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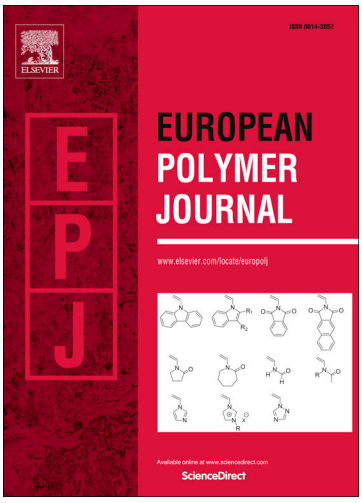

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Non-covalently coated biopolymeric nanoparticles for improved tamoxifen delivery

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Abstract

About one-fifth of cancer patients suffer from breast cancer worldwide. Polymeric nanoparticles play an important role in delivering chemotherapeutic agents in a controlled manner. Polylysine coated tamoxifen loaded poly(lactic-*co*-glycolic acid) nanoparticles were prepared using a single emulsion technique with subsequent non-covalently surface functionalization in order to improve nanoparticle-cell interaction and hence tamoxifen therapeutic effect. The obtained nanoparticles were fully characterized in terms of their physico-chemical properties as well of their *in vitro* performance against human breast adenocarcinoma cells. The successful incorporation of tamoxifen within the hydrophobic matrix of nanoparticles is evidenced by a high loading efficiency (86%). Furthermore, ideal size, morphology and hydrodynamic properties are observed being the proposed nanocarrier capable of display a valuable antiproliferative *in vitro* effect.

Keywords: Tamoxifen, nanoparticles, poly(lactic-*co*-glycolic acid), polylysine, drug delivery, cell viability

1. INTRODUCTION

As reported by the World Health Organization (WHO), cancer figure among the leading causes of morbidity and mortality worldwide, with an estimated number of 14 million new cases and 8.2 million cancer related deaths in 2012 (contemporary global cancer statistics report). Among all cancer types, breast cancer is the most frequent in women with approximately 1.67 million new cancer cases diagnosed per year and representing the 25% of all cancers being also the second most common kind of cancer in the world [1].

The inherent limitations of conventional cancer treatments prompted the advance in the development of new nanosystems for effective and safer oncological therapies, this field of study and application it is known as cancer nanomedicine which possess unique appealing features for drug delivery [2, 3]. The capability of accommodate a broad spectrum of drug molecules offered by biopolymeric nanoparticles (NPs) represents one of the most solid alternatives to improve anticancer therapies, being capable of achieve the desired concentration of drug in the specific site of action, thus, minimizing negative side effects, reducing toxicity, overdosage, etc [4, 5].

Considering the wide range of biopolymers studied for medical applications, poly(lactic-*co*-glycolic acid) (PLGA) stands out due to its associated minimal systemic toxicity supported by the fact that its hydrolysis leads to endogenous and easily metabolized monomers (lactic acid and glycolic acid) which are biologically inert to the growing cells and effectively removed from the body by normal metabolic pathways[6]. Additionally, PLGA, approved by Food and Drug Administration (FDA)[7], possess high biocompatibility, good miscibility with other polymers and adjuvants film, particle and capsule forming properties, etc [8].

Particularly, tamoxifen (TMX), developed in the 1970s and approved by the FDA in 1990s, is a fundamental drug for the treatment of breast cancer conforming to the

WHO [9, 10]. This selective estrogen receptor modulator has been the Trojan horse for the endocrine treatment of all stages of estrogen-receptor-positive breast cancer and it has become the first cancer chemotherapeutic for the reduction of breast-cancer incidence in both pre- and post-menopausal women. Nevertheless, TMX based breast cancer treatment involves an oral administration of its salt form (tamoxifen citrate) up to 5 years. TMX exhibits a very low bioavailability, considerable inter subject variability and an appreciable dose and concentration dependent negative side effects such as oxidative stress mediated hepatotoxicity and propensity to endometrial cancer [11]. Even though TMX is the gold standard for the endocrine treatment of estrogen-receptor-positive breast cancer [12] and the first clinical choice, it is viable to capitalize its therapeutic effect, reduce side effects and improve patients welfare through a biopolymeric nanoparticulated formulation.

It has been reported how important surface properties are when chasing NPs cell internalization [13]. In this sense, positively charged NPs can lead to improved internalization when compared with anionic or neutral NPs due to a superior interaction with the negatively charged cell membrane [14]. Ɛ-polylysine (PLL) is a naturally occurring, biodegradable and nontoxic homopolyamide. Its poly cationic nature at physiological pH makes it very valuable in the field of biomedical applications, particularly as a coating material [15].

On the grounds that the estrogen receptor is located intracellularly, the internalization of TMX loaded NPs may have a great influence over the final therapeutic effect achieved after the administration of the nanoformulation. An attractive strategy to improve cellular uptake of PLGA NPs relies in the fact that the intrinsic anionic surface of this kind of particles facilitates non covalently coating through simply electrostatic anchoring of a cationic molecule or conjugate of interest (**Figure 1**) [16].

The present work has been aimed at obtaining and studying TMX loaded PLGA NPs in terms of loading efficiency, TMX release profile, non-covalently surface coating for improved NPs-cell interaction and *in vitro* performance against breast cancer.

