Araştırma Makalesi/Original Article
Makale Dili "İngilizce"/Article Language "English"

Türk Hijyen ve Deneysel Biyoloji Dergisi

Determination of doxorubicin amount conjugated to mPEG-b-PCL copolymer via pH sensitive hydrazone bond

pH duyarlı hidrazon bağıyla mPEG-b-PCL kopolimerine konjuge edilen doksorubisin miktarının belirlenmesi

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ABSTRACT

Objective: The aim of this study was to find the efficient medium to bind an anticancer drug, Doxorubicin (DOX), to a synthesized polymer, methoxy poly(ethylene glycol)-block-polycaprolactone (mPEG-b-PCL) via pH sensitive hydrazone bonds and to determine the amount of conjugated drug.

Methods: DOX conjugation was carried out in two different media [dimethyl sulfoxide (DMSO) and methanol-trifluoroacetic acid (MeOH-TFA)]. The amount of conjugated drug was determined with two different methods. One method was applied the dissolution of the conjugate in chloroform: methanol (Ch: MeOH, 1: 1 v/v) solution without considering pH responsiveness, and the other method was after breaking pH sensitive hydrazone bonds in acidic medium [using three different media as 0.1 M hydrochloric acid (HCl), concentrated HCl (12 M HCl) and concentrated sulfuric acid (18.3 M H2SO4)].

Results: The highest conjugation efficiency was obtained when the conjugation was achieved in MeOH-TFA solution, and for the polymer-drug conjugates after the treatment with concentrated sulfuric acid.

ÖZET

Amaç: Bu çalışmanın amacı, antikanser bir ilaç olan Doksorubisinin (DOX) sentezlenen metoksi poli(etilen glikol)-blok-polikaprolakton (mPEG-b-PCL) polimerine pH-duyarlı hidrazon bağları aracılığıyla bağlanabileceği etkin ortamı bulmak ve konjuge edilmiş ilaç miktarını belirlemektir.

Yöntem: DOX konjugasyon işlemi iki farklı ortamda [dimetil sülfoksit (DMSO) ve methanol trifloroasetik asit (MeOH-TFA)] gerçekleştirilmiştir. Konjuge ilaç miktarı iki farklı yöntemle belirlenmiştir. Bir metot, ph duyarlılığını göz ardı ederek konjuge yapının kloroform:methanol (Ch:MeOH, 1:1 v/v) çözeltisi içerisinde çözünmesi sonrasında, diğer metot pH'ya duyarlı hidrazon bağlarının asidik ortamda [0.1 M hidroklorik asit (HCl), derişik HCl (12 M HCl) ve derişik sülfürik asit (18.3 M H2SO4) olarak üç farklı ortamda] kırılması sonrasında uygulanmıştır.

Bulgular: En yüksek konjugasyon verimliliği MeOH-TFA çözeltisi içerisinde konjugasyon sağlandığında ve polimer-ilaç konjugatları konsantre sülfürik asit ile muamele edildikten sonra elde edilmiştir.

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Geliş Tarihi / Received : 16.08.2021
Kabul Tarihi / Accepted : 04.01.2022

DOI ID : 10.5505/TurkHijyen.2022.04317

Işık G, Tezcaner A, Hasırcı N, Kızıltay A. Determination of doxorubicin amount conjugated to mPEG-b-PCL copolymer via pH sensitive hydrazone bond. Turk Hij Den Biyol Derg, 2022; 79(2): 279 - 292

Conclusion: It was concluded that, MeOH-TFA method was a good method for conjugation of DOX to mPEG-b-PCL copolymer, and H2SO4 (18.3 M) method was better than any other present in literature for determination of the amount of DOX linked to the polymer via hydrazone conjugation.

Key Words: Doxorubicin, hydrazine bond, mPEG-b-PCL, trinitrobenzene sulfonic acid, drug conjugation

Sonuç: MeOH-TFA yönteminin DOX'un mPEG-b-PCL kopolimerine konjugasyonu için iyi bir yöntem olduğu ve H2SO4 (18.3 M) yönteminin hidrazon konjugasyonu yoluyla polimere bağlı DOX miktarının belirlenmesi için literatürde mevcut diğer yöntemlerden daha iyi olduğu bulunmuştur.

Anahtar Kelimeler: Doksorubisin, hidrazon bağı, mPEG-b-PCL, trinitrobenzen sülfonik asit, ilaç konjugasyonu

INTRODUCTION

Conjugation of drugs to biocompatible polymers responsive to the tumor microenvironment improves their therapeutical activities (1-4). Cancer cells favor glycolysis rather than oxidative phosphorylation in order to supply the required ATP with a fast-metabolic pathway. This reaction results in hypoxia in and around the tumor, and pH value decreases to 6.5-7.2, 5.0-6.5, and 4.5-5.0 for the tumor microenvironment, endosomes and lysosomes, respectively (5). Therefore, drugs which are conjugated to polymers with pH sensitive linkages as hydrazone, disulfide, azo, acetal, ortho ester, vinyl ether, amine, imine, can be targeted to tumor and be effective in that region (6). Hydrazone conjugation of cancer drugs to the polymers is an attractive technique since it can easily break in acidic media (7). Doxorubicin (DOX), which is an effective cancer drug, can be either entrap into nanoparticles (8) or conjugate to polymers to be targeted to tumour area (9,10). Faster release at pH 5.0 than the release at pH 7.4 was reported for the system having conjugated DOX to N-(2-hydroxypropyl) methacrylamide (HPMA) copolymers via hydrazone bonds (9). Similarly, when DOX was conjugated to a pentablock copolymer via hydrazone bonds, the release was reported as 89% at pH 5.0 while it was about 29% at pH 7.4 after 7 days of incubation (10).

