Synthesis and operating optimization of PEG conjugate via CuAAC in scCO$_2$

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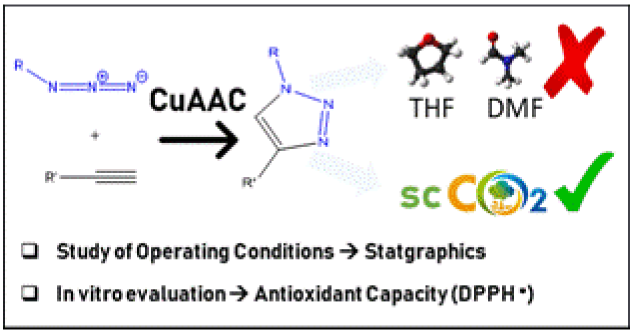


Abstract

A new sustainable green protocol for obtaining polyethylene glycol (PEG) conjugates, with a prototype molecule, which in this work was coumarin, by means of click chemistry. The organic solvents commonly used for this type of reaction were replaced by supercritical carbon dioxide ($scCO_2$). The synthesis and characterization of PEG-coumarin was successfully reported using FTIR, ^{1}H NMR and MALDI TOF. Subsequently, a preliminary study was carried out, using response surface methodology to examine the variables that most affect the use of $scCO_2$ as a reaction medium. The main effects caused by these variables, individually and their binary interaction, have been estimated. Response surface methodology has been used in this work to screen variables, using a factorial design $2^{3.}$ The p-value of temperature and pressure was 0.006 and 0.0117, being therefore the most significant variables of the response surface methodology study.

Subsequently, a more intensive study has been carried out on the variables that have shown the greatest significant effect on reaction performance where a 82.32% synthesis success was achieved, which broadens the scope of the use of $scCO_2$ as a reaction medium. The conjugated coumarin with mPEG-alkyne and coumarin were evaluated for their in vitro antioxidant activity by DPPH radical scavenging assay and was found to exhibit substantial activity. The click product showed comparable or even better efficacy compared to the initial coumarin.

1. Introduction

In the last 15 years, various research teams have carried out the applicability of polymer-drug conjugates to deliver drug combinations. Polymer-drug conjugate is a technology in which a drug is covalently bound to a polymeric carrier. In 1960s, the Prof. Frank Davis proposed to conjugate polyethylene glycol (PEG) with a protein, i.e. "PEGylate" a protein, in order to create a conjugate with a hydrophilic polymer with a new protein. Therefore, the new recombined proteins would be less immunogenic in the body, and therefore improve its circulation and activity during all its life [1,2]. In 1975, a rational model for pharmacologically active polymers was first proposed by Helmut Ringsdorf [3,4]. His concept of covalently bound polymer-drug conjugates still forms the basis for much of the work in this area performed today[5].

The Huisgen and co-workers in 1960 studied the cycloaddition using azides and alkynes is an important method for the synthesis of 1,2,3 triazoles [6]. Independently, Sharpless and Meldal in 2002, discovered that copper catalysis could increase its reaction rate by up to 10^7 [7,8]. The copper (I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction is generally considered as the most remarkable example of click chemistry, which has emerged as a prominent organic transformation. Polymer science has profited from CuAAC by its simplicity, ease, scope, applicability, and efficiency. In addition, click chemistry has found wide application in drug discovery bioconjugation reaction, polymer chemistry with pharmaceutical and biomedical applications [9–16]. Furthermore, it has greatly facilitated the overall drug discovery process by providing easy access for the synthesis of building blocks for new molecular entities. CuAAC reaction is usually carried out with organic solvents, such as dimethylformamide (DMF), toluene or tetrahydrofuran (THF) [17–19]. Traditional organic solvents could potentially cause various health and environmental concerns due to their volatility and toxicity. Recently the use of supercritical fluids as environmentally benign solvents for chemical synthesis is one of the new approaches as a green chemistry. Carbon dioxide is the most widely used gas for supercritical fluid studies because of its moderate critical constants ($P_c = 73.8$ bar; $T_c = 31.1$ ºC), it is nontoxic, non-flammable, inexpensive, relatively inert, its removal after the chemical transformation is not energy consuming and its recycling [20,21]. There are a variety of contributions which have studied CuAAC reaction with organic solvents and at high temperatures. However, there are only four papers that use $scCO_2$ to carry out CuAAC reactions. Therefore, the CuAAC reaction is yet far from being fully developed in $scCO_2$ [22–26].

The functionalized polymer-drug conjugates are, with steady increase, being utilized to obtain biodegradable systems in an effort to enhance localized drug delivery and easy removal[5]. Although different polymer compositions have been synthesized and studied, some of the simplest polymers, such as poly (ethylene glycol) (PEG), maintain widespread use and versatility. Furthermore, the use of PEG has been established clinically and approved by the Food and Drugs Administration (FDA) and Evaluation of Medical Product in Europe (EMEA) [27]. Because of PEG only contains two functional groups limiting the scope for further derivation with targeting ligands, methoxy-poly(ethylene glycol) alkyne (mPEG-alkyne) was used in this [28–31]. The bioactive agent chosen for PEG conjugation is the coumarin. Natural coumarins or synthetic analogues are of great interest due to their pharmacological properties. Their physiological, bacteriostatic and anti-tumour activity makes these compounds interesting as novel therapeutic agents [32,33].

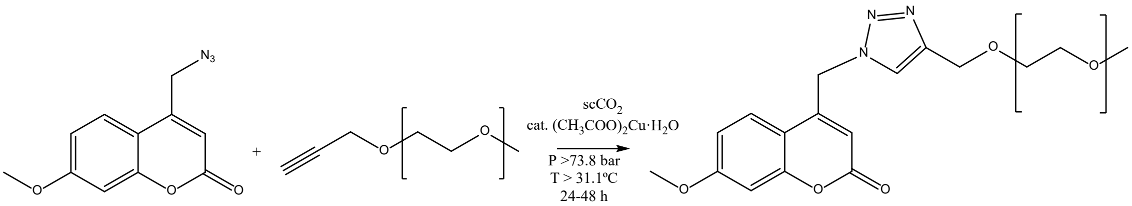
“In the present work, the main objective was to obtain a conjugate polymer-drug, in which the interaction has been achieved through a covalent bond in $scCO_2$ by means of click chemistry. This research has been initiated by recent interest in click products which have a broad spectrum of applications in the emerging fields such as drug discovery, chemical biology and materials science[9–12]”.The functionalized product was characterized by ^{1}H NMR spectroscopy, MALDI-TOF MS and FTIR spectroscopy. The next step was to optimize the operating conditions using a factorial design 2^3, where the most significant influence of the process variables was observed. Once this information was obtained, an additional study was carried out on the most influential variables on the reaction yield. Finally, the antioxidant activity of the click conjugate, the raw coumarin and the modified coumarin with the azide group was evaluated.

2. Results and Discussion

2.1. Synthesis of mPEG-coumarin using scCO$_2$

The initial working conditions were above 31.1 ºC and pressures above 73.8 bar with the intention of working in the supercritical solvent region. The molar ratio between alkyne and azide groups was maintained in 1. Copper (II) acetate monohydrate was chosen as catalyst to conjugate coumarin with mPEG-alkyne (Scheme 1). The choice of this catalyst was derived from its high efficiency in scCO$_2$[24].

Scheme 1. Synthesis reaction scheme for mPEG-coumarin in scCO$_2$.