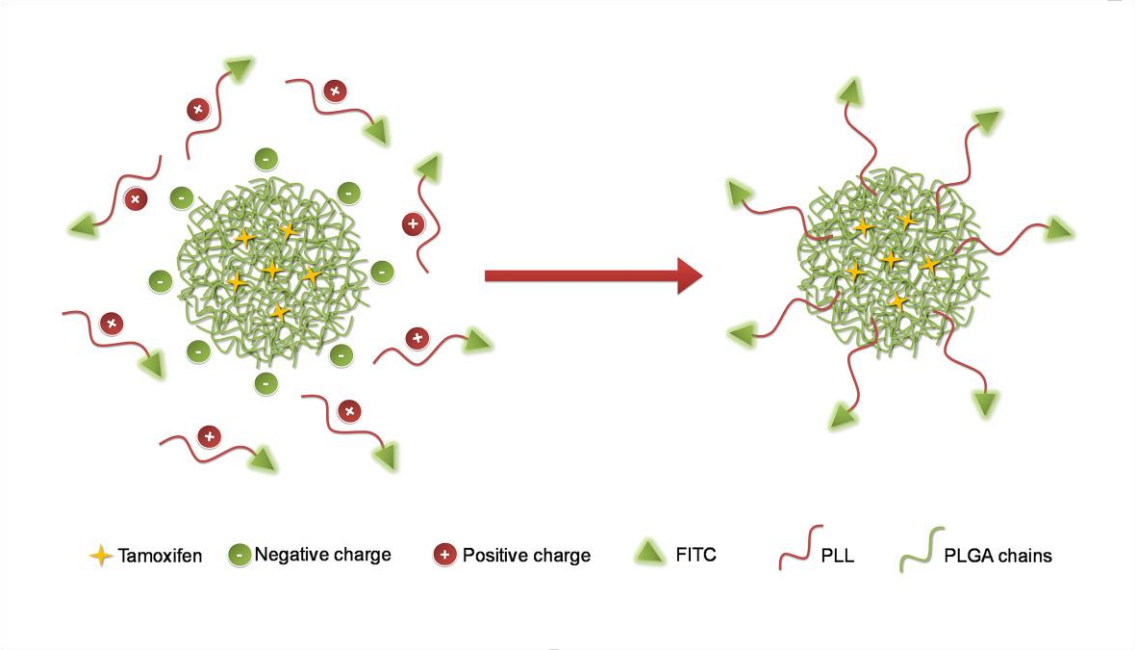

Figure 1. Scheme of proposed non-covalently functionalization to obtain PLL coated TMX loaded PLGA NPs.

2. MATERIALS AND METHODS

2.1. Materials

Poly (D,L-lactic-*co*-glycolic) acid 50:50 (RESOMER RG 504), inherent viscosity 0.45–0.60 dl/gr (0.1 wt.% in $CHCl_3$, 25 C^0), and Mw: 38,000–54,000 Da was purchased from Boehringer–Ingelheim (Germany). Polyvinyl alcohol, >89% hydrolyzed (PVA), Mw: 31,000–50,000 was purchased from Sigma–Aldrich. Tamoxifen free base was purchase from Sigma-Aldrich (Prod. No. T5648). Polylysine labeled with fluorescein (PLL-FITC) sequence: FITC - C6 - Lys - Lys - Lys – Lys - Lys - Lys - Lys - Lys - Lys – Lys was purchased from Americ Peptide Company. Phosphate buffered saline (SigmaAldrich), methanol, HPLC grade (J.T.Baker), deionized water was purified on-site using a Milli-Q Plus purification system (Millipore Corp., USA).

2.2. Preparation of TMX loaded PLGA NPs

TMX loaded PLGA NPs (NP-TMX), were prepared by single emulsion method with subsequent solvent evaporation. Briefly, 100mg of PLGA was dissolved in 5 mL of ethyl acetate with the drug using magnetic agitation. This solution was emulsified with 2% w/v of PVA aqueous solution using a tip sonicator (VIBRA CELL Sonics mod. VC 750 USA, 39% Amp.) for 20 min. The resulting emulsion was transferred into 0.2% w/v PVA aqueous solution, which was then magnetically stirred at room temperature to enhance complete solvent evaporation. The NPs were collected by centrifugation at 11.000g after four washes with deionized water to remove excess of surfactant. Unloaded PLGA NPs (NP-0) were obtained using the same technique [17, 18].

2.3. Preparation of PLL coated TMX loaded PLGA NPs

NP-TMX, having negative surface charges, were coated with positively charged PLL via ionic interactions in an aqueous phase [16], PLL coated NP-TMX (PLL-NP-TMX) were prepared by mixing 500 µL (1mg/ml) of NP-TMX with 50 µL, 100 µL and 200 µL of an aqueous solution of PLL (1mg/ml)and left at room temperature for 15 min, corresponding to a PLL/ NP-TMX ratio of 0.1, 0.2 and 0.4 respectively.

2.4. NPs characterization

The morphology of the polymeric NPs was analyzed by Field Emission Scanning Electron Microscope (FESEM, Hitachi SU 8000 TED). Samples were prepared by deposition of one drop of the NPs suspension over a small glass disk with subsequent evaporation at room temperature. Finally samples were coated with gold palladium alloy (80:20).

Size distribution was also evaluated trough FESEM analysis; a histogram extracted from NP-TMX FESEM micrographs was built from 300 nanoparticle diameters measured using ImageJ software. Furthermore, the mean diameter (d) ± standard

deviation (σ) value and the polydispersity index (PDI) according Equation 1 were calculated.

Equation 1. $PDI = \left(\frac{\sigma}{d}\right)^2$

The particle size distribution of the NPs suspensions was determined by Dynamic Light Scattering (DLS) using a Malvern Nanosizer. Measurements of NPs dispersions were performed in square polystyrene cuvettes (SARSTEDT) and the temperature was kept constant at 25 °C. The zeta potential (ζ) was determined for NPs formulations at 1 mg/ml PBS concentration and pH= 7,4 and was automatically calculated from the electrophoretic mobility using the Smoluchowski´s approximation. Evolution of mean diameter with temperature from 24 to 40ºC was also evaluated in the same apparatus. For each sample, the statistical average and standard deviation of data were calculated from 6 different samples (3 measurements of 20 runs each one).

The loading efficiency (LE%) of TMX in the NP-TMX was determined by High Performance Liquid Chromatography (HPLC) according to MacCallum*et al*.[19].Briefly, the amount of entrapped TMX was calculated indirectly by measuring the difference between the initial amount of TMX(TMX_{in}) and the amount of free TMX (TMX_{free}) in the supernatant during the washing steps. The LE% was expressed according to the **Equation 2**.

Equation 2. $LE\% = (TMXin - TMXfree)/TMXin\, x\, 100\%$

2.5. Thermal analysis

Thermogravimetric Analysis (TGA) was performed in a Q500TA Instruments TGA system. Three milligrams of free TMX, and NP-TMX samples were hermetically sealed in an aluminum pan. The dynamic scans were taken in nitrogen atmosphere (flow rate

of 50mL/min) at the heating rate of 10°C/min. The TGA curves were obtained in a temperature range of 25-600 °C, respectively.

Differential Scanning Calorimetry (DSC) was performed in a Mettler Toledo 822e model apparatus. Ten milligrams of dried samples were placed in covered aluminum sample pans and then placed in the DSC sample holder. The samples were then heated at 10°C/min, in the -25 to 250°C temperature range and in a nitrogen atmosphere.

