Tamoxifen-Loaded Microspheres Based on Mixtures of Poly(D,L-lactide-*co*-glycolide) and Poly(D,L-lactide) Polymers: Effect of Polymeric Composition on Drug Release and *In Vitro* Antitumoral Activity

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ABSTRACT: Mixtures of different bioerosionable polyesters were used to prepare microparticulated tamoxifen delivery systems to achieve anticancer effects in breast malignant cancer cells. Tamoxifen (TMX) was included into microspheres (MS) formulated via spray-drying. Mixtures of poly(D,L-lactide-*co*-glycolide) (PLGA) of different lactide/glycolide proportions (50 : 50 and 75 : 25) and poly(D,L-lactic acid) (PLA) were used. The average diameter of the resultant TMX-loaded microparticles was in the range 1.04 ± 0.51–1.55 ± 0.95 μm. The encapsulation efficiency of TMX was between 97.8% [48.9 ± 0.1 TMX (μg)/MS (mg)] and 69.6% [36.6 ± 0.1 TMX (μg)/MS (mg)] depending on the polymeric composition of the formulation. Drug burst effect was not observed. TMX was released from the polymeric matrices in a sustained release manner between 11 and 58 days depending on polymeric composition of microspheres. TMX-loaded microspheres showed high efficacy in causing cell death in MCF7 breast malignant cancer cells. Thus, these TMX-loaded PLGA-based microspheres hold potential to treat breast malignant cancer cells. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 124: 2987–2998, 2012

Key words: tamoxifen; drug delivery; polyesters; microspheres; breast cancer

INTRODUCTION

Chemotherapy causes notoriously undesirable side-effects in many patients, and some of them may die because of the side-effect of the anticancer drugs rather than the cancer itself. Due to these harmful effects, clinical application of some anticancer drugs is limited. In this way, drug delivery systems aiming to overcome side effects.[1] Among biodegradable polymers, poly(D,L-lactic acid), poly(D,L-lactic-*co*-glycolic acid), and poly(ε-caprolactone) have been extensively used to form microparticles and nanoparticles to encapsulate a variety of therapeutic compounds.[2] The low toxicity of these polyesters and their degradation products[3,4] make possible to obtain biodegradable drug delivery systems, whose properties can be modified as a function of the polymer molecular weight and copolymerization composition of the polymeric matrices used for drug encapsulation.[5] Furthermore, the methodology chosen to the preparation of polyester microspheres, as well as slight changes of the different parameters of the selected methodology, can have radical effects on microsphere characteristics and, in turn, drug release.[5,6]

The treatment of choice for patients with estrogen receptor (ER) positive breast cancer is tamoxifen (TMX).[7,8] This anticancer drug is mainly employed for long-term prophylactic therapy in high risk and postmenopausal women[9,10] by oral administration. Among side effects of TMX, endometrial cancer, due to agonist activity of the drug in the uterus, and drug resistance, which may lead to further progression of the tumor, are the major ones.[11] These undesirable effects can be minimized by maximized drug concentration in the target tissues and maintained it at minimal level in blood stream and nonspecific organs, which could be achieved by TMX administration by drug delivery systems.

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Here, we reported PLGA based microparticles prepared by spray-drying technology from polymeric feed mixtures of different proportions of PLGA 50 : 50, PLGA 75 : 25 and poly(D,L-lactic acid) (PLA), as drug delivery systems for TMX. The physicochemical properties of the microparticles were characterized. The action of these TMX-loaded microspheres on viability of human breast adenocarcinoma (MCF7) cells was investigated via the methylthiazoletetrazolium (MTT) method.

MATERIALS AND METHODS

Materials

Poly(D,L-lactide-*co*-glycolide) (PLGA) [lactide : glycolide 50 : 50, M_w = 34,400 and lactide : glycolide 75 : 25, M_w = 57,600] (Sigma-Aldrich Barcelona, Spain), PLA (M_w = 40,400) (Sigma-Aldrich Barcelona, Spain), potassium monohydrogen phosphate (K_2HPO_4) (Panreac, Barcelona, Spain), potassium dihydrogen phosphate (KH_2PO_4) (Panreac), dichloromethane (DCM) (Panreac), chloroform (Panreac), methanol HPLC quality (Panreac), dimethyl sulfoxide (DMSO) (Sigma-Aldrich), dodecyl sulfate sodium salt (SDS) (Merck, Darmstadt, Germany), TMX minimum 99% (Sigma-Aldrich), penicillin (50 U/mL), streptomycin (50 μg/mL), 0.05% trypsin/0.53 m*M* EDTA (Invitrogen Life Technologies, Grand Island, NY), gentamicin (50 μg/mL; Sigma-Aldrich Company, UK), MTT (Sigma-Aldrich), and Dulbecco's modified Eagle medium (DMEM) + GlutaMax-I (supplemented with 10% heat inactivated fetal bovine serum) (FBS) (Invitrogen Life Technologies) were used as received. Milli-Q® water (Millipore, Madrid, Spain) was used.

Methods

Preparation of microspheres

Preparation of microspheres was carried out by the spray-drying process (Mini Spray-dryer B-191, Büchi, Switzerland). To obtain microspheres without drug, polymers (2 wt %) were dissolved in dichloromethane.[5,12–14] The following polymers and polymer mixtures were used: PLGA 50 : 50, PLGA 75 : 25, PLGA 50 : 50 + PLGA 75 : 25 (50/50 wt %), PLA + PLGA 50 : 50 (25/75 wt %), and PLA + PLGA 75 : 25 (25/75 wt %). Microspheres with TMX were prepared from 1.9 wt % polymer to 0.1 wt % of drug, both dissolved in dichloromethane. The polymeric solution with and without drug (100 mL) were maintained under constant stirring (900 rpm) and sprayed through the nozzle (0.7 mm diameter) of the spray-dryer. Assay conditions were: inlet air temperature 52–60°C and outlet temperature were between 34 and 37°C; spray flow 5 mL/min and compressed spray air flow (represented as the volume of the air input) 700 Nl/h. Microspheres were collected from the spray-dryer cyclone separator and then they were stored under vacuum condition.