A first preliminary study was carried out at 130 bar, 0.5 molar ratio between the catalyst and alkyne group (C/A), 35 ºC and 24 hours. The evidence for click reaction between the 4-azidomethyl-7-methoxycoumarin and mPEG-alkyne can be proved by FTIR. The FTIR spectra of click product, mPEG-alkyne and 4-azidomethyl-7-methoxycoumarin were shown in Figure 1. The peak at 2167 cm^{-1} corresponds to the alkyne groups of the mPEG-alkyne. The peak at 2110 cm^{-1} corresponds to the azide group of the 4-azidomethyl-7-methoxycoumarin. Both are not observed in the spectra of the mPEG-coumarin click product, which means the azide and alkyne groups disappear completely due to the coupling reaction between them. In addition, the appearance of characteristic peaks of triazole ring at 1464 and 1615 cm^{-1} were observed. Therefore, the click reaction between the 4-azidomethyl-7-methoxycoumarin and mPEG-alkyne was carried out successfully.

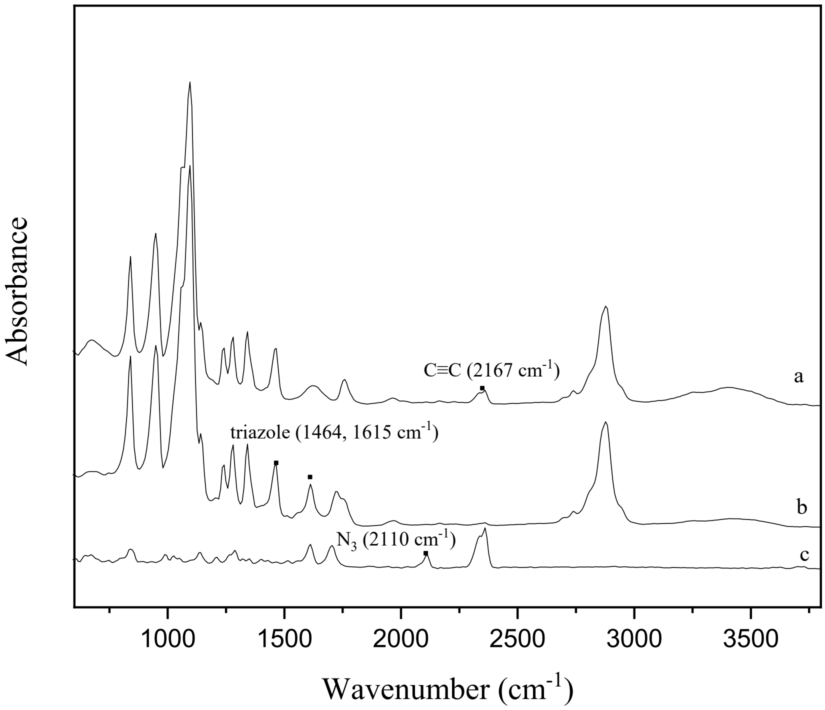


Figure 1. FTIR spectrum where: a) mPEG-alkyne; b) click product mPEG-coumarin in $scCO_2$; c) 4-azidomethyl-7-methoxycoumarin.

With the aim of getting to know the structure of the mPEG-coumarin product and identify more clearly the presence of the triazole ring and coumarin group in the polymer structure, ^{1}H NMR analysis was used. As shown in Figure S6, the protons of PEG chain were observed in the range of δi 3.5-3.8. The triazole proton appeared as a singlet at δ_g 7.85. The aromatic proton of coumarin were observed with a doublet δ_c 7.59,7.61 and δ_b 6.83, 6.84 and multiplet around δ_e 5.76. The presence of the chemical shifts of proton corresponding to PEG, triazole and coumarin confirmed that the conjugate has been successfully synthesized.

Finally, MALDI-TOF mass spectroscopy was used to complete the characterization of the click product. Through this characterization technique, it will be possible to determine both: the structure of the polymer and the synthesized click product as well as, the yield achieved in the reaction. Figure 2 (a) shows the mass spectrum for mPEG-alkyne, where it can be verified that the monomer unit of the polymer corresponds to 44 g mol^{-1}

(ethylene oxide), and an average molecular weight of 2122 g/mol^{-1}. In this procedure, the exact distribution of the polymer and its molecular weight are known. Therefore, it will be possible to compare the displacement of the mPEG-alkyne molecular weight signal with the click product (mPEG-coumarin) molecular weight signal and determine the yield of reaction through the equation (1), where $I_{PEG.\text{-}alkyne}$ represent the intensity of polymer, and I_{Click} represent the intensity of mPEG-coumarin. In the Figure 2 (b), a displacement of 232 um was observed, which corresponds to the molecular weight of azide coumarin. Therefore, the reaction was successfully carried out.

$$\eta = 100 - \left(\frac{I_{PEG-alkyne}}{I_{PEG-alkyne} + I_{Click}}\right) \cdot 100 \tag{1}$$

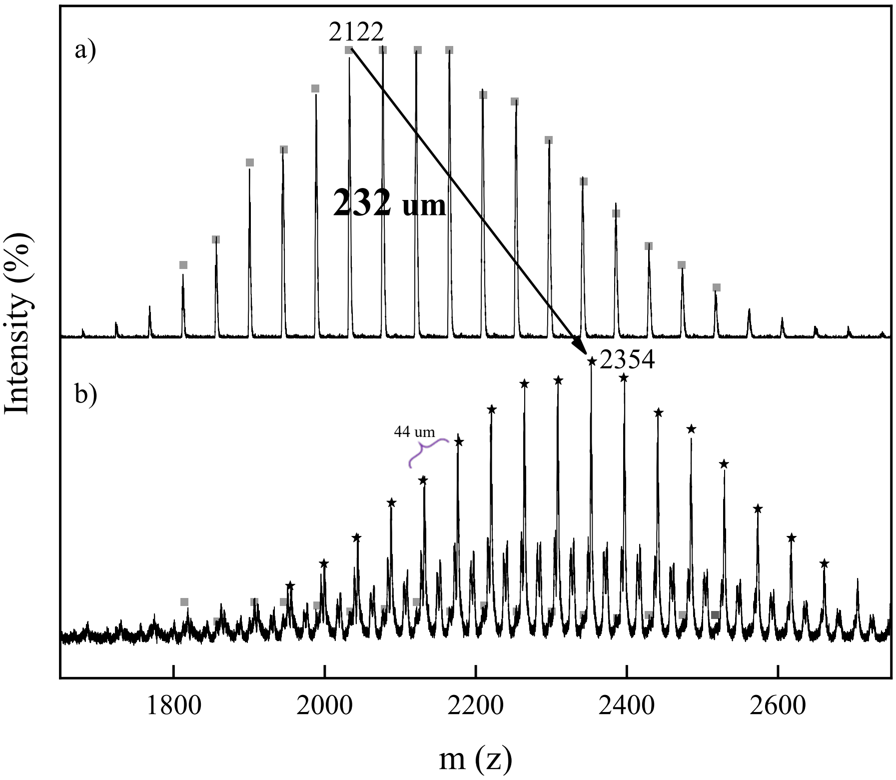


Figure 2. MALDI TOF MS spectra: a) mPEG-alkyne; b) mPEG-coumarin. ■ Peaks of mPEG-alkyne ★ Peaks of mPEG-coumarin.

The yield obtained was 82.32 % for 130 bar, 0.5 molar ratio of catalyst/alkyne, 35 ºC and 24 hours. An additional experiment was carried out in order to optimize the reaction time. In previous studies of CuAAC reactions, the absence of ligand of catalyst, caused a reduction in the observed reaction rate[34]. The time was increased from 24 to 48 hours, getting a yield of 87.14 %. When the time is increased, there is a very low increase in yield. In other words, most of the reaction takes place in the first 24 hours.