2.6. In vitro TMX release

In vitro TMX release from NP-TMX was investigated in PBS suspensions under physiological conditions for a total period of 21 days. Briefly, 5 mL of NP-TMX suspension with 15 u/mL of esterase from porcine liver (Sigma-Aldrich) were dialyzed against 15 mL of PBS at 37 °C using a 3.5kDa MWCO membrane (Spectrum Laboratories). After certain time periods, 2 mL of the dialyzing medium was withdrawn and the same volume (2 mL) of PBS was replenished. TMX concentration was measured by HPLC (Shimadzu) according the validated method described by Mac Callum *et al.* [19]. The experiment was carried out in triplicate.

2.7. In vitro PLGA NPs biodegradation studies

In vitro biodegradation of PLGA matrix was investigated during the aforementioned experiment carried out to study *in vitro* TMX release. The morphology of the nanoparticles and the molecular weight of the polymer was systematically analyzed using FESEM, and Gel Permeation Chromatography (GPC, refractor index detector Waters 2414 with HR4, HR1 and HR0.5 Waters Styragel columns of 7,8x300mm) respectively.

In order to visualize NP-TMX morphological biodegradation progress through FESEM samples were deposited onto a small glass disk (12 mm diameter) and coated with gold palladium alloy (80:20).

For the determination of PLGA molecular weight variation trough GPC after 7, 14 and 21 days of biodegradation, the samples were referenced to polystyrene standards between 4000 and 400.000 Da. Briefly, 4 mg of sample were dissolved in 1 mL of filtered tetrahydrofuran (THF). Before measurements, the diluted polymer was filtered off using a NYLON 0.2µm filter. The molecular weight averages Mn (number molecular weight), Mw (average molecular weight) and IP (polydispersivity index) calculated are different statistical representations of the molecular weight. Each of the averages is related to different physical properties and processing parameters of the polymer.

2.8. Cell culture

Human mammary adenocarcinoma cells, MCF-7(ECACC) cells, were cultured in Dulbecco's modified Eagle's medium(DMEM), supplemented with 10% fetal bovine serum (FBS), 2L-glutamine, 1% penicillin/streptomycin, and incubated at 37 °C and 5%CO2.

2.9. In vitro cytotoxicity and antiproliferative effect

Cell viability in the presence of different concentrations of NPs (2.500, 1.250, 0.625, 0.312, 0.156, 0.078, 0.039, 0.019, 0.009, 0.004 mg/mL) was measured using Alamar Blue assay. Briefly, $8x10^4$ live cells/ml were seeded in 96-well plates to obtain a 100% of confluence. After 24 hours of incubation the medium was replaced with the corresponding NPs dispersed in PBS (50 µL of the NPs suspension and 50 µL of completed DMEM).

The plates were incubated at 37 °C in a humidified air with 5% CO_2 for 24 and 72 h. Afterwards, AlamarBlue® (Invitrogen) was carried out to determine cell viability using a Multi-Detection Microplate Reader Synergy HT(BioTek Instruments; λabs = 570 nm).

3. RESULTS AND DISCUSSION

3.1. NPs characterization

NP-TMX were successfully obtained through single emulsion/solvent evaporation method. As can be observed in **Figure 2A**, FESEM images of the obtained NP-TMX exhibited a quasi-spherical regular shape. This resulting morphology is associated with an effective processing without any loss of the droplet structure during the first stage of the freeze drying process, demonstrating that the selected experimental conditions allow control of the formation of NPs, their dimensions, and ergo their properties [21]. A narrow size distribution profile was observed in both FESEM and DLS extracted histograms (**Figure 2B** and **D**). The same results were obtained for NP-0. (data not shown).

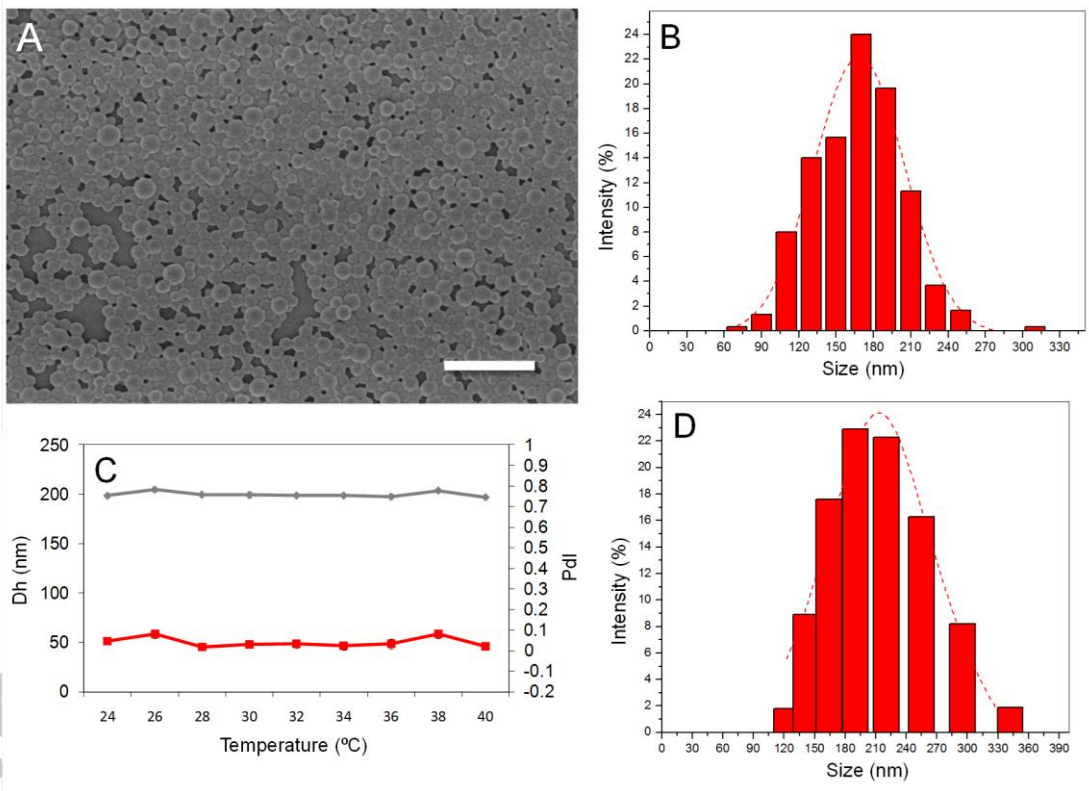

Figure 2. (A) FESEM images of obtained NP-TMX; Scale bar: 1µm. (B) Representative FESEM extracted histogram of NP-TMX size distribution. (C) NP-TMX Dh (grey line) and PDI (red line) evolution with temperature. (D) Representative DLS extracted histogram of NP-TMX size distribution.