Polyethylene glycol (PEG) and polycaprolactone (PCL) are biocompatible polymers and widely used in the production of drug delivery systems and tissue engineering scaffolds (11). However, PCL is highly hydrophobic, its degradation is too slow for drug delivery applications, and it can be recognized by immune system rapidly (12). On the other hand, PEG is hydrophilic and can escape from the immune system. Scientists prepared or used copolymers or tri-block-polymers of PEG and PCL and prepared either nanoparticles or micelles as drug delivery vehicles and evaluated *in vitro* and *in vivo* properties (13). Gong et al studied affinity of PEG-PCL-PEG micelles (14). Hu et al prepared micelles and polymersomes from PCL-PEG-PCL polymers to be used as drug carriers (15). Cytotoxic properties of Doxorubicin loaded PEG-PCL micelles prepared with functionalized PEGs were evaluated by Chen et al. and promising results were reported (16). An accurate determination of drug conjugation is important since higher dose given to a patient may create some unwanted symptoms like cardiomyopathy.

In this study, methoxy poly(ethylene glycol)-b-

polycaprolactone block copolymer (mPEG-b-PCL) was synthesized to form micelles due to hydrophobic-hydrophilic properties of the copolymer. The mPEG-b-PCL copolymer was functionalized with hydrazone bonds by using hydrazine hydrate, and then a chemotherapeutic drug, DOX, was conjugated by using two different media, either dimethyl sulfoxide (DMSO), or methanol-trifluoroacetic acid (MeOH-TFA). The conjugated drug content of the copolymer was determined in two methods by measuring UV-visible absorbances of the solutions: 1) after dissolving the drug-polymer conjugate in chloroform:methanol (Ch:MeOH, 1:1 v/v) solution without considering pH responsiveness of the chemical bond, 2) breaking the pH sensitive bonds in three different acidic media (as hydrochloric acid (0.1 M HCl), concentrated hydrochloric acid (12 M HCl) and concentrated sulfuric acid (18.3 M H_2SO_4)).Ch:MeOH was chosen for the dissolution of the polymer-drug conjugates since chloroform and methanol are solvents for mPEG-b-PCL copolymer and DOX, respectively. Therefore, Ch:MeOH solution was used to compare its effectiveness with the data obtained via breaking the conjugation bonds in acidic media. Results demonstrated that, H_2SO_4 method is more effective to break the hydrazone bonds than using 0.1 M or 12 M HCl as given in this study and also compared to the techniques given in the literature. To the best of our knowledge, this is the first study showing the efficiency of conjugation of DOX to mPEG-b-PCL copolymer in MeOH-TFA media and determination of the conjugated amount by H_2SO_4 method.

MATERIAL and METHOD

Materials

Adriamycin (trade name of DOX) was purchased from Deva Holding (Turkey) as lyophilized powder of 10 mg of Doxorubicin hydrochloride in injection vials. ε-Caprolactone (ε-CL) was purchased from Acros Organics (USA). Methoxy poly(ethylene glycol) (mPEG) (Mn=5000 Da), tin(II) 2-ethylhexanoate, succinic anhydride, dimethylaminopyridine, dicyclohexylcarbodiimide, N-hydroxysuccinimide (NHS), hydrazine hydrate, trinitrobenzene sulfonic acid (TNBSA) (5%), sodium bicarbonate, glycine and DMSO were obtained from Sigma-Aldrich (Germany). Dichloromethane, ethanol, tetrahydrofuran, diethyl ether, chloroform, methanol, TFA, 35% HCl concentrated sulfuric acid were purchased from Merck (Germany).

Methods

Synthesis of mPEG-b-PCL Copolymers

mPEG-b-PCL copolymer was synthesized by ring opening polymerization of PCL according to the method previously carried out by our group (17). Shortly, predetermined amount of ε-caprolactone was added to a 3-necked flask containing mPEG (Mn = 5000 Da) and tin(II) 2-ethylhexanoate (0.1% of ε-caprolactone in molar amount). The flask filled with argon gas was sealed, and the reaction was carried out in an oil bath at 120°C for 6 hours. The product obtained was dissolved in dichloromethane, precipitated in cold ethanol and then dried under vacuum at 40°C.

Synthesis of mPEG-b-PCL-COOH

mPEG-b-PCL, succinic anhydride and dimethylaminopyridine were dissolved (1:2:0.5 molar ratio) in tetrahydrofuran (50 mL) in a three-neck flask and reacted under N_2 atmosphere for 24 hours at 30°C (10). The product (mPEG-b-PCL-COOH) was precipitated in cold diethyl ether, dried under vacuum and examined by 1H-NMR (by Bruker Avance 400 DPX (USA) working at 400 MHz and using deuterated chloroform) and Fourier Transform Infrared (FT-IR) (by Perkin Elmer Spectrum 65, Perkin Elmer Inc., USA) analyses.

Synthesis of mPEG-b-PCL-CO-NH-NH_2

mPEG-b-PCL-COOH, dicyclohexylcarbodiimide and NHS were dissolved (1:1:1 molar ratio) in tetrahydrofuran (50 mL) and reacted under N_2 atmosphere for 24 hours at 30°C (10). Hydrazine hydrate was then added to this mixture and the

reaction was continued for 12 hours. The product (mPEG-b-PCL-CO-NH-NH_2) was filtered, precipitated in cold diethyl ether, and the precipitate was dried under vacuum. FT-IR spectrum was recorded (by Perkin Elmer Spectrum 65, Perkin Elmer Inc., USA).

For colorimetric detection of primary amine groups attached to the copolymer, TNBSA method was used. For this purpose, different concentrations of glycine solutions (4 - 20 µg/mL) were prepared in sodium bicarbonate buffer (0.1 M, pH 8.5) to construct a calibration curve. Then, 5% TNBSA was diluted 500-fold with 0.1 M sodium bicarbonate buffer. Then, 1 part of TNBSA solution was mixed with 2 part of glycine solutions having different concentrations (4 - 20 µg/mL; or 0.53×10^{-7} - 27×10^{-7} mol amine/mL). The solutions were incubated at 37°C for 2 hours and absorbances were measured at 335 nm using a plate reader (SpectraMax iD3, Molecular Devices, USA). Meanwhile, 1 mg/mL mPEG-b-PCL-CO-NH-NH_2 was suspended in 0.1 M sodium bicarbonate buffer and 1 part of TNBSA was mixed with 2 parts of mPEG-b-PCL-CO-NH-NH_2 suspension. The absorbance of the solution was measured at 335 nm and the amount of primary amine was determined using the calibration curve.