2.2. Preliminary study with factorial design 2^3

Once the feasibility of the click product formation process has been demonstrated using supercritical technology with CO_2 as solvent, the operation conditions have been optimized through a 2^3 full factorial design. The yields were determined with MALDI TOF-MS, as mentioned previously, and the spectrum of each run are included in Supplementary Material (Table S1). The studied variables have been the pressure, the molar ratio of catalyst/alkyne and the temperature. The standard experimentation matrix is shown in Table 1.

Table 1. Experimental matrix and results for the full 2^3 factorial design[a].

Run	P (bar)	T (ºC)	C/A molar ratio	Density of $scCO_2$[b] (g/l)	Yield (%)
1	1	-1	-1	769	67.67
2	-1	1	1	560	40.90
3	0	0	0	675	60.23
4	-1	-1	-1	680	45.52
5	1	1	1	725	59.52
6	1	1	-1	725	45.62
7	1	-1	1	769	82.32
8	-1	-1	1	680	50.89
9	-1	1	-1	560	37.45
10	0	0	0	675	57.96

[a] Experimental conditions: azide to alkyne molar ratio, 1:1; reaction time, 24 h.
[b] Density of $scCO_2$ determined by equation of Bender[35]

The analysis of the main effects and their interactions for the chosen response are shown in Table 2. Statistically significant effects are underlined according to p-values calculated.

According to the results shown in Table 2, the yield of click reaction is mainly affected by the pressure and temperature.

Table 2. Estimated effects, interactions and ANOVA analysis from 2^3 factorial design for click reaction in $scCO_2$.

Factor of interaction	Yield (%)	
	p-Effects (± s)	p-Value
Pressure	20.025 ± 2.86369	0.0060
Temperature	-15.795 ± 2.86369	0.0117
C/A molar ratio	9.275 ± 2.86369	0.0479
Pressure - Temperature	-6.765 ± 2.86369	0.0992
Pressure - C/A molar ratio	4.865 ± 2.86369	0.1879
Temperature – C/A molar ratio	-0.435 ± 2.86369	0.8140

The yield of reaction was investigated by mean of a Pareto chart (Figure 3). The length of each bar indicates the standardized effect of the selected factor on the different responses and its colour represents if the contribution was positive or negative. The positive effects (grey colour) presented a favourable effect on the response while the negative effects (black colour) shown an antagonistic effect on it. The effect of increasing the C/A molar ratio, increasing the pressure and both, promote the increase of the yield. The temperature has a negative effect in the yield.

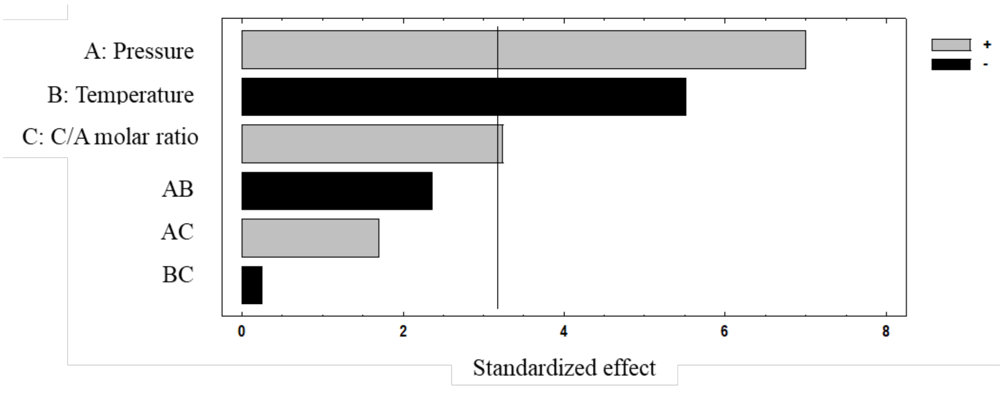


Figure 3. Standardized pareto chart for the yield.

CO_2 density is directly dependent on pressure and temperature. The density of $scCO_2$ was calculated as a function of pressure and temperature with the equation of Bender[35]. The density of CO_2 increases between 100 and 130 bar and decreases between 35 and 45 ºC the density decreases, therefore CO_2 density increases with the pressure and decreases with the temperature, as shown the Table 1. Slight changes in pressure and temperature in the process with CO_2 will produce significant variations in its density and hence in the solubility of CO_2 with different kinds of compounds. According to literature, in the case of polymers such a high molecular weight, which are generally insoluble in CO_2, a gradual change in the solubility of the CO_2 polymer mixture was reported, when sudden changes in CO_2 density[36–38]. On the other hand, CO_2 is also able to plasticize many polymers owing to its capability to solubilize into the polymer. This effect appears when the CO_2 density increases into polymer causing the plasticisation effect. A pressure increase or a temperature decrease favour the density upgrade. This effect improves the yield of reaction due to a better interaction between polymer and organic compound[39,40]. On this basis, temperature has a global negative effect and pressure has a positive effect in the experimental range analysed according to pareto chart. Therefore, an increase in pressure and a decrease in temperature, at constant C/A molar ratio, acted increasing the yield of reaction.

The effect of catalyst was evaluated for 0.1 and 0.5 C/A molar ratio. The minimum value of C/A chosen in the factorial design was 0.1, as a large decrease in yield was observed when the ratio decreases. The ratio is not increased up to 0.5 because it will later present the problem of removing it from the click product. Figure 4 shows the MALDI TOF-MS spectrum for click reaction product using both ratio catalyst and in absence of it. It is possible to observe when the C/A molar ratio was zero, the reaction was not carried out. Furthermore, the increase in the C/A molar ratio causes an increase in performance, as shown in Figure 4, as the intensity of the peaks corresponding to click product (mPEG-coumarin) and the signal corresponding to the mPEG.

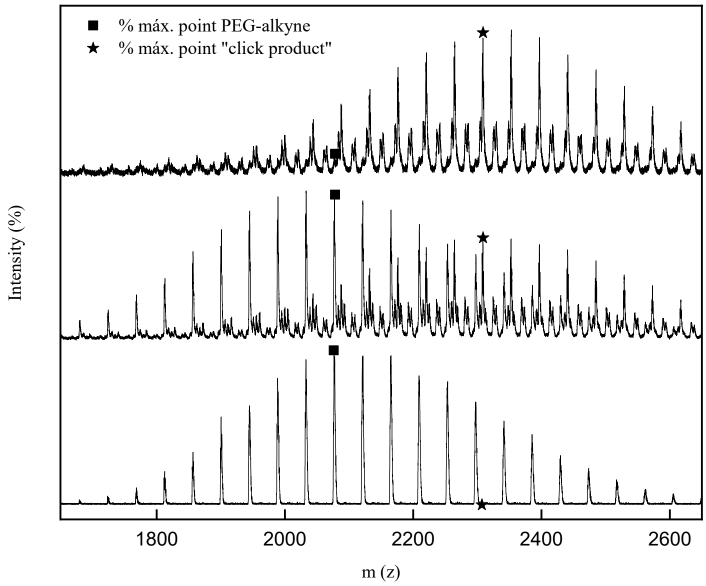


Figure 4. MALDI TOF-MS spectrum: a) 0.5 C/A molar ratio (Run 5); b) 0.1 C/A molar ratio (Run 4); c) Absence of catalyst. ■ Peaks of polymer ★ Peaks of click product.

For the purpose of determining whether the association between the response and each term included in the model is statistically significant, compare the p-value of the term with the significance level for assessing the null hypothesis. Binary interaction between pressure and temperature had a p-value of 0.09, binary interaction between pressure and molar C/A ratio had a value of 0.1879 and the binary interaction between temperature and molar C/A ratio 0.8140. These binary factors are not statistically significant at confidence level (0.05) with the terms of the current model. Thus, these values indicate that binary interaction between pressure, temperature and C/A molar ratio respectively had not a statistically significant association with the yield. In fact, evidence, it could be more clearly observed in pareto chart (Figure 3).