Additionally, the evolution of hydrodynamic diameter (Dh) which is related to the size of particulated systems in suspension and polydispersity index (PDI) was evaluated by DLS considering a temperature range according the biological human milieu, the results proves that NP-TMX possess an adequate thermal behavior for the proposed application (**Figure 2C**). NP-TMX showed a narrow size distribution when were analyzed both by FESEM and DLS as can be seen in **Figure 2B** and **D** respectively.

NP-TMX and NP-0 were characterized in terms of Dh, PDI, zeta potential (ζ) by DLS, and, in case of NP-TMX loading efficiency (LE%) was determined by HPLC, the collected data regarding these parameters is summarized in **Table 1**.

Particularly, NP-TMX exhibited a Dh of 196.9 ± 0.2 nm and a PDI of 0.040 ± 0.014. Considering size values extracted from FESEM images the mean diameter and the PDI were 166.3 ± 34.7 nm and 0.043 respectively. Thus, both FESEM and DLS results confirm the monodispersity of NP-TMX which positively influence their colloidal stability. Regarding the differences observed between NP-TMX average sizes measured by FESEM imaging and DLS, there are a result of the hydration and swelling of the NPs in aqueous suspension. The obtained size values are highly adequate for potential biomedical applications[22]. For instance, in order to enhance tumor penetration it is important to pay attention in physicochemical properties of NPs to penetrate diffusion barriers in the interstitial matrix. Smaller NPs could more easily diffuse throughout the tumour tissue, but very small particles would be rapidly cleared by renal filtration[2]. Taking into consideration another clinical scenario in which TMX is a maintenance treatment and there is no tumor presence it is crucial the size control in order to avoid the reticuloendothelial system (RES), NPs larger than 200 nm will be accumulate in liver and spleen previous elimination as a result of RES action.[23] This would result in the loss of drug cargo and hence in the loss of therapeutic effect.

The size and shape of NPs are influenced by the selected surfactant, PVA in this case, which main function is to stabilize the emulsion[24]. The observed parameters for NP-TMX support their stabilization through steric repulsion due to PVA hydrophobic acetate moieties adsorption at PLGA matrix surface [25].

Meanwhile, the observed zeta potential value is directly related with the intrinsic negative charges in PLGA structure and with the performed sequential washes to remove excess of PVA as well as expose the negative charges for the subsequent surface electrostatic functionalization. A residual mass of PVA remains associated with the NPs despite repeated washing due to the interconnected network that this polymer forms with the PLGA at the interface [21]. However, it was possible to reach a value of-13.5±-0.7 mV which allowed subsequent surface modification with positively charged PLL chains.

The ability of load a wide range of hydrophobic molecules has been extensively reported for PLGA [26]. This behavior was confirmed when TMX loading efficiency in the obtained NP-TMX was measured resulting in an 86% over the initial amount of the drug. The obtained loading efficiency value can be explained as a consequence of the higher affinity of TMX for PLGA matrix compared to that for the aqueous media containing the emulsifier. This is directly related with the hydrophobic nature of both polymer and drug.

Table 1. Hydrodynamic diameter (Dh), polydispersity index (PdI), zeta potential (ζ), and loading efficiency (LE%) of obtained NPs.

System ID	Dh (nm)	PDI	Z (mV)	LE (%)
NP-TMX	196.9 ± 0.2	0.040 ± 0.014	-13.5 ± -0.7	86

NP-0	170.4 ± 0.8	0.036 ± 0.020	-18.9 ± -1.2	-

Thermoanalytical methods are of great value regarding polymer-based material study, and they have been used to investigate interactions between polymer and drugs in several micro and nanosystems in drug delivery. The thermal characteristics of the samples as well as the physical interaction between PLGA and TMX constituting the NP-TMX was verified by TGA and DSC (**Figure 3 and Figure 4**). From collected thermograms all degradation temperatures were obtained and reported in **Table 2** as well as T_{05} values which is a more accurate indicator of the degradation temperature of this type of materials and represents the temperature when 5% of the material was degraded.

Regarding thermogravimetric analysis, TGA-DTGA curve of neat PLGA demonstrated that the polymer starts to degrade at 225 ºC and presents thermal stability until 250 ºC. The thermal decomposition of PLGA appears with a peak centered at 370 ºC with a shoulder (311 ºC). On the other hand, the thermal decomposition of NP-0 begun at a lower temperature (228 ºC) than that of the pure polymer. The NPs are more exposed to the thermal degradation because their nanometric size makes the superficial area larger than the one of the neat polymer, since particles show wider superficial area they degrade easier [27, 28]. Free TMX starts to degrade at 150 ºC exhibiting one degradation peak centered at around 280 ºC with a sharp shoulder (around 198 ºC). Meanwhile, NP-TMX degrades near 200 ºC, this behavior confirm the aforementioned capability of biopolymers in improving thermal stability of drugs.

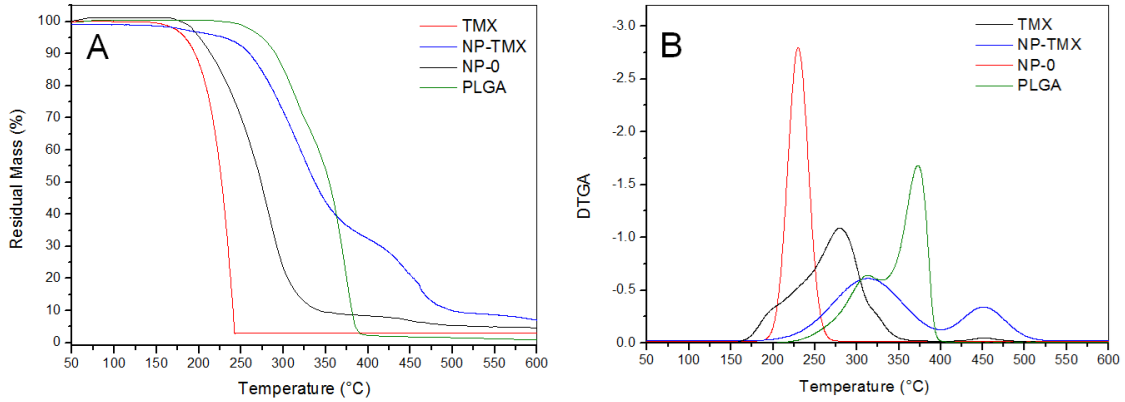

Figure 3. (A) TGA and (B) DTGA curves of TMX, neat PLGA, NP-0 and NP-TMX.

Table 2. Degradation temperatures and T_{05} values of TMX, neat PLGA), empty NP-0 and NP-TMX.