Morphology studies

The morphology, average diameter as well as the size distribution of unloaded and drug-loaded microspheres was studied by field emission scanning electron microscopy (FEG-SEM; Jeol JSM-6330F Electron Microscope, resolution 36 mm of Centro de Microscopia y Citometría, UCM). The samples were fixed with an adhesive sheet on a rigid support and coated with gold for their later visualization. Micrographs were recorded in randomly selected particle populations. The diameter of 500–900 microspheres of each type was measured using enlargements of FEG-SEM photographs. The counted particles were used to check the convergence of the polydispersity index (U). The combined diameters were used to calculate the number-average diameter (D_n) and the weight-average diameter (D_w) using the following equations[15]:

$$U = D_w/D_n$$
$$D_n = \Sigma N_i D_i/\Sigma N_i \quad (1)$$
$$D_w = \Sigma N_i D_i^4/\Sigma N_i D_i^3$$

N_i is the number of particles measured, and D_i the diameter of the measured particle. The particle distribution is considered to be monodisperse when the polydispersity index is between 1.0 and 1.1.[16]

TMX loading studies

All the experiments with TMX were performed under subdued light, since the drug is highly photosensitive. To determine the amount of TMX included in the polymeric microparticles, 30 mg of microspheres was dissolved in 3 mL of chloroform. The amount of TMX was determined by UV/V spectroscopy (UNICAM 8700 Spectrophotometer) at 277.6 nm using a microcell (50 μL) due to polymers as well as the drug are soluble in chloroform and UV/V spectrum of TMX shows a maximum absorbance at 277.6 nm; however, the polymer solutions did not absorb at this wavelength. The absorbance of the samples was interpolated in a calibration curve, which was obtained from TMX solutions in the range of 1–60 μg TMX/mL chloroform. Furthermore, UV/V spectra (200–600 nm) of the samples were carried out to evaluate whether alterations of the drug in the microspheres preparations process have taken place. It was previously verified that the absorbance of TMX was not modified when the polymers and

TABLE I
Coefficients of Variation (CV) of TMX Indicating the Precision of the HPLC Assay

Interday		Intraday	
TMX (ng/mL)	CV	TMX (ng/mL)	CV
25	5.1%	25	4.1%
500	3.3%	500	2.3%
5000	5.5%	5000	0.5%

the drug were dissolved in freshly prepared solutions.

The samples were also analyzed by high-performance liquid chromatography (HPLC) assay to confirm the absence of chances in TMX (Spectra System P2000 HPLC pump, Spectra System FL3000 fluorescence detector and Spectra System SCM1000 degasser; Thermo Electron Corp.) using a postcolumn photochemical reactor supplied with a 5-m reaction coil and a 254-nm UV lamp (AURA Industries, NY) that converted TMX to a highly fluorescent phenanthrene derivative. The HPLC method was based on those developed by MacCallum et al.[17] and by Lee et al.[18] To determine the amount of TMX-loaded into microspheres, 3 mg of microspheres was dissolved in 200 μL of dichloromethane by stirring, then 3 mL of methanol were added and the mixture was shaken during an hour to obtain a homogeneous dissolution due to both are miscible solvents. The dichloromethane was evaporated at room temperature and polymers were eliminated by precipitation, so the amount of TMX in methanol could be determinate by HPLC. The stationary phase was Spherisorb ODS2, C_{18}, 5 μm (25 × 0.46 cm; Waters). The mobile phase consisted of triethylamine (1% in H_2O, pH 8)/methanol (11/89 v/v). The flow rate was set at 1 mL/min. The fluorescence detector was set at an excitation wavelength of 250 nm and emission wavelength of 370 nm. Peak area of each sample was generated through the computerized software (ChromQuest™). The calibration curve was obtained from TMX solutions between 50 and 5000 ng/mL in methanol and a good linear correlation ($r^2 = 0.99$) was obtained. The TMX retention time was 8.92 ± 0.1 min at 27°C. The validity of the method was investigated by the determination of precision of the assay based on the reported guidelines.[19] Five replicates of control samples at each concentration of 25, 500, and 5000 ng TMX/mL were used to determine the interrun and intrarun validity. The precision was demonstrated by the coefficient of variation (CV) values (Table I).

The experiments were carried out in triplicate in both methods. The amount of drug entrapped per weight of microspheres was calculated (drug loading = weight of TMX in microspheres/microsphere sample weight). The percentage of entrapment efficiency was expressed by relating the actual drug entrapment to the theoretical drug entrapment.[20]

Thermal analysis

TGA curves of unloaded and TMX-loaded microparticles as well as pure TMX, PLA, PLGA 50 : 50 and PLGA 75 : 25 were obtained using a Mettler Toledo thermal analyzer (TGA-SDTA 851). The mass of the samples was 3 mg. The sample pan was placed onto the balance and the temperature rose from 25 to 600°C at a heating rate of 10°C/min under nitrogen atmosphere (N_2 flow rate: 60 cm^3/min). The mass of the sample pan was continuously recorded as a function of temperature.

In vitro drug release studies

For TMX release studies, 30 mg of TMX-loaded microparticles were added to 50 mL PBS/SDS (0.5 wt/v), which was placed in an Erlenmeyer covered with Parafilm® at a constant temperature (37°C) and at a constant shake (100 rpm) in a horizontal shaking water bath. At intervals, 100 μL samples were withdrawn from the solution to follow the change in TMX concentration by UV/V spectroscopy at 277.6 nm. The volume removed from the Erlenmeyer was replaced with PBS/SDS (0.5 wt/v). SDS was added to the release medium to increase the solubility of TMX in aqueous solutions and prevent adsorption of the drug on the surface of the vessel.[21] In drug release experiments, sink conditions were maintained,[22,23] since TMX concentration in the release medium was always very much lower than its solubility in aqueous medium (<1 mg/mL at 20°C). The experiments were carried out in triplicate.

From plots of cumulative TMX released versus time, release rates were calculated. For a specific time interval, a straight line was obtained by using a least square fit and the release rate was determinate from the slope.

Mathematical modeling of release kinetic

The *in vitro* drug release data were fitted to various release kinetic models[24] that can define mechanism of the release: Higuchi,[25] Korsemeyer - Peppas,[26] and Hopfenberg[27] models employing the following set of equations:

$$\text{Higuchi model} : M_t = K \times t^{1/2} \quad (2)$$

$$\text{Korsmeyer} - \text{Peppas model} : M_t/M_\infty = k \times t^{\text{n}} \quad (3)$$

$$\text{Hopfenberg} : M_t/M_\infty = 1 - [1 - (K_1 t/C_0 R)]^3 \quad (4)$$

In these equations M_t and M_∞ correspond to the amount of drug dissolved at a particular time (t)

TABLE II
Concentration of Microparticles Needed to Obtain 50, 30, and 10 μM of TMX in the Cell Culture Medium

FORMULATION	Concentration of microparticles (μg/mL) to obtain different concentrations of TMX in the cell culture medium		
	50 μ*M* TMX	30 μ*M* TMX	10 μ*M* TMX
PLGA 50 : 50	390	234.2	78.1
PLGA 75 : 25	380	227.8	75.9
PLGA mixture[a]	383	229.8	76.6
PLA + PLGA 50 : 50	432	259.1	86.4
PLA + PLGA 75 : 25	474	284.6	94.9

[a] PLGA mixture: PLGA 50 : 50 + PLGA 75 : 25 (50/50 wt %).

and at infinite time, respectively. Parameters K, k, and K_1 are the release kinetic constants obtained from the linear curves. C_0 is the initial concentration of drug, R represents the radius of particles, and n is the release exponent.

