Research Article

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pH-responsive polymer micelles for methotrexate delivery at tumor microenvironments

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Abstract: Methotrexate (MTX) anticancer drug was successfully loaded and released in a controlled manner from polymer micelles made of a diblock copolymer of poly(monomethoxy ethylene glycol)-b-poly(ε-caprolactone) (mPEG-PCL). The empty and MTX-loaded micelles (MTX/mPEG-PCL) were characterized by electron microscopy. The drug release dependence upon pH 5.4, 6.5, and 7.4 for 30 days was proven and characterized by UV-Vis spectroscopy. The cytotoxic effect of MTX/mPEG-PCL micelles on MCF-7 breast cancer cells was evaluated through an MTT assay. The morphological analysis indicated the successful formation of micelles of 76 and 131 nm for empty and MTX-loaded micelles, respectively. An encapsulation efficiency of 70.2% and a loading capacity of 8.8% were obtained. The in vitro release of MTX showed a gradual and sustained profile over 22 days, with a clear trend to much higher release at acidic pH (80 and 90% for pH 6.7 and 5.5, respectively). The MTX/mPEG-PCL micelles showed an IC_{50} of MCF-7 cells at 30 μg mL^{-1}. The results suggested that MTX/mPEG-PCL could be a promising drug delivery system for cancer treatment.

Keywords: cancer treatment, copolymer micelles, drug delivery system, encapsulation efficiency, methotrexate

1 Introduction

Polymeric micelles are created from amphiphilic copolymers that spontaneously form nanometric aggregates above a threshold concentration referred to as the critical micellar concentration (CMC) (1). Polymeric micelles have been used as vehicles for the solubilization of poorly water-soluble molecules, delivering drugs specifically to the site of action, improving the pharmacokinetics of the loaded drug, and reducing cytotoxicity outside the target (2), thus reducing side effects in patients. Diblock copolymer micelles of poly(ethylene glycol)-b-poly(ε-caprolactone) (PEG-PCL) (Figure 1a) have received attention for loading therapeutic drugs (3,4). Because of their amphiphilic character, diblock copolymers form micellar systems that can interact with hydrophilic or hydrophobic compounds such as anti-cancer drugs. Moreover, these copolymers have shown biocompatibility, biodegradability, and low toxicity (4). Size is an important criterion in micelle design for successful drug delivery, which must be small enough (~10–200 nm) for the following reasons: (a) to be effective to penetrate the tissue, (b) to be unrecognizable by the mononuclear phagocyte system for a sufficient time to allow the accumulation in the target tissue, (c) to be removed from the organism either after degradation or dissolution, (d) to be located to interact with the target cells, (e) to be stable, (f) for improved pharmacokinetic profile of the encapsulated drug shipment, (g) for high load capacity, and (h) to be a reproducible, easy, and reasonably cheap synthetic method (5,6).

The controlled administration of water-insoluble drugs has a great interest in pharmacology because in this way the dosage frequency is reduced and thus the drug-related toxicity and side effects are decreased (7). For instance, PEG-PCL micelles loaded with paclitaxel showed a constant reduction in the volume of MDA-MB-468 breast tumors in mice (8). A similar system loaded with the anti-inflammatory drug dexamethasone was effective for reducing the symptoms of arthritis using low doses (9).
Figure 1: Scheme of (a) synthesis and structure of the poly(ethylene glycol)-b-poly(ε-caprolactone) monomethoxy copolymer (mPEG-PCL) and (b) structure of methotrexate (2,4-diamino-N10-methylpropylglutamic acid).

Methotrexate (2,4-diamino-N10-methyl propylglutamic acid) (MTX) (Figure 1b) is a last generation antimetabolite frequently used to treat various types of cancers, such as breast (10), lung (11), non-Hodgkin lymphoma (12), head and neck (13), and osteosarcoma (14). This drug has been approved to treat solid tumors (15), hematology (16), malignant (17), and autoimmune diseases (18).