MATERIAL	T_{05} (ºC)	Td PEAK (ºC)
PLGA	275.0	311.4 – 371.6
TMX	183.4	197.5 – 279.2
NP-0	203.2	228.3
NP-TMX	229.0	311.5 – 450.5

As can be seen in DSC analysis (**Figure 4**) the free TMX shows a sharp endothermic peak that corresponds to melting at 98 °C, indicating its crystalline nature[20]. However, no peak related with any crystalline drug material was detected when NP-TMX were tested. This is a clear indication of the successful incorporation of the drug in the polymer matrix, in an amorphous or disordered-crystalline phase of a molecular dispersion or a solid solution state. Other authors reported similar results when hydrophobic drugs loaded PLGA matrix was studied [27, 28].

Neat PLGA exhibit an endothermic event near to 60 °C which is related to the relaxation peak that follow the glass transition, it is possible to observe the same event for NP-0. As PLGA is semi-crystalline but mainly amorphous, no melting point was observed. NP-TMX exhibit an endothermic event at 50 °C referring to the relaxation peak that follows the glass transition and no melting point was observed. Thus, the NPs preparation process did not affect the polymer structure because the pure polymer presented the same value for relaxation enthalpy.

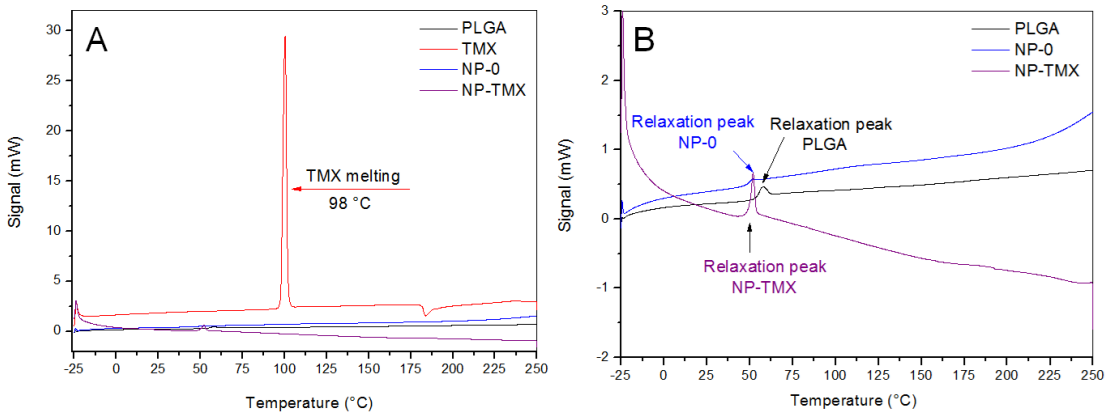

Figure 4. (A) Heating thermograms TMX, neat PLGA, NP-0 and NP-TMX. (B) Magnified neat PLGA, NP-0 and NP-TMX heating thermograms.

Surface PLL modification over NP-TMX was successfully achieved leading to a change in zeta potential values up to 4.5 ± 1.8 mV when a ratio PLL/ NP-TMX of 0.2 was tested. All the data regarding the fundamental characterization of obtained PLL-NP-TMX is presented in **Table 3**.

Labeled PLL was anchored on the surface of NP-TMX via ionic interactions. Three different PLL/ NP-TMX ratio were assayed in order to determine the optimum conditions to achieve PLL coating. It was observed that PLL-NP-TMX obtained using a PLL/ NP-TMX weight ratio of 0.2 displayed the highest positive charge (zeta-potential value increased from -13.5±-0.7 mV to 4.5 ± 1.8). This phenomenon arises from

electrostatic interactions between the negatively charged groups of PLGA and the positively charged PLL which is in this manner adsorb onto the NP-TMX surface. Only a portion of labeled PLL chains were required to neutralize the negative charges of PLGA, whereas the remaining free labeled PLL were responsible for the resulting positive zeta potential. Additionally, a considerable shrinkage of the NP-TMX was observed after PLL coating when PLL/ NP-TMX weight ratio of 0.2 and 0.4 were tested. This behavior as well as this kind of non-covalently surface modification was previously reported and it was suggested that the ionic anchoring of the positively charged coater in the negatively charged surface is able to induce partial shrinkage of the surface layer [16]. This phenomenon is likely to be due to the reduction of the mutual repulsion forces associated with the negative charges present in all the PLGA chains constituting the NPs. As a result of surface neutralization with PLL the size of NP-TMX decreases since it allows the PLGA chains to get closer. Moreover, size values after coating highlight no aggregation of the PLL-NP-TMX which is a very valuable feature when chasing long systemic circulation, especially in the PLL/ NP-TMX weight ratio of 0.2, with an average size of 158.1 nm (**Table 3**). The lowest PDI value (0.100 ± 0.080) was also found when PLL/ NP-TMX weight ratio of 0.2 was tested. Considering these features PLL/ NP-TMX weight ratio of 0.2 was selected to perform cell culture assays.

Table 3. Ratio PLL/NP-TMX, hydrodynamic diameter (Dh), Polydispersity Index (PDI), zeta potential (ζ), of PLL-NP-TMX.

System ID	Ratio PLL/ NP-TMX	Dh (nm)	PDI	Z (mV)
PLL-NP-TMX	0.1	201.2 ± 4.4	0.205 ± 0.040	3.3 ± 0.6
	0.2	158.1 ± 1.7	0.100 ± 0.080	4.5 ± 1.8

	0.4	168.8± 7.2	0.150±0.040	2.5± 0.8

3.2. In vitro TMX release and biodegradation assay

TMX release from NP-TMX was assayed in physiological conditions and measured trough HPLC validated method. After 7, 14 and 21 days of incubation 51%, 56% and 74% of the initial amount of TMX where respectively released (**Figure 5**).