2.3. Study of the most influential variables of the cycloaddition in $scCO_2$

Since pressure is the operational variable most easily controllable by the experimental setup, further experiments were carried out to obtain more detailed information within the pressure range chosen for the factorial design. Temperature was not considered in this additional study, since its increase causes a negative effect on the yield and it could lead out of the supercritical region. The effect of pressure was re-evaluated because it was the most significant variable in the yield according to pareto chart (Figure 3), widening the range between 80 and 170 bar. In addition, the $scCO_2$ density for pressure and temperature conditions was determined with the Bender equation as shown in Table 3.

Table 3. Effect of pressure in the cycloaddition reaction[a].

Run	Press. (bar)	Density of $scCO_2$[b] (g/l)	Yield (%)
11	80	332.90	14.30
12	90	602.95	32.55
13	150	801.66	83.59
14	170	826.55	84.47

[a] Experimental conditions: azide to alkyne molar ratio, 1:1; reaction time, 24 h; temperature, 35 ºC; C/A molar ratio, 0.5.

[b] Density of $scCO_2$ determined by equation of Bender[35].

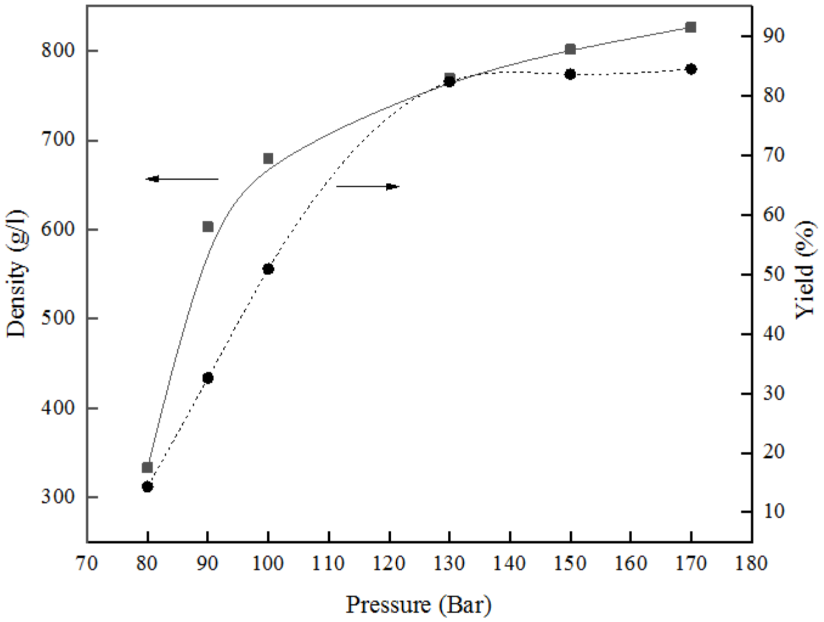


Figure 5. Effect of pressure and density on the yield of cycloaddition reaction. Experimental conditions: azide to alkyne molar ratio, 1:1; reaction time, 24 h; temperature, 35 ºC; C/A molar ratio, 0.5 ■ Values of density of scCO2 determined by equation of Bender [35] and ● Values of the yield of reaction.

With increasing pressure of CO_2, the CO_2 is diffused between the polymer chains and its sorption into polymer increases the free volume and mobility of the polymer segment, making it more accessible to carry out functionalization with the natural drug[41]. However, changes in CO_2 density values from 130 bar are gradually smaller, as shown in Figure 5. For this reason, when the pressure is increased above 130 bar, the yield of cycloaddition reaction is relatively constant. In numerous studies of sorption of CO_2 into polymer[42–44], have shown that by increasing the pressure values close to critical region the sorption values incresase. However, once the critical region is exceeded, these values remain practically constant. From which the performance remains practically constant.

In consequence of this study of response surface, which is carried out for first time for polyethylene glycol, the optimal conditions were 130 bar, 0.5 C/A molar ratio, 35 ºC and 24 hours, obtaining a yield of 82.32 %.

Finally, the yield of cycloaddition reaction using supercritical fluid was compared with a work published recently using the same polymer selected in this work and a similar coumarin. Behl et al.[33] using a THF, as a solvent, and N, N, N′, N″, N″-Pentamethyl diethylenetriamine (PMDTA), as a ligand, in order to carry out the click reaction getting a yield of 73%. Therefore, use of supercritical fluids is a promising way to carried out CuAAC reaction without necessity of ligand and organic solvents.

2.4. In vitro antioxidant evaluation

In the present study, antioxidant activity of the synthesized coumarin and conjugated coumarin has been assessed in vitro by the 1,1-dipheyl-2-picrylhydrazyl (DPPH) radical scavenging assay, and ascorbic acid was employed as a reference standard. The test compounds and the standard ascorbic acid were tested at five different test concentrations of 10, 20, 30, 40 and 50 μM. All the analysed compounds be shown significant scavenging activity ranging from 33.96 % to 78.18 %, in comparison to the standard drug ascorbic acid (95.41% at 50 µM), and the results of antioxidant capacity exhibited significant activity as shown Figure 6.

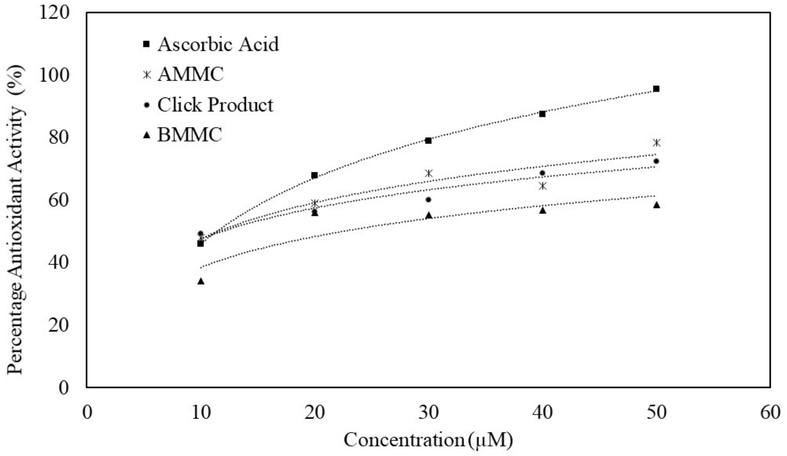


Figure 6. Increase in percentage antioxidant activity of different compound with increase in test concentrations.

Click product derived from 4-azidomethyl-7-methoxycoumarin show potent activity (IC$_{50}$ 11.95 µM) as compared to the standard. However, the coumarin azide (AMMC) shows a higher IC$_{50}$ value compared to the click product, although the different is practically negligible. The compound BMMC (4-bromomethyl-7-methoxycoumarin) with bromo- substituent on coumarin exhibit less activity as compared to AMMC showed an activity very close to the IC$_{50}$ value of the clicked product [45].

The antioxidant activity of the 4-bromomethyl-7-methoxycoumarin, 4-azidomethyl-7-methoxycoumarin and click product by the DPPH assay method, expressed as the mean ± SD (Standard Deviation), along with IC$_{50}$ values obtained by regression analysis, are shown in Table 4.

Table 4. In vitro antioxidant evaluation of 4-bromomethyl-7-methoxycoumarin, 4-azidomethyl-7-methoxycoumarin and click product.