Concerning breast cancer, it is one of the most commonly diagnosed cancers worldwide, generally known as metastatic cancer resistant to chemotherapy (19,20). Therefore, many investigations are focused on finding new drugs and drug delivery systems to fight it. The use of polymeric materials to develop drug delivery systems is of interest because of its biocompatibility and variety of compounds (21–23). Previous research reported the loading of MTX in diblock PEG-PCL copolymer micelles functionalized with folic acid and with diameter size in the range of 100–200 nm. Those micellar systems released the drug at a physiological pH of 7.4, but releasing data at acidic pHs were not reported (24). The release of cancer cytotoxic drugs at physiological pH is not desirable given that healthy cells would be exposed to these drugs too. In another study of MTX-conjugated poly(monomethoxy ethylene glycol)-b-poly(ε-caprolactone) (mPEG-PCL) micelles, with an average diameter of 72 nm, the release of MTX was reported as dependent on the pH, with higher release at acidic pH than in the neutral environment. Likewise, the maximum MTX release of around 63% on day 5 was found (25).

Herein, we report the loading of MTX into mPEG-PCL copolymer micelles of uniform nanometric size, capable of releasing the drug in response to acidic pH, characteristic of lysosomes, or tumor microenvironments, thus providing a more specific drug release regularly for 22 days. The space-time control of drug release is desirable for drug nanocarriers to contribute to reduce the toxicity and improve the therapeutic efficacy of drugs (26). The characterization of empty micelles and loaded micelles with MTX is also presented, as well as their cytotoxic effect toward MCF-7 breast cancer cells compared to free MTX to assess their potential as a treatment against breast cancer.

2 Experimental

2.1 Materials

Methoxy poly(ethylene glycol) (mPEG, $M_n = 2,000$ g mol$^{-1}$), 1.0 M hydrochloric acid (HCl) in diethyl ether, triethylamine, dichloromethane anhydride, and MTX (99% purity) were acquired from Sigma-Aldrich, USA. Diethyl ether and ε-caprolactone were purchased from Alfa Aesar. Hexane and phosphate-buffered saline (PBS) solution were supplied by Fermont Co. and Fluka, respectively.

2.2 Synthesis of the mPEG-PCL copolymer

The mPEG-PCL copolymer was synthesized by ring-opening polymerization using HCl as the catalyst (27). mPEG (1 mmol) was dissolved in dichloromethane (24 mL) and subsequently the ε-caprolactone (8.76 mmol) was added. The polymerization was initiated by adding 1.0 M HCl in diethyl ether (6 mL). The reaction was maintained under magnetic stirring at 30°C. After 24 h, 0.5 mL of triethylamine was added to neutralize the HCl, and the salt formed was separated through a filter paper (Whatman 4). Next, a mixture of diethyl ether:hexane, 1:1 volume ratio, was added to precipitate the copolymer. Finally, the solvent was decanted, and the residual solvent evaporated to
recover the copolymer. The polymerization efficiency (% Yield) was calculated by Eq. 1:

\[
\% \text{Yield} = \frac{W_f}{W_0} \times 100,
\]

where \(W_0\) is the initial weight of the monomer and \(W_f\) is the weight of the copolymer after the purification process.

### 2.3 mPEG-PCL copolymer characterization

#### 2.3.1 Chemical structure

The copolymer chemical structure was identified by proton nuclear magnetic resonance spectroscopy (\(^1\)H NMR) using deuterated chloroform (CDCl\(_3\)) as the solvent and tetramethyldisilane as the internal reference at 90 MHz (Eft-90 NMR; Anasazi Instrument). Also, Fourier-transform infrared spectroscopy (FTIR) (Affinity 1S; Shimadzu) was used to obtain spectra by reflectance mode with the help of a Quest ATR accessory (attenuated total reflectance), using a one-step diamond window. Each spectrum was the average of 45 scans performed with a resolution of 4 cm\(^{-1}\).

#### 2.3.2 Molecular weight

The copolymer molecular weight was determined using a gel permeation chromatography equipment (GPC 1260; Infinity) with a refractive index detector and a PLgel 5 \(\mu\)m MIXED-D column of 300 mm \(\times\) 7.5 mm. The sample was dissolved in 5 mL of tetrahydrofuran of HPLC grade (Fisher Scientific) at 40\(^\circ\)C and then 50 \(\mu\)L was injected at a flow rate of 1.0 mL min\(^{-1}\).