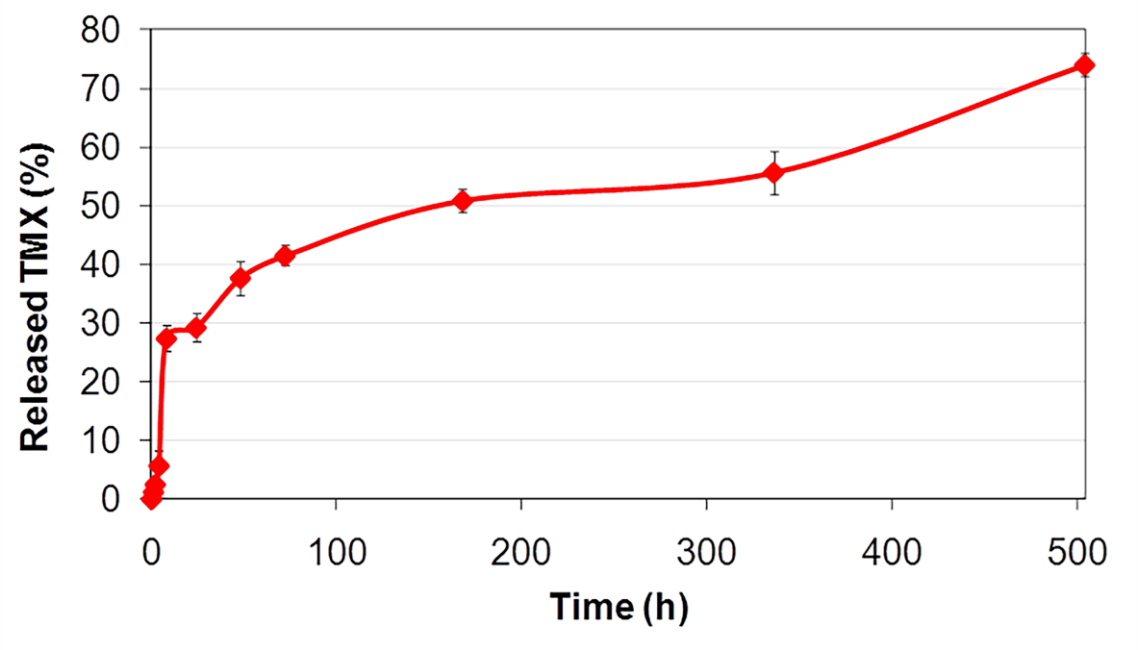

Figure 5. *In vitro* release TMX profile of obtained NP-TMX in PBS at 37 Cº.

As degradation proceeds, the spherical morphology of NP-TMX as well as the general nanostructure was lost after 21 days of incubation. A non-regular porous matrix-like appearance combined with remaining PBS crystals can be observed in **Figure 6**.

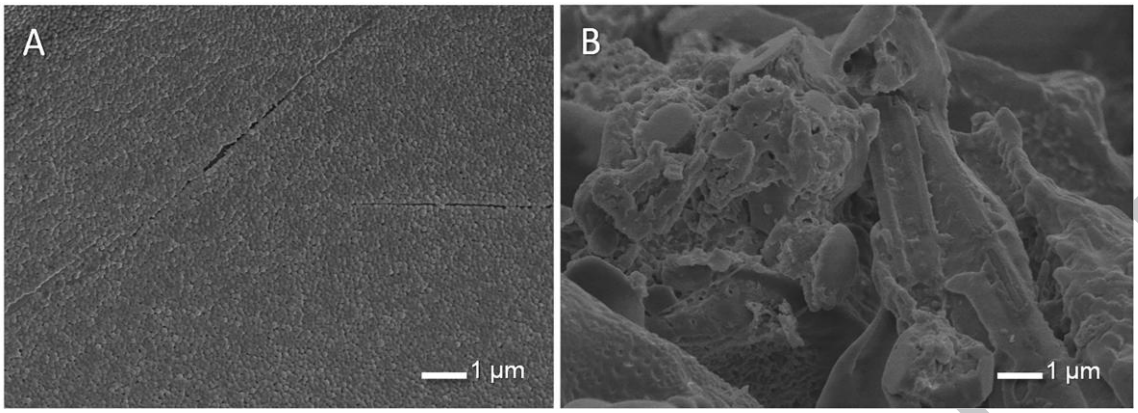

Figure 6. FESEM micrograph of (A) NPs before *in vitro* biodegradation and (B) remaining polymer after 21 days of *in vitro* biodegradation.

Regarding NP-TMX biodegradation study, after of 7 days of incubation a considerable decrease in PLGA molecular weight was observed (**Table 4**). Moreover, after 14 and 21 days it was not possible to measure polymer molecular weight due to a non-detectable PLGA signal.

He et al. reported the degradation process of these biopolymeric systems began with a decrease of PLGA molecular weight as a consequence of the hydrolysis of the polymer ester bonds [29]. In according, after 7 days of incubation a decrease in PLGA M_n from 39,748 to 32,127 Da was observed. However, after 14 and 21 days the chromatographic signal of PLGA was too low to assess molecular weight quantification. These results are in agreement with the fact that PLGA degrades through the hydrolysis of backbone ester groups, which leads to acidic monomers that are responsible for subsequent autocatalyzed degradation by carboxylic end groups [30]. Thus, once NP-TMX start to degrade an acidic environment is generated as a result of the accumulation of the degradation products which in this case are lactic and glycolic acid. These small polymer chains are capable to catalyze remaining polymer matrix that originally has formed the NPs.

Rescignano *et al*. have demonstrated that PLGA NPs suffer from heterogeneous degradation and that particle agglomeration with subsequent fragmentation lead to

cargo release after 21 days of incubation [30]. *In vitro* TMX release assay revealed a profile which is in coincidence with this previous study. After 7 days of NP-TMX esterase-mediated physiological incubation a release of 51% of the TMX content from the polymer matrix was observed. Moreover, during the last 2 weeks of the assay TMX was released in a sustained manner reaching 74% of the original cargo. This profile proves a correlation between polymer matrix degradation and drug release.

Table 4. PLGA molecular weight measured by GPC after 7, 14 and 21 days of *in vitro* physiological incubation.

System	Degradation Time (Days)	Mn (Da)	Mw (Da)
NP-TMX	0	39,748	67,213
	7	32,127	56,240
	14	-	-
	21	-	-

3.3. In vitro cytotoxicity and antiproliferative effect

In vitro biological activity of polymeric NPs was evaluated using human breast adenocarcinoma cell line (MCF-7) in order to determine their therapeutic effect toward cancer cells. **Figure 7** shows that relative cell viability decreased in a dose-dependent manner only when PLL-NP-TMX was added to the cell culture after 24h. NP-0 was non cytotoxic after 24 h. Non statistically significant differences ($P^* \leq 0.05$) was achieved between NP-0 and NP-TMX (**Figure 7**). A significant improvement in cytotoxic effect was achieved when cells were treated with PLL-NP-TMX. These systems decreased cell viability below 30% after 24 h of exposure to NPs (**Figure 7**). However, only slightly differences between cells treated with NP-0, PLL-NP-0 and NP-TMX was achieved. Particularly, cell viability was reduced below 80% at 0.250 mg/ml NPs concentration

and lower than 20% at 0.500 mg/mL of PLL-NP-TMX, showing a big improvement in cytotoxicity after 24 hours of NPs exposure. The negatively charged surface of NP-0 and NP-TMX could interact with negatively charged cell membrane to a lesser extent limiting their chance of cellular uptake [16]. By the addition of PLL NPs surface charge becomes near to neutral, providing a better scenario for an adequate interaction with the cells, improving their internalization and thus their cytotoxic effect. Indeed, the cytoxicity of these NPs was strongly influenced by the TMX cargo, as could be seen with the non-cytotoxic effect of PLL-NP-0 systems (100% cell viability in all doses tested), showing that PLL remains to be non-cytotoxic and only enhances NP-cell interaction [15].