Conjugation of DOX to mPEG-b-PLC-CO-NH-NH_2

For conjugation of DOX to hydrazone group, two different methods were used as given below:

DMSO method: mPEG-b-PCL-CO-NH-NH_2 copolymer and DOX were dissolved (1:1 molar ratio) in DMSO (50 mL) and kept in dark, for 72 hours, under N_2 gas, at 30°C for reaction (9). The product was purified by dialysis (MWCO 3500, Spectrum Laboratories, USA) against deionized water at room temperature for 3 days by changing the water in every 6 hours. DOX conjugated mPEG-b-PLC-CO-NH-NH_2 was obtained after lyophilization. The yield of the synthesis was determined with the equation below;

$$\text{Eq. 1: } \textit{The yield } (\%) = \frac{\textit{Total amount of DOX conjugated polymer}}{\textit{The sum of DOX and the polymer used for the synthesis}} \times 100$$

MeOH-TFA method: mPEG-b-PCL-CO-NH-NH_2 copolymer and DOX were dissolved (1:1 molar ratio) in methanol (15 mL), a drop of TFA was added, left overnight at 60°C (18). After the removal of methanol under vacuum, the residue was suspended in water and dialyzed (MWCO 3500, Spectrum Laboratories, USA) against deionized water at room temperature for 3 days by changing the water in every 6 hours. DOX conjugated mPEG-b-PLC-CO-NH-NH_2 was obtained after lyophilization. The yield of the synthesis was determined with using Eq. 1.

Determination of Conjugated DOX Content of the Copolymer

DOX conjugation to the copolymer was characterized with using 1H-NMR [by Bruker Avance 400 DPX (USA) working at 400 MHz and using deuterated DMSO].

DOX conjugation efficiency was determined by using the following two methods:

1. Dissolution of the drug-polymer conjugate (Ch:MeOH (1:1) Method): DOX (10 mg) was dissolved in chloroform:methanol (1:1, v/v) and a calibration curve was constructed (1 - 70 µg DOX / mL) by measuring absorbances at 440 nm with UV-visible spectrophotometer. 10 mg of pristine mPEG-b-PCL-CO-NH-NH_2 copolymer (as blank solution) and/or 10 mg of DOX conjugated copolymer were dissolved in 1 mL of Ch:MeOH (1:1, v/v). DOX content of the DOX-copolymer conjugate was calculated by using the following equation;

$$\text{Eq. 2: Conjugated drug content } (\mu g/mg \text{ polymer}) = \frac{(\text{Abs of DOX conj poly} - \text{Abs poly}) / X}{10}$$

where; Abs of DOX conj poly = Absorbance of DOX conjugated copolymer,

Abs poly = Absorbance of pristine copolymer.

X = The slope of the calibration curve is 0.014 for Ch:MeOH

10 = Dilution factor (the samples were diluted 10 times before the measurements).

Conjugation efficiencies of the copolymer was calculated with the equation below;

$$\text{Eq. 3: Conjugation efficiency} = \frac{\textit{Total conjugated DOX to the polymer}}{\textit{DOX content used for the synthesis}} \times 100$$

2. Breaking the pH sensitive bond in acidic media: Three different media [HCl (0.1 M), concentrated HCl (12 M) and concentrated H_2SO_4 (18.3 M)] were used to determine the conjugated DOX content. HCl (0.1 M) was used to compare the results with literature (10). For each, the wavelength having maximum absorbance values was determined, and the calibration curves were prepared accordingly. Pristine copolymer (10 mg) having no drug was also dissolved or dispersed in the mentioned media and used as blank solution (in order to eliminate any effect caused by the polymer). DOX-polymer conjugates (10 mg) were dissolved in the solutions and conjugated drug content of the copolymer was determined spectroscopically by using the following equation;

$$\text{Eq. 4: Conjugated drug content } (\mu g/mg \text{ polymer}) = \frac{(\text{Abs of DOX conj poly} - \text{Abs poly}) / X}{10}$$

where; Abs of DOX conj poly = Absorbance of DOX conjugated copolymer,

Abs poly = Absorbance of pristine copolymer.

X = The slope of the calibration curves, which are 0.0211, 0.02 and 0.0415 for HCl (0.1 M), HCl (12 M) and H_2SO_4, respectively.

10 = Dilution factor (the samples were diluted 10 times before the measurements).

Conjugation efficiencies of the copolymer was calculated with using Eq. 3.

The procedures of each method are given below.

a) HCl (0.1 M and 12 M) method: DOX (10 mg) was dissolved in 0.1 M HCl or 12 M HCl separately. First, wavelength values where the maximum absorption was observed (λ-max) were determined as 480 nm for 0.1 M HCl and at 504 nm for 12 M HCl media. Then calibration curves were constructed (using concentrations as 1 - 50 µg DOX/mL for 0.1 M HCl and 1-55 µg DOX/mL for 12 M HCl) by measuring absorbances at with UV-visible spectrophotometer at determined λ-max (10). Similarly, 10 mg DOX conjugated copolymer was suspended in 5 mL of 0.1 M HCl or in 12 M HCl, separately and probe sonication with 10% amplitude for 5 min was applied. The suspensions were kept at 37°C for 48 hours, copolymers were separated by centrifugation (14000 rpm for 10 min), and the absorbances of the supernatant at 480 nm for 0.1 M HCl and at 504 nm for 12 M HCl were measured by UV-visible spectrophotometer. As blank solution, 10 mg pristine copolymer prepared in the same way in 0.1 M HCl or 12 M HCl was used. The amount of the DOX in the supernatant was calculated from the formula given above.

b) H_2SO_4 (18.3 M) method: DOX was dissolved in concentrated (18.3 M) sulfuric acid. A calibration curve was constructed with different concentrations of the DOX (1 - 40 µg/mL) by measuring absorbances at 543 nm with a UV-visible spectrophotometer. 1 mg of DOX conjugated mPEG-b-PCL-CO-NH-NH_2 was dissolved in 1 mL of concentrated sulfuric acid. The same amount of pristine copolymer dissolved in 1 mL of concentrated sulfuric acid was used as blank solution and the conjugated DOX content was calculated from the formula given above.