#### 2.3.3 CMC

For the CMC determination, Lugol’s iodine method was used, which is based on the detection of the iodine that dissociates when the micellar aggregates are formed. Iodine has the advantage of having a small molecular size, avoiding to affect micelle formation (28,29). Aqueous dilutions of the copolymer (1, 3, 5, 10, 15, 20, and 30 \(\mu\)g mL\(^{-1}\)) were placed in 15 mL tubes to a final volume of 10 mL, and 25 \(\mu\)L of Lugol’s iodine solution (1% I\(_2\) and 2% KI) was added to each tube. Then, all the tubes were sonicated for 10 min and were incubated overnight in the dark at room temperature. After incubation, each dilution was analyzed using a UV-Vis spectrophotometer (Evolution 220; Thermo Scientific) at a wavelength of 366 nm. The polymer concentration versus absorbance was plotted, and CMC was determined at the concentration from which absorbance started to increase (30).

### 2.4 Micelle preparation and MTX loading

The MTX-loaded micelles were prepared by the dialysis method (31). Briefly, 10 mg of the mPEG-PCL copolymer and 1 mg of MTX were dissolved in 1.5 mL of dimethyl sulfoxide (DMSO), under magnetic stirring for 2 h at 25\(^\circ\)C. This was the optimal time to achieve both polymer solvation and drug dispersion to obtain the maximum loading of MTX. The DMSO and unencapsulated drug were removed by dialysis, placing the solution in a 2,000 NMWCO pore size cellulose tube (Sigma-Aldrich). The cellulose tube was sealed at both ends and immersed in 1 L of distilled water, under moderate magnetic stirring for 3 days; the water for dialysis was changed by fresh-water at 12, 24, and 48 h. This progressive incorporation of water allowed the spontaneous formation of the micelles with the consequent capture of the drug. When the dialysis was completed, the suspension was filtered using a 0.45 \(\mu\)m pore size filter to remove the aggregates, and the filtrate was lyophilized. The lyophilized product was weighed and analyzed by UV-Vis spectroscopy to assess drug encapsulation efficiency (EE).

### 2.5 Micelle characterization

#### 2.5.1 Particle morphology

The micellar morphology was evaluated using a field emission scanning electron microscope in transmission mode (STEM, JSM 7401F; JEOL). For empty micelles, an aqueous solution with a copolymer concentration of 100 \(\mu\)g mL\(^{-1}\) was prepared; then, 20 \(\mu\)L of the sample was deposited on a carbon-coated copper grid and left to dry. Regarding characterization of MTX-loaded micelles, a sample of the lyophilized dialysis product was deposited on a grid in the same way as for empty micelles.

In addition, to determine the average particle size of the mPEG-PCL and MTX/mPEG-PCL micelles, the diameter of at least 700 particles was measured (from STEM images) using the Image-Pro Plus software. Also, the average particle size of micelles in the suspension was analyzed.
using a particle size analyzer (Zeta NanoSizer ZS; Malvern Instruments) under a backscatter configuration (633 nm) and copolymer refractive index of 1.38, at 25°C and in triplicate.

### 2.5.2 Melting and crystallization temperatures

The melting ($T_m$) and crystallization ($T_c$) temperatures of the MTX/mPEG-PCL micelles were characterized using a differential scanning calorimeter (DSC 2920 Modulated; TA Instruments). Unloaded mPEG-PCL copolymer, PCL and mPEG homopolymers, and a physical mixture of mPEG-PCL and MTX were also analyzed as reference. Samples of 10 mg were tested in the range 0–100°C, with a heating rate of 10°C min$^{-1}$ in a static air atmosphere. $T_m$ and $T_c$ were taken from the first heating cycle.

### 2.6 Drug loading and EE

The percentage of drug loading (%DL), Eq. 2, and EE of MTX (%EE), Eq. 3, were determined based on the weight of loaded micelles in their lyophilized form (32). The weight of encapsulated MTX was obtained by relating the absorbance values to the regression curve for the standard MTX measured under the same conditions.

$$\% DL = \frac{\text{Weight of the drug in the micelles}}{\text{Weight of the micelles}} \times 100, \quad (2)$$

where %DL is the DL ratio trapped in the micelles.

$$\% EE = \frac{\text{Weight of the drug in the micelles}}{\text{Initial drug weight}} \times 100, \quad (3)$$

where %EE is the effectiveness of encapsulation concerning the initial drug.