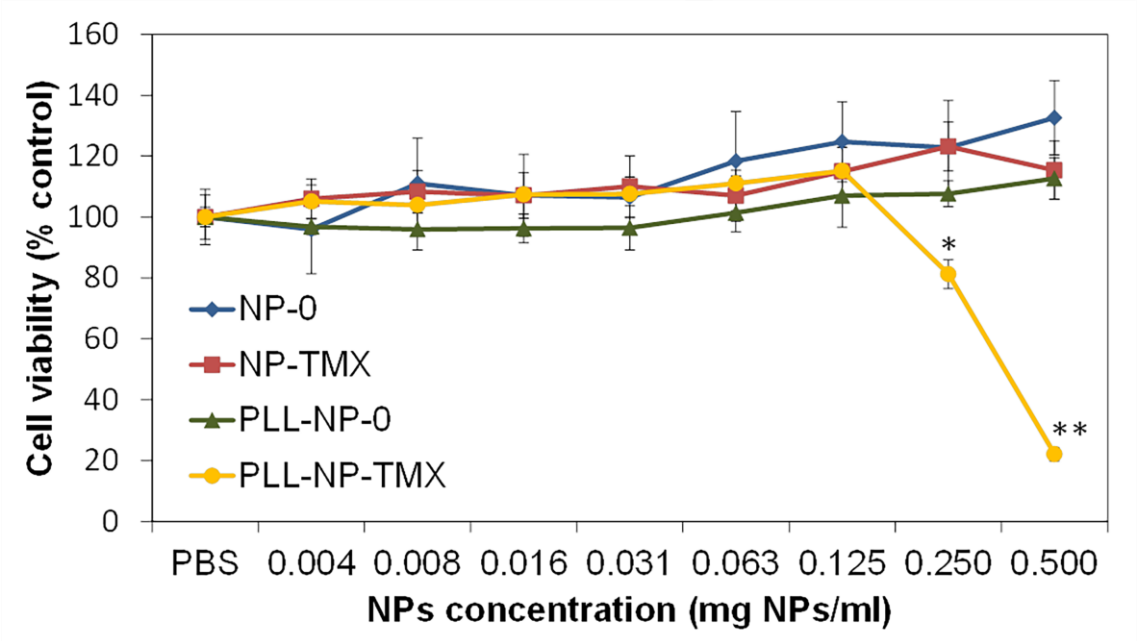

Figure 7. Cell viability of MCF7 treated with different concentrations of NP-0, NP-TMX and PLL-NP-TMX at 24 h. The data are presented as the mean ± standard deviation (n = 8), and the ANOVA results* P≤0.05 with respect to the control, ** P≤0.01 with respect to the control.

Antiproliferative effect of NPs systems was measured after 72 h of NPs addition. That improvement in the interaction between cells and PLL-NP-TMX was confirmed after

72h of exposure, when PLL-NP-TMX reduce cell viability near to 0 at the higher concentration, showing statistically significant difference (*P≤0.05) with respect the non-coated formulation NP-TMX in 0.250 and 0.500 mg/ml concentrations (**Figure 8**). Relative cell viability decreased in cells treated with NP-0 below 64%, near to cytotoxic limit only with the higher NPs concentration (viability <70%; ISO 10993-5:2009). Statistically significant difference (*P≤0.05) was achieved in NP-TMX treated cells if compared with NP-0 treated MCF-7 (**Figure 8**). Moreover, cell viability decreased over 47% when cells were treated with 0.250 mg/ml and over 23% when treated with 0.500 mg/ml of NP-TMX. Relative cell viability descend below 10% when treated with PLL-NP-TMX, being this the formulation displaying the most considerable antiproliferative effect (**Figure 8**). These results support the fact that PLL coating assist TMX delivery to cells, probably due to the improvement in the interaction with the cell membrane. This phenomenon was particularly important in the first 24h, when the non-coated formulation NP-TMX showed non cytotoxicity against MCF-7 line. The observed PLL-NP-TMX *in vitro* performance, and the fact that D_h was significantly reduced (from 196 to 158 nm), could be promising for the use of PLL-NP-TMX in *in vivo* models.

