURE AND SCHEME LEGENDS

Scheme 1: Steps of the different copolymer synthesis routes: a) common first step, b) route A, c) route B1 and d) route B2

Scheme 2: Click reaction between synthesized copolymer and azide-coumarin

Figure 1: FTIR spectra of the GPE and the copolymers without and with GPE

Figure 2: 1H-NMR spectra of (a) pure GPE and (b) 2nd and 3rd steps of the PEO-b-PPO-b-GPE copolymer synthesis reaction for a 10 wt % of GPE content

Figure 3: Determination by UV-vis/KI/I2 method of CMC value for a PEO-b-PPO copolymer without GPE

Figure 4: FTIR spectra of bromo-coumarin and azide-coumarin

Figure 5: 13C-NMR spectrums of bromo-coumarin and azide-coumarin

Figure 6: FTIR spectra of products before and after click reactions at P_{atm} for PEO-b-PPO-b-GPE copolymers with (a) 3 wt % and (b) 10 wt % of GPE

Figure 7: 1H-NMR spectra of products after click reactions at P_{atm} for PEO-b-PPO-b-GPE copolymers with 3 wt % and 10 wt % of GPE content

Figure 8: (a) FTIR spectra of products before and after click reaction at Patm and in scCO2 conditions for PEO-b-PPO-b-GPE copolymer with 10 wt % of GPE and (b) 1H-NMR spectrums of products after click reaction in $scCO_2$ for PEO-b-PPO-b-GPE copolymers with 3 wt % and 10 wt % of GPE content

Figure 9: HRSEM image of coumarin-loaded micelles obtained by physical entrapment for the PEO-b-PPO-b-GPE copolymer with 3 wt % of GPE