### 2.7 In vitro release of MTX

The in vitro release of MTX from the micellar system was performed using the dialysis method (25,26). The MTX-loaded micelles were deposited in a cellulose bag for dialysis (2,000 NMWCO). The bag was placed in a vial with 15 mL of PBS at pH 6.7, simulating a tumor microenvironment, under magnetic stirring (60 rpm) at 37 ± 1°C. For every 24 h, 2 mL of PBS was taken and the same volume was replaced with new PBS. The aliquots were analyzed by UV-Vis spectroscopy at 303 nm, and the amount of MTX released was determined using a calibration curve of MTX in PBS (7, 15, 31, 62, 125, 250 µg mL$^{-1}$). The procedure was repeated for physiological pH (7.4) and lysosome or advanced tumor microenvironment pH (5.5) (33). The concentration of released MTX was used to estimate the percentage of cumulative drug release (Cr), according to Deng et al. (34) (Eq. 4):

$$\text{Cr} (%) = \frac{\sum_{i=1}^{n} V_i C_i + V_0 C_n}{m_{MTX}} \times 100, \quad (4)$$

where $m_{MTX}$ is the amount of MTX in the micelles, $V_0$ is the total volume used in the dialysis procedure ($V_0 = 15$ mL), $V_i$ is the volume of the replaced media ($V_1 = 2$ mL), and $C_n$ is the concentration of MTX in the sample.

### 2.8 MTX/mPEG-PCL cytotoxicity in MCF-7 cells

The cytotoxicity of MTX, mPEG-PCL, and MTX/mPEG-PCL was evaluated in human breast cancer MCF-7 cell line (ATCC® HTB-22™) using the 3-(4, 5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were cultured in DMEM supplemented with 10% fetal bovine serum and incubated at 37°C in 90% humidified 5% CO2/95% air. Media was replaced every 24–48 h, until 80–90% confluence was reached for seeding treatments. MCF-7 cells were seeded in 96-well microplates to the final concentration of 1 × 10$^4$ cells per well and incubated under the same conditions overnight for adhesion. Then, old media was replaced with new media, and a proper volume of stock solutions of mPEG-PCL copolymer and MTX or MTX/mPEG-PCL was added in media to obtain the MTX concentrations assayed (5, 20, 30, 60, and 90 µg mL$^{-1}$) and the respective concentrations of the copolymer (57, 171, 342, 685, and 1,028 µg mL$^{-1}$). Untreated cells were used as reference controls. All treatments were performed in triplicates and the microplate was incubated for 48 h. After incubation, media was removed completely and cells were gently washed with PBS, pH 7.4. Then, 180 µL of media and 20 µL of MTT (5 mg mL$^{-1}$) were added to each well. The microplate was incubated for another 4 h. The media/MTT was removed and 200 µL of acidified isopropanol was added to each well to dissolve the MTT metabolized by the cells. The absorbance of each well was measured at 570 nm using a microplate reader (Varioskan Lux VLBLATD2; Thermo Scientific). Cell viability relative to untreated cells (control) was determined by Eq. 5:

$$\text{Cell viability} (%) = \frac{\text{OD (Test)}}{\text{OD (Reference)}} \times 100, \quad (5)$$
where OD (Test) and OD (Reference) represent the mean absorbance of treated cells and absorbance of reference cells, respectively.

3 Results and discussion

3.1 mPEG-PCL block copolymer

Figure 2 shows the FTIR spectrum of the mPEG-PCL diblock copolymer. The stretching vibrations of the carbonyl (C=O) and ether (CO) groups were shown at 1,719 and 1,106 cm\(^{-1}\), respectively, evidencing the copolymer formation. The signal at 3,471 cm\(^{-1}\) was assigned to the terminal hydroxyl (O–H) group in the copolymer. The symmetric and asymmetric stretching vibration bands of the C–H bonds of methylene groups were shown at 2,952 and 2,884 cm\(^{-1}\), respectively, whereas the peak at 1,339 cm\(^{-1}\) was ascribed to the twisting vibration of these same bonds. The signals present in the spectrum coincided with those reported for equivalent copolymers (35).