Cell culture studies

Human breast adenocarcinoma (MCF7) cells were obtained from Dr. von Kobbe. Cells were maintained in DMEM + GlutaMax-I supplemented with 10% heat inactivated fetal bovine serum, penicillin (50 U/mL), streptomycin (50 μg/mL), and gentamicin (50 μg/mL) in a humidified incubator at 37°C and 5% CO_2 atmosphere (HERA cell, Sorvall Heraeus, Kendro Laboratory Products Gmbh, Hanau, Germany). Cells were plated in 75 cm^2 flask (Sarstedt Ag and Co., Barcelona, Spain) and were passaged when reaching 95% confluence, by gentle trypsinization (0.05% trypsin/0.53 m*M* EDTA).

Cell viability was evaluated by using the MTT method. All experimental conditions were performed in quintuplicate. Each experiment was carried out in triplicate. In preliminary experiments, increasing concentrations of TMX from 0.01 m*M* to 1 m*M* were tested in culture. The selected TMX concentrations were 10, 30, and 50 μ*M*. Cells were seeded in 96-well flat-bottom plates at 5000 cells/well. Twenty-four hours later, the medium was replaced with 100 μL medium with 1% FBS containing unloaded microspheres, TMX-loaded microspheres or the drug in solution. The concentration of microspheres was between 75.9 and 474 μg/mL (Table II), which was in accordance with the concentration of TMX used in the experiment, considering the amount of TMX incorporated in microparticles. After 2, 5, and 6 days, each well was added with 10 μL MTT solution (5 mg/mL). After 2 h incubation at 37°C, 5% CO_2, each well was replaced with 100 μL DMSO.[28] The cell viability was determined by measuring the absorbance at 570 nm using a spectrophotometer (Varioskan, Thermo Fisher Scientific, Barcelona, Spain). Results are presented as the percentage survival in relation to untreated control cells.

Statistical analysis

The data were expressed as mean ± S.D. from at least three independent experiments. Statistical comparisons were performed with one way analyses of variance (ANOVA test). A value of $P \leq 0.05$ was considered significant.

RESULTS AND DISCUSSION

In vitro studies

Antineoplasic drugs have been included in drug delivery systems to improve their pharmacological efficiency and decrease their side effects.[21,29] In this study, the polymer and drug concentration, as well as assay conditions and solvent were chosen based on previously published data on preparation and optimization of PLA and PLGA microspheres by spray drying.[5,12,14,30] Experiments carried out using spray drying have shown that a higher polymer concentration produces more particles with a large particle size,[14] and the preparation of PLA microparticles by this technique have shown that low PLA concentration (<1.5%, wt/v) tended to form poor spherical particles, whereas high PLA concentration (>3%, wt/v) produced fibrin products.[13] Furthermore, studies carried out with chlorambucil indicated that higher drug loading decreased the encapsulation efficiency of the drug in PLA microspheres.[13] On account of these spray drier assay conditions, a polymer concentration of 2% wt/v for preparing unloaded microspheres, and 1.9% wt/v polymer plus 0.1% wt/v TMX for preparing TMX-loaded microspheres were chosen. These delivery systems were prepared for drug administration during a long period, and one of the most important points of the process is the absence of modifications of the drug. One of the parameters that can cause changes on a drug is a high temperature. The temperature of the microspheres obtained by spray drying technique is 15–20°C lower than the outlet temperature.[31] The experimental conditions used in the spray drying process allow obtaining outlet temperatures between 33 and 42°C, which means very probably that the temperature of TMX in the experiment was 16–25°C. This range of temperature does not induce alterations in the drug. These temperatures were possible due to the use of dichloromethane, solvent with a low boiling point (39.8°C), which is a very good solvent of PLGA, PLA, and TMX. Although dichloromethane is a class-2 solvent

according to ICH classification and its administration should be limited (PDE 6 mg/day; concentration limit 600 ppm), it is considered one of the halogen solvents that cause less toxicity.[32]

The yield of microspheres was between 44.3 ± 6% (TMX-loaded microspheres) and 52 ± 7% (unloaded microspheres), which is similar to that obtained by other authors[5,14] using the spray drying technique at laboratory scale. Some studies have been already carried out with PLGA and TMX using different method of synthesis like emulsion-solvent evaporation technique and interfacial deposition of performed polymer.[33–35] There are some commonly methods used to fabricate micro/nanospheres like solvent evaporation/solvent extraction method, phase separation method and spray dryer,[36] but the spray dryer technique is the most simple, fast and reproducible method of them[37] and allows the use of mild conditions obtaining high encapsulations efficiency and product yield.[38]

FEG-SEM photographs of all types of microparticles showed individual and independent particles of a very small size (Fig. 1). Their surface was rough and slightly porous, some hollows, or deformations, which are common characteristic of microspheres obtained by spray-drying process,[39] were observed. This morphology is mainly determined by the solvent evaporation process; dichloromethane evaporation rate is fast and this allow achieving microspheres more spherical than those obtained using solvent with higher boiling points.[14] The average diameter of unloaded microspheres was between 1.62 ± 0.8 and 1.93 ± 0.74 μm (Table III), whereas a smaller average diameter was mainly determined for TMX-loaded microspheres, between 1.04 ± 0.51 and 1.55 ± 0.95 μm (Table III). Differences in appearance between TMX-loaded and unloaded microspheres were observed. TMX-loaded microsphere surfaces were less rough than those of microspheres without drug. Thus, the presence of the drug in the feed mixture influences the size and morphology of the microspheres making microspheres smaller, less rough, and more compact.

The size distribution of unloaded-microspheres was similar to that of TMX-loaded microspheres, and most of the particles were the same size in both groups. All the systems synthesized were polydisperse (Table III), with a polydispersity index overcome 1.1.[16] Furthermore, the percentage of nanoparticles (average diameter between 250 nm and 1 μm) in the formulations was high (between 23.91 and 77.73%).

TMX-loaded microspheres included between 48.9 ± 0.1 and 36.6 ± 0.1 μg TMX/mg microspheres and the percentage of entrapment efficiency was between 97.8 ± 0.1 and 69.6 ± 0.2% depending on the polymer composition of the microspheres (Table IV).