	DPPH method – Mean percentage free radical scavenging activity ±SD[a]											
Compound	**10 µM**		**20 µM**		**30 µM**		**40 µM**		**50 µM**		**IC$_{50}$ ± SD**	
BMMC[b]	33.96	±0.14	55.85	±0.05	55.20	±0.02	56.66	±0.01	58.53	±0.05	22.55	±0.05
AMMC[c]	47.76	±0.14	58.83	±0.36	68.49	±0.05	64.51	±0.05	63.39	±0.05	11.57	±0.24

Click Product	49.11	±0.01	56.13	±0.10	59.85	±0.01	68.56	±0.05	72.34	±0.05	11.95	±0.56
Ascorbic acid	45.78	±0.37	67.73	±0.29	78.91	±0.98	87.45	±0.41	95.42	±0.45	11.42	±0.17

[a] The results are expressed as the mean values ±SD (Standard deviation), n=3, $p < 0.05$.

[b] 4-bromomethyl-7-methoxycoumarin.

[c] 4-azidomethyl-7-methoxycoumarin.

3. Conclusions

In conclusion, the synthesis and characterization of PEG-alkyne with a prototype molecule has been demonstrate by copper-mediated alkyne-azide cycloaddition via $scCO_2$ was successfully reported in which the power of different conditions in $scCO_2$ was studied. The optimization was carried out demonstrating the influence of pressure, temperature and C/A molar ratio on the yield of click reaction in $scCO_2$. Temperature and pressure played a key role in the process and have a strong significant effect on reaction yield. Finally, temperature of 35 ºC and pressure of 130 bar were selected as the optimal experimental variables in order to maximize the yield of reaction. The synthesized compound showed promising antioxidant activity (IC_{50} = 11.95 µM) when compared to ascorbic acid.

The advantage of the presented eco-friendly approach of CO_2 based supercritical technologies show an enormous potential in the production of different enhanced drug formulations. The pharmaceutical industry must take advantage of the opportunities that these technologies offer.

4. Materials and Methods.

4.1. Materials

The following materials were used to carry out the different synthesis: 4-bromomethyl-7-methoxycoumarin (97 %, Sigma Aldrich), sodium azide (>99.5 % sigma Aldrich), methoxy polyethylene glycol (mPEG, Mn=2000 g/mol, Sigma Aldrich), propargyl bromide (80 % in toluene, Sigma Aldrich), sodium hydride (NaH, 60 % in mineral oil, Sigma Aldrich), ethyl acetate (anhydrous, 99.8 %, Sigma Aldrich) and carbon dioxide (Industrial grade ≥ 99 %, Carburos Metálicos). The catalyst used to carry out the cycloaddition reaction was Copper (II) acetate monohydrate (Sigma Aldrich).

4.2. Synthesis of 4-azidomethyl-7-methoxycoumarin

The synthesis of this compound was carried out according to bibliography[46–49]. A mixture of NaN_3 (1.2 g) and 4-bromomethyl-7-methoxycoumarin (1 g) in acetone/acetonitrile (1:1, 120 mL) solution was added to a 250 mL flask (Supplementary Material, Scheme S1). The mixture was stirred at 50 ºC for 48 h. Then, solvents were removed under vacuum. The organic extracts were washed with water to precipitate the 4-bromomethyl-7-methoxycoumarin which did not react. The product was filtered and washed with heptane and dried under vacuum (81 % yield). FTIR and ^{1}H-NMR spectrum are included in Supplementary Material (Figure S1 and Figure S2). The characteristics peaks of FTIR appearing at 2110, 1694, 1604, 1432, 1135, 670 cm^{-1} belong to the C-N_3, C=O, C=O, ether groups, Br-CH, respectively. The FTIR spectra were consistent with the structure of coumarin and confirmed the results of ^{1}H-NMR. ^{1}H-NMR ($CDCl_3$, ppm, 500 MHz): δ_a 3.89 (s, 3H), δ_b 6.87-6.9 (dd, J=8.5Hz, 2.5Hz, 3H), δ_c 7.44-7.45 (d, J=8.5Hz, 1H), δ_d 6.863-6.868 (d, 2.5Hz, 1H), δ_e 6.37 (s, 1H), δ_f 4.51(s, 2H).

4.3. Synthesis of methoxy-PEG alkyne (mPEG-alkyne)

The synthesis was performed using a reactor with 250 mL of capacity. The first step was to add dropwise mPEG to a mixture of sodium hydride (610.5 mg, 15.4 mmol) in 100 mL dry THF at room temperature. The first stage was ended when the gas formation stopped, around 30 minutes. Once the formation of hydrogen has been consumed, the propargyl bromide (80 % in toluene 1.71 mL, 15.4 mmol) was added (Supporting Information, Scheme S2). Operation conditions were 50 ºC and 100 rpm for 24 h in order to assure that hydroxyl groups lost the proton and the alkoxide was formed correctly[33]. Ending the synthesis, the solvent was removed easily on the rotary evaporator. Ethyl acetate was used as wash liquid and, finally, the product was dried under vacuum to get a white solid (62 % yield). The characteristics peaks of FTIR appearing at 2167, 1108, 1750, 2885 cm^{-1} belong to the C≡C, C-O, CH_2 respectively (Supporting Information, Figure S3). The FTIR spectra were consistent with the structure of polymer and confirmed the results of ^{1}H-NMR (Supporting Information, Figure S4). ^{1}H-NMR ($CDCl_3$): δ_a 2.42 (s, 1H), δ_b 3.31 (s, 2H), δ_a 3.5-3.8 (m, 154H), δ_a 4.17 (s, 1H). The MALDI TOF of mPEG-alkyne is shown in Figure S5 (Supporting Information).

4.4. Synthesis of click product at supercritical CO_2

The procedure for synthesizing the click product (mPEG-coumarin) in supercritical media consisted of introducing the reagents into the reactor. An equimolar amount of mPEG-alkyne and 4-azidomethyl-7-methoxycoumarin were added with a molar ratio of catalyst/alkyne (C/A) 0.1 and 0.5. In Figure 7, the schematic diagram of the experimental setup is shown. The scCO2 was pumped out after the reactor was hermetically sealed. Afterwards, the system was heated up within the temperature range studied. Once the reaction is complete, the heating was switched off and the reactor depressurized with a flow rate of 3 l/min.

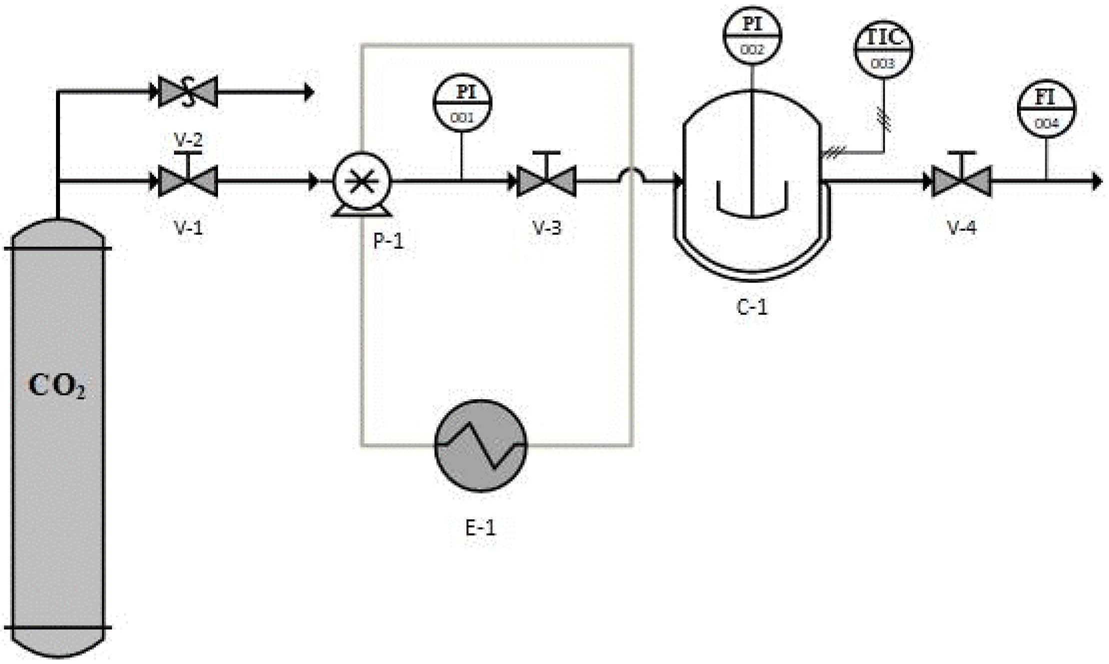


Figure 7. Schematic diagram of the experimental set-up for reaction in supercritical medium. V-1, V-3: Check valves; V-2: Purge valve. P-1: Pump; E-1: Cooler; C-1: Batch reactor; V-4: Valve of depressurization; TIC: Temperature digital controller; FI: Flow indicator; PI: Pressure indicator.