Statistical Analysis

All data are presented as mean ± standard deviation. Results were analyzed using one-way analysis of variance (ANOVA) and Tukey's multiple comparison test.

RESULTS

Characterization of mPEG-b-PCL Copolymers

The number average molecular weight (Mn) of the mPEG-b-PCL copolymer synthesized was determined as 16 kDa using ^{1}H NMR data. This is exactly the same as the value determined by gel permeation chromatography (GPC) for the same polymer synthesized previously (17).

Characterization of Functionalized mPEG-b-PCL Copolymers

mPEG-b-PCL copolymer was functionalized with succinic anhydride to obtain carboxyl ended mPEG-b-PCL-COOH copolymer so that hydrazine hydrate can bind to the copolymer via peptide bond. Then further functionalization was achieved with hydrazine hydrate to obtain mPEG-b-PCL-CO-NH-NH_2 (Figure 1).

^{1}H NMR spectrum of mPEG-b-PCL-COOH copolymer was obtained (Figure 2). The peak at 2.64 shows the -CH_2-CH_2- group from succinic anhydride (10). The peaks at 1.36-1.43 ppm (multiplet), 1.56-1.70 ppm (multiplet), 2.31 ppm (triplet), 4.06 ppm (triplet) and 4.23 ppm (triplet) belong to the methylene protons of PCL block in the copolymer. The peak at 3.65 ppm (triplet) was assigned to the methylene protons of mPEG block in the copolymer (16).

FT-IR spectra of mPEG-b-PCL, mPEG-b-PCL-COOH and mPEG-b-PCL-CO-NH-NH_2 polymers are given in Figure 3. The absorbance peaks observed at 3443 cm^{-1} belong to the stretching vibrations of -NH_2 bonds (10); peaks at 2866 cm^{-1} and 2945 cm^{-1} are belong to C-H stretching vibrations. The absorbance peaks at 1722 cm^{-1} was evaluated as C=O stretching vibrations of PCL unit, and the peak at 1103 cm^{-1} was ascribed to C-O stretching vibrations of mPEG unit in the copolymer (16).

Hydrazide functionalization in the mPEG-b-PCL-CO-NH-NH_2 copolymer was determined by colorimetric primary amine detection assay. The hydrazide amount in mPEG-b-PCL-CO-NH-NH_2 polymer was calculated as 45.6 mol% (100 mol polymer contains 45.6 mol of -NH_2 group defined as hydrazide group) from the calibration curve (Figure 1).

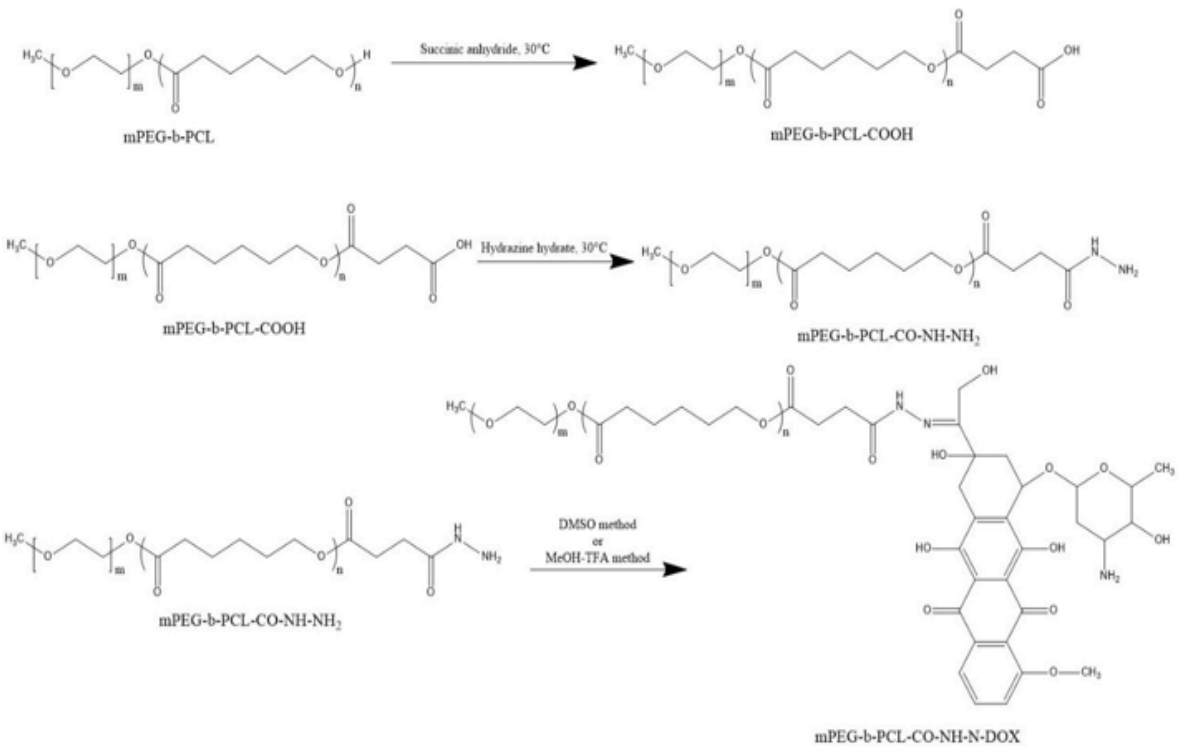

Figure 1. Reaction scheme for hydrazide functionalization of mPEG-b-PCL copolymer and conjugation of DOX