Figure 3 illustrates the H\(^1\) NMR spectrum of the copolymer. The spectrum presents the signal at 3.6 ppm, which is characteristic of mPEG methylene (a and b in the molecular structure). Also, at 3.3 ppm the protons of the methoxy group appear. The last two signals corresponded to the mPEG pre-polymer (33). The signal of methylene α-carbonyl group (c) appears at 2.3 ppm, whereas the signal of methylene protons α to the oxygen of a carboxylic group is at 4.0 ppm, and these two signals appeared at lower field because of the inductive effect of the substituent neighbors. The rest methylene protons of PCL (d, e, f) appear between 1.1 and 1.8 ppm (38). The spectrum showed all proton signals of PCL and mPEG, supporting block copolymer formation.

3.2 Polymerization efficiency and molecular weight

The copolymer was synthesized by ring-opening polymerization of ε-CL initiated on the hydroxyl group of the mPEG, using HCl as the catalyst. According to Eq. 1, the polymerization efficiency (% yield) was 67.3.

On the contrary, the molecular weight was characterized by GPC, yielding a molecular weight of the peak maxima \(M_p\) of 5,360 g mol\(^{-1}\), and a number \(M_n\) and weight \(M_w\) average molecular weight of 4,680 and 5,101 g mol\(^{-1}\), respectively, with a dispersity index of 1.09. The molecular weight of the PCL block was determined by subtracting the molecular weight of the mPEG block (2,000 g mol\(^{-1}\)) from the \(M_w\) of the copolymer, obtaining a value of 2,680 g mol\(^{-1}\). The copolymer molecular weight is in the recommended range of polymers for drug release, as reported by Murata et al. (37), who worked with micelles of PEG-PCL copolymer of 4,600 g mol\(^{-1}\). It is highlighted that the low dispersity index has shown to favor the formation of micelles of uniform size, which provides a controlled drug load and release (38).

3.3 CMC

The copolymer CMC was determined by Lugol’s iodine methodology. Figure 4 shows the absorbance (\(\lambda_{max}\))
increase in response to the copolymer concentration. The increase in $\lambda_{\text{max}}$ indicates that the dye adsorbed to the disassembled copolymer chains is being released. Based on that, the CMC observed was 5 $\mu$g mL$^{-1}$. At this concentration, the copolymer molecules precipitate from the aqueous phase generating molecular assemblies known as polymeric micelles (1). Other methods based on pyrene as a fluorescence probe indicated a CMC of 18 $\mu$g mL$^{-1}$ for a PEG-PCL copolymer of 12,000 g mol$^{-1}$ (39) and 7.2 $\mu$g mL$^{-1}$ for a copolymer of 7,000 g mol$^{-1}$ (9). As noted, the results of this research are approximated to those reported using the pyrene method. The differences may be related to various factors, including the molecular weight of the copolymer and the molecular weight of each block, which affects its solubility in water and therefore the CMC.

### 3.4 Micellar morphology and stability

TEM analysis showed that the mPEG-PCL copolymer produced spherical micelles in aqueous solution with a size smaller than 100 nm (Figure 5a). Size and spherical structure agreed with the reported for analog copolymers (40). For MTX/mPEG-PCL (Figure 5b), spherical aggregates were also observed but with an increase in diameter, which is attributed to the drug absorbed into the micellar nucleus (31).

From microscopy images, the size of empty and loaded micelles was estimated counting at least 700 particles. For empty micelles, an average diameter of 76 ± 16.30 nm was determined, whereas for the loaded micelles an increase to...
131 ± 13.13 nm was observed (Figure 5c and d). This diameter increment was an indication that the drug was encapsulated, as reported for similar copolymers (31). It should be emphasized that the micellar size obtained in this study is in the range recommended for the drug delivery system with acceptable performance (1,41).