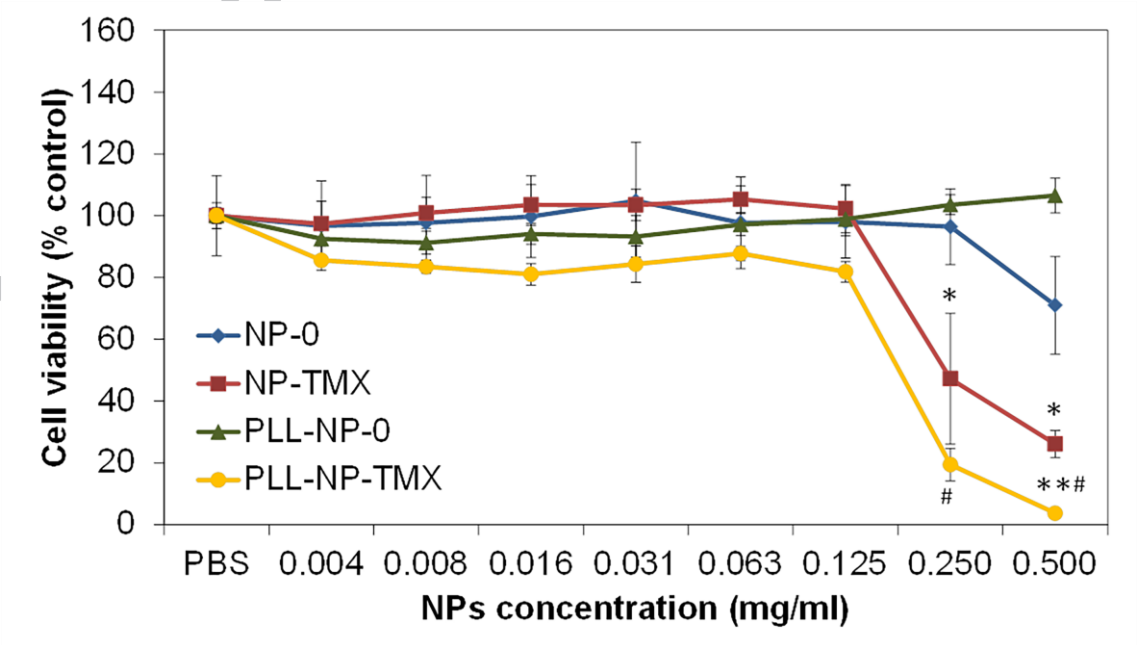

Figure 8. Antiproliferative effect of NPs in MCF7 treated with different concentrations of NP-0, NP-TMX, PLL-NP-0 and PLL-NP-TMX at 72 h. The data are presented as the mean ± standard deviation (n = 8), and the ANOVA results * P≤0.05 with respect to the control, ** P≤0.01 with respect to the control, # P≤0.05 PLL-NP-TMX against NP-TMX.

4. CONCLUSION

New PLL coated TMX loaded PLGA NPs were successfully developed, obtained and characterized. TMX was accommodated within the hydrophobic matrix of PLGA exhibiting appropriate hydrodynamic properties, high loading efficiency and a suitable release profile for sustained breast cancer treatment. *In vitro* cytotoxicity assays fulfill the hypothesis in which PLL was proposed as good candidate to assist TMX delivery by enhance NP-TMX interactions with estrogen positive breast cancer cells. Our results indicate that PLL coating strongly capitalize NP-TMX cytotoxic and antiproliferative effect over MCF7 cell line and that new treatment opportunities could rely in this strategy.

5. ACKNOWLEDGMENTS

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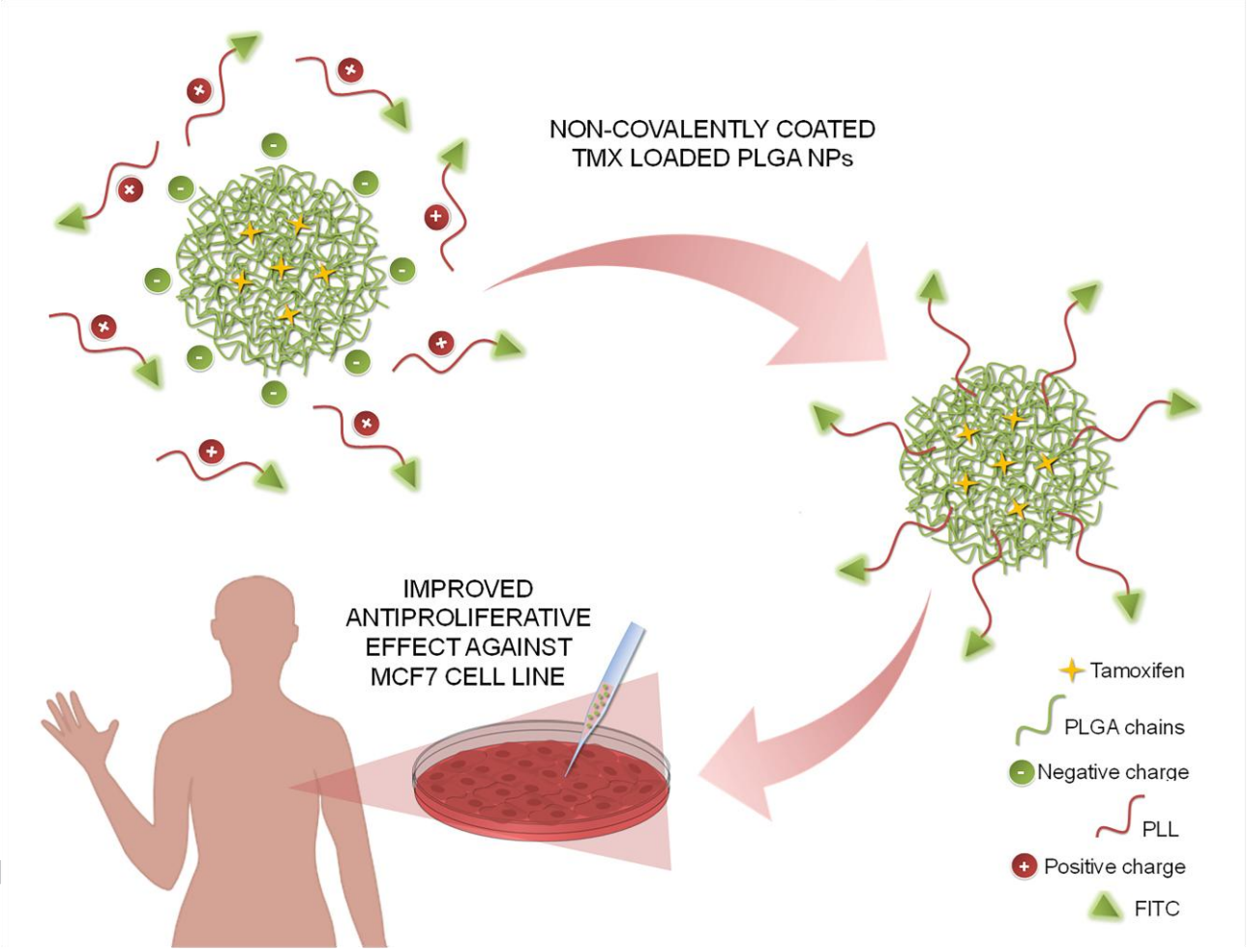
Graphical abstract. Polylysine coated tamoxifen loaded poly(lactic-co-glycolic acid) NPs were succesfully obtained and fully characterized exhibiting appropriate hydrodynamic properties, high loading efficiency and a suitable tamoxifen release profile aimed to adress sustained breast cancer treatment. In vitro cytotoxicity assays fulfill the hypothesis in which polylysine was proposed as good candidate to assist tamoxifen delivery since a remarkable cytotoxic and antiproliferative effect was observed over human breast adenocarcinoma cell line (MCF7).



Highlights

- PLL coated TMX loaded PLGA NPs were successfully obtained.
- NPs exhibit appropriate hydrodynamic properties, high loading efficiency and a suitable release profile for sustained breast cancer treatment.
- PLL coating strongly capitalize NPs cytotoxic and antiproliferative effect over human breast adenocarcinoma cell line (MCF7).