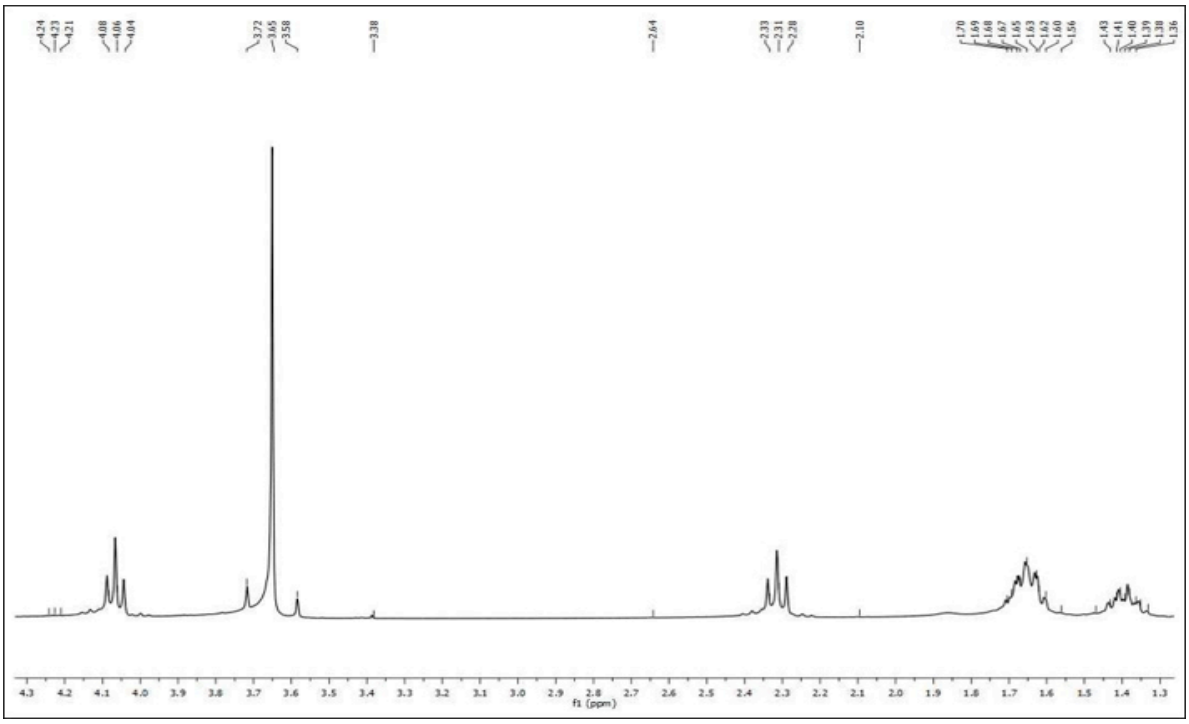

Figure 2. ^{1}H NMR spectrum of mPEG-b-PCL-COOH in $CDCl_3$

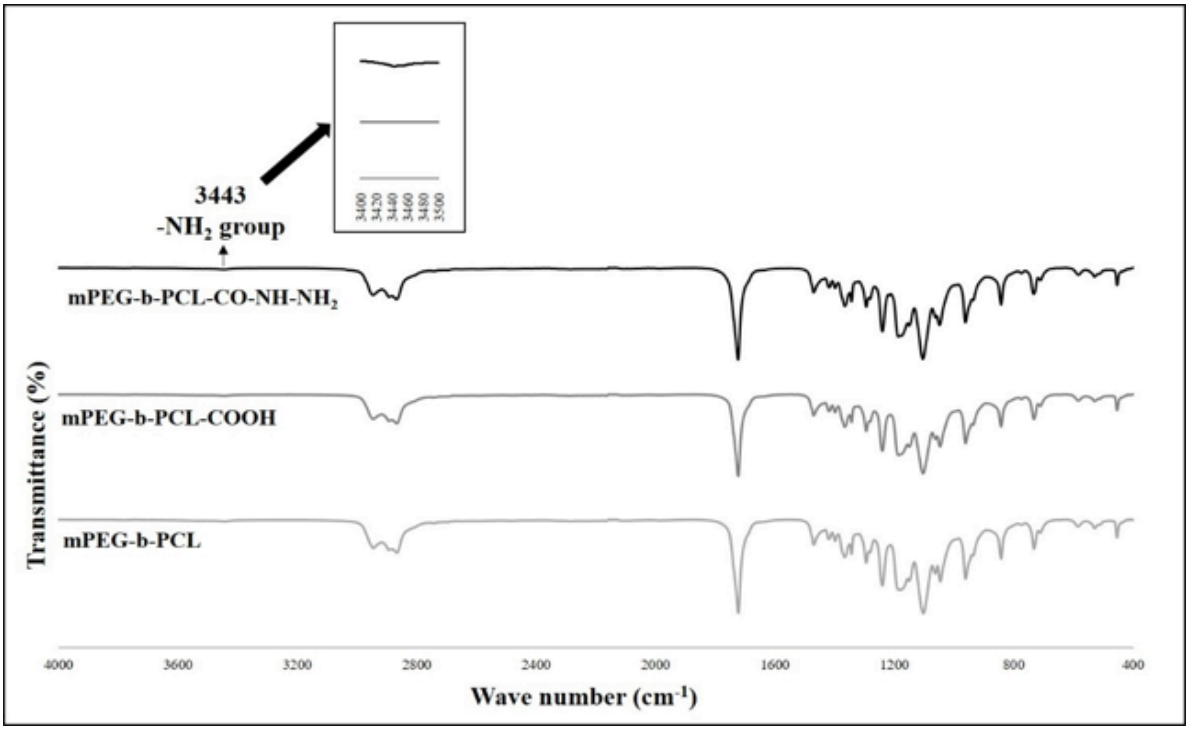

Figure 3. FT-IR spectra of mPEG-b-PCL, mPEG-b-PCL-COOH, mPEG-b-PCL-CO-NH-NH_2.