The stability of the MTX/mPEG-PCL system was evaluated by dynamic light scattering (DLS) in an aqueous micelle dispersion kept up to 60 days at room temperature protected from light. The micelles were analyzed on day 5 and day 60 of storage from their synthesis, showing 167.6 and 185.8 nm, respectively. It is noted that the hydrodynamic size obtained by DLS was greater than that calculated from the microscopy images (131 nm). This behavior was observed in other studies (42), which was attributed to the shrinkage of the PEG shell induced by the evaporation of water before the TEM measurement. Thus, the larger diameter given by DLS is because of the hydration of the PEG shell. On the contrary, an increase in the diameter of PEG-PCL polymersomes was reported after 90 days by DLS. In that study, the size increase was not attributed to particle aggregation, but a swelling/hydration effect as a result of the hydrophilic block of PEG (43). Thus, it is concluded that MTX/mPEG-PCL micelles were stable for 60 days.

3.5 DSC analysis of MTX/mPEG-PCL nanoparticles

Figure 6 shows the DSC traces of the MTX-loaded nanoparticles, pure copolymer, mPEG, and PCL homopolymers, as well as a physical mixture of copolymer and MTX at a weight ratio of 10:1 (8–0.8 mg). Figure 6a denotes the $T_m$ for MTX/mPEG-PCL (52.4°C), for copolymer (53.8°C), and for the physical mixture of copolymer and MTX (50.4°C). The synthesized copolymer was built with two blocks of different lengths; PCL ($M_n = 2,680 \text{ g mol}^{-1}$) is larger than mPEG ($M_n = 2,000 \text{ g mol}^{-1}$) and also with different $T_m$ peaks of 52.1°C and 56.1°C, respectively (Figure 6c). Thus, the sign of only one peak at 53.8°C for the copolymer is explained by a signal overlapping influenced by the larger block, and it also suggests the assembly of the diblock structure. Analogously, the crystallization peaks can be observed in Figure 6d.

On the contrary, the two systems with MTX showed lower $T_m$ compared to the pure copolymer (Figure 6a), indicating that MTX modifies the crystalline structure of the copolymer. Because of MTX hydrophobicity, a major interaction with the PCL block either in the micellar or in the bulk form of the copolymer is expected. According to this, the lower temperature showed by the physical

Figure 6: DSC traces of (a) fusion and (b) crystallization of copolymer (mPEG-PCL), physical mixture (mPEG-PCL + MTX), and methotrexate-loaded micelles (MTX/mPEG-PCL) and (c) fusion and (d) crystallization of polycaprolactone (PCL) and m-polyethylene glycol (mPEG).
mixture indicated that MTX produced higher disorder in the bulk form than in the micellar form.

Figure 6b illustrates the $T_c$ of the copolymer, physical mixture, and MTX-loaded nanoparticles. Important differences were also observed; the copolymer showed two $T_c$ at 13.9°C and 34.2°C, attributed to the PCL and mPEG blocks, respectively (44). The physical mixture showed a shift toward a lower $T_c$ of 21.8°C for the mPEG block and splitting of the $T_c$ signal for PCL as noted at 10.8°C and at 6.6°C. The two $T_c$ signals were related to the formation of two PCL phases, and probably the one with lower temperature may contain more MTX. The MTX/mPEG-PCL showed a $T_c$ of 21.2°C for the mPEG block, which was very similar to that of the physical mixture, in which the $T_c$ for PCL was divided into two signals (24.2°C and 25.7°C) but at a higher temperature, suggesting that MTX favors the ordering of PCL blocks in the micellar structure.

During drug encapsulation (Figure 7), chemical interactions between MTX and PCL block could occur. Yang et al. suggested the formation of hydrogen bonds between the carbonyl groups in PCL and the hydroxyl or amine groups of the drug when they were encapsulated in polymeric micelles. Because of the formation of intermolecular hydrogen bonds, the drug molecules could act as a physical side group of the PCL block in the micelles (45). This intermolecular interaction improved the copolymer crystallization, producing the two crystallization peaks at higher temperatures, in contrast with the analogous phases in the physical mixture. It should be mentioned that in the encapsulation of MTX, it is common for this drug to cause disorder in the micellar structure, manifesting a reduction of the crystallization; for example, in the encapsulation of MTX in Pluronic micelles, it was observed that MTX affected crystalline phase formation (46). Also, nanoparticles loaded with MTX presented disordered recrystallization caused by a strong interaction of the drug with the polymer, obtaining an amorphous structure of the system (47). Moreover, for blends of MTX-loaded poly(lactic acid)/poloxamer, the melting point of the system was increased with the addition of MTX, but a lower percentage of crystallinity was observed (48).