4.5. Fourier-transform infrared spectroscopy (FTIR)

Infrared (IR) spectra were recorded on a Varian 640-IR Fourier transform IR spectrophotometer with 16 scans per experiment at a resolution of 32 cm^{-1} in the range 4000–400 cm^{-1}, using the software Varian Resolution.

4.6. Nuclear magnetic resonance of proton (^{1}H NMR)

^{1}H NMR was measured with Varian Gemini FT-500 spectrometer using $CDCl_3$ as solvent. NMR spectra were acquired at 25 ºC. Chemical shifts are given in ppm relative to TMS (^{1}H, 0.0 ppm) or $CDCl_3$ (^{1}H, 7.2 ppm). The NMR measurement were performed in the Department of Organic Chemistry at University of Castilla-La Mancha.

4.7. MALDI TOF-MS.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF-MS) was carried out using a Bruker Autoflex II TOF/TOF spectrometer (Bremen, Germany) using $CDCl_3$ as solvent and dithranol (1,8,9-trihydroxyanthracene) as matrix material. The MALDI TOF-MS measurements were performed in the Department of Organic Chemistry at University of Castilla-La Mancha.

4.8. Experimental design

A statistical experimental design based on central composite design (CCD) was used. The effect of the operating variables in the cycloaddition reaction in $scCO_2$ (pressure, temperature and catalyst to alkyne groups molar ratio) and their possible interactions on the yield as response variable were studied using a 2^3 full factorial design with two central points [50,51]. The levels of each factor are indicated in Table 5 and were selected on the basis of preliminary studies of click chemistry in $scCO_2$ [22].

Table 5. Levels of factors in the experimental design

Factor	Lower level (-1)	Higher level (+1)
Temperature (ºC)	35	45
Pressure (bar)	100	130
C/A molar ratio	0.1	0.5

A statistical analysis was performed for these results using the commercial software *Statgraphics 5.1* Plus (Manugistics, Inc. Rockville, MD, USA). The analysis of variance (ANOVA) provided a study of the variation present in the results of the experiments carried out. The test of statistical significance, p-value, was determined according to the total error criteria considering a confidence level of 95 %. The influence of a factor will be significant if the value of critical level (p) is lower than 0.05.

4.9. *In vitro* antioxidant activity

The in vitro radical scavenging of the newly synthesized compounds was carried out by 1,1-diphenyl -2-picrylhydrazyl (DPPH) radical. The hydrogen atom or electron donating ability of the compounds was measured from the bleaching of the purple-coloured methanol solution of 1,1-diphenyl-1-picrylhydrazyl (DPPH). where 2 mL of each methanolic solution of two compounds is tested with various concentrations (10, 20, 30, 40 and 50 μM) that are mixed with 2 mL of a methanolic solution of DPPH (0.1, μM) was employed as the control, as indicated Venkata et al.[52]. The solution was incubated at 37 ºC, since this is approximately the corporal temperature and for 30 min and the measurement were done at λ=516 nm because the sorption spectrum gave a at this wavelength (Figure S6). The percentage free radical scavenging activity was calculated according to Equation (2).

$$\% \text{ Scavenging} = \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}} * 100 \qquad (2)$$

The IC_{50} values for each drug compounds as well as standard preparation were calculated. The IC_{50} is the concentration of drug required for 50% inhibition. IC_{50} (μM) value was calculated interpolation from linear regression analysis[53].

The measurement of the samples at different concentrations was done in triplicate.

Acknowledgments

We gratefully acknowledge funding from the Ministry of economy and competitiveness through funding the projects Ref. CTQ2016-79811-P. The authors also acknowledge the support of the Ministry of economy and competitiveness for the fellowship of Ms. López Quijorna Ref. BES-2017-079770. The authors state that they have not conflict of interest with any public or private body.

Supporting Information.

Full experimental details and synthesis and characterizations of synthesized conjugates (FTIR, ^{1}H NMR, and MALDI TOF-MS spectra).

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References

(1) Davis, F. F. The Origin of Pegnology. *Adv. Drug Deliv. Rev.* **2002**, *54* (4), 457–458. https://doi.org/https://doi.org/10.1016/S0169-409X(02)00021-2.

(2) Hoffman, A. S. The Early Days of PEG and PEGylation (1970s–1990s). *Acta Biomater.* **2016**, *40*, 1–5. https://doi.org/https://doi.org/10.1016/j.actbio.2016.05.029.

(3) Ringsdorf, H. Structure and Properties of Pharmacologically Active Polymers. *J. Polym. Sci. Polym. Symp.* **1975**, *51* (1), 135–153. https://doi.org/10.1002/polc.5070510111.

(4) Ringsdorf, H. Ringsdorf, H.: Structure and Properties of Pharmacologically Active Polymers. J. Polym. Sci. Polym. Symp. 51, 135-153. *J. Polym. Sci. Polym. Symp.* **2007**, *51*, 135–153. https://doi.org/10.1002/polc.5070510111.

(5) Larson, N.; Ghandehari, H. Polymeric Conjugates for Drug Delivery. *Chem. Mater.* **2012**, *24* (5), 840–853. https://doi.org/10.1021/cm2031569.

(6) Huisgen, R.; Mloston, G.; Langhals, E. The First Two-Step 1,3-Dipolar Cycloadditions: Non-Stereospecificity. *J. Am. Chem. Soc.* **1986**, *108* (20), 6401–6402. https://doi.org/10.1021/ja00280a053.

(7) Tornøe, C. W.; Meldal, M. Peptidotriazoles: Copper(I)-Catalyzed 1,3-Dipolar Cycloadditions on Solid-Phase. In *Peptides: The Wave of the Future: Proceedings of the Second International and the Seventeenth American Peptide Symposium, June 9--14, 2001, San Diego, California, U.S.A.*; Lebl, M., Houghten, R. A., Eds.; Springer Netherlands: Dordrecht, 2001; pp 263–264. https://doi.org/10.1007/978-94-010-0464-0_119.

(8) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. A Stepwise Huisgen Cycloaddition Process: Copper(I)-Catalyzed Regioselective “Ligation” of

Azides and Terminal Alkynes. *Angew. Chemie - Int. Ed.* **2002**, *41* (14), 2596–2599. https://doi.org/10.1002/1521-3773(20020715)41:14<2596::AID-ANIE2596>3.0.CO;2-4.

(9) van Dijk, M.; Rijkers, D. T. S.; Liskamp, R. M. J.; van Nostrum, C. F.; Hennink, W. E. Synthesis and Applications of Biomedical and Pharmaceutical Polymers via Click Chemistry Methodologies. *Bioconjug. Chem.* **2009**, *20* (11), 2001–2016. https://doi.org/10.1021/bc900087a.