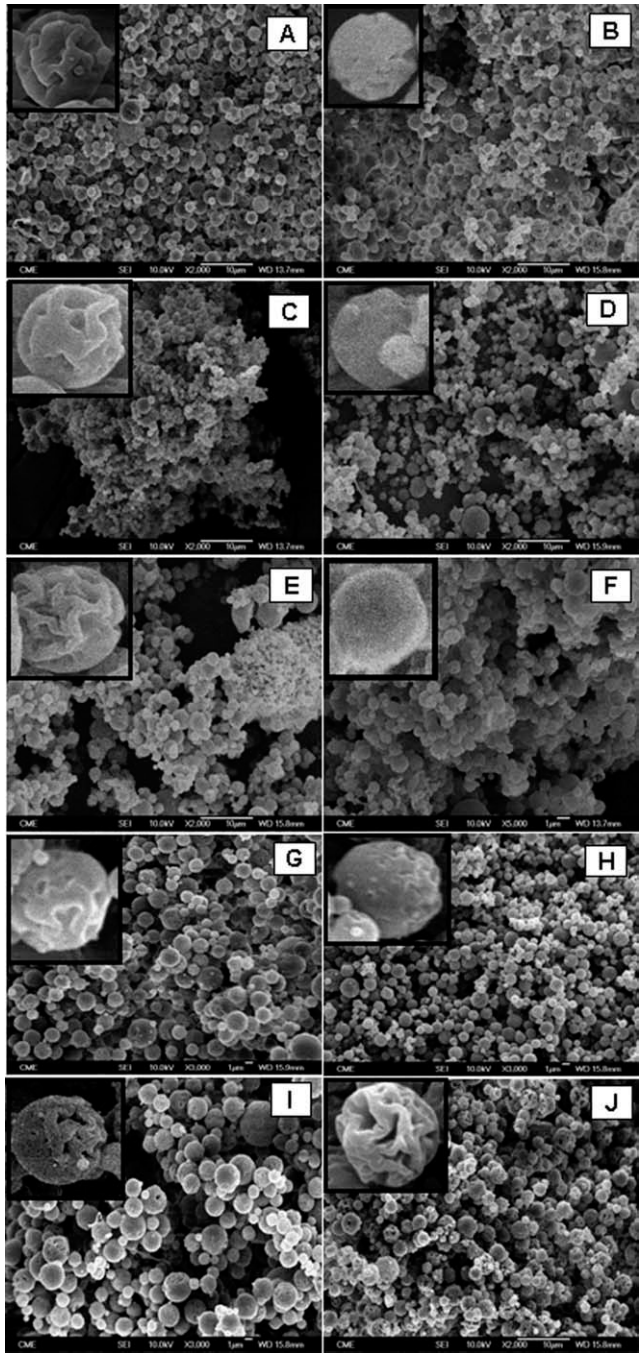

Figure 1 FE-SEM photographs of microspheres: (A) PLGA 75 : 25; (B) TMX-loaded PLGA 75 : 25; (C) PLGA 50 : 50; (D) TMX-loaded PLGA 50 : 50; (E) PLGA 50 : 50 + PLGA 75 : 25; (F) TMX-loaded PLGA 50 : 50 + PLGA 75 : 25; (G) PLA + PLGA 50 : 50; (H) TMX-loaded PLA + PLGA 50 : 50; (I) PLA + PLGA 75 : 25; (J) TMX-loaded PLA + PLGA 75 : 25.

These values were higher than others already reported using the emulsion-solvent evaporation or nanoprecipitation technique with PLGA and TMX. Cirpanli et al.[40] reported a efficiency of encapsulation of 38.99% using PLGA 50 : 50 while Rafati and Mirzajani[41] and Mirzajani et al.[42] obtained 82% for

TABLE III
Average Diameter, Percentage of Nanometer Particles, Technique Yield, and Polydispersity Index (*U*) of Microparticles

Formulation		*U*	Percentage of Nanoparticles (250 nm–1 μm)	Average diameter (μm)	Yield (%)
PLGA 75 : 25	With TMX	2.23	42.86%	1.55 ± 0.95	37.73
	Unloaded	1.62	28.79%	1.62 ± 0.80	46.73
PLGA 50 : 50	With TMX	2.22	40.82%	1.40 ± 0.71	36.21
	Unloaded	1.43	77.73%	0.80 ± 0.31	62.91
PLGA 50 : 50 + PLGA	With TMX	1.43	26.76%	1.49 ± 0.56	53.11
75 : 25 (50/50 wt %)	Unloaded	1.60	23.91%	1.71 ± 0.81	45.57
PLA + PLGA	With TMX	1.69	55.96%	1.04 ± 0.51	46.07
50 : 50 (25/75 wt %)	Unloaded	1.47	23.09%	1.72 ± 0.78	48.00
PLA + PLGA 75 : 25 (25/75%)	With TMX	1.67	48.16%	1.13 ± 0.57	48.28
	Unloaded	1.67	11.25%	1.93 ± 0.74	60.25

PLGA 50 : 50 particles and Sahana et al.[43] using PLGA 85 : 15 obtained entrapment efficiencies between 18.6 and 71.98%. The encapsulation efficiency obtained can be considered high due to dichloromethane is a very good solvent of polymers and drug therefore both components were dissolved in the solvent medium,[44] whereas the entrapment efficiency reported for the encapsulation of drugs that are not soluble in the solvent medium using the spray-drying technique is lower.[45,46] All the TMX entrapment efficiencies were over 95% except that of microspheres with PLA polymer in the polymeric matrix, which have the lower encapsulation efficiency (77–69.6%); this fact can be attributed to the hydrophobic characteristics of PLA and TMX, which seemed to induce a competition between them regarding their presence in the final formulation, and the amount of TMX decreases.

The thermal stability of unloaded microspheres and TMX-loaded microspheres along with that of the drug and the polymers was studied. The TGA first derivate of the formulations is plotted in Figure 2 and the parameters derived from them are collected in Table V. Pure TMX and raw PLGA 75 : 25 were degraded in one-step process at a maximum temperature of 267.9 and 350.61°C, respectively, whereas raw PLA and raw PLGA 50 : 50 showed two steps of thermal degradation (Table V), with peaks at 305.7 and 353.8°C for PLA, and at 322.9 and 367.9°C for PLGA 50 : 50. These data are consistent with those reported by Penco and coworkers[47,48] for PLA, PLGA 75 : 25, and PLGA 50 : 50 in studies of thermal degradation carried out at a heating rate of 20°C/min. When unloaded and TMX-loaded microspheres were studied, only thermal decomposition of formulations with a large amount of PLGA 50 : 50 showed a two-steps process. In all cases, the presence of TMX made the formulations more stable, and a specific peak corresponding to the drug was not observed (Fig. 2), which seemed to indicate a strong interaction between the drug and polymers in the formulations.