Determination of Conjugated DOX Content of the Copolymer

DOX conjugation was verified using ^{1}H NMR spectroscopy (Figure 4) and results showed that hydrazide functionalization of mPEG-b-PCL copolymer was achieved successfully (19-22). ^{1}H NMR results also showed that MeOH-TFA method (Figure 4F) was more efficient than DMSO method since the yield for DOX conjugated polymer synthesis was higher in MeOH-TFA method (%92.1) than DMSO method (%78.9 (Figure 4E and 4F). In ^{1}H NMR spectrum of DOX conjugated copolymer which was prepared with DMSO method, some impurities which can come from the DOX we used were observed. The DOX we used contains methyl paraben and lactose monohydrate as excipients. Some peaks in the NMR spectra could not be detected due to these impurities.

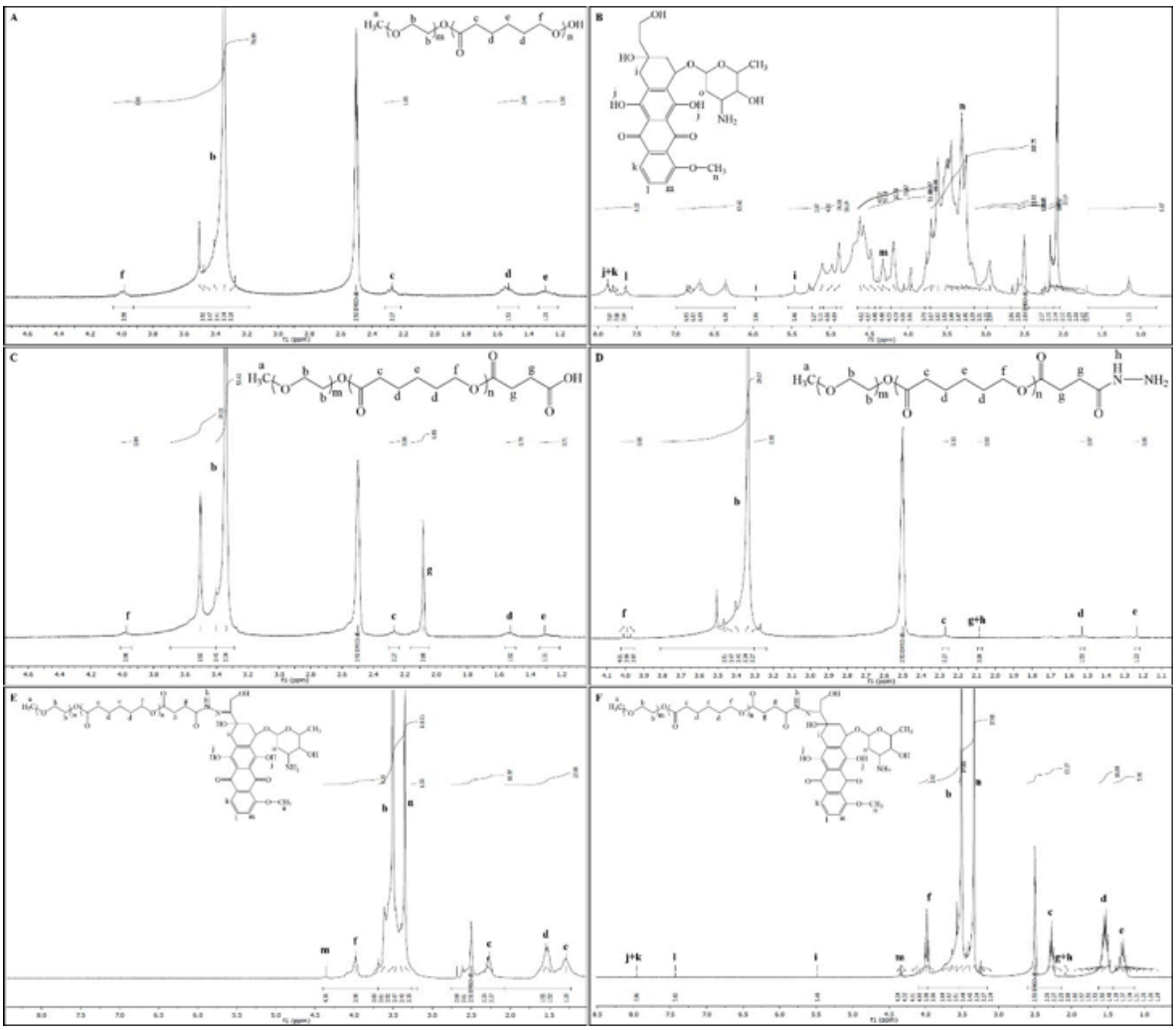

Figure 4. ^{1}H NMR spectra of
A) mPEG-b-PCL in $CDCl_3$, B) DOX in $CDCl_3$, C) mPEG-b-PCL-COOH in $CDCl_3$, D) mPEG-b-PCL-CO-NH-NH_2 in $CDCl_3$,
E) mPEG-b-PCL-CO-NH-N-DOX with DMSO method in DMSO-d6, and F) mPEG-b-PCL-CO-NH-N-DOX
with MeOH-TFA method in DMSO-d6.

DOX was conjugated to mPEG-b-PCL-CO-NH-NH_2 copolymer either in DMSO or in MeOH-TFA solutions, and the DOX contents of the DOX-copolymer conjugate were measured spectroscopically with two different methods: 1. dissolution of the polymer-drug conjugates in Ch:MeOH (1:1) and 2. breaking the pH sensitive bonds between the polymer and the drug using three different acidic media [HCl (0.1 M), HCl (12 M) and H_2SO_4 (18.3 M)]. The results demonstrated that, MeOH-TFA was better than the DMSO, since the amount of DOX conjugation was found to be higher in this media. Meanwhile, when two methods were compared, the results obtained for Ch:MeOH (1:1) and HCl (0.1 M) in DMSO media did not show any significant difference (Table 1 and Table 2). On the other hand, when the medium was changed to MeOH:TFA, a significant difference between Ch:MeOH (1:1) and HCl (0.1 M) was observed. Therefore, using acidic media to determine the conjugation content and conjugation efficiency resulted in higher amount conjugated. In the second method, acidity of the media was changed to compare the conjugation efficiencies and H_2SO_4 (18.3 M) method resulted in higher DOX amount than the ones obtained for HCl (0.1 M) and HCl (12 M) methods (Table 1 and Table 2).