Based on these observed behaviors, it is clear that the encapsulation of MTX in micellar systems generates a dynamic system, as a result of the chemical interactions between the drug and the lipophilic moiety of the copolymers, which would impact the release behavior of the drug from micelles subject to environments that destabilize the associations.

### 3.6 DL and EE

The micellar DL and EE were calculated from the concentration of MTX released, as obtained from the regression equation ($y = 0.0133x + 0.0433; R^2 = 0.999$) of the standard MTX curve. Based on Eq. 2 and 3, the MTX/mPEG-PCL system achieved a DL of 8.8% and an EE of 70.2%.

Another study, using worm-like nanoparticles of PEG-PCL copolymer loaded with MTX, reported a DL of 3.5% and an EE greater than 65.6% (49), whereas for PLA micelles a DL from 3.7% to 12.8% and an EE from 17% to 47% were reported (31). Pluronic micelles also showed a DL of 2.83% and an EE of 84.98% (46). Finally, Song et al. loaded polyurethane micelles with an analog of MTX (folic acid), reporting a DL from 3.19% to 7.68% and an EE from 27.72% to 32.95% (32). Compared with similar drug delivery systems, it can be concluded that the mPEG-PCL system reported here has an adequate loading capacity to be used as a drug delivery system for MTX.

### 3.7 In vitro release of MTX

To determine the influence of a simulated pH for tumor microenvironments or lysosomes on the behavior of MTX/PEG-PCL micelles, MTX release experiments were achieved in PBS solutions at pH 6.7 and 5.5. pH 7.4 was also assayed to determine the behavior under normal physiological conditions (Figure 8).

The maximum MTX release at pH 7.4 was around 10% in 30 days, whereas at pH 6.7 a release of 15% was recorded within the first 2 days, followed by a gradual increase, achieving a maximum of 80% of drug release.

**Figure 7:** Schematic representation of (a) mPEG/PCL copolymer micelles and (b) methotrexate-loaded micelles (MTX/mPEG-PCL). The interaction by hydrogen bonds between the OH groups of methotrexate and C=O of the hydrophobic block of the copolymer is illustrated.
after 25 days, which remained constant until day 30. At pH 5.5, an initial release of 20% was observed on the first day, with a progressive increase until day 22 to a maximum release of 90%, which was kept until the end of the assay. The release kinetics suggests that a tumor microenvironment with pH values of 6.7 and 5.5 would gradually receive 80% and 90% of the drug captured by the micelles, for around 25 days. The gradual MTX released may be enough to eliminate cancer cells, considering that the approximated IC$_{50}$ for cancer cell lines as MCF-7 is 34 µg mL$^{-1}$, reported by Nogueira et al., at 24 h (50), whereas the present system in 24 h released 136 and 83 µg mL$^{-1}$ at pH 5.5 and 6.7, respectively.

The kinetics at pH 7.4 indicated that the micellar system remained stable as it released a very low amount of the drug during the 30 days assayed, which suggests that the system would not importantly affect microenvironments under normal conditions. Rostamizadeh et al. reported that MTX-conjugated PEG-PCL micelles were sensitive to pH 5.5 during the release assay, attributing this to the sensitivity of the ester linkages to cleavage because of pH effect. They also claimed that the release was faster in acidic pH than in neutral (25). This behavior was consistent with the stability and drug release observed in this research because micelles remained stable at pH 7.4 for 60 days. Duan et al. also evaluated the release of MTX from MTX-mPEG micelles in PBS at pH 7.4 and pH 6, reporting that the ester bonds of micelles were slowly hydrolyzed in an acidic environment, hence releasing the MTX slowly, and that at pH 7.4 the bonds were not hydrolyzed (51). On the contrary, D’souza and Shegokar reported that the high polarity of PEG increases hydrophilicity and, therefore, improves water solubility (52). This high solubility of PEG has also been observed in most organic and inorganic solvents (53). Taking these observations into consideration, it can be suggested that the release pattern presented by the mPEG-PCL micelles is caused by the hydrophilic moiety of the copolymer, which is solubilized in an acidic environment, causing a gradual drug release as it is dissolved.