TMX release from microspheres was determined by the polymer composition of the formulations (Fig. 3). Microspheres of PLGA 50 : 50 and those of PLA + PLGA 50 : 50 (25/75 wt %) showed the quicker drug release, whereas the lowest TMX release took place from PLA + PLGA 75 : 25 (25/75

TABLE IV
TMX Load, Entrapment Efficiency of the Drug, and Amount of Drug Residual after Drug Release Experiments

Formulation	TMX load [drug (μg)/ MS (mg)]	Entrapment efficiency (%)	Amount of TMX after drug release	
			drug (μg)/MS (mg)	%
PLGA 50 : 50	47.6 ± 0.5	95 ± 1[a,b]	0.23 ± 0.02	0.49 ± 0.05[c,d]
PLGA 75 : 25	48.9 ± 0.1	97.8 ± 0.1[a,b]	0.12 ± 0.02	0.25 ± 0.03[c,d]
PLGA mixture[e]	48.5 ± 1.4	97 ± 2[a,b]	1.03 ± 0.44	2.13 ± 0.91
PLA + PLGA 50 : 50	40.3 ± 1.1	77 ± 2	0.21 ± 0.02	0.53 ± 0.06[c]
PLA + PLGA 75 : 25	36.6 ± 0.1	69.6 ± 0.2	0.65 ± 0.11	1.77 ± 0.31

[a] Significant difference with respect to PLA + PLA 50 : 50 ($P \leq 0.05$).
[b] Significant difference with respect to PLA + PLA 75 : 25 ($P \leq 0.05$).
[c] Significant difference with respect to PLGA mixture ($P \leq 0.05$).
[d] Significant difference with respect to PLA + PLGA 75 : 25 ($P \leq 0.05$).
[e] PLGA mixture: PLGA 50 : 50 + PLGA 75 : 25 (50/50 wt %).

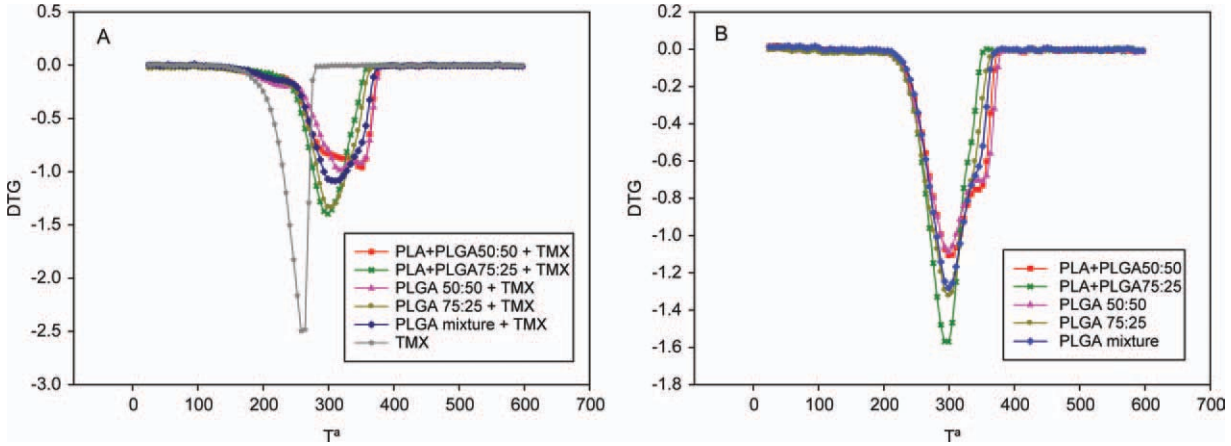

Figure 2 DTG curves of TMX-loaded (A) and unloaded (B) microspheres. PLGA mixture: PLGA 50 : 50 + PLGA 75 : 25 (50/50 wt %). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

wt %) formulation. When microspheres were formulated using an unique polymer, TMX release took place at 54 days from PLGA 75 : 25 microspheres and the maximum drug release was 47.67 μg TMX/mg microspheres (97.45 ± 4.27% of drug load), whereas drug release was faster from PLGA 50 : 50 microspheres, maximum release of TMX was 45.03 μg TMX/mg microspheres (94.64 ± 0.67% of drug load) at 14 days. Microspheres formulated with polymer mixtures showed different periods of maximum TMX release as a function of their hydrophobic characteristics. Thus, microspheres of PLGA 50 : 50 + PLGA 75 : 25 (50/50 wt %) exhibited a maximum TMX release of 43.94 μg TMX/mg microspheres (90.50 ± 2.46% of drug load) at 28 days, period of time intermediate between that corresponding to pure microparticles. TMX release took place at 11 days from PLA + PLGA 50 : 50 (25/75 wt %) microspheres and the maximum drug release was 40.15 μg TMX/mg microspheres (99.55 ± 0.59% of drug load). The PLA + PLGA 75 : 25 (25/75 wt %) microspheres showed the lowest drug release; maximum TMX release was 36.24 μg TMX/mg microspheres (98.93 ± 0.3% of drug load) at 58 days.

Constant release rates for, at least, two different periods of time were observed (Table VI) in all cases; and three stages of drug release were determined for polymer mixture formulations with PLGA 50 : 50. In all formulations, TMX release rate was quicker during the first stage in comparison with that of the second or third stage. First stage of TMX release took place during the first 9 h from formulations with PLGA 50 : 50 in their composition, and the TMX release rate was quicker in the order: PLGA 50 : 50, PLGA 50 : 50 + PLGA 75 : 25 (50/50 wt %), and PLA + PLGA 50 : 50 (25/75 wt %); thus, as the

TABLE V
Phenomenological Data for Steps of Thermal Decomposition of Unloaded and TMX-Loaded Microparticles, as well as TMX and Raw Polymers

		First step of thermal decomposition		Second step of thermal decomposition	
		Peak temperature in DTG (°C)	Mass loss (%)	Peak temperature in DTG (°C)	Mass loss (%)
PLGA 50 : 50	**unloaded**	305.88	44.96	357.93	88.72
	TMX-loaded	323.42	52.05	356.68	83.08
PLGA 75 : 25	**unloaded**	305.37	54.04		
	TMX-loaded	310.01	58.63		
PLGA 50 : 50 + PLGA 75 : 25	**unloaded**	306.58	44.98		
	TMX-loaded	317.93	53.70		
PLA + PLGA 50 : 50	**unloaded**	308.60	45.33	352.24	84.73
	TMX-loaded	330.95	60.63	357.55	84.65
PLA + PLGA 75 : 25	**unloaded**	303.28	55.64		
	TMX-loaded	304.78	52.02		
PLA		305.67	50.09	353.81	89.49
PLGA 75 : 25		350.61	73.51		
PLGA 50 : 50		322.90	44.40	367.91	84.71
TMX		267.94	78.09		