(10) Crescenzi, V.; Cornelio, L.; Di Meo, C.; Nardecchia, S.; Lamanna, R. Novel Hydrogels via Click Chemistry: Synthesis and Potential Biomedical Applications. *Biomacromolecules* **2007**, *8* (6), 1844–1850. https://doi.org/10.1021/bm0700800.

(11) Nwe, K.; Brechbiel, M. Growing Applications of “Click Chemistry” for Bioconjugation in Contemporary Biomedical Research. *Cancer Biother. Radiopharm.* **2009**, *24*, 289–302. https://doi.org/10.1089/cbr.2008.0626.

(12) Kolb, H. C.; Sharpless, K. B. The Growing Impact of Click Chemistry on Drug Discovery. *Drug Discov. Today* **2003**, *8* (24), 1128–1137. https://doi.org/https://doi.org/10.1016/S1359-6446(03)02933-7.

(13) Zou, Y.; Zhang, L.; Yang, L.; Zhu, F.; Ding, M.; Lin, F.; Wang, Z.; Li, Y. “Click” Chemistry in Polymeric Scaffolds: Bioactive Materials for Tissue Engineering. *J. Control. Release* **2018**, *273*, 160–179. https://doi.org/https://doi.org/10.1016/j.jconrel.2018.01.023.

(14) Lallana, E.; Fernandez-Trillo, F.; Sousa-Herves, A.; Riguera, R.; Fernandez-Megia, E. Click Chemistry with Polymers, Dendrimers, and Hydrogels for Drug Delivery. *Pharm. Res.* **2012**, *29* (4), 902–921. https://doi.org/10.1007/s11095-012-0683-y.

(15) Hein, C. D.; Liu, X.-M.; Wang, D. Click Chemistry, a Powerful Tool for

Pharmaceutical Sciences. *Pharm. Res.* **2008**, *25* (10), 2216–2230. https://doi.org/10.1007/s11095-008-9616-1.

(16) Jain, Y.; Gupta, R.; Yadav, P.; Kumari, M. Chemical Waltz of Organic Molecules "On Water": Saline-Assisted Sustainable Regioselective Synthesis of Fluorogenic Heterobioconjugates via Click Reaction. *ACS Omega* **2019**, *4* (2), 3582–3592. https://doi.org/10.1021/acsomega.8b03167.

(17) Dadashi-Silab, S.; Kiskan, B.; Antonietti, M.; Yagci, Y. Mesoporous Graphitic Carbon Nitride as a Heterogeneous Catalyst for Photoinduced Copper(i)-Catalyzed Azide–Alkyne Cycloaddition. *RSC Adv.* **2014**, *4* (94), 52170–52173. https://doi.org/10.1039/C4RA09954K.

(18) Díaz, D.; Finn, M.; Sharpless, K.; Fokin, V.; Hawker, C. Cicloadicción 1,3-Dipolar de Azidas y Alquinos: I: Principales Aspectos Sintéticos. *An. la Real Soc. Española Química, ISSN 1575-3417, Nº. 3, 2008, pags. 173-180* **2020**.

(19) Ellanki, A. R.; Islam, A.; Rama, V. S.; Pulipati, R. P.; Rambabu, D.; Krishna], G. [Rama; Reddy], C. [Malla; Mukkanti, K.; Vanaja, G. R.; Kalle, A. M.; Kumar], K. [Shiva; Pal, M. Solvent Effect on Copper-Catalyzed Azide–Alkyne Cycloaddition (CuAAC): Synthesis of Novel Triazolyl Substituted Quinolines as Potential Anticancer Agents. *Bioorg. Med. Chem. Lett.* **2012**, *22* (10), 3455–3459. https://doi.org/https://doi.org/10.1016/j.bmcl.2012.03.091.

(20) Kazarian, S. Polymer Processing with Supercritical Fluids. *Polym. Sci.* **2000**, *42*, 78-.

(21) Shieh, Y.-T.; Su, J.-H.; Manivannan, G.; Lee, P.; Sawan, S.; Spall, W. Interaction of Supercritical Carbon Dioxide with Polymers. I. Crystalline Polymers. *J. Appl. Polym. Sci.* **1996**, *59*, 695–705. https://doi.org/10.1002/(SICI)1097-4628(19960124)59:4<695::AID-APP15>3.0.CO;2-P.

(22) Gracia, E.; García, M. T.; Borreguero, A. M.; Lucas], A. [De; Gracia, I.; Rodríguez, J. F. Functionalization and Optimization of PLA with Coumarin via Click Chemistry in Supercritical CO2. *J. CO2 Util.* **2017**, *20*, 20–26. https://doi.org/https://doi.org/10.1016/j.jcou.2017.04.008.

(23) Grignard, B.; Calberg, C.; Jerome, C.; Detrembleur, C. "One-Pot" Dispersion ATRP and Alkyne-Azide Huisgen's 1,3-Dipolar Cycloaddition in Supercritical Carbon Dioxide: Towards the Formation of Functional Microspheres. *J. Supercrit. Fluids* **2010**, *53* (1), 151–155. https://doi.org/https://doi.org/10.1016/j.supflu.2009.12.014.

(24) Zhang, W.; He, X.; Ren, B.; Jiang, Y.; Hu, Z. Cu(OAc)2·H2O—an Efficient Catalyst for Huisgen-Click Reaction in Supercritical Carbon Dioxide. *Tetrahedron Lett.* **2015**, *56* (19), 2472–2475. https://doi.org/https://doi.org/10.1016/j.tetlet.2015.03.102.

(25) Grignard, B.; Schmeits, S.; Riva, R.; Detrembleur, C.; Lecomte, P.; Jérôme, C. First Example of "Click" Copper(I) Catalyzed Azide-Alkyne Cycloaddition in Supercritical Carbon Dioxide: Application to the Functionalization of Aliphatic Polyesters. *Green Chem. - GREEN CHEM* **2009**, *11*. https://doi.org/10.1039/b822924d.

(26) Meghani, N. M.; Amin, H. H.; Lee, B.-J. Mechanistic Applications of Click Chemistry for Pharmaceutical Drug Discovery and Drug Delivery. *Drug Discov. Today* **2017**, *22* (11), 1604–1619. https://doi.org/https://doi.org/10.1016/j.drudis.2017.07.007.

(27) Kemptner, J.; Marchetti-Deschmann, M.; Siekmann, J.; Turecek, P. L.; Schwarz, H. P.; Allmaier, G. GEMMA and MALDI-TOF MS of Reactive PEGs for Pharmaceutical Applications. *J. Pharm. Biomed. Anal.* **2010**, *52* (4), 432–437.

https://doi.org/https://doi.org/10.1016/j.jpba.2010.01.017.

(28) D'souza, A. A.; Shegokar, R. Polyethylene Glycol (PEG): A Versatile Polymer for Pharmaceutical Applications. *Expert Opin. Drug Deliv.* **2016**, *13* (9), 1257–1275. https://doi.org/10.1080/17425247.2016.1182485.

(29) Sherman, M. R.; Williams, L. D.; Sobczyk, M. A.; Michaels, S. J.; Saifer, M. G. P. Role of the Methoxy Group in Immune Responses to MPEG-Protein Conjugates. *Bioconjug. Chem.* **2012**, *23* (3), 485–499. https://doi.org/10.1021/bc200551b.

(30) Thomas, A.; Müller, S. S.; Frey, H. Beyond Poly(Ethylene Glycol): Linear Polyglycerol as a Multifunctional Polyether for Biomedical and Pharmaceutical Applications. *Biomacromolecules* **2014**, *15* (6), 1935–1954. https://doi.org/10.1021/bm5002608.

(31) Zacchigna, M.; Cateni, F.; Drioli, S.; Bonora, G. M. Multimeric, Multifunctional Derivatives of Poly(Ethylene Glycol). *Polymers (Basel).* **2011**, *3* (3), 1076–1090. https://doi.org/10.3390/polym3031076.