### 3.8 Cell viability

The cytotoxic effect of mPEG-PCL micelles, MTX/mPEG-PCL micelles, and free MTX was evaluated by an MTT assay, at which the sodium dodecyl sulfate (0.1%) and untreated cells were included as positive (viability reduction) and reference controls, respectively (Figure 9). From the figure, it is noted that cell viability tends to decrease as the concentration of free MTX or loaded MTX in micelles increases (MTX/mPEG-PCL). However, the MTX/mPEG-PCL micelles showed to decrease more cell viability. From percentage viability values, the concentration that inhibits 50% of the cells (IC$_{50}$) was estimated to be 30 µg mL$^{-1}$ for MTX loaded in the MTX/mPEG-PCL micelles, whereas the IC$_{50}$ value for free MTX was not reached at the assayed concentrations. Previous studies have also reported higher cytotoxicity of MTX loaded in PEG-PCL copolymer polymersomes or micelles than free MTX (43,49). Gharebaghi et al. related the greater effect of micelles loaded with MTX to that micelles can bind to the surface of cells, penetrate, and release high levels of the

Figure 8: MTX release in PBS at pH 5.5, 6.7, and 7.4 for 30 days.

Figure 9: Cytotoxic effect of free MTX, MTX/mPEG-PCL, and mPEG-PCL micelles to MCF-7 cells, determined by MTT assay at 48 h of incubation. The data are presented as mean ± SD.
drug inside. In contrast, free MTX can be only transported into cells by passive diffusion. The IC$_{50}$ value obtained from the MTX/mPEG-PCL system was somewhat higher than that reported for another mPEG-PCL system as an MTX carrier (12.5 µg mL$^{-1}$), using MCF-7 cells at 48 h exposure (49). This can be explained by the differences in the structural arrangement of the polymer particles and/or their interaction with the drug, which influenced the drug release rate as noted for the MTX/mPEG-PCL micelles, which released MTX more slowly. Thus, further assays are considered to evaluate the cytotoxic effect of the MTX/mPEG-PCL system at longer times as 72 h. On the contrary, the empty mPEG-PCL micelles were assayed at the equivalent mass provided by the MTX/mPEG-PCL system, observing a slight cell viability decrease at the mass to load up to 30 µg mL$^{-1}$ MTX (342 µg mL$^{-1}$ of the copolymer), whereas the equivalent copolymer mass (685 and 1,028 µg mL$^{-1}$) for the highest MTX loads (60 and 90 µg mL$^{-1}$) decreased cell viability by 50%, indicating the toxic effect of copolymers at these concentrations. Previous studies have reported nontoxic effects at lower concentrations of PEG-PCL polymer particles to MCF-7 cells (43,49,54), which agrees with the nontoxic effects of the lower concentrations of mPEG-PCL particles evaluated in this work.

4 Conclusion

Diblock copolymer synthesis by ring-opening polymerization using HCl as the catalyst allowed obtaining amphiphatic chains with a monodisperse molecular weight (dispersity of 1.09). This copolymer spontaneously self-assembled in aqueous solution forming polymeric micelles of homogeneous nanometric size. The dialysis method used to capture MTX in the micellar nucleus achieved an efficiency of 70% and loaded micelles with a diameter of 131 nm. The drug release dependence upon pH was proven since the MTX/mPEG-PCL system showed a clear trend to release much higher amounts of the drug at lower pH compared to the physiological pH of 7.4, where a very low amount of MTX was released. Likewise, the MTX/mPEG-PCL system showed to be more effective to fight MCF-7 breast cancer cells than free TMX. It can be concluded with certainty that the system showed effectiveness in maintaining the encapsulated drug until it received the acidic stimulus, and the release was sustained for 22 days. This selective and sustained release mechanism for tumoral environments could help to lessen side effects in the patients. The space-time control of drug release observed in the MTX/mPEG-PCL micellar system, its pH-controlled release, and its effectiveness against breast cancer cells suggest it as a promising strategy for cancer treatments.

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