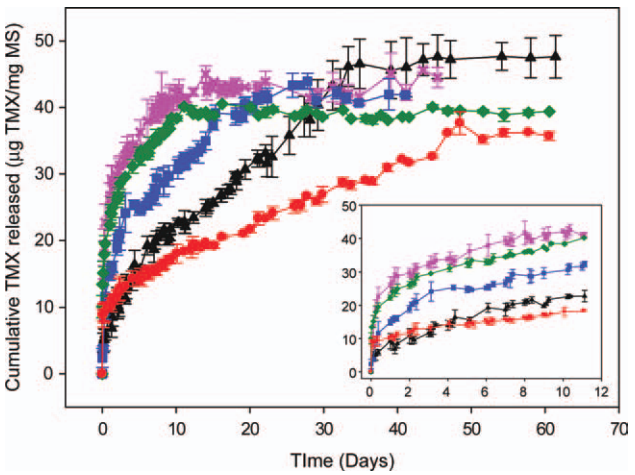

Figure 3 Cumulative amount of TMX released from TMX-loaded microspheres. Inset: First stage of cumulative TMX release. (▲)PLGA 75 : 25; (**X**) PLGA 50 : 50; (■) PLGA 75 : 25 + 50 : 50; (◆) PLA + PLGA 50 : 50; (●) PLA + PLGA 75 : 25. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

amount of PLGA 50 : 50 increase in the formulation, quicker TMX release. Second TMX release rate was similar from PLGA 50 : 50 + PLGA 75 : 25 (50/50 wt %) to PLA + PLGA 50 : 50 (25/75 wt %), although it was longer from the first composition. The third TMX release rate from PLGA 50 : 50 + PLGA 75 : 25 (50/50 wt %) to PLA + PLGA 50 : 50 (25/75 wt %), and the second drug release rate from PLGA 50 : 50 were very similar. With regarding formulations with PLGA 75 : 25, two TMX release rates were calculated; the first one took place during the first 200 and 390 h from PLGA 75 : 25 to PLA + PLGA 75 : 25 (25/75 wt %) formulations, respectively. TMX release was quicker from PLGA 75 : 25 at two stages.

In general, the presence of PLGA 50 : 50 in the polymer composition of microspheres caused quicker release rates of TMX, which can be related with a higher hydrophilicity of the polymeric matrices. Degradation studies of PLGA 50 : 50, PLGA 75 : 25, and PLA microspheres[49] indicated that the degradation of these polyesters is due to the hydrolysis of backbone ester groups; however differences in hydrophobicity determine the rate of *in vitro* degradation. Thus, as larger the percentage of glycolic acid in the copolymer as higher the hydrophilicity of the microspheres and, as a consequence, a larger water uptake and quicker hydrolysis of the ester bonds take place. The hydrolytic effect of the aqueous medium on the polymeric chains allows the formation of larger pores and channel inside the microspheres, which makes drug release more favorable.[30]

The presence of PLA in PLA + PLGA 75 : 25 (25/75 wt %) formulation made TMX release slower and slightly longer than that from PLGA 75 : 25 microspheres, probably due to a slower degradation of the polymeric matrix. The presence of PLA in the formulations decreased the amount of TMX load in microspheres (Table IV) and caused a slower TMX release (Table VI) regarding the values obtained for the corresponding formulations without PLA.

The mechanism of drug release through mathematical modeling of dissolution data for all drug delivery systems was evaluated. Regression parameters obtained after fitting the release kinetic models to the *in vitro* release data are given in Table VII. Thus, the formulations were observed to yield statistically valid correlations with various models. The formulations prepared with TMX showed good correlation for the Higuchi model, because the cumulative amount of drug that was released from these drug delivery systems was proportional to the square root of time, so the polymeric matrix would swell and the drug release would be diffusion controlled. The values of the diffusional exponent "*n*," obtained from the slopes of the fitted Korsmeyer-Peppas model, ranged between 0.44 and 0.22. In general, the values of "*n*" for TMX release from drug-loaded formulations prepared by spray-drying process were different than the standard value for declaring Fickian release behavior, that is, 0.43.[26,50] The exception was TMX release from PLGA 75/25 microspheres whose "*n*" value was very close to 0.43, and Fickian release behavior can be considered in this case. For drug release from spherical particles of a wide size distribution, the value of the exponent "*n*" for Fickian diffusion depends on the width of the distribution[51,52] and "*n*" values lower than 0.43

TABLE VI
Stages of Tamoxifen Release from Microspheres and the Corresponding Release Rate "*K*" [TMX (µg)/MS (mg)/h]

Formulation	1° Stage			2° Stage			3° Stage		
	T (h)	*K*	r^2	*T* (h)	*K*	r^2	*T* (h)	*K*	r^2
PLGA 50 : 50	0–9	73.36	0.90	24–336	1.68	0.91	–	–	–
PLGA 75 : 25	0–200	2.61	0.94	216–836	1.24	0.99	–	–	–
PLGA mixture	0–9	36.66	0.95	24–103	4.31	0.94	120–667	1.15	0.96
PLA + PLGA 50 : 50	0–9	28.93	0.91	9–54	4.56	0.92	57–266	1.59	0.97
PLA + PLGA 75 : 25	0–390	0.87	0.95	459–1306	0.52	0.98	–	–	–

T: time (hours); r^2: regression coefficient; PLGA mixture: PLGA 50 : 50 + PLGA 75 : 25 (50/50 wt %).

TABLE VII
Statistical Parameters of Various Formulations Obtained after Fitting the Drug Release Data to the Release Kinetic Models

		Formulation				
		PLGA 50 : 50	PLGA 75 : 25	PLGA mixture	PLA + PLGA 50 : 50	PLA + PLGA 75 : 25
Higuchi	K_0 ($h^{-1/2}$)[a]	1.51 (0.97)	1.35 (0.98)	1.57 (0.97)	1.63 (0.97)	0.81 (0.98)
Korsmeyer-Peppas	n	0.22 (0.97)	0.44 (0.96)	0.32 (0.99)	0.18 (0.98)	0.31 (0.96)
Hopfenberg	K[a] (first step)	0.741 (0.94)	0.028 (0.96)	0.08 (0.90)	0.556 (0.99)	0.0094 (0.95)
	K[a] (second step)	0.049 (0.97)	0.023 (0.95)	0.033 (0.96)	0.297 (0.96)	0.0081 (0.98)

Model fitting was reattempted until a minimum of 80% of drug was released.
PLGA mixture: PLGA 50 : 50 + PLGA 75 : 25 (50/50 wt %).
[a] Values in parentheses are the values of r^2. In Hopfenberg model, first step was ranged between 0 and 50% TMX released, and second step was ranged between 50 and 95% TMX released. Units of K are [TMX (μg)/MS (mg) μm^{-1}]/h.

can be obtained. Hopfenberg model allows for a quantitative description of drug release from degradable drug delivery systems, and it exhibits a release rate, which is proportional to the surface area of the device. Hopfenberg model showed two different release rates of TMX, the first one was faster for all microparticles. The polymer composition of the formulations influenced the value of the kinetic constants of Higuchi's model and Hopfenberg's model.

After 2 months of drug release, microparticles showed an advanced degradation in all formulations, with the exception of formulations with PLA + PLGA 75 : 25 (25/75 wt %), where the degradation state was no so evident; this fact can be correlated with the hydrophobicity of this polymeric matrix (Fig. 4). The amount of TMX in these polymeric rests was determined. TMX-remaining in the polymeric rests (2.13 ± 0.91 − 0.25 ± 0.03%) was very low in all cases (Table IV); however, its presence in the polymeric rests indicated a significant interaction between polymers and drug in the microspheres.