In Ch:MeOH (1:1, v/v) method, the conjugated DOX contents of the copolymer using DMSO and MeOH-TFA media were found as 1.91 ± 0.01 and 4.15 ± 0.03 µg DOX/mg polymer, respectively, and these values were lower than the other methods. In case of HCl (0.1 M) method, the conjugated DOX contents were found as 2.08 µg ± 0.05 and 6.45 ± 0.10 µg DOX/mg polymer, for the samples prepared by DMSO and MeOH-TFA methods, respectively. In HCl (0.1 M) and HCl (12 M) methods, the polymer obtained after centrifugation was still pink in color (Figure 7) which shows the presence of DOX which still stays in conjugated form in the polymer.

Table 1. DOX conjugation content on copolymer synthesized.

Conjugation Media*	Conjugation contents (µg DOX/mg polymer)*			
	Ch:MeOH (1:1) Method	HCl (0.1 M) Method	HCl (12 M) Method	H_2SO_4 (18.3 M) Method
DMSO, at 30°C	1.91 ± 0.01	2.08 ± 0.05	3.24 ± 0.14[a]	3.93 ± 0.12[a]
MeOH-TFA, at 60°C	4.15 ± 0.03[b]	6.45 ± 0.10[b]	7.99 ± 0.05[b]	12.87 ± 0.07[b]

* n=3,
[a]shows the significant differences between conjugation content of DMSO media,
[b] shows the significant differences between conjugation content methods of MeOH-TFA media.

Table 2. DOX conjugation efficiencies.

Conjugation Media*	Conjugation efficiencies found with different methods (%)*			
	Ch:MeOH (1:1)	HCl (0.1 M)	HCl (12 M)	H_2SO_4 (18.3 M)
DMSO, at 30°C	4.59 ± 0.03	5.00 ± 0.13	7.77 ± 0.33[a]	9.44 ± 0.29[a]
MeOH-TFA, at 60°C	11.61 ± 0.09[b]	18.05 ± 0.27[b]	22.36 ± 0.14[b]	36.04 ± 0.20[b]

* n=3,
a shows the significant differences between conjugation content of DMSO media,
b shows the significant differences between conjugation content methods of MeOH-TFA media.

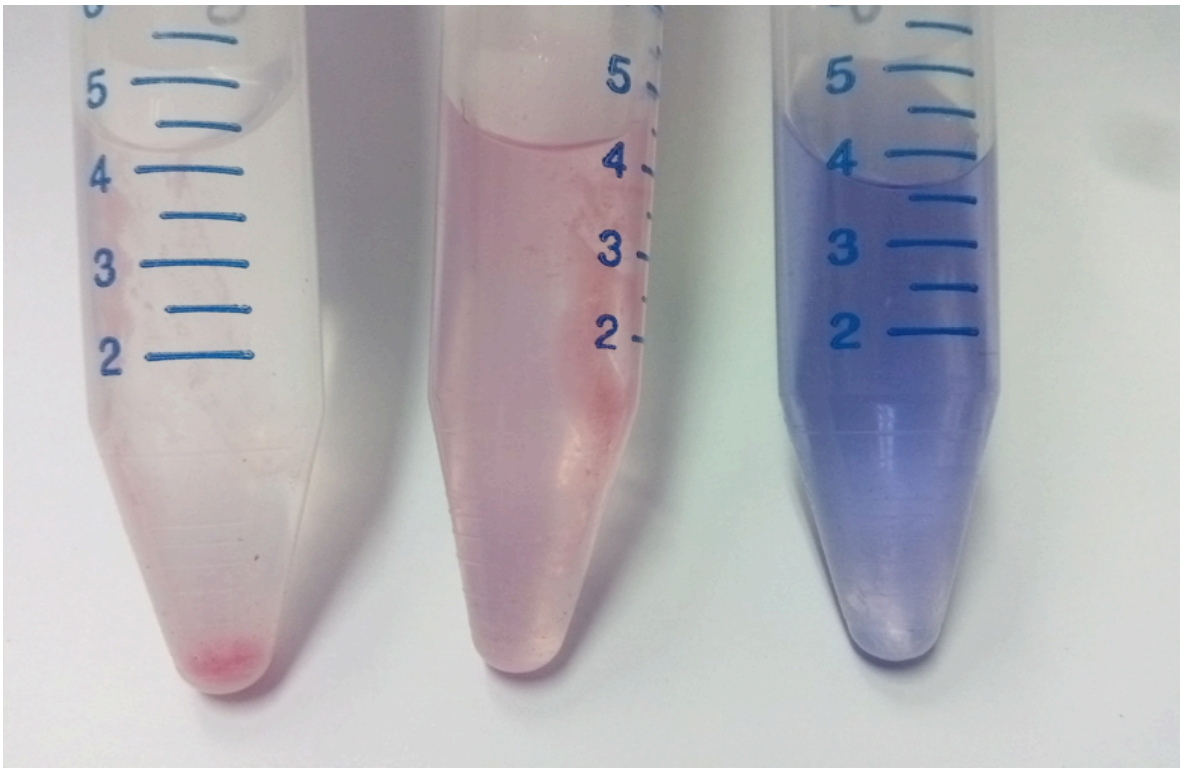

Figure 7. Image of DOX conjugated polymers in,
a) 0.1 M HCl, b) 12 M HCl, and c) 18.3 M H_2SO_4.
DOX conjugated polymers that are not dissolved in 0.1 N HCl and 12 M HCl are shown with arrows.
Polymer dissolved completely in concentrated H_2SO_4.