(32) Hu, Y.; Chen, W.; Shen, Y.; Zhu, B.; Wang, G.-X. Synthesis and Antiviral Activity of Coumarin Derivatives against Infectious Hematopoietic Necrosis Virus. *Bioorg. Med. Chem. Lett.* **2019**, *29* (14), 1749–1755. https://doi.org/https://doi.org/10.1016/j.bmcl.2019.05.019.

(33) Behl, G.; Sikka, M.; Chhikara, A.; Chopra, M. PEG-Coumarin Based Biocompatible Self-Assembled Fluorescent Nanoaggregates Synthesized via Click Reactions and Studies of Aggregation Behavior. *J. Colloid Interface Sci.* **2014**, *416*, 151–160. https://doi.org/https://doi.org/10.1016/j.jcis.2013.10.057.

(34) Tale, R.; Gopula, V.; Toradmal, G. "Click" Ligand for "Click" Chemistry: (1-(4-Methoxybenzyl)-1-H-1, 2, 3-Triazol-4-Yl) Methanol (MBHTM) Accelerated

Copper-Catalyzed [3+2] Azide-Alkyne Cycloaddition (CuAAC) at Low Catalyst Loading. *Tetrahedron Lett.* **2015**, *56*. https://doi.org/10.1016/j.tetlet.2015.09.010.

(35) Bender, E. Equations of State for Ethylene and Propylene. *Cryogenics (Guildf).* **1975**, *15* (11), 667–673. https://doi.org/https://doi.org/10.1016/0011-2275(75)90100-9.

(36) Sato, Y.; Fujiwara, K.; Takikawa, T.; Sumarno; Takishima, S.; Masuoka, H. Solubilities and Diffusion Coefficients of Carbon Dioxide and Nitrogen in Polypropylene, High-Density Polyethylene, and Polystyrene under High Pressures and Temperatures. *Fluid Phase Equilib.* **1999**, *162* (1), 261–276. https://doi.org/https://doi.org/10.1016/S0378-3812(99)00217-4.

(37) Choo, Y.-S.; Yeo, W.-H.; Byun, H.-S. Phase Equilibria and Cloud-Point Behavior for the Poly(2-Phenylethyl Methacrylate) in Supercritical CO2 with Monomers as Co-Solvent. *J. CO2 Util.* **2019**, *31*, 215–225. https://doi.org/https://doi.org/10.1016/j.jcou.2019.03.008.

(38) Panayiotou, C.; Sanchez, I. C. Swelling of Network Structures. *Polymer (Guildf).* **1992**, *33* (23), 5090–5093. https://doi.org/https://doi.org/10.1016/0032-3861(92)90064-4.

(39) Trupej, N.; Hrnčič, M. K.; Škerget, M.; Knez, Ž. Solubility and Binary Diffusion Coefficient of Argon in Polyethylene Glycols of Different Molecular Weights. *J. Supercrit. Fluids* **2015**, *103*, 10–17. https://doi.org/https://doi.org/10.1016/j.supflu.2015.04.022.

(40) Gutiérrez, C.; Garcia, M. T.; Curia, S.; Howdle, S. M.; Rodriguez, J. F. The Effect of CO2 on the Viscosity of Polystyrene/Limonene Solutions. *J. Supercrit. Fluids* **2014**, *88*, 26–37. https://doi.org/https://doi.org/10.1016/j.supflu.2014.01.012.

(41) Martín, A.; Cocero, M. J. Micronization Processes with Supercritical Fluids:

Fundamentals and Mechanisms. *Adv. Drug Deliv. Rev.* **2008**, *60* (3), 339–350. https://doi.org/https://doi.org/10.1016/j.addr.2007.06.019.

(42) Pasquali, I.; Andanson, J.-M.; Kazarian, S. G.; Bettini, R. Measurement of CO2 Sorption and PEG 1500 Swelling by ATR-IR Spectroscopy. *J. Supercrit. Fluids* **2008**, *45* (3), 384–390. https://doi.org/https://doi.org/10.1016/j.supflu.2008.01.015.

(43) Daneshvar, M.; Kim, S.; Gulari, E. High-Pressure Phase Equilibria of Polyethylene Glycol-Carbon Dioxide Systems. *J. Phys. Chem.* **1990**, *94* (5), 2124–2128. https://doi.org/10.1021/j100368a071.

(44) Markočič, E.; Škerget, M.; Knez, Ž. Measurement and Modeling of the CO2 Solubility in Poly(Ethylene Glycol) of Different Molecular Weights. *J. Chem. Eng. Data - J CHEM ENG DATA* **2007**, *53*. https://doi.org/10.1021/je700467p.

(45) Shaikh, M. H.; Subhedar, D. D.; Shingate, B. B.; Kalam Khan, F. A.; Sangshetti, J. N.; Khedkar, V. M.; Nawale, L.; Sarkar, D.; Navale, G. R.; Shinde, S. S. Synthesis, Biological Evaluation and Molecular Docking of Novel Coumarin Incorporated Triazoles as Antitubercular, Antioxidant and Antimicrobial Agents. *Med. Chem. Res.* **2016**, *25* (4), 790–804. https://doi.org/10.1007/s00044-016-1519-9.

(46) Oh, C.; Yi, I.; Park, K. P. Nucleophilic Vinylic Substitution of Halocoumarins and Halo-l,4-Naphthoquinones with Morpholine. *J. Heterocycl. Chem.* **1994**, *31* (4), 841–844. https://doi.org/10.1002/jhet.5570310426.

(47) Chaurasia, C. S.; Kauffman, J. M. Synthesis and Fluorescent Properties of a New Photostable Thiol Reagent "BACM." *J. Heterocycl. Chem.* **1990**, *27* (3), 727–733. https://doi.org/10.1002/jhet.5570270347.

(48) Sivakumar, K.; Xie, F.; Cash, B. M.; Long, S.; Barnhill, H. N.; Wang, Q. A

Fluorogenic 1,3-Dipolar Cycloaddition Reaction of 3-Azidocoumarins and Acetylenes. *Org. Lett.* **2004**, *6* (24), 4603–4606. https://doi.org/10.1021/ol047955x.

(49) Kusanur, R. A.; Kulkarni, M. V; Kulkarni, G. M.; Nayak, S. K.; Guru Row, T. N.; Ganesan, K.; Sun, C.-M. Unusual Anisotropic Effects from 1,3-Dipolar Cycloadducts of 4-Azidomethyl Coumarins. *J. Heterocycl. Chem.* **2010**, *47* (1), 91–97. https://doi.org/10.1002/jhet.273.

(50) Cabezas, L. I.; Mazarro, R.; Gracia, I.; Lucas, A. De; Rodríguez, J. F. Optimizing the Bulk Copolymerization of D , L-Lactide and Glycolide by Response Surface Methodology. **2013**, *7* (11), 886–894. https://doi.org/10.3144/expresspolymlett.2013.86.

(51) Parameswaran, R. Statistics for Experimenters: An Introduction to Design, Data Analysis, and Model Building. *JMR, J. Mark. Res.* **1979**, *16* (000002), 291.

(52) Venkata Sairam, K.; Gurupadayya, B. M.; Vishwanathan, B. I.; Chandan, R. S.; Nagesha, D. K. Cytotoxicity Studies of Coumarin Analogs: Design{,} Synthesis and Biological Activity. *RSC Adv.* **2016**, *6* (101), 98816–98828. https://doi.org/10.1039/C6RA22466K.

(53) Rajesh, P.; Natvar, P. In Vitro Antioxidant Activity of Coumarin Compounds by DPPH, Super Oxide and Nitric Oxide Free Radical Scavenging Methods. *JAPER* **2010**, *1*.