TMX release kinetics and the mathematical modeling study showed that there was a combination of two different processes in TMX release from these polymeric formulations. At the beginning, the release of the drug was favored by the swelling capability of the microspheres, which make easier the diffusion of the entrapped TMX; thus, the release was quicker from the most hydrophilic matrix, which means from PLGA 50 : 50-based formulations. The hydrolytic effect of the aqueous medium on the polymeric chains allows the formation of larger pores and channels inside the microspheres, which makes drug release more favorable.[12] The erosion process of the particles, which is more slowly in more hydrophobic systems, exerted mainly the control on TMX release at second and third stages of drug release. So in this way, these systems present a variety of characteristics and properties, such as controlled release profile, encapsulation efficiency and degradation behavior, that are able to be modificated by changing the polymer composition and polymer mixture increasing the versatility of the microspheres.

Cell culture studies

In general, the cytotoxicity of the microparticles was low. The range of cell survival, in the presence of various concentrations (75.9–474 μg/mL) of unloaded microparticles, was between 139 ± 6 and 72 ± 5% for MCF7 cells (Fig. 5). At low concentration (75.9–94.9 μg/mL) of unloaded microparticles [Fig. 5(A)], the different formulations seemed to induce a slight cell proliferation at short times; this effect was more significant for PLGA 50 : 50 and PLGA 50 : 50 + PLGA 75 : 25 (50/50 wt %) formulations, and it was maintained at the shortest exposure time when the particle concentration increased [Fig. 5(B,C)].

Three concentrations of TMX (10, 30, and 50 μ*M*) were administered to MCF7 cells by dissolutions of the drug and by TMX-loaded microparticles. These concentrations were chosen since previous studies using different concentrations of TMX in solution (from 0.01 μ*M* to 1 m*M*) demonstrate that those concentrations were the most optimal for the cellular line MCF7. Besides these concentrations were in between of other concentrations that have been used in other studies.[21,53,54] In the case of TMX include in the particles, it was not in contact with MCF7 cells until its release from the system so its concentration in the cell medium was increasing as it was released. *In vitro* drug efficacy of TMX-loaded microspheres against MCF7 cells was determined for 2, 5, and 6 days. As *in vitro* release studies demonstrated that, the TMX released at the second and sixth day was between 22–64 and 39–82%, respectively, can be considerer that in only 6 day there is enough TMX released to have a cytotoxic effect in cells. The viability of MCF7 cells in the presence of 10 μ*M* TMX

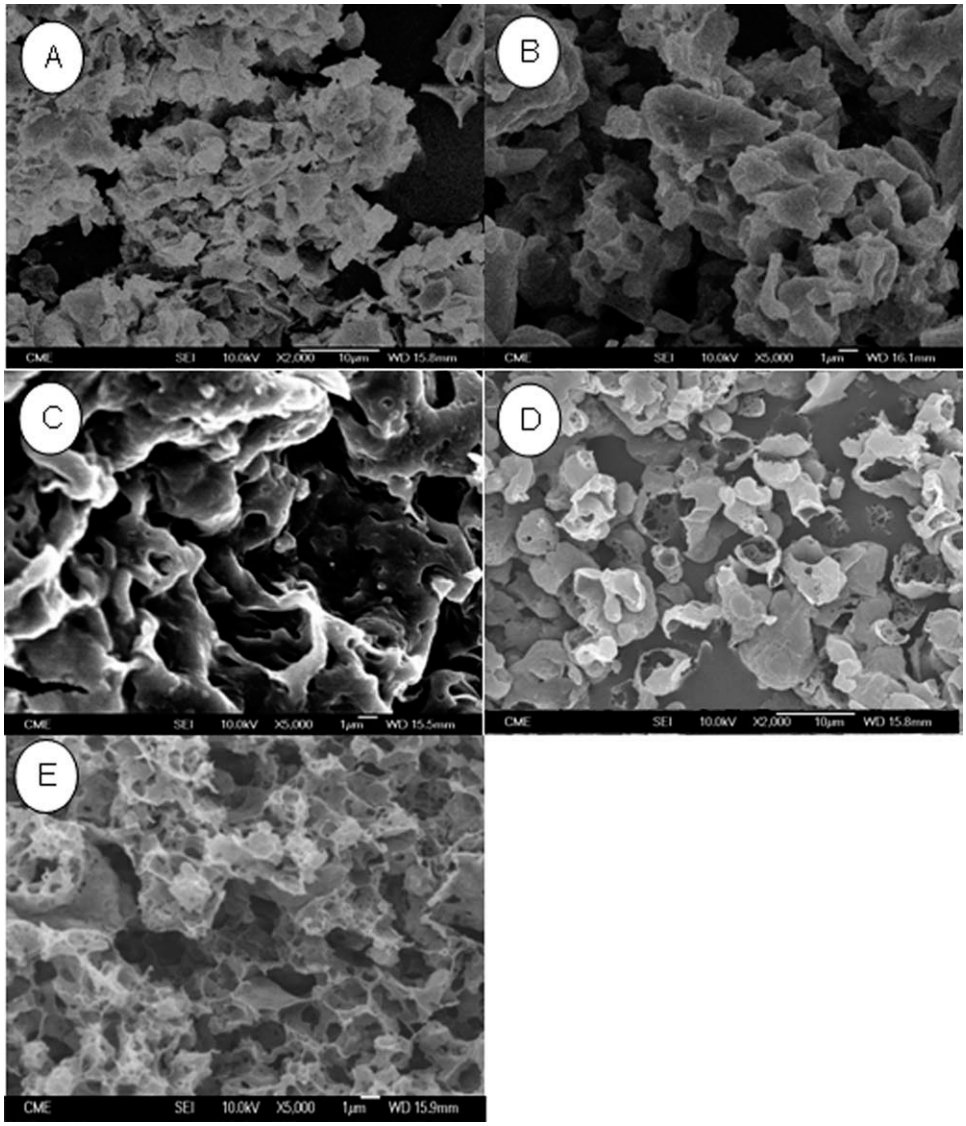

Figure 4 FE-SEM photographs of microspheres after two months of drug release. (A) PLGA 75 : 25; (B) PLGA 50 : 50; (C) PLGA 75 : 25 + PLGA 50 : 50; (D) PLA + PLGA 75 : 25; (E) PLA + PLGA 50 : 50.

administered by TMX-loaded microparticles at all exposure times [Fig. 6(A)] was similar to that observed when TMX was administered in solution. Only, PLA + PLGA 75 : 25 (25/75 wt %) and PLA + PLGA 50 : 50 (25/75 wt %) induced a slightly lower cytotoxicity at 6 days of exposure. When TMX was administered at 30 μ*M* in dissolution [Fig. 6(B)], no cell survival was observed from the first time of study. The drug administration by the polymeric formulations showed time dependent cell viability. Furthermore, in general a correlation with the release rate of TMX from the formulations can be observed, mainly at longer time exposure. Thus, microparticles of PLGA 50 : 50, which showed the quickest TMX release (Table VI), were the most efficient in decreasing cell viability; and, in general, cell viability was increasing in the same order than the TMX release rate from the formulations decreased. The largest TMX concentration studied, 50 μ*M* [Fig. 6(C)], caused an equivalent effect to the above described on cell viability; whereas the drug in solution caused total cell death, the administration of TMX by the polymeric formulations caused a continuous decrease in cell viability, which was time and polymeric composition dependent. Thus, for MCF7 cells the cytotoxic efficacy of these TMX-loaded microparticulated polymeric formulations was confirmed.