DISCUSSION and CONCLUSION

The solvent choice is an important role in determination of drug conjugation efficiency since hydrazone bond is cleaved at acidic pH. In the current study, we compared two different methods to find the most efficient medium to determine the conjugated drug content: 1. dissolution of the drug-polymer conjugates, and 2. breaking the pH sensitive bond in acidic media. For this purpose, DOX conjugation efficiencies were obtained with two different methods, the polymer-drug conjugates were subjected to Ch:MeOH (1:1) for the first method and to 0.1 M HCl, 12 M HCl and 18.3 M H_2SO_4 separately to break linkages between the polymer and the drug. 0.1 M HCl was used as a control to compare the results with the literature. The higher bond cleavage efficiency was obtained with H_2SO_4 since the molarity of sulphuric acid was higher than that of HCl.

The hydrazide yield can vary depending on polymer and the crosslinker used. For example, Etrych et al. (9,23) used N-(2-hydroxypropyl) methacrylamide (HPMA) copolymers and hydrazide amount was determined as 8.2 mol% and 5.6 mol% in their polymer in two different studies while del Rosario et al. (24) used amphiphilic macromolecule and quantified the hydrazide amount as 63%.

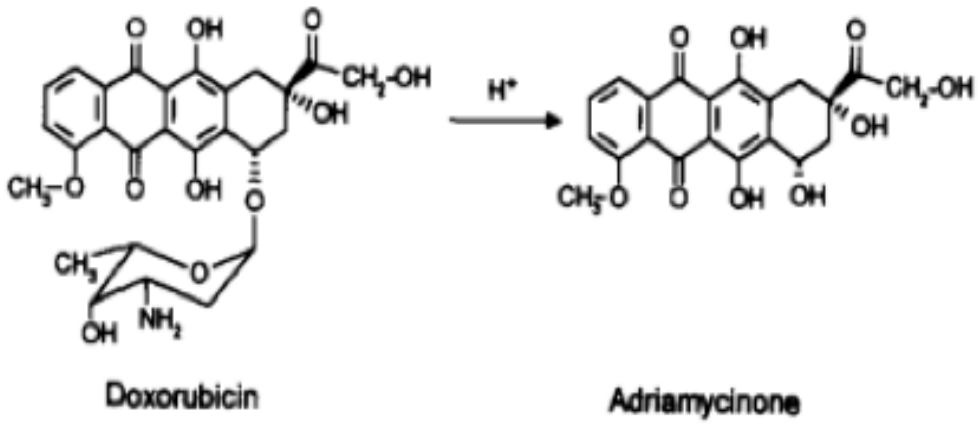

Figure 5. Acid hydrolysis of doxorubicin (25).

DOX.HCl has a characteristic absorbance peak at 480 nm. However, the the maximum absorbance wavelength (λ_{max}) was observed at 504 nm when the calibration curve was prepared using concentrated acid (12 M HCl). It is possible that DOX might have different conformational forms depending on the acidity of the medium, or some by-products of DOX might be formed in very high acidic medium, which might cause peak shifting (480 nm to 504 nm) in the absorption of DOX in UV-visible radiation. Dox conjugation content and conjugation efficiency were measured highest in H_2SO_4 . It is highly possible that DOX can degrade in H_2SO_4 but calibration curve of free DOX in H_2SO_4 shows that R^2 is 0.9936 which means that even if the DOX is degraded in H_2SO_4 we can still measure it. Thus, we assumed that degradation products of DOX gave max absorption at 543 nm. It is known that DOX is not stable in solutions with a pH less than 3; DOX breaks up into a red-colored, water insoluble aglycone (adriamycinone) (Figure 5) and a water soluble, reducing amino sugar (daunosamine) (25). For example, treatment of DOX with mild acids (e.g. 1 N HCl) selectively cleaves the glycosidic bond between the aglycone and the sugar group components. Thus, quantification of conjugated DOX after mild acid hydrolysis is based on released adriamycinone component (26).

According to the statistical analysis, for DMSO method, there was no significant difference between Ch:MeOH (1:1, v/v) method and HCl (0.1 M) method, while 12 M HCl and H_2SO_4 (18.3 M) methods were significantly different from the others. The statistical analysis was also showed that H_2SO_4 (18.3 M) method was also significantly different from 12 M HCl method. The statistical analysis also showed that, for MeOH-TFA method, all conjugated drug contents obtained with the four methods were significantly different from each other. These statistical results proved that, MeOH-TFA method is preferable for DOX conjugation to the copolymer and concentrated sulfuric acid is in determination of the conjugated drug content.

In literature, there are studies using UV-spectrophotometer (at 488 nm) for determination of conjugated DOX content to hydrazide groups. Etrych et al. (9,23) used N-(2-hydroxypropyl) methacrylamide polymer to conjugate DOX via hydrazone bond, and reported 12 wt%, and 9 wt% conjugation contents in two different studies. The reported values are lower than the ones we found in our study. That might be because of the use of different polymers.

In conclusion; in this study, a functional copolymer having hydrazine end groups (mPEG-b-PCL-CO-NH-NH_2) was synthesized, and DOX was linked via acid sensitive hydrazone conjugation with application of two different media mentioned as DMSO or MeOH-TFA methods. Conjugated drug contents of the copolymer were determined as 1.91 to 12.87 µg DOX/mg polymer and was found to be higher for MeOH-TFA compared to DMSO method.

Two different methods were applied to determine the amount of DOX: 1. dissolution the polymer-drug conjugates in MeOH and 2. breaking the pH sensitive bonds in acidic media. Conjugated DOX contents of the copolymer were found to be higher when H_2SO_4 (18.3 M) method was applied. This method was applied the first time in this study and found to be more effective than the ones present in the literature and achieved in this study such as Ch:MeOH (1:1, 1/1), HCl (0.1 M) and HCl (12 M) methods. The results suggest that acidic strength of the solution plays an important role in the determination of conjugated drug contents bound via hydrazone linkages, and the use of concentrated H_2SO_4 is suggested as a new and efficient method for this purpose.

ACKNOWLEDGEMENTS

The authors acknowledge BIOMATEN Center of Excellence in Biomaterials and Tissue Engineering and Central Laboratory of Middle East Technical University.

FUNDING

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the Middle East Technical University [DKT-310-2018-3580].

ETHICS COMITTEE APPROVAL

*This study does not require Ethics Committee Approval.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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