Different studies have shown the interaction of PLGA nanoparticles with tumoral cells, allowing the uptake of the particles into the cells.[55,56] Two distinct but not exclusive pathways can justify the therapeutic activity of drug incorporated into nanoparticles: (a) nanoparticles can absorb onto the cell membrane,

leading to an increase in drug concentration near the cell surface, thus generating a concentration gradient that would favor a drug influx into the cell; (b) tumor cells can internalize polymeric nanoparticles, allowing the drug to be released into the interior of the cells, thus contributing to an increase in the drug concentration near its site of action.[21,56] In this way, the higher sensitivity of MCF7 cells to TMX-loaded Nanoparticles (NPs) may be related to the adsorption onto the cell membrane and accumulation of NP in the cells, where TMX-loaded NPs should release the drug, thus increasing the intracellular concentration of TMX and enhancing the cytotoxicity effect as it is released.

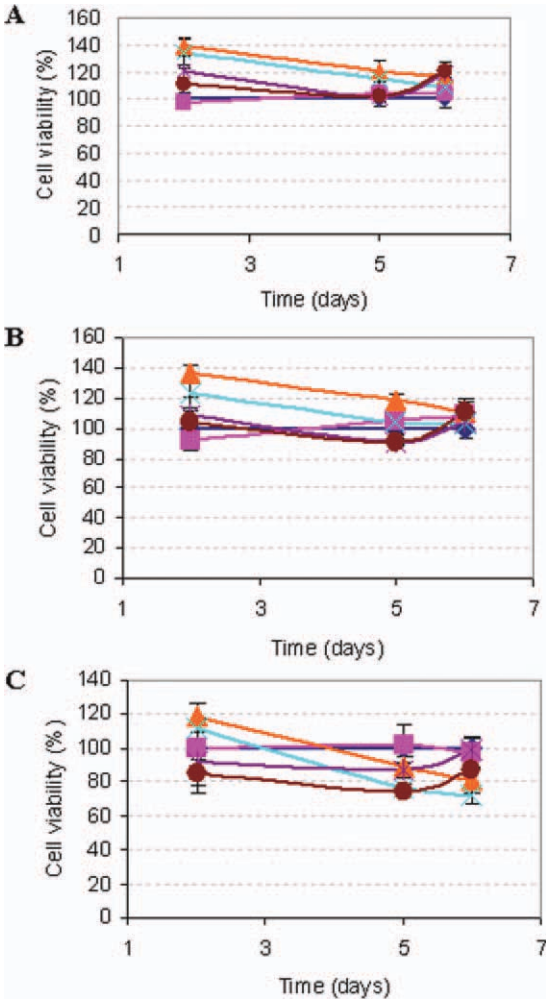

Figure 5 Cytotoxicity of unloaded microspheres. Cell viability of MCF7 in correlation of the quantity of microspheres added in the experiment (Table VII): (A) concentration of microspheres between 75.9 and 94.9 μg/mL; (B) concentration of microspheres between 227.8 and 284.6 μg/mL; (C) concentration of microspheres between 380 and 474 μg/mL. Without microspheres (◆) or with PLGA 75 : 25 (■); PLGA 50 : 50 (▲); PLGA 75 : 25 + PLGA 50 : 50 (50/50 wt %) (x); PLA + PLGA 75 : 25 (25/75 wt %) (★); PLA + PLGA 50 : 50 (25/75 wt %) (●) microspheres. Data were shown as mean ± SD ($n = 15$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

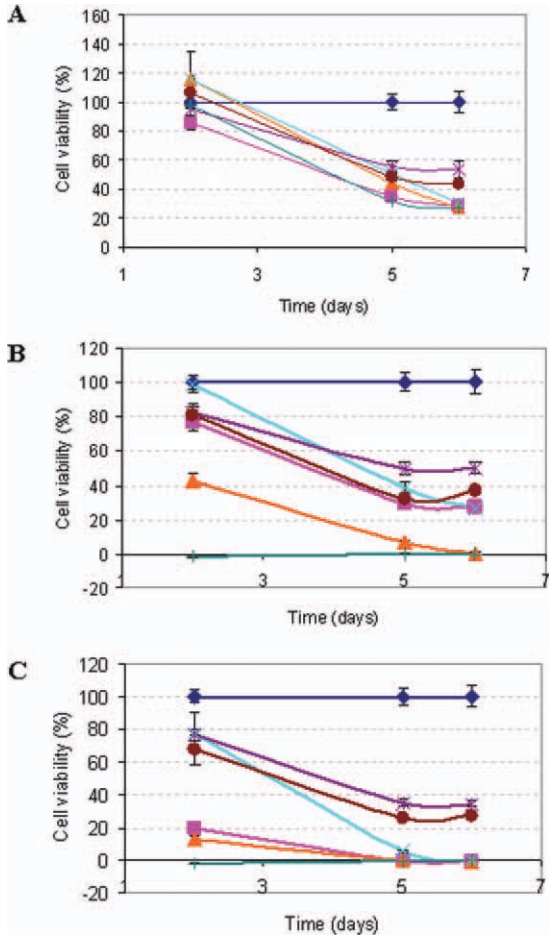

Figure 6 Cell viablitiy of MCF7 in the presence of 10 μ*M* (A); 30 μ*M* (B) and 50 μ*M* (C) of tamoxifen (TMX): without drug (◆); TMX dissolution (+); with TMX-loaded microspheres of PLGA 75 : 25 (■); PLGA 50 : 50 (▲); PLGA 50 : 50 + PLGA 75 : 25 (50/50 wt %) (x); PLA + PLGA 75 : 25 (25/75 wt %) (★); PLA + PLGA 50 : 50 (25/75 wt %) (●). Data were shown as mean ± SD ($n = 15$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

CONCLUSIONS

The present investigation suggests that TMX release can be tailored significantly by varying the polymer composition of microparticles. Changes in copolymer composition of PLGA and in its proportion regarding that of PLA in the formulations produced different TMX release behavior. Thus, a vast range of TMX release rates as well as of time needed to obtain the maximum amount of released TMX (between 11 and 58 days) was modulated with the polymeric composition of the microspheres. The microparticles were well tolerated by human cell line model MCF7. TMX-loaded polymeric formulations showed to be efficacy to cancer cells. Thus,

these TMX delivery systems are good candidates for anticancer therapy. Further *in vivo* studies are planned to demonstrate the *in vivo* application of these TMX sustained release formulations.

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